

Hyperimmunization of Steers with J5 *Escherichia coli* Bacterin: Effects on Isotype-Specific Serum Antibody Responses and Cross Reactivity with Heterogeneous Gram-Negative Bacteria

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ABSTRACT

Isotype-specific antibody responses and cross reactivity were profiled following hyperimmunization of steers with J5 *Escherichia coli* bacterin. The vaccine was administered at time 0, 30 d later, and every 2 wk for 10 subsequent immunizations. Blood was collected preimmunization and multiple times following each immunization. Isotype-specific anti-J5 *Escherichia coli* antibody response profiles in diluted sera harvested from each sample were assayed by ELISA and recorded as optical density. Selected sera were assayed for anti-J5 *Escherichia coli* antibody titers and used to determine cross reactivity against a variety of gram-negative bacteria. Immunization number and day postimmunization influenced response profiles for anti-J5 *E. coli* IgM, IgG₁ and IgG₂ antibodies. Two immunizations increased mean serum IgM and the IgG₁ antibody profiles above preimmunization levels, but 5 immunizations were required to detect significant IgG₂ antibody responses that were above preimmunization levels. Isotype-specific cross reactivity of the serum antibodies with a variety of heterologous gram-negative bacteria was also increased by hyperimmunization. However, no cross reactivity was observed for *Staphylococcus aureus*, purified lipopolysaccharide, or lipid A. Our results indicate that multiple booster doses of J5 *E. coli* bacterin may be required to elicit high levels of cross-reactive serum IgG₂ antibodies.

(Key words: J5 *Escherichia coli*, hyperimmunization, antibody response, cross reactivity)

Abbreviation key: FBS = fetal bovine serum, INF- γ = interferon-gamma, OD = optical density, OMP = outer membrane protein, TSB = trypticase soy broth.

INTRODUCTION

Immunization of cattle with J5 *Escherichia coli* bacterin has been used as a method to enhance immune resistance against gram-negative bacteria, including mastitis-causing coliforms. Commercial J5 bacterins contain a mutant strain of *E. coli* 0111:B4 (Rc mutant) lacking the “O” antigen capsular portion of the bacterial cell wall but with core LPS and lipid A antigens intact. These core antigens are highly conserved across all gram-negative bacteria (Gonzalez et al., 1989; Tyler et al., 1990b; Cullor, 1991) and are thus considered as being primarily responsible for eliciting cross-reactive antibodies in J5 vaccinated cows (Tyler et al., 1990a; 1992; Tomita et al., 1995).

Some studies have reported that cows vaccinated with J5 bacterin using the manufacturer’s recommended protocols have relatively adequate IgM and total IgG antibody responses in serum and milk (Tyler et al., 1991, 1992; Tomita et al., 1995). However, only a few studies have monitored responses of the main opsonizing antibody in J5 immunized cattle, namely IgG₂. In 2 such studies, J5 *E. coli*-specific IgG₂ antibodies were increased modestly in conventionally vaccinated dairy cows vs. unvaccinated cows, but the vaccinated animals were not protected from experimental coliform mastitis (Hill, 1991; Tomita et al., 2000). This may have been due to the dose of bacteria used for the experimental infections or an indication that the serum antibody responses elicited by recommended use of J5 bacterins were not strong enough to be protective, even though they were detectable.

Gram-negative bacterins have traditionally been labeled as weakly immunogenic in cattle because they elicit relatively poor anamnestic (IgG₁ and IgG₂) antibody responses (reviewed by Kehrl and Harp, 2001). Published studies exploring the potential of improving IgG₁ and IgG₂ immune responses using J5 dosing protocols different from those currently recommended by vaccine manufacturers are scarce. To our knowledge, no studies have been published in which the goal was

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to optimize the J5 *E. coli* immunization schedule to maximize serum antibody concentrations in cattle. This was one objective of the current study. In light of the key role as an opsonin that IgG₂ plays in efficient clearance of invading pathogens by bovine neutrophils (McGuire et al., 1987; Burton and Erskine, 2003), the second objective of this study was to determine whether J5 bacterin elicits gram-negative cross-reactive IgG₂ antibodies. Based on the notion that J5 *E. coli* bacterins are lowly immunogenic, our hypothesis was that multiple booster doses of commercial J5 vaccine would be needed to elicit strong serum IgG₁ and IgG₂ antibody responses that cross-react well with heterologous gram-negative bacteria.

MATERIALS AND METHODS

Animals

Five 5-mo old Holstein steers were used for this study. Steers were owned by a private dairy in Michigan, and housed and cared for at the farm for the duration of the study. Animals were group-housed with free access to water and feed. In preparation for entry into our study, the animals were immunized twice for bovine viral diarrhea, infectious bovine rhinotracheitis, parainfluenza 3, bovine respiratory syncytial virus, and 5 serovars of *Leptospira* (triangle 9; Fort Dodge Animal Health, Fort Dodge, IA), and dewormed (Ivermectin, Merck & Co., Inc., Rahway, NJ) at 3 mo of age (i.e., before the trial). All steers were deemed healthy at the start of the study, and remained healthy throughout the study.

Immunization Schedule

All experimental animals entered the trial, were immunized and bled on the same days. A total of 12 immunizations were administered to each steer by subcutaneous injection of 5-mL doses of a commercially available vaccine (J5 *E. coli* bacterin; Pharmacia Animal Health, Kalamazoo, MI). The first immunization was administered at study time 0. The second immunization was administered 30 d later. The subsequent 10 immunizations were administered every 2 wk, with the exception of an 8-wk rest period between the sixth and seventh immunizations. This was done because the PCV of the steers had decreased after the sixth, bleeding to the point where it was considered necessary to suspend bleeding to insure anemia did not develop.

Blood was collected from each steer immediately before the first immunization (preimmunization), on d 4, 6, and 11 postimmunization for doses 1 through 6, and on d 4, 6, 11, and 13 postimmunization for doses 7 through 12. The additional blood sample was collected

on d 13 postimmunization for the last 6 doses to maximize the harvest of serum antibodies for isolation and purification. Blood samples (150 mL) were collected from the jugular vein into 50-mL sterile conical centrifuge tubes using 14 gauge, 3.75-cm needles. Samples were allowed to clot overnight at 4°C and were then centrifuged (2400 × *g* for 30 min at 4°C) for sera harvesting. Sera were stored in ~25-mL aliquots at -20°C until assayed by ELISA.

