ABSTRACT

A change in the epidemiology of mastitis in recent years has emphasized the role of the udder immune system in the pathogenesis of *Staphylococcus aureus*. Therefore, if the bovine or udder immune capability could be enhanced, susceptibility to *Staph. aureus* could be reduced and antibiotic efficacy could be increased. Immune system defense mechanisms could be enhanced by vaccination and by biological response modifiers. Within this latter group, a biological response modifier obtained from *Parapox ovis* that was attenuated over 200 tissue culture passages was recently developed and commercialized in some European countries. This study reports the results of a field trial on the efficacy of this biological response modifier in reducing *Staph. aureus* intramammary infection (IMI) after calving in primiparous and pluriparous cows. The trial included 106 cows sampled six times (55 cows from herd A and 51 from herd B) for a total of 2544 quarter milk samples. The analysis of IMI prevalence showed that 25.09% of samples were bacteriologically positive in the placebo group, and 23.17% of the positive samples were observed in the biological response modifier group. *Staphylococcus aureus* IMI had a frequency of 11.44% in the placebo group and 6.00% in the biological response modifier group. The dynamic of the hazards showed significantly lower rates in the biological response modifier group than in the placebo group (risk ratio = 0.47). Treatment with the parapox-containing biological response modifier showed significant reduction of *Staph. aureus* IMI around calving, and this reduction was attributed to an increase in immune defenses.

(Key words: bovine response modifier, Parapox virus, *Staphylococcus aureus* mastitis, prevention)

Abbreviation key: BRM = bovine response modifier, PLC = placebo, PR = prevalence ratio, QMS = quarter milk samples.

INTRODUCTION

Mastitis is still the most important disease of dairy herds. The epidemiology of this infection has changed in the last 20 yr; the prevalence of *Streptococcus agalactiae* has declined, and the frequencies of *Staphylococcus aureus*, environmental pathogens (streptococci other than *Strep. agalactiae* and coliforms), and coagulase-negative staphylococci have increased (13, 18, 21, 25).

A change in the epidemiology of mastitis emphasizes the role of the udder immune system in the pathogenesis of these infections. Indeed, *Staph. aureus* has broad interaction with the immune system not only during invasion and colonization phases, but the cure-rate also depends on the activity of immune cells (2, 9). Therefore, if the bovine or udder immune capability of cows could be enhanced, susceptibility to *Staph. aureus* could be reduced, and the effectiveness of antibiotics could be increased.

Immune system defense mechanisms could be enhanced in at least two ways: by vaccination and by biological response modifiers. The latter mechanisms are either chemical or biological substances that could modify the host's nonspecific response to pathogens (7). Within this general definition, biological response modifiers (BRM) could be differentiated in substances that induce immune reactions (paraimmunization) and substances that substitute primitive immune reactions (i.e., cytokines) (4).

Recently a BRM obtained from *Parapox ovis* (strain D1701) that had been attenuated over 200 tissue cultures passages was developed. This virus is a powerful stimulator of nonspecific immune reactions mediated mainly by virus envelope epitopes (4). The use of parapox viruses as immunomodulators is advantageous because they do not need a specific receptor to enter into the cells and because virus multiplication is not a prerequisite for paraimmunization (5). This BRM is an interferon-inducer, as shown by different studies and field trials (5, 7, 12, 16, 24).

Within the different phases of lactation, the drying off period is very likely the best phase in which to clear...
the mammary gland from infection. Indeed, this is probably the only period when there are reliable chances to eliminate *Staph. aureus* IMI. However, bacteria that survive in the udder after drying off are in a favorable environment near the time of parturition when a decrease in immune defenses is observed and more nutrients are available for bacterial growth, which allows a continuation of the infection process (6, 14). Therefore, preventing the impairment of immune defenses could reduce the IMI rate after calving.

The goal of this study was to assess the efficacy of a parapox-based BRM in reducing *Staph. aureus* IMI after calving in primiparous and pluriparous cows.

**MATERIALS AND METHODS**

**Design of the Study**

The study was a controlled field trial with a 2-mo follow-up period; the study was started in September 1995 and ended in January 1997. The last treatment was administered in November 1996.

