Prevention of Clinical Coliform Mastitis in Dairy Cows by a Mutant Escherichia coli Vaccine

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ABSTRACT

A prospective cohort study was undertaken in two commercial California dairies. The treatment group, 246 cows, received three doses of a whole cell bacterin of J5 Escherichia coli (mutant of E. coli O111:B4) plus Freund's incomplete adjuvant vaccine (two in the dry period and one after calving) while 240 unvaccinated cows served as controls. Thirty-five cases of clinical coliform mastitis were diagnosed, six in vaccinated cows and 29 in unvaccinated cows. Bacteria isolated from the clinical cases included 15 E. coli five Klebsiella pneumoniae, three K. oxytoca, three K. ozaenae, five Enterobacter aerogenes, three Serratia marcescens and one Serratia spp. Four control cows were culled, three of them because of chronic coliform mastitis and one because of postcoliform infection agalactia. Incidence rate of clinical gram-negative mastitis was 2.57% in vaccinated cows and 12.77% in unvaccinated cows. The estimated risk ratio, the measure of risk of having clinical gram-negative mastitis for vaccinated cows to unvaccinated cows, was 0.20 (p < 0.005), indicating a strong relation between vaccination and lack of clinical gram-negative mastitis. The results of this trial indicate that the administration of the E. coli J5 vaccine is protective against natural challenge to gram-negative bacteria, and reduces the incidence of clinical gram-negative mastitis in dairy cows during the first three months of lactation.

RÉSUMÉ

Cette expérience portait sur deux troupeaux laitiers de la Californie et elle impliquait 486 vaches, dont 246 reçurent trois doses d'une bactérie, préparée avec des cellules bactériennes complètes de la souche J5 d'Escherichia coli, un mutant de la souche O111:B4, et enrichie de l'adjuvant incomplet de Freund. Elles reçurent les deux premières doses de ce vaccin, au cours de leur période de tarissement, et la troisième, après le vêlage. Les 240 autres vaches servirent de témoins. Les auteurs diagnostiquèrent six cas de mammite clinique à coliformes, chez les vaches vaccinées, et 29, chez les témoins. Un examen bactériologique permit d'isoler E. coli, dans 15 de ces cas; Klebsiella pneumoniae, dans cinq; K. oxytoca, dans trois; K. ozaenae, dans trois; Enterobacter aerogenes, dans cinq; Serratia marcescens, dans trois; Serratia spp., dans un. Il fallut réformer quatre témoins: trois, à cause d'une mammite chronique à coliformes, et l'autre, à cause d'une agalactie consécutive à une infection à coliformes. Le taux d'incidence de la mammite clinique à gram-négatifs fut de 2.5%, chez les vaches vaccinées, et de 12.77%, chez les témoins. Le taux de risque approximatif, ou la mesure du risque d'enregistrer une mammite clinique à gram-négatifs chez les vaches vaccinées, par rapport aux témoins, fut de 0.2 (p < 0.005), indice d'une relation étroite entre la vaccination et l'absence de mammite clinique à gram-négatifs.

INTRODUCTION

Bovine mastitis produced by gram-negative bacteria, usually called coliform mastitis (1-5), may range in severity from fatal peracute cases to chronic and subclinical infections (2,5,6). The principal organisms involved in coliform mastitis are Escherichia coli, Klebsiella spp. and Enterobacter aerogenes (2,4-6), E. coli being most prevalent (4,6). These microorganisms are widely disseminated in the environment of the dairy cow (2,5,7), especially in bedding material, manure and water (7-11). Coliform mastitis is most frequent during the first three months, and especially the first two weeks, of lactation (2,5,12). It is a multifactorial disease for which no program of prevention or control has proved to be entirely successful (3), and is becoming more important as the prevalence of mastitis from Streptococcus agalactiae and Staphylococcus aureus infections is reduced (6,12,13).

A review of the literature on immunization against different agents of bovine mastitis shows that advances have been made (14), but as yet no
vaccination has proven to be effective in practice (15-17). However, it is thought that immunoglobulins produced in response to vaccination could protect the host against mastitis by acting as bacterial toxin neutralizers, providing opsonins to enhance the phagocytic activity of polymorphonuclear leukocytes and macrophages, and preventing colonization of mammary tissues by the invading organisms (14,18).