Preparation of J5 *E. coli* Whole Cell Antigen for Use in ELISA

J5 *E. coli* (0111:B4, Rc mutant; donated by James Cullor, University of California, Davis, CA) was plated on 5% sheep blood agar and incubated overnight at 37°C. The plate was checked for culture purity and single colonies from pure cultures selected and inoculated in 15 mL of trypticase soy broth (TSB) for overnight culture at 37°C in an orbital shaker (120 rpm; J. T. Baker, Phillipsburg, NJ). To establish purity of the cultures, 10-μL aliquots were collected from each inoculum and plated on 5% sheep blood agar overnight at 37°C. The cultures were stored at 4°C during this incubation. If the overnight cultures proved to be pure, each culture was transferred into 1 L of TSB and incubated while shaking at 120 rpm for 18 h at 37°C. These preparations were then stored at 4°C while purity was assured by culture, and the pure bacteria were killed by adding 15 mL of a 99% liquid phenol solution (Sigma Chemical, St. Louis, MO) per liter of the bacterial preparation. This solution was incubated with shaking for 1 h at 37°C and 120 rpm. The phenol-killed whole cell bacteria were centrifuged (1000 × *g* for 12 min at 4°C), pellet washed twice in 500 mL of sterile 0.9% NaCl, and suspended in the same to an optical transmission of 13% (~1 × 10⁹ cfu/mL) using a spectrophotometer (model DU 650; Beckman Coulter, Inc., Fullerton, CA). This whole cell J5 antigen solution was stored in 12-mL aliquots at -20°C until used in ELISA.

ELISA to Monitor Isotype-Specific Anti-J5 *E. coli* Antibody Response Profiles

Serum anti-J5 *E. coli* IgM, IgG₁, and IgG₂ antibody response profiles (Burton et al., 1993) were determined using an ELISA protocol described in Tyler et al. (1990a). Briefly, 100 μL of J5 *E. coli* whole cell antigen was used to coat wells of 96-well ELISA plates (ProBind Flat Bottom plates, Fisher Scientific). Normal saline (100 μL/well) was added to 2 wells of each plate for later blanking during plate reading (see below). Three plates were coated at a time, one for analysis of IgM antibody responses and the other 2 for analysis of Ig-

IgG₁ and IgG₂ antibody responses. The plates were covered and left to incubate on a flat surface at room temperature for 15 h, after which unbound antigen was removed by 6 washes using 200 μ L/well of biowash solution (0.9% saline containing 0.05% Tween 20).

Test sera were prepared for ELISA using separate 96-well, U-bottom polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ). Individual samples were placed in the top well of each column and diluted twofold down the 8 rows of the plate, starting with a 1:50 dilution in row A and ending with a dilution of 1:6400 in row H. The sample diluent was PBS (pH 7.3) with 0.5% Tween 20. A positive control consisting of pooled sera from blood samples collected following the 5th immunization was similarly diluted down rows of one column of each plate. The negative control in this assay was fetal bovine serum (FBS, endotoxin \leq 10 EU/mL; Life Technologies, Rockville, MD) and was diluted in the same fashion in one column of each plate. Additional controls in the assay included quadruplicate wells of premade 1:400 dilutions of the positive and negative control sera, duplicate wells that received only the coating antigen, and duplicate wells that received all reagents except test sample. Samples contained in each well of the dilution plates were transferred (100 μ L/well) to washed ELISA plates using a multi-channel pipetter (Costar Corporation, Cambridge, MA).

Once all samples were delivered to appropriate wells, ELISA plates were sealed and incubated at 37°C for 45 min. Following incubation, the plates were washed 6 times (as above) and 100 μ L/well of detection antibody (1:25,000 in sample diluent; horseradish peroxidase-conjugated sheep anti-bovine IgM, IgG₁, or IgG₂; Bethyl Laboratories, Montgomery, TX) added to wells of the appropriate plates. Plates were again sealed and incubated for 30 min at 37°C, washed 6 times, and 125 μ L/well of substrate (hydrogen peroxide-azino-bis-3-ethylbenzthiazoline sulfonic acid; Sigma) added for 45 min at 37°C.

The levels of isotype-specific antibodies in the variously diluted samples were recorded as optical density (OD) following spectrometric analysis at dual wavelength (405 nm normalized against 450 nm) using an ELISA plate reader (Benchmark, Bio-RAD Laboratories, Hercules, CA). The plate reader was calibrated against the 2 blank wells of each plate before each was read. The IgG₁ antibodies are always highest in concentration in bovine serum (Butler, 1998). We thus used the daily IgG₁ plates to monitor repeatability of each ELISA performed. We found that the greatest and most repeatable differences in IgG₁ OD between the positive and negative control sera always occurred at the 1:400 dilution, whether the control sera were diluted that day or used as the premade controls. Thus, if OD values of

the 1:400 dilutions of positive and negative control sera in the IgG₁ plate were not within preestablished ranges ($0.99 \leq OD \leq 1.10$ and $OD \leq 0.09$, respectively), all samples in each isotype-specific plate were repeated on a subsequent assay day. Once all OD data were acquired for each isotype, antibody response profiles were checked for parallelism across all dilutions used. This was done by correlation analysis using SAS (SAS/STAT, 1999). Parallelism was considered significant when OD profiles of 3 consecutive doubling dilutions of samples within isotype yielded $r > 0.90$ with $P < 0.0001$ and resulted in identical response curves over time. According to these criteria, we selected the 1:100 dilution for statistical analysis of the serum IgM anti-J5 *E. coli* antibody response profile and 1:400 for analysis of the serum IgG₁ and IgG₂ anti-J5 *E. coli* antibody response profiles.

Isotype Specific Anti-J5 *E. coli* Antibody Titers in Selected Serum Samples

ELISA was used to determine endpoint titers of isotype-specific anti-J5 *E. coli* antibodies in selected sera. This was done to identify samples to be used in the subsequent antibody cross reactivity experiment (see below). Based on the response profiles already determined by the protocol described above, samples selected for titer analysis were preimmunization and 6 d post 3rd, 6th, 9th, and 12th immunizations. These sera were serially twofold diluted from 1:4 through 1:131,072 down columns of a series of U-bottom plates per isotype before addition to J5 *E. coli* coated ELISA plates, and the remainder of the ELISA performed as described above (including blanks and controls). Endpoint titers were recorded as the last dilution to yield an $OD > 0.09$, which was considered zero-titer because the OD for IgG₁ in the 1:400 dilution of FBS was always < 0.09 .

