Physiology and Management

Antigenic Homology of Endotoxin with a Coliform Mastitis Vaccine Strain, Escherichia coli O111:B4 (J5)

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Abstract

This study examined recognition of heterologous Gram-negative endotoxin by antibodies recognizing common lipopolysaccharide core antigens. Gram-negative endotoxins from 11 heterologous bacterial strains were tested for recognition by antibodies against common lipopolysaccharide core antigens. Serum was harvested from a calf immunized with the Rc mutant, Escherichia coli O111:B4 (J5), and affinity purified against endotoxin derived from an Ra mutant, Salmonella typhimurium, producing an antibody reagent recognizing homologous Gram-negative core antigens present in the Rc mutant vaccinal antigen. This reagent demonstrated reactivity against 11 chemically purified Gram-negative endotoxins. Included were endotoxins derived from 3 smooth E. coli species, 2 Salmonella spp., Shigella flexneri, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, and lipid A. Endotoxin derived from K. pneumoniae had significantly higher ELISA reactivity with core antigen specific antibodies than did endotoxin derived from either E. coli O111:B4 (J5) or P. aeruginosa. These results suggest immunization with R mutant bacteria may have utility in the prevention of Gram-negative mastitis even when whole bacteria react poorly with antibodies recognizing common core antigens.

(Key words: mastitis, endotoxin, antigenic homology)

Abbreviation key: LPS = lipopolysaccharide, OD = optical density.

Introduction

The lipopolysaccharide (LPS) component of the Gram-negative cell wall is composed of three layers: 1) an outermost somatic antigen (O antigen), linked to 2) the innermost lipid A layer by 3) a series of oligosaccharides (8, 13, 24). Variations in constituent hexoses, their arrangement, and the manner in which they are linked create the potential for innumerable, distinct somatic antigens. Diversity among somatic antigens has permitted the development of the antisera used to identify Gram-negative bacteria in clinical settings. The innermost portion of the LPS is composed of lipid A, the toxic moiety of Gram-negative endotoxin (14). The two inner parts of the LPS layer, containing glucose, galactose, N-acetyl-glucosamine, heptoses, pentoses, ketodeoxyoctonate acid, and lipid A, are structurally similar in diversely related Gram-negative bacteria (8, 13, 24).

The R (rough) mutant bacteria lack enzymes required for normal cell-wall LPS synthesis. These enzymatic deficiencies result in an incomplete assembly of either somatic antigens or the oligosaccharide bridge between somatic side chains and lipid A, exposing the highly homologous cell-wall core antigens. The Ra mutant lacks the enzyme necessary for somatic antigen attachment and, consequently, has nearly complete LPS synthesis. Rough mutants with successive subscripts have less complete LPS synthesis. An Re mutant LPS contains only lipid A and the innermost ketodeoxyoctonate acid residue (4, 8, 13, 22).

The presence of common antigenic structures in unrelated bacteria has raised the possi-
bility of developing antisera and vaccines with broad-spectrum therapeutic and prophylactic merit (4, 21). Naturally occurring serologic recognition of common Gram-negative core antigens has been associated with a decreased incidence of clinical Gram-negative bacterial mastitis (20). Active immunization with *Escherichia coli* O111:B4 (J5), an Rc mutant, has been associated with decreased incidence and severity of several Gram-negative diseases, including *Salmonella typhimurium* endotoxemia in calves (6), *Actinobacillus* (*Hemophilus*) *pleuropneumoniae* in swine (9), and clinical coliform mastitis of dairy cattle (10).

A technique recently has been reported by which antibodies produced following vaccination with the Rc mutant, *E. coli* O111:B4 (J5), are affinity purified against LPS derived from the Ra mutant, *S. typhimurium* TV119. Antibodies purified by crossreactive affinity chromatography readily recognized heterologous Gram-negative bacteria (20, 23). Immunoglobulins purified in this manner were tested against a variety of Gram-negative and Gram-positive pathogens in whole cell ELISA. Although recognition of whole heterologous Gram-negative bacteria was observed, variations in the degree of crossreactivity were noted.