Spink et al (19) observed that 12 out of 24 Mexican patients affected with Brucella melitensis infections and treated with aureomycin experienced shock with high fever, tachycardia and a drop in blood pressure. It was suggested that an endotoxin was released by the action of the antibiotic on the bacteria (20). Later, Carroll et al (21,22) postulated that endotoxin release through death of bacterial cells during phagocytosis by udder leukocytes was the causative mechanism of coliform mastitis. Research indicates that antibodies to the lipopolysaccharide (LPS) core antigens of gram-negative bacteria may mitigate the severe systemic effect of endotoxemia (23-26). It has been reported in humans and experimental animals that antibodies to the rough mutant E. coli O111:B4 (27), commonly called J5, confer cross-protection against diverse gram-negative septicemias and endotoxic shock (25,26,28-30). Our prospective cohort study was undertaken to evaluate the potential ability of an E. coli J5 vaccine given in multiple doses to pregnant and freshly calved dairy cows to prevent clinical coliform mastitis (CCM) during the first 90 days of lactation. There are no reported studies in dairy animals that elucidate the efficacy of this vaccine preparation under natural challenge conditions.

MATERIALS AND METHODS

DAIRIES

This study was conducted in two commercial cooperator dairies, milking averages of 700 and 470 Holstein-Friesian cows respectively and located in the Central Sacramento-San Joaquin Valley of California. The two dairies were randomly selected, by drawing numbers from a box, from a group of seven dairies wanting to participate in the trial. Consent was obtained from the owners of each dairy following their review of the written objectives, methods, risks, possible benefits and specific responsibilities and tasks of cooperators in the trial. Both herds were participating in surveillance and control programs against S. agalactiae and S. aureus mastitis being conducted by the Veterinary Extension Service, University of California, Davis.

The average milk production for 305 days and the monthly mean somatic cell count obtained from the Dairy Herd Improvement Association records (DHI Computing Service, Provo, Utah) were 8,307 kg (SD = 1,762) and 200,000 cells per mL (SD = 12,650) for one dairy, while for the other dairy the figures were 8,368 kg (SD = 1,795) and 162,500 cells per mL (SD = 7,540).

ENROLLMENT

The experimental unit was the individual cow. Cows selected for the trial were recently dried off, confirmed pregnant and had completed one or more lactations. Both dairies routinely dried off cows on the 10th, 20th and 30th day of each month.

VACCINE

The J5 antigen was prepared as described by Ziegler et al (26). Heat-killed boiled cells of a 24 h culture of E. coli J5 were resuspended in sterile 0.9% sodium chloride to a spectrophotometrically adjusted concentration of 1.5 x 10⁹ bacteria per mL (27% light transmission at 610 nm, model 6/20, Junior II, Coleman Instrument Co., Maywood, Illinois). This bacterial concentration had been shown to increase serum titers in vaccinated calves (28), and in several vaccinated cows before starting the trial. Phenol was added to a final concentration of 0.05 mg/mL and the bulk antigen was tested for sterility, bottled in 50 mL aliquots and stored at -30°C. Before use, the antigen was thawed, mixed thoroughly, and sonicated to emulsify the cell suspension (Heat Systems-Ultrasonics, Inc., Plainview, New York). Each vaccine dose was composed of 5 mL of the emulsified E. coli J5 antigen together with 1 mL of Freund’s incomplete adjuvant (Sigma Chemical Co., St. Louis, Missouri) in a 10 mL sterile capped tube. Immediately before vaccination, the vaccine mixture was emulsified by repeated withdrawal and expulsion using a 12 mL sterile vaccination syringe and needle.

SAMPLE SIZE

A sample size of 218 cows per group (vaccinated and unvaccinated) was calculated (31) to be the required sample size to demonstrate efficacy with level of significance (α) of 0.05 and power (1-β) of 0.90, assuming an annual incidence of clinical coliform mastitis (CCM) in dairies in the area of 25% and an anticipated vaccine effectiveness of 50%. Additional cows were enrolled to allow for normal attrition.

GROUP ASSIGNMENT

Cows, each identified by an ear tag number, were assigned to the vaccinated group or to the control (unvaccinated) group by chance possession, as determined by the flip of a coin, of an even or an odd ear tag identification number. Both groups shared the same facilities in each dairy, so that feeding, housing, bedding and climate were the same. A placebo was not used in the unvaccinated group because it was considered not practical under these field conditions. Neither owners nor dairy personnel were informed which cows were vaccinated or unvaccinated.

FOLLOW-UP

The trial started on October 7, 1985 and sample collection ended on March 2, 1987. The immunized group received three doses of the E. coli J5 vaccine subcutaneously: the first at drying off, the second 28 days later and the third within 14 days after calving. Each animal that was taken out of milk production or culled because of any disease process other than mastitis was dropped from the trial.