Cross Reactivity of the Isotype-Specific Serum Antibodies Measured by ELISA

The antibodies in sera from preimmunization and 6 d post-3rd, -6th, -9th, and -12th immunizations were assessed for cross reactivity against multiple heterologous gram-negative antigens by ELISA. The whole cell antigens used contained ($\sim 1 \times 10^9$ cfu/mL of relevant bacteria (i.e., 13% optical transmissions) and included J5 *E. coli* as a gram-negative positive control, *Staphylococcus aureus* as a gram-positive negative control, and *E. coli* McDonald 487, *Pseudomonas* ssp., *Serratia* ssp., *Klebsiella* ssp., *Salmonella newport*, and *Salmonella typhimurium* as test antigens. The *S. newport* and *S. typhimurium* were donated by the Animal Health Diagnostic Laboratory (Michigan State University,

East Lansing). The other bacteria originated from milk cultured in our laboratory from cows with clinical mastitis. Each was cultured and killed with phenol to make antigen solutions as described above for J5 *E. coli*. Purified LPS (from J5 *E. coli* 0111:B4 Rc mutant; Sigma) and lipid A (monophosphoryl from *S. minnesota*, Re 595; Sigma), both at 10 $\mu\text{g}/\text{mL}$, were prepared as described (Freundenburg et al., 1989) and used as additional antigens for plate coating. Test sera were diluted 1:50 (for IgG₂ and IgM) or 1:200 (for IgG₁) in sample diluent for determining isotype-specific antibody cross reactivity for the test and control antigens, which was recorded as OD.

Cross Reactivity of the Isotype-Specific Serum Antibodies Determined by Immunoblotting

The various bacteria described above were grown on 5% sheep blood agar overnight at 37°C. Single colonies were inoculated in 15 mL of TSB and incubated while shaking (225 rpm) for 18 h at 37°C. Purity of culture was confirmed by overnight culture at 37°C on 5% sheep blood agar. The cultures were centrifuged (8000 $\times g$ for 15 min at 4°C) to collect bacterial cell pellets, which were then suspended in 3.0 mL of lysing buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 5% glycerol. Using a probe sonicator (Sonifier), the bacterial cells were disrupted with multiple short bursts of 15-s pulses with 15-s rests for 1 to 2 min and kept on ice at all times (Harlow and Lane, 1999; Promega, 1996). Resulting crude lysates were cleared of cell debris by centrifugation (10,000 $\times g$ for 15 min at 4°C). Trichloroacetic acid was added to reconstitute the pellet to a final concentration of 10% (wt/vol) and placed on ice for 5 min to precipitate proteins (Promega, 1996). The TCA-treated mixture was centrifuged (12,000 $\times g$ for 2 min at 4°C) to collect the protein pellets, which were washed twice (12,000 $\times g$ for 2 min at 4°C) and suspended in ice cold 0.9% sterile NaCl. Protein concentrations were determined spectrophotometrically ($\lambda = 260, 280, \text{ and } 320 \text{ nm}$) and stored at -20°C until used for immunoblotting.

Sodium dodecyl sulfate-PAGE was used to separate antigens contained in the crude bacterial lysates based on molecular weight. Precision Plus All Blue (size range 10 to 250 kDa; BioRad) was the molecular weight marker used on all gels. Bacterial lysates (5 μg each) were prepared using a 1:2 ratio of sample in loading buffer, which was 95% (vol/vol) of Laemmli sample buffer (BioRad) that contained 5% (vol/vol) β -mercaptoethanol (Sigma Chemical Co., St Louis, MO). The mixed samples were heat-denatured at 95°C for 4 min and placed on ice prior to loading on precast Tris-HCl polyacrylamide gels (12.0%, 160 \times 160 \times 0.75 cm; BioRad).

The electrophoresis buffer consisted of 1 M Tris/glycine/SDS, pH 8.3. Separated proteins were transferred (Trans-Blot Cell with plate electrode, BioRad) to nitrocellulose membranes (0.45 μm ; Pierce, Rockford) at 25 V (PowerMac 300, BioRad) for 6 h at 4°C. The membranes were incubated in Super Blocking buffer in Tris (Pierce) that contained 0.1% added Tween 20 for 1 h at room temperature, and were then probed with diluted (1:1000 in sterile milli-Q water) test sera (pooled across the 5 steers from samples collected 6 d post 3rd or 9th immunization). After a 60-min incubation at room temperature, the blots were washed 6 times [BupH Tris Buffer Saline (Pierce) containing 0.05% Tween 20] and probed further with anti-bovine HRPO-conjugated isotype-specific detection antibodies (IgM, IgG₁, or IgG₂, heavy chain specific, Bethyl Laboratories, Inc., Montgomery, TX) for 60 min at room temperature. After 6 more washes, substrate was added (Super Signal West Pico Chemiluminescent Substrate system; Pierce) for 5 min and the membranes exposed to X-ray film (Kodak BioMax MS; Fisher Scientific) for 2 min. Films were developed (Futura 2000 E Automatic X-Ray film processor; Fischer Industries Inc., Geneva, IL) and photographed (Gel Doc 2000 Fluor-S MultiImager; BioRad) for data presentation.

Statistical Analyses

The OD data from the antibody profiling experiment were normally distributed and thus analyzed statistically without transformation. For statistical analyses of these data, immunization number and sample day nested within immunization number were included in a mixed model as fixed effects along with assay day, assay plate nested within assay day, and animal as random effects. In addition, autoregressive order one covariance structure (based on Schwarz' Bayesian model-fit criterion) was specified in the REPEATED statement of a model covariance structure (SAS/STAT, 1999) within animal. Statistical differences in isotype-specific mean OD over days following each immunization, and specific differences between the mean immunization number OD for immunizations 1 through 3 versus the preimmunization mean OD, and for immunizations 4 through 12 vs. immunization 3, were analyzed using LSMEAN and ESTIMATE statements of SAS (SAS/STAT, 1999) with Tukey-Kramer adjustment and declared significant when $P \leq 0.05$.

Repeated measures analysis of variance was used to analyze the isotype-specific anti-J5 *E. coli* IgM, IgG₁, and IgG₂ end-point titer and cross reactivity data sets. The end-point titer data were log₂-transformed prior to statistical analyses to normalize distributions, but the cross reactivity data (as OD) were normally distributed

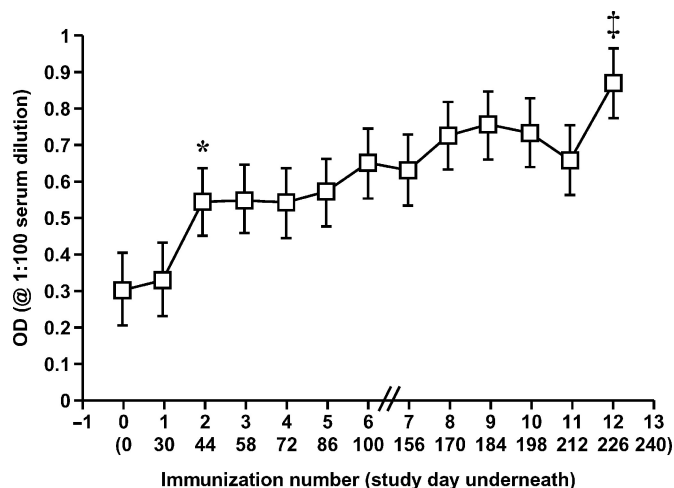


Figure 1. Mean serum anti-J5 *Escherichia coli* IgM antibody response profile for 5 Holstein steers immunized 12 times with J5 bacterin. Each data point is an immunization number LSMean (\pm SEM) from sera collected either on d 4, 6, and 11 postimmunization (first 6 immunizations) or on d 4, 6, 11, and 13 postimmunization (last 6 immunizations), expressed as optical density (OD \pm SEM) when sera were diluted 1:100. *Indicates the point at which mean OD was significantly higher than the preimmunization mean ($P \leq 0.05$). ‡ Indicates the point at which mean OD was significantly higher than after the third immunization ($P \leq 0.03$).

