Antigenic Homology Among Gram-Negative Organisms Isolated from Cattle with Clinical Mastitis

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ABSTRACT

This study examined the degree of serologic homology among mastitis pathogens. Antibodies were raised against the Rc mutant, *Escherichia coli* O111:B4 (strain J5) and affinity purified against lipopolysaccharide derived from the Ra mutant, *Salmonella typhimurium* TV119. These antibodies reacted with a battery of unrelated Gram-negative bacteria in whole cell ELISA. Bacteria with strong cross-reactions included a heterologous, smooth *E. coli*, *Salmonella dublin*, *S. typhimurium*, *Salmonella newport*, and *Pseudomonas aeruginosa*. Recognition of *Klebsiella pneumoniae* and *Bordetella bronchisepticum* was observed, but reactions were weaker than with the other isolates. The reduced recognition of these isolates probably reflects a masking effect of the bacterial capsule and variations in lipopolysaccharide structure. The polyclonal antibody did not recognize a Gram-positive isolate, *Staphylococcus aureus*.

These immunoglobulins were then tested using whole cell ELISA against a panel of bacteria recovered from the mammary glands of cattle with clinical mastitis. Marked reactivity was noted against a variety of Gram-negative pathogens. Gram-positive isolates had lower recognition by Gram-negative core antigen specific immunoglobulin. The results suggest immunization with rough mutant bacteria may have broad application in the prevention of coliform mastitis.
(Key words: mastitis, Gram-negative homology)

INTRODUCTION

Unlike contagious mastitis caused by *Streptococcus agalactiae* and *Staphylococcus aureus*, mastitis caused by environmental microorganisms resists the management intervention strategies available to dairy farmers (2, 4, 5, 6, 7, 10, 11, 12). Proper milking practices, teat dipping, and dry cow therapy usually result in gradual reductions in the prevalence and incidence of mastitis caused by *Strep. agalactiae* and *Staph. aureus* (4). The same statement cannot be made in the case of the environmental mastitis pathogens (2, 4, 5, 6, 7, 10, 11,

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12, 21, 22). Here the reservoir and source of infectious organisms include bacteria capable of surviving and multiplying in the farm environment (5, 6). Additionally, many infections occur between milkings (5, 6, 10, 11, 12, 21). Mastitis control programs emphasizing the identification of infected cattle followed by either treatment or segregation have minimal effect upon the prevalence of mastitis caused by environmental contaminants (4, 11). Consequently, mastitis caused by environmental bacteria remains an important problem even after the common contagious mastitis syndromes have been eradicated. Approximately 50% of the cows in well-managed dairies experience intramammary infection each year (12, 14, 19, 20, 27). Gram-negative bacteria, typically called coliforms, are the bacteria most frequently isolated from clinical mastitis cases in these herds (14, 19, 20).

Clinical Gram-negative mastitis is noteworthy because of its peracute onset, poor response to therapy, and relatively higher mortality (1). Agalactia of the affected quarter of varying duration is a frequent sequela to Gram-negative mastitis. Following the introduction of an infectious inoculum through the teat sphincter, coliform mastitis follows a well-described course (1, 10, 11). Bacterial proliferation and release of bacterial endotoxins by either live or dying bacteria activate inflammatory cascades, releasing potent vasoactive mediators (6, 17). Against this backdrop, the necessity for alternative control measures for Gram-negative mastitis is apparent. One promising approach is immunoprophylaxis targeting common Gram-negative core antigens.

Crossreactive immunoprophylaxis targeting Gram-negative bacteria has been extensively reviewed (3, 30). Passive and active immunization of laboratory animals against antigens common to Gram-negative bacteria have since demonstrated value in the prevention and treatment of experimental disease caused by heterologous bacteria (3, 30). The first reports of such cross-protective immunity in livestock included protection against *Salmonella typhimurium* enteritis, endotoxemia in calves, and *Actinobacillus (Hemophilus) pleuropneumoniae* in swine (8, 15, 16).

