Effect of a *Staphylococcus aureus* Bacterin on Serum Antibody, New Infection, and Mammary Histology in Nonlactating Dairy Cows

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ABSTRACT

The influence of a *Staphylococcus aureus* mastitis vaccine on immunologic status and rate of new IMI was evaluated. At drying off, cows were vaccinated, either intramuscularly or subcutaneously in the area of the supramammary lymph node, or were left as unvaccinated controls; vaccinates received booster injections at 6 wk. Serum antibody concentrations, bacteriologic status, and SCC of quarter milk samples were determined. Four weeks after revaccination, cows were challenged by intramammary infusion of *S. aureus* and then killed 24 to 72 h later. Mean serum antistaphylococcal antibody titer of vaccinated cows during the trial was 4.7-fold that of controls. Challenge resulted in IMI rates of 92, 36, and 60% for control cows, cows vaccinated intramuscularly, and cows vaccinated in the area of the supramammary lymph node. Vaccination by either route had no influence on mammary parenchymal tissue components compared with controls; however, leukocyte infiltration was greater in quarters from cows vaccinated in the area of the supramammary lymph node than in quarters from unvaccinated controls. Plasma cell populations producing IgG1, IgG2, IgA, and IgM were greatest in quarters of cows vaccinated in the area of the supramammary lymph node followed by those in quarters of cows vaccinated intramuscularly and control cows.

(Key words: *Staphylococcus aureus*, bacterin, vaccination)

INTRODUCTION

Vaccination against mastitis has been attempted to increase antibody titers in blood and in milk to a specific organism, thereby promoting immunity by enhancing phagocytosis of bacteria and neutralizing toxins. Because *Staphylococcus aureus* mastitis is a major threat to the dairy industry and is difficult to treat, most mastitis vaccine research has been directed against this organism. Typically, organisms were cultured in vitro, killed, and injected systemically with or without toxoids and immunologic adjuvants (1, 2, 6). These studies demonstrated that vaccination increased the spontaneous cure rate of IMI and lessened its severity but did not prevent new IMI.

Vaccination by intramuscular and intramammary infusion with killed *S. aureus* at cessation of lactation and prepartum increased serum antibody titers, but concentrations in milk were unaffected (6). In another study (18), an intramuscular injection of bacterin at cessation of lactation plus a booster injection into the area of the supramammary lymph node (SMLN) prepartum resulted in high antibody titers during the subsequent lactation and the seeding of sensitized lymphoid cells in involuting mammary tissue, producing large amounts of IgA and IgM.

*Staphylococcus aureus* vaccines have been improved using a formulation developed by Watson (16). *Staphylococcus aureus* grown in vivo express pseudocapsular antigens (19, 21) that are not produced in vitro using standard laboratory media. The pseudocapsule inhibits opsonization and phagocytosis by blocking C3b molecule attachment to bacteria (17). Cows immunized with a live vaccine produced
from *S. aureus* that were grown in vivo produced antipseudocapsular antibodies that promoted phagocytosis of *S. aureus* (17). This vaccine was modified by incorporation of staphylococcci that were cultured in enhanced growth media that promoted synthesis of pseudocapsular components. In addition, organisms were killed, and toxoids and an adjuvant were added. In a subsequent study (20), the modified vaccine induced an antibody response similar to the live vaccine that was produced in vivo (15). Use of this vaccine in commercial dairies in Australia demonstrated that new IMI rate with natural *S. aureus* IMI was reduced 25% (*P* < .01) and that clinical mastitis was reduced 45% (*P* < .001) in certain herds (20).

Because new *S. aureus* IMI during the dry period and early lactation may occur despite dry cow therapy, this study was undertaken to evaluate the effects of the vaccine developed by Watson (16) on the immunologic status during the nonlactating period. Objectives were to monitor serum antibody titers during the trial, to determine the influence on development of new *S. aureus* IMI after challenge, and to quantify histologic and cytologic changes in mammary tissues after vaccination.