Whole bacterial cells of some common mastitis pathogens had less pronounced crossreactivity with core antigen-specific Ig (23), raising serious questions concerning the potential spectrum of protection provided by an Rc mutant vaccine for coliform mastitis. These differences in ELISA reactivity may have resulted from either structural differences in LPS composition or the masking of LPS antigens by bacterial capsules and somatic antigens. Measurement of reactivity against chemically purified LPS removes variables associated with capsular and somatic antigen masking of underlying homologous antigenic structures. The purpose of the current study was to examine the recognition of Gram-negative endotoxins by similarly purified antibodies.

**MATERIALS AND METHODS**

**Purification of Ig Recognizing Common Gram-Negative Core Antigens**

Briefly, antisera was harvested from an adolescent Holstein calf vaccinated with the Rc mutant, *E. coli* O111:B4 (J5). This antiseraum was then purified by affinity chromatography. The capture antigen used was LPS derived from an Ra mutant, *S. typhimurium*, covalently bound to a solid matrix. This procedure served to isolate antibodies recognizing homologous antigenic structures present in both the vaccine and capture antigen. Vaccination and purification procedures are discussed elsewhere in greater depth (20, 23).

**ELISA Procedure**

The ELISA reagents and solutions used were as follows: 1) diluent was isotonic phosphate-buffered NaCl, pH 7.20, .05% polyoxyethylene-sorbitan monolaurate (Sigma); 2) substrate was .05 M citric acid, pH 4.00, .2 mM 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid, diammominium salt (Sigma Chemical Co., St. Louis, MO), 2 M H2O2; 3) wash solution was isotonic NaCl, .1% polyoxyethylene-sorbitan monolaurate; and 4) stopping reagent was 4 mM sodium azide.

The ability of affinity-purified Ig to recognize heterologous LPS was measured using adaptations of previously reported techniques (17, 18). Lipopolysaccharide (5.0 µg/ml) from Gram-negative bacteria dissolved in distilled water was bound passively to a polystyrene solid phase by incubating 100 µl overnight at 37°C in flat-bottomed microtiter plates (Probind, Becton Dickinson Labware, Lincoln Park, NJ). Plate antigens (all from List Biologicals, Campbell, CA unless specified otherwise) included LPS derived from *E. coli* J5 (the Rc mutant of *E. coli* O111:B4), *E. coli* O111:B4, *E. coli* O55:B5, *E. coli* O26:B6, *S. typhimurium* (ATCC 14028), *Salmonella typhosa*, *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (Fischer-Devlin Immunoype 1, ATCC 27312), *Serratia marcescens* (ATCC 14756), *Shigella flexneri* (serotype 1A) (Sigma), and lipid A from *Salmonella minnesota* (ATCC 9700) (Sigma). All LPS were derived by phenol-chloroform-ether extraction and had <3% protein or RNA content. Unbound antigen was removed by washing each well three times. Affinity-purified Ig recognizing homologous core antigens (100 µl) was added to wells following a 1:100 dilution. Plates were incubated at 37°C for 30 min.
Unbound Ig was removed by washing wells three times. Antisera specific for gamma chain and of goat origin linked to horseradish peroxidase recognizing bovine IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) (100 μl diluted 1:3000) were added to each well, and the incubation was repeated. Plates were washed five times to remove unbound conjugate. Substrate solution (125 μl) was added to test wells and allowed to incubate at 37°C for 30 min. Plates were read spectrophotometrically on a 410 nm setting. Sixteen identical samples were processed for each LPS or lipid A plate antigen. All optical density (OD) readings were blanked against the mean of 8 negative control wells containing no LPS plate antigen located on the same plate. Results were reported as the percentage of the mean ELISA OD observed with 8 positive control wells with E. coli LPS as a capture antigen on the same plate [(100 x (sample ELISA OD − mean no-antigen control ELISA OD))/(mean E. coli J5 LPS ELISA OD − mean no-antigen control ELISA OD)].

Statistical Analysis

Antigen recognition by the affinity-purified antisera was compared by one-way analysis of variance. Comparisons of group mean ELISA reactivities were performed following appropriate adjustments made for multiple comparisons using the Bonferroni technique. The null hypothesis, that mean ELISA reactivity did not differ among endotoxin sources, was rejected when $P < .05$. Calculations were assisted by a statistical software program (BMDP Statistical Software, Berkeley, CA).