CLINICAL MASTITIS CASE DEFINITION

Clinical cases of mastitis were defined as those in which the milkers found a cow at milking time shedding abnormal milk (flakes, clots, watery or discolored milk), or having swollen or hard mammary quarters. Approxi-
mately 10 mL of milk or quarter secretions were collected into sterile tubes from each quarter showing signs of clinical mastitis after cleaning and disinfecting the teats with pads saturated with 70% isopropyl alcohol (Clinipad Corporation, Guilford, Connecticut). Sample tubes were labelled with the cow ear tag number, quarter and date, and immediately stored at 4°C until picked up once per week to be delivered to the laboratory in iced containers.

**BACTERIOLOGICAL PROCEDURES**

Aliquots of 50 μL of each milk sample were streaked onto the surface of trypticase soy (BBL Microbiological Systems, Cockeysville, Maryland) blood agar plate containing 5% washed bovine red blood cells (32) and onto a MacConkey agar (Difco Laboratories, Detroit, Michigan) plate. Inoculated agar plates were placed in an incubator at 37°C and were examined for bacterial growth at 24 and 48 h. Milk samples yielding no growth on plates were incubated overnight at 37°C with equal amounts of brain heart infusion broth and recultured on blood agar and Mac-Conkey agar plates. Gram-negative isolates were identified by using the API 20E system (Analytab Products, Plainview, New York). The California mastitis test (CMT) (33,34), an indirect measure of leukocyte concentration, was performed on each milk sample. All samples were tested under a code unknown to the bacteriologist throughout the trial.

A quarter was diagnosed as having coliform mastitis if a pure culture of any gram-negative bacteria was obtained from a fresh or enriched sample and the sample had a CMT score of 1, 2 or 3. For a previously infected quarter to be counted as a new infection with a different pathogen, the old pathogen first had to be eliminated; this required one intermediate sample from which neither pathogen was isolated. The API 20E system was used to identify whether successive isolates did or did not represent a different pathogen. For a previously infected mammary quarter to be counted as a new infection with the same pathogen, two intermediate samples free of the pathogen were required. Otherwise, it was regarded as a chronic infection and counted only once.

**STATISTICAL ANALYSIS**

Evaluation of the protective efficacy of the vaccine was made using a 2 x 2 table of frequencies to compare the incidence rate of cases of CCM in the vaccinated and unvaccinated groups, and applying the chi-square test for independence. Differences between the vaccinated and control groups were considered significant at the level of p < 0.05.

**RESULTS**

**STUDY POPULATION**

A total of 486 cows were enrolled in the study between October 7, 1985 and June 30, 1986. The treatment group, 246 cows, received three doses of the *E. coli* 35 vaccine while 240 unvaccinated cows served as control. The mean lactation numbers when cows entered in the trial were 2.2 (SD = 1.2) for vaccinated cows and 2.1 (SD= 1.4) for unvaccinated cows, while for the other dairy the numbers were 2.7 (SD = 1.8) for vaccinated cows and 2.7 (SD = 1.4) for unvaccinated cows. The mean 305-day milk production, representing milk yield when cows entered the trial, was similar for the vaccinated and unvaccinated groups in each dairy.

Fifteen percent of the cows developed small lumps at the injection site probably due either to the adjuvant or to a deeper administration of the vaccine as a consequence of cows moving their necks in the stanchions at vaccination time. These lumps lasted an average of three weeks without causing discomfort to the cows.

Sixty-three of the cows, including 33 vaccinated and 30 unvaccinated cows, were culled or died during the trial from causes other than mastitis (16 in the dry period and 47 in lactation).

**CASES OF CCM**

Thirty-five cows, including 29 unvaccinated cows and six vaccinated cows, were diagnosed as having CCM (Table I). Milk from 19 cows had a CMT-2 score and eight each had CMT-1 and CMT-3 scores. Milk with CMT-2 scores from three unvaccinated cows were negative on primary culture, but when enriched, *K. pneumoniae* was isolated from two samples and *E. aerogenes* from one sample. Five strains of *E. coli*, with different API 20E profile numbers, and two each of *K. pneumoniae, K. oxytoca, E. aerogenes* and *Serratia marcescens* were isolated from cases of mastitis in the two dairies. Three unvaccinated cows were culled because of chronic coliform mastitis with *E. coli, K. pneumoniae* and *K. ozanae* being involved in one case each, while another unvaccinated cow was culled due to post-*K. pneumoniae* infection agalactia. The infections were always confined to only one mammary quarter.