**MATERIALS AND METHODS**

Twelve nonpregnant Jersey cows from the Hill Farm Research Station dairy herd were used. Unbred cows were used because they were to be slaughtered for mammary tissue evaluation after the trial. The vaccine was a cell-toxoid adjuvanted preparation produced from *S. aureus* strain JG80 (ATCC 53486) (16). Bacteria were cultured in nutrient broth (CM1; Oxoid USA, Inc., Columbia, MD) to which was added 10% (vol/vol) sterile ovine milk whey prepared by rennet precipitation. Cultures were grown for 24 h at 37°C on an orbital shaker (60 rpm) and sterilized by addition of formalin to 1% (vol/vol). After sterility was ensured, cells were harvested by centrifugation (6000 × g, 4°C, 1 h) and suspended in sterile PBS (pH 7.2). Bacterial concentrations were determined spectrophotometrically, adjusted to 1010 bacteria/ml with sterile PBS containing .015% (wt/vol) thimerosal, and stored at 4°C as the bacterin.

For the toxoid component, bacteria were grown for 48 h at 37°C in brain-heart infusion broth (Oxoid) on an orbital shaker (30 rpm). Bacteria were deposited by centrifugation, and the supernatant was concentrated by lyophilization to 12% of the original volume. Formalin was added to 1% (vol/vol), and toxoiding occurred over 48 h at 4°C. Each dose of the vaccine consisted of 1 ml of bacterin, 1 ml of toxoid, and 50 mg of dextran sulfate (Pharmacia, Piscataway, NJ), which were emulsified with 2 ml of Freund's incomplete adjuvant, for a total dose of 4 ml.

The 12 cows selected for this study had been sampled every 2 wk during lactation to determine quarter IMI status and SCC. Similarly, prior to drying off, duplicate quarter samples were collected to determine bacteriologic status and SCC. All quarters were determined to be free of major mastitis pathogen IMI. One cow had a nonfunctional quarter. Cows did not receive dry cow therapy.

One day after drying off, 4 cows were vaccinated intramuscularly into the gluteal muscles, 4 cows were vaccinated subcutaneously in the area of the SMLN, and 4 cows served as unvaccinated controls. After 6 wk, vaccinated cows received booster injections, and, 4 wk later, all cows were challenged with *S. aureus* Newbould 305 via intracisternal inoculation of approximately 8 × 103 cfu per eligible quarter. Three quarters of each cow were challenged in the a.m. at 72, 48, and 24 h prior to commercial slaughter. The remaining quarter of each cow was not challenged. Cows were challenged in this manner to assess histologic response to *S. aureus* IMI at various times after challenge. Two quarters of 2 cows in the group vaccinated in the area of the SMLN were ineligible for challenge because of a nonfunctional quarter and because of a new coliform IMI that occurred after drying off, and 1 quarter in the group that was vaccinated intramuscularly was ineligible because of a new *Streptococcus uberis* IMI that occurred after drying off.

During the trial, aseptic duplicate secretion samples were taken from quarters biweekly to determine electronic SCC (A/SN Foss Electric, Hillerød, Denmark) and bacteriologic status (4). Raw SCC of undiluted samples were transformed to log10 for statistical analysis. For cases in which only small amounts of secretion were obtained, even after hand massage to move fluids ventrally into the gland and teat.
cisterns, samples were used to determine bacteriologic status only, and SCC were not performed.

After S. aureus challenge at the end of the trial, duplicate samples were collected twice daily: at approximately 7 h postchallenge (p.m. sample) and on the following morning (a.m. sample). Samples were used only for bacteriology because volume was minimal, and the presence of clots and flakes in many samples precluded electronic cell counting. Thus, as a minimum, two sets of duplicate samples were used to confirm the IMI status of cows challenged 24 h prior to slaughter.