**Herd Characteristics**

Two commercial dairy herds with *Staph. aureus* IMI were considered. Herd A had about 80 lactating cows, and herd B had about 100 lactating cows. In both herds, cows were housed in loose barns with cubicles; cows did not graze. Milking procedures included cleaning of the teat with a dry paper towel, no predip, and postdipping with a chlorexidine solution (5000 ppm). Separation of *Staph. aureus* infected cows was not applied in both herds.

**Cow Selection and Assignment**

Eligible cows were selected by the following criteria: lactation number (1 to 3), and absence of clinical signs of diseases (mastitis included). The selected cows were stratified by age (primiparous and pluriparous), and, for each strata, treatment randomization was performed by the means of a complete block design including 6 cows per block. Within each block, a definite random sequence of treatment was applied. Cows entered into the block as they started the drying off period.

**Treatment Products**

All of the lactating cows were treated at drying off with a product containing 1000 mg of oxacillin (Stapenor Retard®; Bayer AG, Leverkusen, Germany). Cows selected for inclusion in the treatment group were treated with a new paraimmunity inducer (Baypamun®; Bayer AG), which was already available in some European countries. Baypamun® is a BRM and is manufactured in cell culture on the basis of an inactivated parapox virus. The parapox virus was isolated from the pustules of a sheep suffering from pustular dermatitis and was attenuated in over 200 passages in cell culture on ovine and bovine cells, and was chemically inactivated with β-propiolacton. Control cows received sterile saline solution as a placebo (PLC). Cows were treated systemically with 2 ml of the BRM or PLC according to the following schedule: 7 and 5 d before the expected date of calving and within 24 h after calving.

**Samplings**

Duplicate quarter milk samples (QMS) were taken from all of the lactating cows within 7 d of drying off. After calving, QMS were taken according to the following schedule: within 24 h of calving, 5 to 7 d of calving, 12 to 15 d of calving, 18 to 21 d of calving, 27 to 30 d of calving, and 55 to 60 d of calving.

**Microbiological Assay**

For each sampling, 0.01 ml of milk from the four quarters was separately cultured on a single 5% blood-agar plate. Bacteria were presumptively identified on the basis of colony morphology, pigmentation and hemolysis, Gram-staining, catalase reaction, and a 24-h tube coagulase test. Strains other than *Staph. aureus* were identified by a standardized panel of biochemical tests (API system; Bio-Merieux, Florence, Italy).

A quarter was defined as infected when no more than two types of bacteria were found based on the following criteria: one or more colonies of *Staph. aureus* or *Strep. agalactiae*; environmental bacteria (streptococci other than *Strep. agalactiae* or coliforms), coagulase-negative staphylococci, or other bacteria in pure culture or with more than 1000 CFU/ml.

**Data Handling and Analysis**

The analysis of an association between treatment and IMI prevalence was estimated by prevalence ratios (PR) by means of calculation of relative risk as in a cohort study (15); significance was assessed by a chi-square test [PROC FREQ; (14)]. This approach allows the evaluation of the association of overall data, stratified for sampling, and verifies the homogeneity of PR within samplings.

To assess infection status during the first 2 mo of lactation and the influence of treatment, a different approach was applied. Data were analyzed by survival analysis methods by PROC LIFETEST and PHREG (22), considering as censored (observations that did not
experience the outcome) quarters that remained Staph. aureus negative for the whole follow-up period (60 d). Once Staph. aureus IMI was detected, the quarter was not considered further. Hazard was estimated by a model including treatment, herd, and age. Hazard was defined as the number of IMI per day per 100 cows (1); relative risks caused by treatment, herd, and age were also estimated using the same model.

RESULTS

Complete and incomplete blocks were included; some blocks were not completed mostly because the treatments could not be administered according to the protocol (early parturition, antibiotic treatment during lactation), this fact imbalanced the number of cows in the two experimental groups. At the end of the trial, 106 cows were sampled six times (55 cows from herd A and 51 cows from herd B) and were included in the final analysis for a total of 2544 QMS (1416 quarters from the BRM group and 1128 quarters from the PLC group); the number of samples taken from primiparous cows totaled 936, and those from pluriparous cows totaled 1608. The overall quarter prevalence of Staph. aureus IMI at drying off was 29.61% (17.98% in herd A and 11.63% in herd B); the BRM group showed a prevalence of 32.29%, and the PLC group showed a prevalence of 25.00%. The difference was not statistically significant ($\chi^2 = 0.902; P = 0.342$).