and thus analyzed without transformation. Both analyses were carried out utilizing the MIXED procedure of SAS (SAS/STAT, 1999). Main effects in the model included immunization number for the titer data, and antigen and the interaction of antigen \times immunization number for cross reactivity data, and were assessed using the Type III F-test. Differences between Tukey-Kramer adjusted LSMean from this analysis were assessed by *t*-test and declared significant when $P \leq 0.05$.

RESULTS

Isotype-Specific Anti-J5 *E. coli* Antibody Response Profiles

Day postimmunization significantly ($P < 0.02$) influenced anti-J5 *E. coli* IgM, IgG₁, and IgG₂ antibody responses (not shown). While no differences in mean OD between sampling days were observed following immunizations 1 to 3 or 8 to 12, all other immunizations had highest mean OD either on the 11th day postimmunization (for the 4th, 5th, and 6th doses) or on the 6th and 11th days postimmunization (for the 7th dose). Animal did not contribute ($P > 0.10$) to variation in mean antibody response profiles. On the other hand, immunization number significantly affected ($P < 0.0001$) mean anti-J5 *E. coli* IgM, IgG₁, and IgG₂ antibody responses, which all increased with increasing immunization number (Figures 1 to 3).

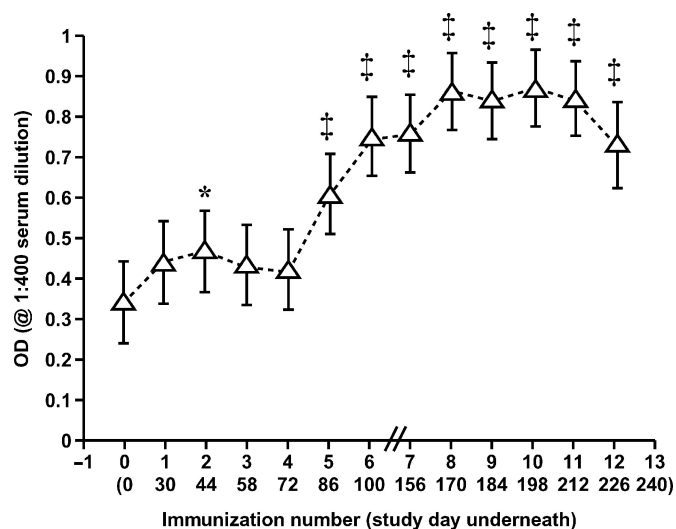


Figure 2. Mean serum anti-J5 *Escherichia coli* IgG₁ antibody response profile of 5 Holstein steers immunized 12 times with a J5 bacterin. Each data point is an immunization number LSMean (\pm SEM) from sera collected either on d 4, 6, and 11 postimmunization (first 6 immunizations) or on d 4, 6, 11, and 13 postimmunization (last 6 immunizations), expressed as optical density (OD \pm SEM) when sera were diluted 1:400. *Indicates the point at which mean OD was significantly higher than the preimmunization mean ($P = 0.004$). ‡ Indicates the point at which mean OD was significantly higher than after the third immunization ($P \leq 0.02$).

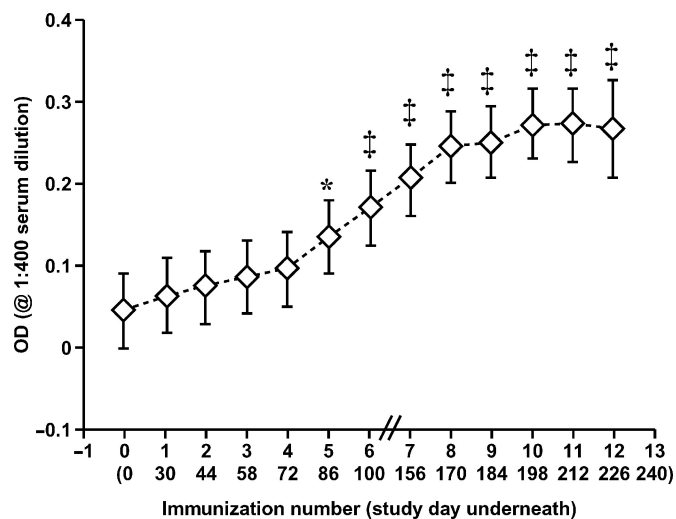


Figure 3. Mean serum anti-J5 *Escherichia coli* IgG₂ antibody response profile for 5 Holstein steers immunized 12 times with a J5 bacterin. Each data point is an immunization number LSMean (\pm SEM) from sera collected either on d 4, 6, and 11 postimmunization (first 6 immunizations) or on d 4, 6, 11, and 13 postimmunization (last 6 immunizations), expressed as optical density (OD \pm SEM) when sera were diluted 1:400. *Indicates the point at which mean OD was significantly higher than the preimmunization mean ($P = 0.004$). ‡ Indicates the point at which mean OD was significantly higher than after the 3rd immunization ($P \leq 0.001$).

Adjusted differences in mean OD between immunization numbers revealed that only 2 doses of J5 bacterin were required to significantly ($P \leq 0.05$) increase serum anti-J5 *E. coli* IgM above preimmunization levels (Figure 1). The IgM response reached a plateau after the second immunization but a sudden increase in the response was observed following the 12th immunization. Like IgM, 2 doses of J5 bacterin were enough to elicit a significant ($P = 0.004$) increase in the anti-J5 *E. coli* IgG₁ antibody response above that observed preimmunization (Figure 2). The IgG₁ response then increased ($P \leq 0.02$) between the fifth and eighth immunizations, after which it reached a plateau and began to decline after the 12th immunization. For IgG₂, no increase in the antibody response was observed until the fifth immunization was administered, when serum anti-J5 *E. coli* IgG₂ antibodies rose significantly ($P < 0.02$) above the preimmunization level (Figure 3). The IgG₂ response continued to increase until the 11th immunization, after which it reached a plateau.