Cows were bled biweekly by jugular venipuncture, and antistaphylococcal serum titers were determined using the indirect ELISA procedure. To prepare the coating antigen from challenge organisms, S. aureus Newbould 305 was cultured under conditions to promote the production of pseudocapsule as described by Watson and Watson (21). The ELISA antigen was prepared from formalin-inactivated (4% vol/vol) bacteria by collection of the cells by centrifugation and resuspension in PBS (7.5-fold concentration). The stock antigen suspension was diluted 1:10,000 in sodium carbonate-bicarbonate buffer (pH 9.6) immediately before the plates were coated. Coated plates were incubated for 1 h at 37°C and then washed three times with 0.01 M PBS containing 0.3% Tween 20 (Sigma Chemical Co., St. Louis, MO). Plates were blocked for 1 h at 37°C with 1% low fat milk containing 5% lamb serum preabsorbed to remove specific S. aureus antibodies and to prevent nonspecific protein binding. Plates were then washed three times as described with 0.01 M PBS containing 0.3% Tween 20. Serum samples for analysis were initially diluted 1:100 and then placed in round bottomed microtiter dilution plates. Twofold dilutions were then made from 1:100 to 1:102,400, and 100 µl of each dilution were transferred to coated blocked plates, incubated at 37°C for 1 h, and washed three times as described. Following washing, 100 µl of conjugate (peroxidase-labeled sheep anti-bovine IgG1 or IgG2) were added to each well, incubated at 37°C for 1 h, and washed as described. Finally, 100 µl of ABTS substrate (2,2'-azino-di[3-ethylbenzthiazolinesulfonate]) were added to each well and incubated at 37°C for 30 min. The optical density of each well was read using a dual wavelength ELISA plate reader. The endpoint titer for each plate was defined as the highest serum dilution with ≥0.05 optical density. Titers were expressed as log₁₀ of the reciprocal of the dilution.

At the end of the trial, cows were slaughtered, and mammary tissues were taken for histologic and cytologic examination as described by Nickerson et al. (7). Parenchymal tissues were analyzed to determine the percentage of mammary tissue components (alveolar epithelium, alveolar lumen, and interalveolar stroma) and degree of leukocytosis in vaccinated and control cows. Teat end tissues were stained immunocytochemically to determine the influence of vaccination on local antibody production in mammary tissues by quantifying concentrations of plasma cells producing IgG, IgG2, IgM, and IgA as described by Nickerson et al. (9). Samples of the right and left SMLN were examined to assess concentrations and classes of Ig-producing plasma cells. Cell concentrations were quantified on a unit tissue area basis at magnification ×600 and scored as 1 = minimal (<10 cells), 2 = moderate (10 to 300 cells), or 3 = marked (>300 cells).

RESULTS

Mean anti-S. aureus IgG antibody titers in serum across the trial for unvaccinated (○) and vaccinated (●) cows are in Figure 1. Data from both vaccinated groups were combined because no significant differences existed be-

Figure 1. Mean serum anti-Staphylococcus aureus IgG antibody titers in unvaccinated (○) and vaccinated (●) cows. Means for each treatment without a common letter (a,b,c) differ (P < .05).
Anti-S. aureus IgG2 titers in vaccinated cows also remained elevated ($P < .05$) over pretreatment and control titers except at wk 4 (Figure 3). The IgG2 titers in vaccinated cows were highest 2 wk after the primary vaccination and at 2 wk and 4 wk after booster injections.

A comparison between IgG1 and IgG2 anti-S. aureus titers is shown in Figure 4. The IgG2 titers tended to remain elevated over IgG1 antibody titers throughout the trial, except at wk 8, but differences were not significant. The IgG1 titers tended to increase more than IgG2 titers 1 wk after booster injections.

