Local Vaccination with Killed *Streptococcus uberis* Protects the Bovine Mammary Gland against Experimental Intramammary Challenge with the Homologous Strain

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The ability of killed *Streptococcus uberis* to induce protection against mastitis when administered either into the cistern of the dry mammary gland (intramammary vaccination) without adjuvant or subcutaneously with adjuvant was investigated. Bacteria were never reisolated from vaccinated quarters following challenge with the same strain during the subsequent lactation, and no inflammatory response was detected. In contrast, following subcutaneous vaccination, milk from challenged quarters contained very small numbers of bacteria, but these quarters did exhibit clinical disease, whereas quarters on nonvaccinated control animals produced discolored, clotted secretion with large numbers of bacteria and somatic cells and required antibiotic therapy by 60 h postchallenge. There was a significant increase in the levels of *S. uberis*-specific immunoglobulin G1 (IgG1), IgG2, and IgM in milk following intramammary vaccination and in the levels of specific IgG1 and IgG2 in milk following subcutaneous vaccination. Levels of specific antibody in serum were also elevated following vaccination by either route. However, despite this, there was no increase in the opsonic activity of serum or milk. Both peripheral blood lymphocytes and dry-period mammary gland lymphocytes showed strong proliferative responses to *S. uberis* in vitro following subcutaneous vaccination, but only mammary gland lymphocytes responded following intramammary vaccination. It was concluded that the protection seen in vaccinated quarters did not appear to be related to levels of specific antibody or neutrophil function and was possibly brought about by the inhibition of bacterial growth.

The failure of existing control measures to significantly reduce new intramammary infections by *Streptococcus uberis* has emphasized the need to investigate an alternative approach. The infiltration of polymorphonuclear leukocytes (PMN) into the mammary gland following bacterial infection has long been recognized as a primary protective mechanism against mastitis caused by coliforms (8, 10) and staphylococci (15); therefore, most attempts to protect ruminants from mastitis by vaccination have focused on optimizing the level of opsonic antibody in milk and potentiating the speed of PMN recruitment into the mammary gland (1, 14, 16). In contrast, recent observations have indicated that the elevation of opsonin levels in milk does not appear to be an appropriate strategy for the control of intramammary infections caused by *S. uberis*. Subcutaneous administration of live *S. uberis* at drying off and at calving was shown to dramatically reduce both the incidence of clinical mastitis and numbers of bacteria recovered from the milk following challenge with the same strain during the next lactation (5). There was no indication that the modification of the disease pathogenesis that was attained was mediated by local inflammation or by infiltration of PMN into the gland. Furthermore, although levels of *S. uberis*-specific immunoglobulin G2 (IgG2) were elevated following vaccination, no opsonic activity could be detected in serum or milk.

Strains of *S. uberis* have been shown to vary in their ability to produce mastitis in dry and lactating cows (4), but there is no indication at present that avirulent strains are available for use as locally administered vaccines. In the absence of knowledge of the protective antigen(s) of the live bacteria, the efficacy of whole killed bacteria administered via the subcutaneous or intramammary route to induce resistance to homologous challenge was examined and the humoral and cellular immune responses in both blood and the mammary gland were assessed.

**MATERIALS AND METHODS**

**Animals.** Twelve Friesian cattle from the Institute dairy herd were used in the trial. All were at the end of their first or second lactation and had no history of clinical *S. uberis* mastitis. They remained with the herd throughout the trial, except for the 7- to 9-day period following intramammary bacterial challenge, when they were housed and milked separately.

**Bacteria.** *S. uberis* 0140J (originally isolated from a cow with bovine mastitis) was used throughout the experiment. The organism was stored in Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom) at -20°C in the presence of 25% glycerol. For the inoculation of defined medium or infection of cattle, bacteria were grown in Todd-Hewitt broth at 37°C for 18 h.

**Vaccine preparations.** Bacteria were grown in a chemically defined liquid medium supplemented with 1% (wt/vol) casein hydrolysate as previously described (11). The cultures were incubated for 18 h at 37°C, harvested by centrifugation at 10,000 × g for 15 min, and washed twice in phosphate-buffered saline (PBS; pH 7.2). Bacterial cells at a concentration of 10⁹/ml were then killed by either heat (i.e., boiled for 5 min, held at room temperature for 5 h, and boiled again) or formalin inactivation (i.e., held at 4°C for 18 h in the presence of formalin [final concentration of 1% formaldehyde] and then washed four times in PBS). Both vaccine preparations were

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adjusted to a bacterial cell concentrations of 10^{10}/ml and stored at 4°C in 1.0-ml aliquots until required.

**Vaccination regimes.** The 12 cows were randomly divided into three groups of 4 animals. One group contained controls, which received no vaccinations prior to intramammary bacterial challenge. Cows in the second group were vaccinated via the intramammary route; two cows received 1.0 ml of the heat-inactivated vaccine, and the other two received 1.0 ml of the formalin-inactivated preparation. Each animal was given six weekly doses of the appropriate vaccine made up in 3.0 ml of PBS into both left mammary quarters starting immediately after the last milking prior to drying off. Cows in the third group were vaccinated subcutaneously; again, two cows received 1.0 ml of the heat-inactivated vaccine and the other two received 1.0 ml of the formalin-inactivated preparation. Each subcutaneous dose (1.0 ml of bacterial suspension) was emulsified in 1.0 ml of incomplete Freund's adjuvant—2.0 ml of 1% (wt/vol) Tween 20 prior to injection. The number of doses and timing were the same as for the cows receiving intramammary vaccinations.