Isotype Specific Anti-J5 *E. coli* Antibody Endpoint Titers

Preimmunization sera contained low but detectable titers of naturally occurring anti-J5 *E. coli* antibodies for all 3 isotypes (Figure 4). Of the selected samples analyzed, titers only reached levels that were significantly higher ($P < 0.005$) than preimmunization after the sixth dose of J5 bacterin was administered, and remained high through the 12th immunization.

Isotype-Specific Cross Reactivity Determined by ELISA

As evidenced by increased OD values, cross reactivity of IgM antibodies rose above preimmunization reactivity after the sixth immunization for *Serratia* spp., *Pseudomonas* spp., and *Klebsiella* spp. ($P < 0.01$), after the ninth immunization for *S. typhimurium* and *E. coli* 487 ($P < 0.01$), and after the 12th immunization for *S. newport* ($P < 0.01$) (top panel of Figure 5). Cross reactivity of IgM with LPS and lipid A was low and did not increase ($P > 0.10$) as the number of immunizations increased (bottom panel of Figure 5). Cross reactivity of IgG₁ antibodies increased significantly ($P < 0.01$) above preimmunization following the sixth immunization for all gram-negative bacteria tested, except for *E. coli* 487, which only required 3 immunizations ($P < 0.001$), and *S. newport*, which required 9 immunizations ($P < 0.05$, top panel of Figure 6). Cross reactivity of IgG₁ with LPS and lipid A was low and did not increase ($P > 0.10$) as the number of immunizations increased (bottom panel of Figure 6). Cross reactivity of IgG₂ antibodies in-

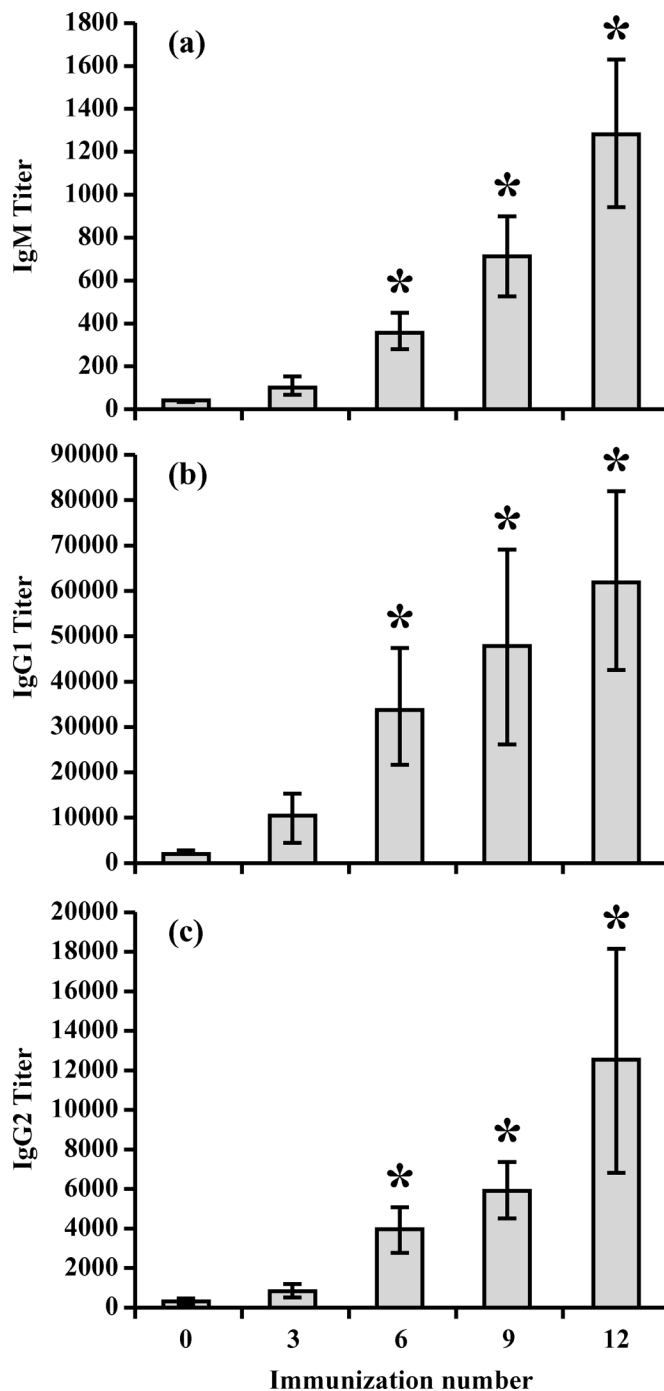


Figure 4. Mean (\pm SEM) isotype-specific anti-J5 *Escherichia coli* IgM (a), IgG₁ (b), and IgG₂ (c) antibody titers in selected sera from J5-hyperimmunized Holstein steers. Sera were from 6 d post each immunization. *Postimmunization titers different than preimmunization titer (immunization number 0) at $P < 0.005$.

creased significantly ($P < 0.01$) after the 6th immunization compared with preimmunization for all gram-negative bacteria tested (top panel of Figure 7). As with IgM and IgG₁, cross reactivity of IgG₂ with LPS and lipid A