The SCC data in the BRM and PLC treated cows, classified by bacteriological status, are reported in Figure 1. Somatic cell counts were $>1 \times 10^6$ at first sampling (postcolostral phase), then the concentrations decreased to values close to $1 \times 10^5$ SCC/ml in bacteriologically negative quarters; values observed for Staph. aureus infected quarters ranged from 0.80 to $3.1 \times 10^5$ SCC/ml in the BRM group and 0.90 to $4.7 \times 10^5$ SCC/ml in the PLC group. The two experimental groups did not statistically differ.

Prevalence of IMI After Calving

The distribution of bacteriological findings is reported in Table 1; 25.10% of the samples were bacteriologically positive in the PLC group, and 23.16% were positive in the BRM group. Staphylococcus aureus was recovered in 11.44 and 6.00% of the samples in the PLC and BRM groups, respectively.

When data were analyzed by herd, the IMI prevalence was lower in BRM-treated cows from herd A (16.37%) than in the corresponding group in herd B (29.30%), and IMI prevalence in the PLC group was 22.99 and 27.92% in herds A and B, respectively. However, the prevalence of Staph. aureus IMI was lower in the BRM group than in the PLC group in both herds. The frequency of Staph. aureus IMI in the BRM-treated group was 4.61% in herd A and 7.26% in herd B, and that in the PLC group was 12.81 and 9.58%, respectively, for the two herds (Table 2).

When data were analyzed by age (number of calvings), the prevalence of infections showed some differences. Primiparous cows in the BRM group had an IMI prevalence of 25.62% versus 22.80% in the PLC group, and pluriparous cows showed prevalences of 21.91 and 26.64%, respectively. The pattern observed for Staph. aureus IMI was different for primiparous cows; the frequency was 3.13% for the BRM group, and the PLC group had a frequency of 5.92%. The corresponding BRM and PLC figures for pluriparous cows were 7.48 and 15.18%, respectively (Table 3).

The analysis of an association between treatment and IMI prevalence, estimated by the calculation of odds ratios and chi-square test, is reported in Table 4. Overall, BRM-treated cows had a 50% less probability of Staph. aureus IMI than cows in PLC group ($PR = 0.5; \chi^2 = 0.001$). Depending on the sampling time, the PR was in the range from 0.2 to 0.9; for samplings taken at 28 and 60 d; significant associations could not be detected.

When data were analyzed by herd, BRM treatment was associated with a significantly lower prevalence of Staph. aureus IMI in herd A (PR range, 0.1 to 0.5), but samples taken at 21 and 28 d were not significant. The PR range for herd B was between 0.4 and 0.5; the only anomaly was the fifth sampling (28 d), which showed a value of $>1$ (higher probability of Staph. aureus IMI).
Table 1. Distribution of bacteria recovered from quarter milk samples taken at six different times after calving in cows treated with a biological response modifier or a placebo.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Biological response modifier group (%)</th>
<th>Placebo group (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 1 d 7 d 14 d 21 d 28 d 60 d Overall</td>
<td>n 1 d 7 d 14 d 21 d 28 d 60 d Overall</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>85 1.27 4.66 3.39 5.51 12.71 8.47 6.00</td>
<td>129 6.38 12.23 10.11 11.70 15.43 12.77 11.44</td>
</tr>
<tr>
<td>Environmental streptococci</td>
<td>34 2.81 2.96 0.84 2.12 2.96 1.70 2.39</td>
<td>26 1.59 1.59 1.06 5.22 2.12 2.13 2.30</td>
</tr>
<tr>
<td>Coliforms</td>
<td>6 1.27 0 0.85 0.42 0 0 0.42</td>
<td>8 1.59 0.53 0.53 0 1.06 0.71</td>
</tr>
<tr>
<td>Other</td>
<td>23 3.81 1.27 0.85 2.54 0.42 0.85 1.62</td>
<td>10 1.06 2.13 0 2.13 0 0 0.89</td>
</tr>
<tr>
<td>Missing</td>
<td>8 0.42 0.42 0.42 0.85 0.85 0.42 0.56</td>
<td>21 2.13 2.13 2.13 1.06 1.06 1.86</td>
</tr>
<tr>
<td>Negative</td>
<td>1080 77.13 74.16 81.79 77.98 68.65 77.98 76.28</td>
<td>824 74.48 71.81 76.06 70.75 72.88 72.34 73.04</td>
</tr>
</tbody>
</table>

1Coagulase-negative staphylococci.