Serum antibody titers recognizing common Gram-negative core antigens were identified as a nouveau risk factor for coliform mastitis (29). More recently, vaccination with an Rc mutant, *Escherichia coli* O111:B4 (strain J5), has been observed to reduce the incidence of clinical Gram-negative mastitis by as much as 80% in a controlled clinical trial (18). *Escherichia coli* (J5) has exposed crossreactive core antigens (13, 24, 31). These core antigens are highly homologous (13, 24, 31) in unrelated Gram-negative bacteria and should provide an appropriate vaccine against a number of disease syndromes caused by unrelated Gram-negative bacteria. A rough mutant *E. coli* (J5) vaccine has recently been granted a license for intrastate use by the state of California.

Recently a technique has been described in which antiserum raised against the Rc mutant, *E. coli* O111:B4 (J5) was affinity purified against lipopolysaccharide derived from the Ra mutant, *Sal. typhimurium* TV119, effectively isolating those immunoglobulins recognizing homologous or common core antigens (28). The objective of this study was to test the immunoglobulins purified in this manner against a variety of Gram-negative and Gram-positive mastitis pathogens, identifying the degree with which mastitis pathogens express common Gram-negative core antigens, and, consequently, to aid in identifying the prophylactic spectrum of an Rc mutant vaccine for coliform mastitis.

MATERIALS AND METHODS

Purification of Immunoglobulin Recognizing Common Core Antigens

A 6-mo-old Holstein calf was inoculated with 5 ml of the *E. coli* (J5) antigen, mixed with 2.0 ml of Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO) four times at 2-wk intervals. Serum was obtained from the vaccinate 2 wk after the last immunization. Using a previously described ELISA, this serum was positive at a 1:6400 dilution for IgG recognizing *E. coli* J5 (29).

Freeze-dried Sepharose 6B, 45 g, (Pharmacia, Uppsala, Sweden) was swollen in distilled water for 15 min and washed with 2 L of distilled water. Lipopolysaccharide (1 g) derived from the Ra mutant of *Sal. typhimurium* (Sigma Chemical Co., St. Louis, MO) was

dissolved in 1 L of distilled water, pH 12. The lipopolysaccharide solution was incubated with the swollen gel for 48 h at 37°C in a shaker water bath. Gel was separated from unbound ligand by filtration. The gel was washed, and remaining active sites were bound by incubating washed gel 4 h at 37°C in a 1 *M* glycine (Sigma Chemical Co., St. Louis, MO) solution, pH 8.0. Immunoglobulin was precipitated by mixing 3 parts hyperimmune sera with 2 parts saturated ammonium sulfate. The mixture was incubated for 15 min at 4°C, and immunoglobulin was precipitated by centrifugation for 15 min, 4°C, at 3000 × *g*. The supernate was discarded, and the immunoglobulin was resuspended in distilled water and desalinized by dialysis at 4°C for 24 h. The dialysis solution, .1 *M* NaCl, pH 7.4, was changed four times during the 24-h period.

An affinity chromatography column was filled with the matrix bound antigen. Immunoglobulin was passed through the column. Elution was accomplished using a continuous gradient of NaCl. The initial osmotic strength was 0 mOsm/L, and the final concentration was 1500 mOsm/L. Eluant fractions were subjected to determinations of IgG concentration, and IgG titer recognizing *E. coli* O111:B4 (strain J5). Eluant protein concentration was assessed by optical density at 280 nm. Eluant immunoglobulin concentration was measured by radial immunodiffusion. Fractions with low protein (low optical density$_{280nm}$), low IgG concentration, and high ELISA optical density recognizing *E. coli* (J5) were pooled and dialyzed to isotonicity in phosphate-buffered NaCl (pH 7.4).