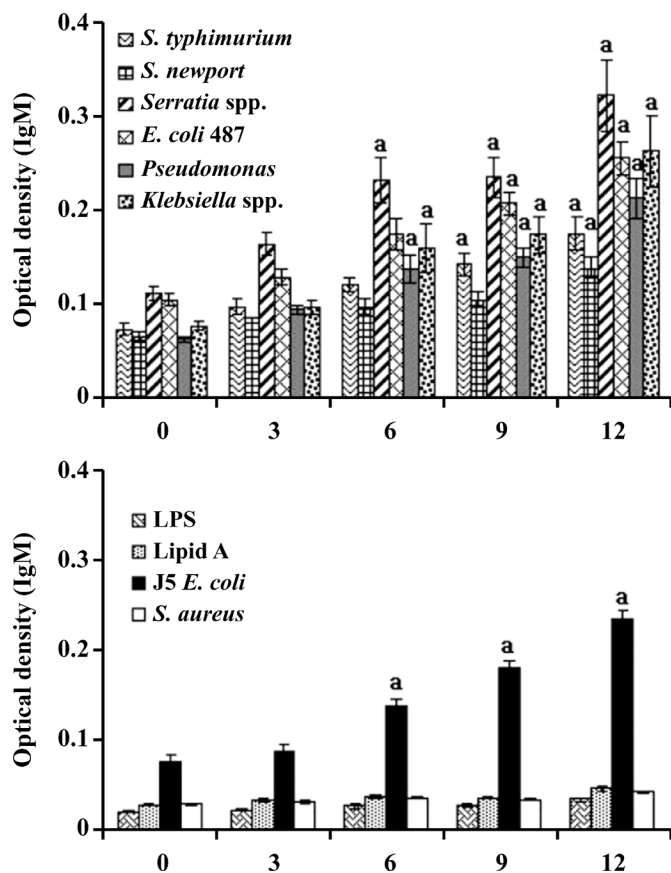


Figure 5. Cross-reactivity of serum IgM antibodies to heterologous gram-negative bacteria. Data are presented as mean optical densities (OD \pm SEM) within J5 immunization number, and represent anti-J5 IgM antibody cross reactivity against different test antigens, with J5 *Escherichia coli* antigen as a positive control and *Staphylococcus aureus* as the negative control. Test antigens included *Staphylococcus typhimurium*, *Staphylococcus newport*, *Serratia* spp., *E. coli* 487, *Pseudomonas* spp., and *Klebsiella* spp. ^a Indicates that cross reactivity was significantly higher than preimmunization at $P < 0.01$.

did not increase ($P > 0.10$) as the number of immunizations increased (bottom panel of Figure 7). No cross reactivity of any antibody isotype with the *S. aureus* negative control was ever observed.

Isotype-Specific Cross Reactivity Observed by Immunoblotting

Immunoblot analysis of antigens present in crude lysates of heterologous gram-negative bacteria recognized by IgM, IgG₁, and IgG₂ antibodies present in J5 immune sera demonstrated 3 key points (Figure 8). First, broad cross reactivity of all 3 antibody isotypes for large antigens (34 to 40 kDa; left panels of Figure 8) and small antigens (6 to 10 kDa; right panels of Figure 8) was observed. Second, at least some isotype switching in response to J5 hyperimmunization must

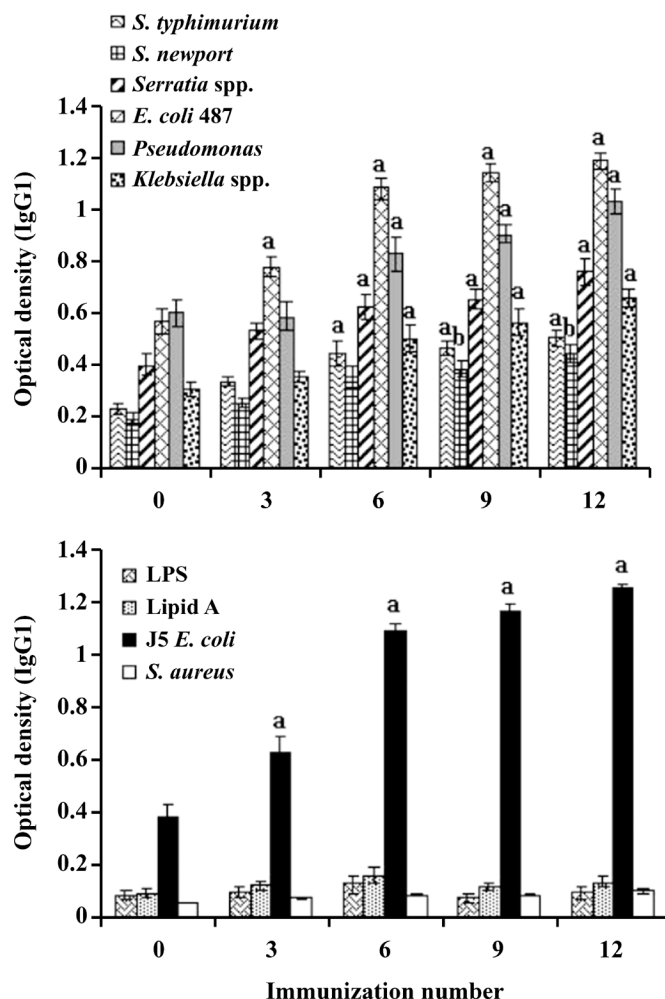


Figure 6. Cross-reactivity of serum IgG₁ antibodies to heterologous gram-negative bacteria. Data are presented as mean optical densities (OD \pm SEM) within J5 immunization number, and represents anti-J5 IgG₁ antibody cross reactivity against different test antigens, with J5 *Escherichia coli* antigen as a positive control and *Staphylococcus aureus* as the negative control. Test antigens included *Staphylococcus typhimurium*, *Staphylococcus newport*, *Serratia* spp., *E. coli* 487, *Pseudomonas* spp., and *Klebsiella* spp. ^a Indicates that cross reactivity was significantly higher than preimmunization at $P < 0.01$ and ^b at $P < 0.05$.

have occurred because IgM and IgG₁ recognition of the 34 to 40 kDa antigens disappeared between the 3rd and 9th immunizations (left panels of Figure 8). On the other hand, IgG₂ recognition of these same antigens remained strong between immunization numbers 3 and 9. Finally, IgG₁- and IgG₂-specific antibody responses against smaller (6 to 10 kDa) antigens appeared to be especially responsive to hyperimmunization because these were negligible in third immunization serum but pronounced in ninth immunization serum (right panels of Figure 8). No cross reactivity of any antibody isotype with antigens *S. aureus* was observed.