In the BRM-treated group than in the PLC group), but the result was not significant. The PR observed within strata were not statistically different.

Primiparous cows had a PR in the range from 0.3 to 0.8, but in none of the samplings could a significant association be observed. However, the overall PR was 0.6, close to the significance level of 0.05 (P = 0.058). Pluriparous cows had a PR in the range from 0.1 to 0.4 (P < 0.05) in the first three samplings; in the last two samplings, the PR was higher and nonsignificant. The PR observed within strata were not statistically different.

Dynamic of Hazards and Risk Ratios

The dynamic of the hazards of a Staph. aureus IMI in BRM-treated and PLC-treated groups is reported in Figure 2. The hazard for the BRM group was significantly lower than that for the PLC group. Only in the last sampling (60 d) were the two curves closer at a level between 0.4 and 0.5 IMI/100 cows per d. The hazard curves stratified by herd showed similar results, including a higher level in PLC groups (Figure 3). The curves for these latter groups were different, and the curves for BRM-treated groups in both herds were nearly the same up to the fifth sampling (28 d). When data were stratified by age, the BRM group was confirmed to have less risk compared with the PLC group for the whole period (Figure 4). The model applied to estimate hazards showed that treatment and age had a significant influence on the regression curve (P < 0.0001). By the same model, risk ratios were estimated, and BRM treatment showed a value of 0.47; age showed a value of 2.48. Therefore, BRM-treated cows had less than half the probability of having a Staph. aureus IMI, and, for pluriparous cows, the risk was more than twice as high as that for primiparous cows.

Discussion

There are many studies on the application of different BRM to potentiate dry cow therapy or to increase resistance to IMI after calving, and those applications were reviewed previously (3, 8). The results obtained from
those studies were promising, but some side effects were also reported (11). In this study, the results showed that the number of *Staphylococcus aureus* IMI after calving was significantly reduced in the BRM-treated group. A similar reduction was observed in a recent study investigating the use of interleukin-2 in addition to dry cow treatment (10). The analysis of hazard curves, which considered only new IMI, confirmed that the quarters of cows in the BRM-treated group had significantly fewer cases of new IMI for the first 21 d. At 28 d, *Staph. aureus* IMI had the highest prevalence within the follow-up period, as a result of increased bacterial shedding. Even at this time, the number of new IMI in the BRM-treated group was significantly less than that in the PLC group. The decrease of IMI observed at 60 d in comparison with 28 d could be explained by a limited self-cure, caused by a balance between host and bacteria, or by a decrease of sensitivity caused by the dilution of bacteria in milk as cows approached peak lactation.

When data were stratified by herd, BRM treatment was associated with a reduction in *Staph. aureus* IMI, but this reduction was significant only for herd A. Therefore, a herd effect could have occurred, but the analysis of new IMI by the survival analysis method did not show significant differences. Indeed, the hazard curves in both herds were nearly the same from 7 to 28 d. Large differences could be observed in control groups.

Primiparous cows had fewer *Staph. aureus* IMI than pluriparous cows. Indeed, primiparous cows in the PLC group had a hazard level as high as that for pluriparous cows in the BRM-treated group for the first 28 d. The curve for BRM-treated primiparous cows was far below 0.1 for the first 21 d, suggesting that if heifer resistance were enhanced, the risk of new IMI could be largely reduced.