Results of challenge exposure to S. aureus (Table 1) demonstrated that, under the criteria used to diagnose IMI, 11 of 12 challenged quarters of control cows (91.7%) became infected, whereas 10 of 21 quarters of vaccinated cows (47.6% across both groups) became infected (different from control; $P < .05$). Approximately 50% of all quarters that became infected exhibited clinical symptoms in secretions, but vaccinates and controls did not differ. Time of challenge relative to trial termination did not influence IMI rate among treatments. Quarters becoming infected were positive for S. aureus by 24 h postchallenge. Among immunized cows, 4 of 11 challenged quarters of the group that was intramuscularly vaccinated (36.4%) became infected (different from control; $P < .005$), and 6 of 10 challenged quarters of cows vaccinated in the area of the SMLN. Titers in vaccinates remained elevated approximately 4.7-fold ($P < .05$) over those of controls and pretreatment titers throughout the trial. At wk 8 and 10 (2 and 4 wk after booster injections), titers in vaccinates tended to be higher than at other times during the trial and were elevated ($P < .05$) over those at wk 4.

Anti-S. aureus IgG1 titers in vaccinated cows were elevated ($P < .05$) over pretreatment titers and those of unvaccinated cows except at wk 4 (Figure 2). The IgG1 titers in vaccinates were highest 2 wk after booster injections (wk 8).
TABLE 1. New IMI in unvaccinated control cows and vaccinated cows following intramammary challenge with *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cows</th>
<th>Eligible quarters</th>
<th>New IMI</th>
<th>Infected quarters</th>
<th>Reduction1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>12</td>
<td>11</td>
<td></td>
<td>91.7</td>
</tr>
<tr>
<td>Vaccinated intramuscularly</td>
<td>4</td>
<td>113</td>
<td>4</td>
<td>36.4**</td>
<td>60.3</td>
</tr>
<tr>
<td>Vaccinated in SMLN2 area</td>
<td>4</td>
<td>104</td>
<td>6</td>
<td>60.0*</td>
<td>34.7</td>
</tr>
<tr>
<td>All vaccinates</td>
<td>8</td>
<td>21</td>
<td>10</td>
<td>47.6*</td>
<td>48.1</td>
</tr>
</tbody>
</table>

1 Percentage of reduction in IMI rate from that of unvaccinated control cows.

2 SMLN = Supramammary lymph node.

3 One of the original 12 quarters became infected with *Streptococcus uberis* after drying off and, therefore, was ineligible for a new IMI during experimental challenge.

4 One of the original 12 quarters was nonfunctional prior to the trial, and 1 quarter became infected with coliform after drying off; therefore, both were ineligible for new IMI.

* Differed from control (P < .05).
** Differed from control (P < .005).

area of the SMLN (60%) became infected (different from control; P < .05). Overall, vaccination by both routes combined reduced new IMI by 48.1% compared with controls. Intramuscular vaccination reduced new IMI by 60.3%, and vaccination in the area of the SMLN reduced new IMI by 34.7%.

After cows were dried off, SCC in all three treatments remained significantly elevated over pretreatment (wk 0) SCC but did not differ significantly (Figure 5). The SCC in cows vaccinated in the area of the SMLN tended to be lowest throughout the trial. The SCC among treatments prior to *S. aureus* challenge varied considerably and were unrelated to susceptibility of quarters to new IMI or severity of IMI.

Mammary tissue response to vaccination was examined to determine whether vaccination affected the normal histological changes following *S. aureus* IMI. Also, leukocyte infiltration was measured to determine whether vaccination influenced the recruitment of phagocytes and the seeding of mammary tissues with lymphoid cells, which may play a role in local immunity. The effects of vaccination on mammary tissue parenchymal tissue components and overall leukocyte infiltration are shown in Table 2. Histological analysis of uninfected quarters among cows vaccinated by either route demonstrated no differences in the structure of milk-producing tissues compared with unvaccinated controls. The leukocyte infiltration score was significantly higher in quarters from cows vaccinated in the area of the SMLN than in quarters from unvaccinated controls but was not different from quarters from cows vaccinated intramuscularly. Among *S. aureus*-infected quarters, tissues from cows vaccinated intramuscularly exhibited less luminal and more stromal area compared with other treatments. Leukocyte infiltration was similar among treatments. Among *S. aureus*-infected quarters across treatments, time of challenge had no influence on the percentages of parenchymal components; however, leukocyte infiltration scores were significantly higher (P < .05).
TABLE 2. Effect of treatment on mammary parenchymal tissue components\(^1\) and degree of leukocyte infiltration\(^2\) in uninfected quarters and in quarters infected with Staphylococcus aureus.