**PROTECTIVE EFFICACY**

Evaluation of the protective efficacy of the vaccine was based on comparison of the incidence rate of cases of CCM in the vaccinated and unvaccinated groups. Thirty-five cases of CCM were included in this analysis. The incidence rate among vaccinated cows was 2.57% (6 of 233), and among unvaccinated cows was 12.77% (29 of 227) (Table II). The estimated risk ratio, the measure of having CCM for

<p>| Table I. Microorganisms Isolated From 35 Cases of Clinical Coliform Mastitis in Vaccinated (E. coli 35) and Unvaccinated Cows |
|-----------------------------------------------|----------|----------|----------|</p>
<table>
<thead>
<tr>
<th>Microorganisms Isolated</th>
<th>Vaccinated</th>
<th>Unvaccinated</th>
<th>Total</th>
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<td>15</td>
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<tr>
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<td>5</td>
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<tr>
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<td>3</td>
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<tr>
<td><em>Klebsiella ozaenae</em></td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
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<td>3</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
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<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

303
vaccinated to unvaccinated cows, was 0.20 (p < 0.005).

**DISCUSSION**

This is the largest reported study in which a vaccine against any kind of bovine mastitis agent has been used. It demonstrates the protective efficacy of the heat-killed *E. coli J5* vaccine against postcalving CCM when administered to pregnant and recently-calved dairy cows. During the first 90 days of lactation, cows vaccinated with the *E. coli J5* vaccine were at five times lower risk of suffering CCM than unvaccinated cows.

Since sanitizing teat dipping and dry cow therapy have failed to control coliform infections (35-39) and treatment therapy is unsatisfactory (38), immunization of dairy cows against coliform mastitis may be a highly valuable procedure to bring these infections under control. Coliform mastitis can be caused by several species and strains of bacteria. The approach using an individual strain or species-specific vaccine will not protect against heterologous strains (40), and is therefore an unsatisfactory method of prevention. Evidence suggests that a common antigenic structure is shared by the potential gram-negative pathogens and that the antigen is immunogenic (41-43). Results from use of the *E. coli J5* vaccine support this new concept in immunization against coliform infections in adult cattle.

Chedd et al (44) considered that core antigens of gram-negative bacteria were responsible for the lack of specificity and broad spectrum activity of natural antibodies. Recently, it was found in a California dairy that cows with naturally occurring serum immunoglobulin G1 enzyme-linked immunosorbent assay (ELISA) titers higher than 1:240 recognizing the gram-negative core antigen (*E. coli J5*) had 5.3 times lower risk of suffering CCM than cows with lower titers (J. W. Tyler, J. S. Cullor, B. I. Osburn, R. B. Bushnell, B. W. Fenwick, unpublished observations). These findings are in agreement with previous speculations that natural antibodies may protect the udder against coliform infection if they cross from serum to milk (40). Jain et al (45) found that serum from a cow vaccinated by a combination of procedures with an *Aerobacter aerogenes* [*K. pneumoniae* (46)] bacterium mixed with Freund’s complete adjuvant had greater bactericidal activity for the organism than did serum from a control cow.

Ziegler et al (26) theorized that during infection there is a rapid multiplication of invading gram-negative bacteria, thereby J5 antibody could bind to exposed core antigens of bacteria before attachment of side-chain is completed. Recent work by McCallus and Norcross (47) suggested the feasibility of the aforementioned theory. They challenged two groups of mice (one group immunized with J5 vaccine and one control group) with LPS from a smooth *E. coli* strain isolated from a cow with clinical mastitis, and grown for 3 or 19 h. The J5 vaccine protected mice challenged with LPS extracted from bacteria grown for 3 h but not from LPS extracted from bacteria grown for 19 h, thus agreeing with Ziegler et al (26) that J5 antibody could bind to invading bacteria at a time when core antigens are most exposed.

The presence of serum antibodies in the milk depends upon increased capillary permeability during inflammatory response to infection (40,45). The study design of this prospective trial did not enable us to demonstrate that the *E. coli J5* vaccine prevented gram-negative intramammary infection. However, we demonstrated that the administration of an *E. coli J5* vaccine is protective against natural challenge to gram-negative bacteria and reduces the incidence of clinical coliform mastitis. This is in agreement with previous speculations on the subject (15,48,49).

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