ELISA

Bacteria used in the initial portions of the study included *E. coli* O111:B4 (strain J5), *E. coli* (smooth), *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolated from the mammary glands of cattle with acute clinical mastitis, *Salmonella dublin* and *Staph. aureus* isolated from the mammary glands of chronically infected cattle, *Salmonella newport* and *Sal. typhimurium* from the feces of cattle with clinical enterocolitis, and *Bordetella bronchiseptica* from the lung of a pig with pneumonia. A single representative isolate of each species was used in this portion of the study.

Following initial growth on blood agar plates, isolates were streaked for isolation and identified based on biochemical characteristics. A single colony was transferred to 50 ml trypticase soy broth (Difco Laboratories, Detroit, MI) incubated 18 h at 37°C, and formalin was inactivated. Antigen preparations were concentrated by centrifugation at 1500 × *g* for 10 min and resuspended in isotonic phosphate-buffered NaCl (pH 7.4) containing .02% thimerosal. The cell washing procedure was repeated twice, and the optical density of the suspension adjusted to 25% transmission at 610 nm.

All bacterial isolates used in subsequent experiments were isolated from the mammary glands of cattle with clinical mastitis. Isolates examined included *E. coli* (n = 52), *Enterobacter* spp. (n = 3), *Proteus* spp. (n = 2), *Pseudomonas* spp. (n = 5), *Serratia* spp. (n = 2), *Citrobacter* spp. (n = 2), *Klebsiella* spp. (n = 3), *Staph. aureus* (n = 7), *Strep. agalactiae* (n = 4).

Descriptions of solutions used are as follows: 1) diluent; isotonic phosphate-buffered sodium chloride, pH 7.20, .05% polyoxyethylene-sorbitan monolaurate (Sigma Chemical Co., St. Louis, MO); 2) substrate; citric acid .05 *M*, pH 4.00, .2 m*M* 2,2′-azinobis-3-ethylbenzthiazoline sulfonic acid, diammonium salt (Sigma Chemical Co., St. Louis, MO), .2 *M* H_2O_2; and 3) wash solution; isotonic sodium chloride, .1% polyoxyethylene-sorbitan monolaurate.

Bacterial antigens were bound by incubating 100 μl of the aforementioned antigen suspensions overnight at 37°C in flat-bottomed microtiter plates (Pro-bind, Becton Dickinson Labware, Lincoln Park, NJ). Unbound antigen was removed by washing each well three times. Affinity purified immunoglobulin recognizing Gram-negative core antigen (100 μl/well) was added following a 1:5 dilution. Plates were incubated at 37°C for 30 min. Unbound immunoglobulin was removed by washing three times. Gamma chain specific goat origin antisera recognizing bovine IgG linked to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) (100 μl of a 1:2000 dilution/well) was added, and incubation was repeated. Plates were washed three times to remove unbound conjugate. Substrate solution (125 μl/well) was added, incubated at 21°C for

TABLE 1. Enzyme-linked immunosorbent assay reactivity of whole cell bacterial preparations to core antigen specific IgG derived by crossreactive affinity purification. Results were obtained using the following formula after blanking all readings against a mean no antigen control reading (100 × mean optical density with test antigen/mean optical density with *Escherichia coli* J5).

Bacteria	Percentage *E. coli* J5 optical density
Escherichia coli O111:B4 (J5)	100.0
Escherichia coli (smooth)	106.83
Salmonella typhimurium	81.26
Salmonella newport	106.30
Salmonella dublin	87.92
Klebsiella pneumoniae	66.02
Pseudomonas aeruginosa	91.42
Bordetella bronchisepticum	25.74
Staphylococcus aureus	6.30

30 min, and plates read on a 410 nm setting. A minimum of three replicates was performed using each antigen preparation. The measured assay result was median optical density after blanking against readings the mean of 6 no antigen control wells. Results were reported as the percentage of mean ELISA optical density observed with an *E. coli* O111:B4 (J5) plate antigen on the same plate [100 × median isolate ELISA optical density/mean *E. coli* O111:B4 (J5) ELISA optical density].