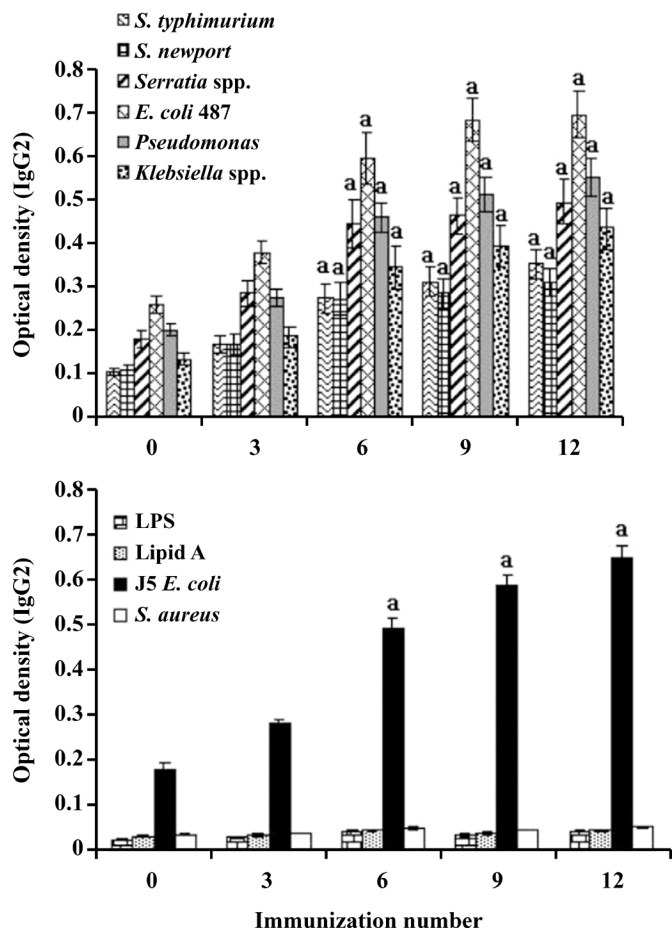


Figure 7. Cross reactivity of serum IgG₂ antibodies to heterologous gram-negative bacteria. Data are presented as mean optical densities (OD ± SEM) within J5 immunization number, and represents anti-J5 IgG₂ antibody cross reactivity against different test antigens, with J5 *Escherichia coli* antigen as a positive control and *Staphylococcus aureus* as the negative control. Test antigens included *Staphylococcus typhimurium*, *Staphylococcus newport*, *Serratia* spp., *E. coli* 487, *Pseudomonas* spp., and *Klebsiella* spp. ^aIndicates that cross reactivity was significantly higher from preimmunization at $P < 0.01$.

DISCUSSION

In our study, hyperimmunization of cattle with a commercial J5 *E. coli* bacterin increased isotype-specific antibody responses, titers and, for some antigens, cross reactivity to heterologous gram-negative bacteria, especially when compared to the naturally occurring antibodies present before immunizations were initiated and after 3 immunizations were given. Although 2 to 3 doses of J5 bacterin were sufficient to increase serum anti-J5 *E. coli* IgM and IgG₁ antibody response profiles above levels present at preimmunization, more than 3 doses were needed to increase anti-J5 *E. coli* endpoint titers and cross reactivity above background for all 3 isotypes studied. Based on endpoint titer data and antibody

cross reactivity to heterologous whole cell gram-negative bacteria, the results of the current study indicate that additional booster doses above the 2 currently recommended may be required to increase cross reactive IgG₁ and especially IgG₂ antibody responses in cattle. Cross reactivity appeared to be specific for gram-negative bacteria because none was observed with *S. aureus* (Figures 5 to 7), as was shown in a previous study (Tyler et al., 1991). Also consistent with previous observations (Gonzalez et al., 1989), we found antibodies in preimmune sera, especially IgG₁, with some cross reactivity against whole cell *E. coli* and *Pseudomonas*. This might be explained through natural exposure of animals to environmental gram-negatives, especially bacteria present in the digestive tract.

Antibodies of the IgM isotype are the first to be produced and secreted by naïve B cells during primary exposure to antigens. With successive exposures to the same antigen, IgM-to-IgG₁ or to-IgG₂ isotype switching can occur in responding B cell clones, effectively shutting down production of IgM by these clones and resulting in massive synthesis and secretion of either IgG₁ or IgG₂. The classical humoral immune response occurs via a specific class of B cells that are characterized by lack of surface CD5 expression (i.e., CD5⁻ B cells) and requires stimulation by specific cytokines elaborated from antigen-reactive helper T cells to stimulate isotype switching. The cytokines interleukin (IL)-4, IL-5, and IL-6 are primarily responsible for promoting isotype switching from IgM-to-IgG₁ in bovine CD5⁻ B cells (Estes, 1996; Haas and Estes, 2000). These cytokines are elaborated from helper T cells known as type 2 cells, or T_H2. The T_H2 response is directed specifically towards antibody production against certain protein antigens, and in the case of IgG₁ antibody production, becomes critical for blocking bacterial colonization and toxins at sites of tissue infection (Butler, 1998). A T_H2-like response must have occurred during the initial phase of our J5 hyperimmunization protocol because the magnitude of the serum anti-J5 *E. coli* IgM response profile did not change between immunizations 3 and 6 (Figures 1 and 4), during which time there was a remarkable increase in the magnitude of the IgG₁ response (Figures 2 and 4). After the sixth immunization, additional isotype switching in existing IgM- and (or) IgG₁-producing B cells must have occurred because a gradual but continuous increase in the magnitude of the J5 *E. coli*-specific serum IgG₂ antibody response was observed (Figures 3 and 4). Also, immunoblot analysis showed that IgM and IgG₁ recognition of 34 to 40 kDa antigens in J5 *E. coli* and heterologous gram-negative bacteria lysates dwindled between immunizations 3 and 9, while IgG₂ recognition of these antigens remained strong and increased remarkably for some