RESULTS

The affinity-purified Ig reagent has marked reactivity against the entire battery of LPS tested (Figure 1). Only 1 of 176 assays with an LPS or lipid A capture antigen had an ELISA OD less than 20% of the mean E. coli J5 LPS OD. All 16 controls uniformly had an ELISA OD less than 20% of the mean E. coli J5 LPS OD. The ELISA reactivity was strongly associated with source and presence of an LPS capture antigen. All group mean comparisons of ELISA reactivity involving controls were highly significant ($P < .0001$). Lipopolysaccharide from K. pneumoniae had significantly higher ELISA reactivity than LPS derived from either E. coli O111:B4 or P. aeruginosa ($P < .0001$). No other group mean comparisons were significantly different.

DISCUSSION

Only 13 comparisons of group means demonstrated significant differences. All 11 of the comparisons between assays with Gram-negative endotoxin or lipid A and controls were significant, an expected result. The increased reactivity of the Ig reagent against K. pneumoniae LPS, compared with the reactivity of LPS derived from P. aeruginosa, may relate to species differences in LPS structure and assembly. The reason for the decreased recognition of E. coli O111:B4 compared with recognition of K. pneumoniae, is unknown. Escherichia coli O111:B4 is the parent strain from which was derived the Rc mutant used as the original vaccinal antigen, E. coli O111:B4 (J5). Consequently, we logically anticipated increased recognition by the affinity-purified reagent when E. coli O111:B4 was compared with other LPS sources. This was not the case.

Previous studies have examined recognition of whole Gram-negative bacteria by antibodies purified using crossreactive affinity chromatography (20, 23). Studies examining the reactivity of core antigen-specific antibodies to laboratory-adapted bacterial isolates have demonstrated broad-spectrum crossreactivity against E. coli (106.83%) and Salmonella spp. (81.26, 106.30, and 87.92% for three isolates) but reduced activity against K. pneumoniae and Bordetella bronchiseptica isolates (20). Antibodies purified in this manner also readily recognized field isolates of mastitis pathogens, including E. coli, Enterobacter spp., Klebsiella spp., Pseudomonas spp., and Serratia spp. Less reactivity was noted against Klebsiella spp. (66.06%) and Pseudomonas spp. (57.14%) than with E. coli (81.16%) isolates (20).

The bacteria responsible for clinical Gram-negative mastitis of dairy cattle are a diverse group. Commonly isolated bacteria include E. coli, Klebsiella spp., Enterobacter spp., Serratia spp., and Pseudomonas spp. (3, 7, 11, 19). Development of efficacious vaccines for coliform mastitis will require the use of immunogens, which have antigenic structures in com-
mon with the variety of potential mastitis pathogens. Furthermore, these antibodies must have access to these structures in vivo. Recognition of masked or hidden antigens will be of minimal benefit in preventing disease.

Immunization with R mutant bacteria may decrease the severity of coliform mastitis by two mechanisms. First, crossreactive antibodies may bind free endotoxin, masking pharmacologically active sites with the LPS moiety. The role of endotoxin in diseases caused by Gram-negative bacteria is well recognized (2, 15, 16), particularly in the case of Gram-negative mastitis (1, 5, 12). Second, these antibodies may target immune effectors (neutrophils and complement cascades) against live bacteria with exposed homologous core antigens.

The high degree of reactivity of the affinity-purified reagent against chemically purified K. pneumoniae and P. aeruginosa LPS in the present study suggests that the low to moderate reactivity of these bacteria to core antigen-specific Ig in previous studies related to structural masking of LPS, rather than compositional differences. The enhanced recognition of LPS observed in the present study may permit successful application of crossreactive immunization against Gram-negative mastitis pathogens, even when homologous Gram-negative core antigens are poorly expressed by mature, stationary phase, Gram-negative mastitis pathogens.

Endotoxin assembly is an ordered process. The innermost portions of the LPS moiety are assembled first, and the outermost portions are attached later in the bacterial cell life cycle (8, 13, 14). Consequently, rapidly proliferating Gram-negative bacteria have enhanced exposure of homologous core antigens (9), permit-

Figure 1. The ELISA reactivity of an affinity-purified bovine Ig reagent (expressed as a percentage of mean outside diameter (OD) using lipopolysaccharide derived from Escherichia coli J5, with standard errors) against Gram-negative endotoxins derived from several sources.
ting serologic recognition of Gram-negative bacteria, which have poorly exposed core antigens in mature, stationary-phase cultures. This hypothesis is substantiated by the decreased incidence of clinical mastitis caused by *Klebsiella* spp. observed in previous R mutant vaccine trials (10).

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