Statistical Analysis

Isolates were grouped on the basis of Gram staining characteristics. Antigen recognition by the affinity purified antisera was compared by one-way ANOVA. Group means were compared by the Tukey method of honestly significant difference. Isolates were later grouped on the basis of isolate genus and species, and similar calculations were repeated. Calculations were assisted by a statistical software program (BMDP Statistical Software, Berkeley, CA).

RESULTS

Of the 8 Gram-negative isolates initially tested, 6 had ELISA reactivity > 80% of the optical density observed in an assay using *E. coli* O111:B4 (J5), the original vaccinal antigen, as a plate antigen (Table 1). Two of the isolates tested, the smooth *E. coli* and *Sal. dublin*, had enhanced serologic recognition in ELISA using the affinity purified antiserum (106% of *E. coli* O111:B4 J5 ELISA optical density). The *K. pneumoniae* and *B. bronchisepticum* isolates had lower reactivity, 66.02 and 25.74%, respectively. The polyclonal antiserum had far less reactivity against the *Staph. aureus* isolate (6% of *E. coli* J5 ELISA optical density).

In the second set of experiments, Gram-positive mastitis isolates had uniformly low ELISA values (mean 28.18%, SD 9.62) (Table

TABLE 2. Enzyme-linked immunosorbent assay crossreactivity with *Escherichia coli* (J5) of 80 bacterial isolates recovered from cattle with clinical mastitis. Results were obtained using the following formula after blanking all readings against a mean no antigen control reading (100 × mean optical density with test antigen/mean optical density with *E. coli* J5).

Isolate	n	Mean percentage *E. coli* J5 optical density	SD
Gram-negative isolates	69	76.03	25.40
Escherichia coli	52	81.16	23.61
Enterobacter spp.	3	61.27	20.99
Proteus spp.	2	91.43	19.65
Pseudomonas spp.	5	57.14	32.71
Serratia spp.	2	45.14	29.52
Citrobacter spp.	2	42.62	8.78
Klebsiella spp.	3	66.06	10.46
Gram-positive isolates	11	28.18	9.62
Staphylococcus aureus	7	29.97	9.73
Streptococcus agalactiae	4	25.08	9.93

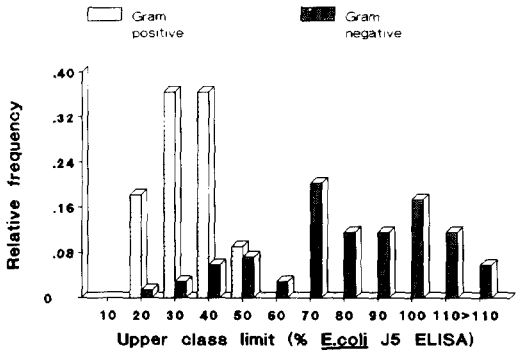

Figure 1. Relative frequency of percentage *Escherichia coli* J5 ELISA values in Gram-negative and Gram-positive bacteria isolated from clinical mastitis in cattle.

2). All Gram-positive isolates had less than 50% the mean *E. coli* J5 optical density reading from the same plate. Gram-negative isolates had higher ELISA values (mean 76.03%, SD 25.40) ($P < .0001$). The distribution of % *E. coli* J5 ELISA values from Gram-negative and Gram-positive isolates demonstrated minimal overlap (Figure 1). Bacteria grouping, either Gram staining characteristics or bacterial genus, were associated with percentage *E. coli* J5 optical density ($P < .0001$). Sample size constraints precluded meaningful comparisons within the larger groups defined on the basis of Gram staining characteristics. The calculated honestly significant difference exceeded all differences in bacterial genus group means.