The reduction of IMI after calving could be the result of an enhancement of dry cow therapy, or a result of the increase of resistance to infections. However, the prevalence of IMI in the BRM-treated group was less than half that in the PLC group at 21 d, supporting the hypothesis of increased resistance rather than an enhancement of antibiotic efficacy. This is also supported by the absence of an increase in SCC; therefore, a bias due to high SCC effect on *Staph. aureus* recovery can be excluded. Actually, the increase in resistance is an explanation for the observed reduction in clinical signs and virus shedding after challenge with IBR viruses in BRM-treated calves (16). Indeed, the parapox virus used as BRM was previously shown to induce the release of different immune components, particularly interferon-α and interleukin-2 (17, 19, 23).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Biological response modifier group (%)</th>
<th>Placebo group (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 1 d 7 d 14 d 21 d 28 d 60 d Overall</td>
<td>n 1 d 7 d 14 d 21 d 28 d 60 d Overall</td>
</tr>
<tr>
<td><strong>Primiparous cows</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>15 1.25 1.25 1.25 3.75 7.50 3.75 3.13</td>
<td>27 0 5.26 3.95 6.58 10.53 9.21 5.92</td>
</tr>
<tr>
<td>Other IMI</td>
<td>98 27.50 27.50 16.25 22.50 21.25 17.50 22.49</td>
<td>77 25.00 11.84 13.15 18.42 13.15 19.74 16.88</td>
</tr>
<tr>
<td>Missing</td>
<td>0 0 0 0 0 0 0 0</td>
<td>4 1.32 1.32 1.32 1.32 0 0 0.88</td>
</tr>
<tr>
<td>Negative</td>
<td>357 70.00 70.00 82.5 73.75 71.25 78.75 74.38</td>
<td>348 73.68 81.58 81.58 73.68 76.32 71.05 76.32</td>
</tr>
<tr>
<td><strong>Pluriparous cows</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>70 1.28 6.41 4.49 6.41 15.38 10.90 7.48</td>
<td>102 10.71 16.96 14.29 15.18 18.75 15.18 15.18</td>
</tr>
<tr>
<td>Other IMI</td>
<td>135 17.31 16.67 13.46 12.18 16.03 10.90 14.43</td>
<td>77 11.61 15.18 10.71 13.39 8.03 9.82 11.46</td>
</tr>
<tr>
<td>Missing</td>
<td>8 0.64 0.64 0.64 1.28 1.28 0.64 0.85</td>
<td>17 2.68 2.68 2.68 2.68 2.68 1.79 2.53</td>
</tr>
<tr>
<td>Negative</td>
<td>723 80.77 76.28 81.41 80.13 67.31 77.56 77.24</td>
<td>476 75.00 65.18 72.32 68.75 70.54 73.21 70.83</td>
</tr>
</tbody>
</table>

1Not estimated.
Figure 2. Dynamic of estimated hazards (± SE) for *Staphylococcus aureus* IMI in cows treated with a biological response modifier (●) or a placebo (○).

The differences observed within age support the hypothesis of an enhancement of immune defenses. Indeed, BRM-treated cows in both herds had the same hazard curves, although the infection pattern and the management were different. Moreover, primiparous cows, when treated with the BRM showed a hazard curve close to 0 for the first 3 wk of lactation. Because no antibiotic had been administered to primiparous cows, the observed reduction in hazard could be only the consequence of increased resistance. The increase of prevalence and hazard ratio observed after 3 wk in the BRM-treated group could be the result of the transmission of the infection during milking or through other sources (20). *Staphylococcus aureus* positive cows were neither segregated nor treated and, therefore, when BRM activity declined, cows became more and more susceptible to infections.

As for any biological outcome, the underlying mechanism should be demonstrated. In this trial, an evaluation on which immune mechanism reduced the susceptibility, directly or indirectly, elicited by BRM treatment is still in progress (26). Previous studies showed that parapox viruses increased lymphocyte and NK cell proliferation (19) and the production of interleukin-2 and interferon-α. Therefore, it can be hypothesized that the BRM treatment could directly modulate the activity of lymphocytes or indirectly enhance nonspecific humoral and cellular immune defenses through cytokine activity (10).

**CONCLUSIONS**

Treatment with an interferon inducer based on a parapox virus significantly reduced prevalence of *Staphylococcus aureus* IMI when administered around calving. The effects of the treatment lasted for 3 wk, as demonstrated by the reduced number of IMI compared with controls. This reduction very likely was due to an enhancement of resistance to IMI. Therefore, the application of a BRM, which decreases the infection risk, could be helpful in *Staphylococcus aureus* control programs, particularly in reducing the incidence of IMI in primiparous cows.

**REFERENCES**

INTERFERON INDUCER TO PREVENT INFECTIONS