<table>
<thead>
<tr>
<th>Tissue component</th>
<th>Uninfected quarters</th>
<th>Quarters infected with S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unvaccinated</td>
<td>Vaccinated intramuscularly</td>
</tr>
<tr>
<td>epithelium</td>
<td>30.3(^a)</td>
<td>29.3(^a)</td>
</tr>
<tr>
<td>lumen</td>
<td>13.2(^a)</td>
<td>12.1(^a)</td>
</tr>
<tr>
<td>stroma</td>
<td>56.5(^a)</td>
<td>58.6(^a)</td>
</tr>
<tr>
<td>infiltration(^1)</td>
<td>1.04(^b)</td>
<td>1.10(^ab)</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means without a common superscript within IMI status differ \((P < .05)\).

\(^1\)Expressed as percentages of parenchymal tissues occupied by alveolar epithelium, lumen, and stroma.

\(^2\)Quantified on a unit tissue area basis and scored as 1 = minimal (<10 cells), 2 = moderate (10 to 300 cells), and 3 = marked (>300 cells).

\(^3\)SMLN = Supramammary lymph node.

An examination of the effects of treatment on plasma cell concentrations in teat end tissues (teat cistern and Fürstenberg's rosette combined) shows that concentrations of IgG\(_1\) and IgG\(_2\) cells were greater \((P < .05)\) in cows vaccinated in the area of the SMLN than in controls (Figure 6). Concentrations of these cell populations in cows vaccinated in the area of the SMLN were also higher, but not significantly greater, than those in cows vaccinated intramuscularly. Concentrations of IgA and IgM cells in cows vaccinated in the area of the SMLN were significantly elevated over those in cows vaccinated intramuscularly and in those of unvaccinated controls. Plasma cell concentrations generally were higher in the Fürstenberg's rosette area than the teat cistern, but the differences were not significant (data not shown). Plasma cells producing IgG\(_1\) were the most numerous, followed by those producing IgG\(_2\), IgM, and IgA (Figure 6). Among S. aureus-infected quarters, plasma cells producing IgG\(_1\), IgG\(_2\), and IgM generally were most numerous across treatments in quarters that were challenged at 72 h prior to slaughter compared with quarters challenged at 48 and 24 h; the concentration of IgA-producing cells did not follow this trend (data not shown).

An examination of tissue obtained from the SMLN to enumerate plasma cells showed no differences among treatments for each Ig class (Table 3). The IgG\(_1\) and IgG\(_2\) cells predominated, followed by IgM and IgA.

| Table 3. Concentrations\(^1\) of plasma cells producing IgG\(_1\), IgG\(_2\), IgA, and IgM in the area of the supramammary lymph node (SMLN) of control and vaccinated cows. |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | Control          | Vaccinated intramuscularly | Vaccinated in SMLN area |
| IgG\(_1\)         | 2.75             | 2.83             | 3.0               |
| IgG\(_2\)         | 2.0              | 1.67             | 2.0               |
| IgA              | 1.25             | 1.0              | 1.0               |
| IgM              | 2.0              | 2.33             | 1.75               |

\(^1\)Plasma cell concentration was quantified on a unit tissue area basis and scored as 1 = minimal (<10 cells), 2 = moderate (10 to 300 cells), or 3 = marked (>300 cells).
DISCUSSION