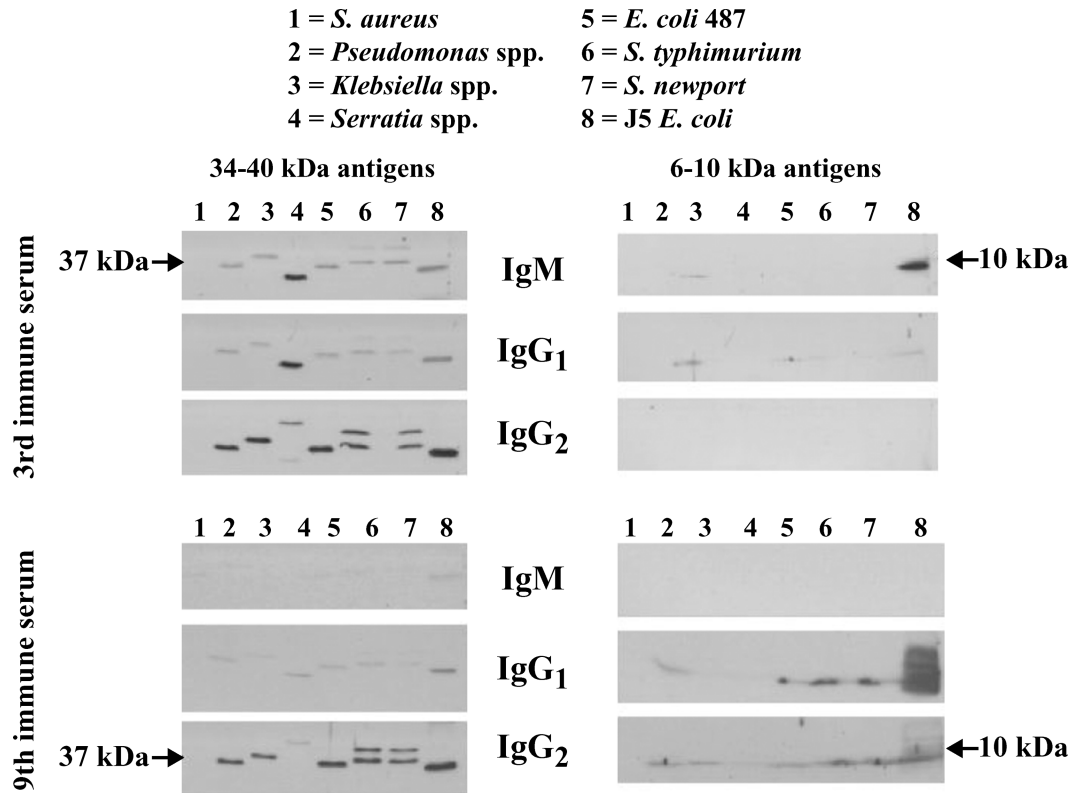


Figure 8. Immunoblot analysis of cross reactivity of IgM, IgG₁, and IgG₂ antibodies present in third and ninth J5 immune sera with large (34 to 40 kDa; left panels) and small (6 to 10 kDa; right panels) antigens contained in crude lysates of heterologous gram-negative bacteria. *Staphylococcus aureus* lysate (lane 1) was used as a negative control and J5 *E. coli* lysate (lane 8) as a positive control. Test lysates included *Pseudomonas* spp. (lane 2), *Klebsiella* spp. (lane 3), *Serratia* spp. (lane 4), *E. coli* 487 (lane 5), *Staphylococcus typhimurium* (lane 6), and *Staphylococcus newport*. (lane 7).

smaller (6 to 10 kDa) antigens (Figure 8). Interferon-gamma (IFN- γ) is primarily responsible for IgG₂ isotype switching in bovine CD5⁻ B cells (Estes, 1996) and is elaborated from helper T cells known as type 1 cells, or T_H1. Our results suggested that 6 or more doses of J5 bacterin may be required to stimulate strong T_H1 responses in cattle because anti-J5 *E. coli* IgG₂ antibodies detected in our study animals did not differ between immunizations 1 to 5 (Figure 3). T_H1 responses are critical to rapid immune defense against acute bacterial infections in cattle because IgG₂ antibodies act as direct opsonins for efficient neutrophil phagocytosis of the pathogens and because IFN- γ potentially activates the neutrophils to kill bacteria that have been phagocytosed (reviewed by Burton and Erskine, 2003). Combined with the significantly enhanced cross reactivity of IgG₂ antibodies in hyperimmune sera for all 6 heterologous gram-negative bacteria tested (Figure 7) and for 6 to 10 kDa antigens present in lysates of these bacteria (right panels of Figure 8), we suggest that at least 6 doses of J5 bacterin may be needed to elicit strong T_H1-based IgG₂ immunity in vaccinated cattle.

None of the antibody isotypes in J5 hyperimmune sera had marked cross reactivity with purified LPS or lipid A in ELISA (Figures 5 to 7). In other studies, antibodies elicited in rabbits with fewer immunizations also showed little cross reactivity with LPS or lipid A (Siber et al., 1985; Warren et al., 1987; Hellman et al., 1997). Core antigens are suggested to be important inducers of protective antibodies in J5-immunized animals (Baumgartner et al., 1987; Pollack et al., 1989; Aydintug et al., 1989; Tyler et al., 1991, 1992). While it is possible that ELISA is not an optimal platform to detect antibody reactivity with purified LPS or lipid A, it is also possible that assumptions of ready recognition and responsiveness of the immune system to these exposed core antigens on J5 *E. coli* are incorrect. It has been suggested that apparent binding by antibodies, raised from J5 *E. coli* immunizations, to LPS may be, in part, a result of LPS-outer membrane protein (OMP) complexes released by bacterial cell walls (Freudenberg et al., 1992). Immunoblot analysis revealed that antiserum raised from 11 immunizations of J5 *E. coli* in rabbits contain high titers of IgG antibodies that bind to

at least 3 major OMP, but not LPS (Hellman et al., 1997). In a model of bacterial sepsis, 3 conserved OMP-LPS complexes were captured from septic rat blood by O-chain-specific anti-LPS IgG (Hellman, et al., 2000a). The OMP have been identified as outer membrane protein A, of 35 kDa, peptidoglycan-associated lipoprotein, of 18 kDa, and murein lipoprotein of 5 to 9 kDa (Hellman et al., 2000b). Thus, J5 *E. coli* immunization may offer protection, in part, from production of IgG antibodies that bind to OMP released during bacterial sepsis.

Unlike protein antigens that induce specific T_{H1} or T_{H2} antibody responses in reactive CD5⁻ B cells, non-protein LPS and lipid A antigens require a different type of immune recognition that can stimulate another class of B cells expressing CD5 on their plasma membranes (i.e., CD5⁺ B cells). CD5⁺ B cells do not require help from T cells and produce mainly IgM antibodies with little to no isotype switching (Hayakawa et al., 1984; Kantor 1991; Mond et al., 1995; Haas and Estes, 2000). During some infections, CD5⁺ B cells can account for up to 30% of bovine peripheral blood lymphocytes and lead to very high titers of pathogen-specific serum IgM antibodies with a notable lack of IgG₁ or IgG₂ antibody responses (Nassens and Williams, 1992; Meiom et al., 1993; Buza et al., 1997; Haas and Estes, 2000). While IgM-producing CD5⁺ B cells may have responded to LPS or lipid A in our J5 vaccinated steers, this response was not detectable in serum using our ELISA. Instead, we detected primarily IgG₁ and IgG₂ antibody responses in hyperimmune sera that were cross reactive with whole gram-negative bacteria and certain 34 to 40 kDa and 6 to 10 kDa antigens but not with LPS or lipid A, suggesting that the majority of J5-responsive B cells were CD5⁻ and responded to protein antigens contained in the bacterin (Hellman et al., 1997). If future studies show this to be true, J5 vaccines may prove more efficacious if modified to include these immunogenic proteins in such a way that they rapidly stimulate production of cross protective IgG₁ and IgG₂ antibodies.

CONCLUSION

Hyperimmunization of steers with a J5 *E. coli* bacterin increased serum responses of IgM IgG₁, and IgG₂ compared with preimmunization levels. However, little or no increase was observed in the IgG₂ response until after the fifth immunization. Increases in cross-reactive antibodies to heterologous gram-negative bacteria were apparent for all 3 isotypes, but significant only after 6 immunizations. Thus, multiple immunizations of this bacterin, beyond the labeled use of 3 doses, were needed to induce significant responses for certain isotypes and cross-reactive antibodies. It is unknown, however, whether this observation would apply to other commer-

cially available gram-negative bacterins, including core-antigen varieties. No cross reactivity with LPS or lipid A was observed for any antibody isotype or any immunization period studied. We suggest that isotype switching, as indicated by increased IgG₂ responses in hyperimmunized steers, could be an important indicator of profound changes in antigen recognition and presentation elicited by multiple dosing of gram-negative core antigen bacterins.

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