DISCUSSION

Escherichia coli O111:B4 (J5) is a uridine diphosphate galactose epimerase deficient mutant (13, 24, 31). A direct consequence of this deficiency is the inability to link exogenous galactose to glucose in the inner core of the Gram-negative cell wall. The enzymatic deficiency present in the Ra mutant, *Sal. typhimurium* TV119, occurs in a later stage of lipopolysaccharide synthesis, and, consequently, only the terminal, variable oligosaccharides are absent (24). The only antigens shared by the whole cell Rc mutant *E. coli* and the chemically extracted Ra lipopolysaccharide should be those included in the inner core and lipid A regions. The large number of shared structures present in Gram-negative cell wall, lipid A, N-acetyl-glucosamine, 2-keto-3-deoxyoctonate, heptose, and glucose insures the presence of multiple crossreactive binding sites (13, 24). The described procedure theoretically should isolate only those immunoglobulins recognizing common Gram-negative core antigens.

The polyclonal antiserum demonstrated reactivity against a broad spectrum of Gram-negative bacteria, including *E. coli*, *B. bronchisepticum*, *K. pneumoniae*, *P. aeruginosa*, and *Salmonella* spp., including *Salmonella* of the B, C, and D serogroups. This wide spectrum of reactivity demonstrates the wide degree of antigenic homology among Gram-negative bacteria. The structure of the Gram-positive cell wall differs markedly from the Gram-negative cell wall. The peptidoglycan layer is more extensive, and the lipopolysaccharide layer characteristic of Gram-negative bacteria is absent. Because the affinity purification procedure isolated antibodies recognizing homologous lipopolysaccharide epitopes, the minimal ELISA recognition of *Staph. aureus* was anticipated.

The reduced recognition of the *K. pneumoniae* isolate (66% of *E. coli* J5 ELISA reactivity) may reflect a partial masking of homologous core antigens by the bacterial capsule (9). The presence of a capsule has been described as an intrinsic virulence factor, permitting bacteria to escape humoral immune surveillance despite the presence of specific immunoglobulin. This mechanism may also function in the case of crossreactive immunity recognizing common core antigens (31).

Two possibilities may explain the limited recognition of *B. bronchisepticum* by the core antigen specific antiserum. The structure and biological activity of lipopolysaccharide derived from *Bordetella* spp. reportedly varies greatly from the enterobacteriaceae (23, 25). Consequently, the vaccinal antigen, the capture antigen, or both may have limited crossreaction with the test antigen. Additionally, the presence of a cell capsule, which has been reported in *B. bronchisepticum*, may mask common core epitopes (26). The use of clinical isolates may have further limited the potential for serologic crossreactions. *Bordetella* spp. have been reported to lose their capsule following repeated in vitro cultures (23, 25, 26). Potentially, the use of a laboratory adapted strain might have enhanced the potential heterologous recognition of common core antigens.

The low but detectable optical density readings against Gram-positive isolates probably reflects passive adsorption of either the affinity purified antibody or the conjugate to the solid phase. Such background reactions are common in immunoassays.

Antibodies recognizing homologous core antigens reacted readily with a variety of unrelated Gram-negative mastitis pathogens. Although variable crossreactivity was noted among Gram-negative isolates, all genus groups of Gram-negative bacteria exceeded the mean percentage *E. coli* (J5) ELISA readings observed with both *Staph. aureus* and *Strep. agalactiae*. These Gram-positive isolates were included as an additional experimental control. Our findings suggest that immunization against homologous core epitopes will induce humoral immune responses against a wide array of potential Gram-negative intramammary pathogens.

The method of antigen production may have affected the expression of homologous core antigens. An 18-h incubation was chosen to insure a standardized culture maturity before harvesting antigen. Aged cultures of this type will have greater opportunity for somatic side chain synthesis and capsular development. Either of these two processes may limit the exposure of crossreactive core antigens. Cultural maturity has previously been observed to effect the ability of antibodies raised to *E. coli* (J5) to recognize heterologous Gram-negative bacteria (15). Cultures of *Actinobacillus pleuropneumoniae* have markedly greater affinity for antibodies targeting homologous core antigens during the logarithmic growth phase. Superior crossreactivity might have been observed with rapidly replicating bacteria. Rapid bacterial replication is anticipated in the time span following initial intramammary infection. Consequently, the potential for crossreactive immunity may in fact be greater than that suggested by the present study.