In the present study, serum antibody titers increased significantly following immunization and remained significantly elevated over those of controls. These findings support those of Sears et al. (12). At the time of S. aureus challenge, anti-S. aureus IgG1 and IgG2 titers in the present study were greater (P < .05) in blood sera of vaccinated cows than in controls. Similarly, Watson (15) observed a significant increase in circulating IgG1 (threefold) and IgG2 (eightfold) antibody titers in heifers that were vaccinated and received booster injections prepartum with a live attenuated strain of S. aureus, which appeared to provide protection against challenge postpartum. In addition, serum from vaccinated cows exhibited elevated opsonizing capacity for S. aureus. Neutrophils armed with cytphilic IgG2 may have extravasated into milk in response to S. aureus challenge, placing IgG2 at IMI sites (15). Thus, in immunized cows, large amounts of specific IgG2 is transported cytphilically to inflamed tissue to promote opsonization and phagocytosis by neutrophils, which was demonstrated in vitro (14). In addition, because IgG1 is quantitatively the major isotype and because macrophages are the predominant leukocyte type in normal milk and in dry secretions, macrophages with surface receptors for IgG1 may play a role in defense before the arrival of the neutrophils transporting IgG2.

Sears et al. (12) studied a vaccine that was similar to that used in the present study and was formulated to enhance production of cell surface pseudocapsular antigens. Bred heifers were immunized and revaccinated subcutaneously in the area of the SMLN prior to parturition and challenged with S. aureus by intramammary infusion at calving. In that study (12), new IMI in vaccinated heifers was reduced 52% from incidence of IMI in unvaccinated controls, which is similar to the 48.1% reduction in the present study.

The same vaccine used in the present study was evaluated previously in a field trial using five commercial dairies (20). Cows were injected intramuscularly with either the vaccine or a placebo at 8 and 4 wk prior to parturition. In comparison with placebo controls, incidence of clinical S. aureus mastitis was reduced approximately 45%, overall prevalence of subclinical IMI was reduced 18%, and number of new subclinical S. aureus IMI was reduced 25% in vaccinated cows.

Teat end and SMLN tissues were evaluated immunocytochemically to determine any effect of vaccination of plasma cell populations. Although vaccination did not influence IgG-producing cell populations in the SMLN, concentrations of all isotypes were greater in teat end tissues of vaccinated, especially in tissues of cows vaccinated in the area of the SMLN, suggesting that subcutaneous vaccination in the area of the SMLN enhanced local production of antibody in the udder. Similarly, previous cytological analysis of leukocytes infiltrating tissues of the teat cistern and Fürstenberg's rosette demonstrated an increase in plasma cells in tissues from S. aureus-vaccinated cows compared with those from unvaccinated controls (9); infiltration was greatest in tissues of cows immunized in the area of the SMLN compared with cows vaccinated intramuscularly. Likewise, Lee et al. (5) observed a marked increase in B-cell concentrations in ovine mammary glands after infusion of killed S. aureus and, 14 d later, observed numerous plasma cells, the majority of which were IgA cells, followed by IgG1 cells, with smaller numbers of IgM and IgG2 cells. Protective lymphoid cells also preferentially infiltrate teat end tissues, especially those exposed to S. aureus antigens (10), and in quarters infected with Mycobacterium bovis (8).

As observed previously (3), IgG1 and IgG2 cells predominated in the present study, followed by cells producing IgA and IgM. Likewise, Sheldrake et al. (13) enumerated IgG-producing cells in mammary tissue and in the SMLN after infusion of antigen into nonpregnant sheep mammary glands and found that IgG1 cells were the most prevalent plasma cell types, followed by IgG2 cells.

CONCLUSIONS

The role of vaccination in the present study and in recent investigations (12, 20) in reducing incidence of new IMI is greater than that observed previously (11) with cows vaccinated in the area of the SMLN with a protein A vaccine or intramuscularly using a commercial bacterin (Somatostaph®; Anchor Laboratories, St. Joseph, MO). Bacteriologic and SCC data analyzed after the three-lactation trial (11) re-
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revealed no reduction in the number of new IMI with *S. aureus* in cows vaccinated with the protein A vaccine or commercial bacterin, although the spontaneous cure rate was greater for immunized cows. Results of the present study suggested that vaccination provided partial protection (48.1%) against *S. aureus* challenge and that the elevation in serum antibody titers and local antibody synthesis may be responsible, in part, for the reduction in the new IMI rate.

REFERENCES