The potential diversity of opportunistic Gram-negative bacteria has slowed the impetus for the development of vaccines for coliform mastitis. As recently as 1984, it was definitively stated that the bacteria responsible for coliform mastitis were a heterogeneous group, lacking known common antigenic structures and, consequently, available immunization pro-

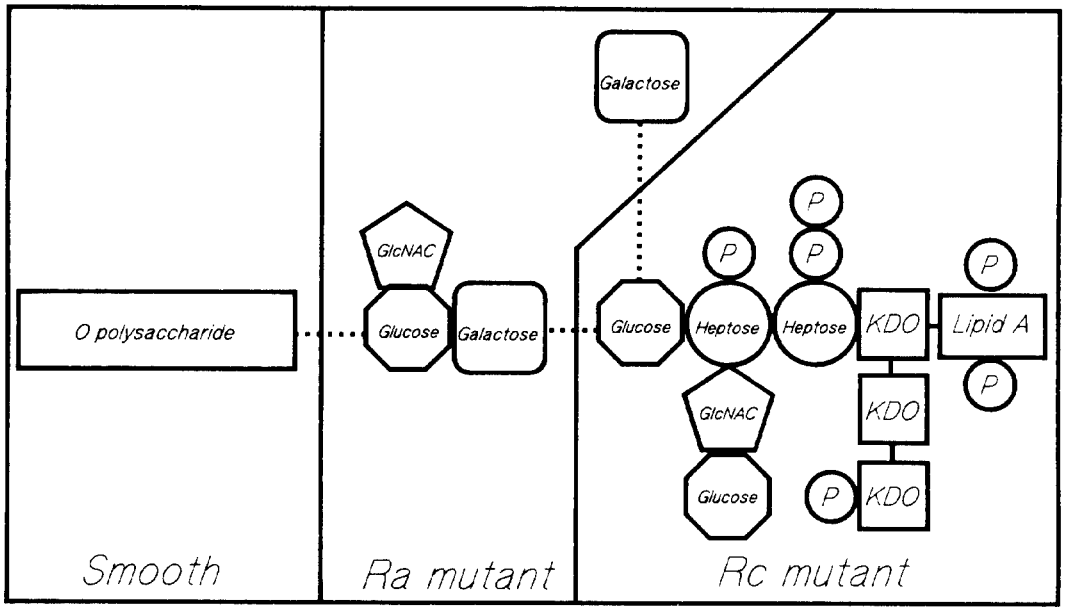

Figure 2. Schematic representation of Gram-negative cell wall lipopolysaccharide structure in smooth and rough mutant bacteria (GlcNAC = N-acetyl glucosamine, P = phosphate esters, KDO = keto-deoxyoctonate).

cedures were probably not effective (11). Studies in humans and laboratory animals and recent field studies using R mutant bacterins in livestock would tend to contradict these assertions (3, 19, 29, 30).

Successful immunization hinges upon satisfying three basic criteria: 1) the vaccine must contain antigens common to all potential pathogens; 2) the common antigenic structure must be immunogenic, capable of inducing an immune response; and 3) this immune response must protect the host (29). The requirements of immunogenicity and protective effects have already been demonstrated by previous studies (3, 16, 18, 30).

Although differences in composition, order, and linkage of carbohydrate subunits creates a wide array of potential somatic antigens, underlying portions of Gram-negative cell wall lipopolysaccharide are relatively uniform (13, 24). Structures present in the inner core and lipid A portions of the cell wall are promising candidates as crossreactive immunogens (Figure 2). Crossreactive antibodies induced by immunization with an Rc mutant *E. coli* were demonstrated in the present study to recognize unrelated Gram-negative bacteria.

Although the initial vaccinal antigen was relatively complex, affinity purification using chemically purified heterologous lipopolysaccharide as a capture antigen should serve to isolate only those immunoglobulins recognizing homologous core antigens. Based on our results, immunization with homologous core antigens may have utility in the prevention of coliform mastitis.

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