**Measurement of specific antibody by ELISA.** The levels of specific antibody were determined by enzyme-linked immunosorbent assay (ELISA) as previously described by Hill et al. (5), except that the microtiter plates were pretreated for 1 h with 100 μl of 0.025% glutaraldehyde diluted in 0.05 M carbonate buffer (pH 9.6) and coated overnight with live *S. uberis* at a concentration of 10^{5} CFU per well in 100 μl. Antibody titers were determined to be the highest dilution of serum or milk that gave an optical density reading which was 1.5 times that of the negative control sample.

**Measurement of the opsonic capacity of serum and milk.** The ability of isolated peripheral PMN to kill *S. uberis* when suspended in either milk or serum was used to assess opsonic activity by the method previously described (13). Briefly, bacteria were mixed with the appropriate samples of serum or milk for 90 min at 37°C in either the presence or absence of PMN. The numbers of viable bacteria were then determined following overnight culture on blood agar containing 1% (wt/vol) esculin.

**Isolation of lymphocytes from blood and mammary secretions.** Heparinized blood samples were collected prior to vaccination and before the third administration of vaccine. Mononuclear cells were isolated by density gradient centrifugation (19). The cells were resuspended to a final concentration of 2 × 10^3/ml in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum.

Mammary secretions were collected from all four quarters of each cow in the mid-dry period, prior to the third administration of vaccine. The secretions were diluted in 3 volumes of warm (37°C) PBS and centrifuged at 500 × g for 10 min at 20°C. The resulting cell pellets were pooled and washed three times in warm PBS, and lymphocytes were isolated by density gradient centrifugation followed by separation on a Sephadex G-10 column as described by Collins and Oldham (2) and resuspended to 2 × 10^3/ml of RPMI 1640 medium.

**Quantification of bacteria in milk.** Triplicate cultures were established in 96-well, round-bottom microtiter trays. Cells (100 μl) were cultured in the presence of an equal volume of killed *S. uberis* bacteria containing approximately 2 × 10^6 bacteria per ml. Control cultures received an equal volume of RPMI 1640 medium alone or phytohemagglutinin (5 μg/ml; Wellcome Diagnostics), which provided a positive control for the assay. Cultures were maintained at 37°C in 5% CO_2 in air for 5 days, pulsed with tritiated thymidine (1 μCi of [3H]Tdr per well; Amersham), and harvested onto fiberglass discs as described previously (5). Thymidine incorporation was measured by liquid scintillation counting, and results were expressed as counts per minute.

**Intramammary bacterial challenge.** All animals were challenged 2 to 6 weeks into lactation by the infusion of 1 ml of skimmed milk (Oxoid) containing approximately 300 CFU of *S. uberis* 0140J into two mammary quarters (left fore and right hind) immediately after an afternoon milking. Quarter milk samples were collected aseptically at each milking for bacteriological and cytological testing, and the appearance of the milk, the condition of the udder, and the animal's general state of health were recorded. Quarters producing milk with a high bacterial count (>10^5/ml) during the previous day and clinical signs throughout the drawing of 25 ml of fore-milk were given a course of antibiotic therapy (Orbenin QR; SmithKline Beecham) at the end of that milking, and regular sampling was discontinued. The experiment continued for 12 milkings postchallenge, after which antibiotic was administered to all quarters known to be bacteriologically positive and not previously treated.

**Counting of somatic cells in milk.** Milk samples were fixed with Somafix (Coulter Electronics), and the somatic cells were counted electronically by using a Model F4 counter (Coulter Electronics). Results were expressed as the log_{10} somatic cells per milliliter of milk.

**Bacteriological examination of milk.** Bacterial numbers were determined by diluting the milk samples in physiological saline (0.9% [wt/vol] NaCl), plating them onto nutrient agar (Oxoid) supplemented with 5.0% (vol/vol) washed bovine erythrocytes and 0.1% (wt/vol) esculin, and incubating them for 18 h at 37°C. The number of colonies present was counted, and results are expressed as the log_{10} bacteria per milliliter of milk.

**Statistical analysis.** Student's *t* tests were performed on the geometric means of the logarithmically transformed data and on the arithmetic means of untransformed data by using the statistical package from Minitab Inc. Means were considered to be significantly different when *P* < 0.05.

**RESULTS**

**Bacterial recovery from milk and clinical signs following experimental intramammary challenge.** Nonvaccinated control animals were found to have large numbers of bacteria in the first milk samples collected after challenge (Fig. 1), although at this stage no clinical signs were seen. Bacterial numbers increased at each milking, with the appearance of discoloration, clots, and edema by the second or third milking. All control quarters had received antibiotic therapy by the fifth milking postchallenge. In contrast, quarters given intramammary vaccination were never bacteriologically positive following challenge (Fig. 1) and showed no clinical signs throughout the experiment. With the exception of the first milking, all challenged quarters on animals which had received subcutaneous vaccinations displayed small or undetectable numbers of bacteria (Fig. 1), yet these were often associated with clotted and decolored milk. Because of the low bacteriological status of the milk, antibiotic therapy was not administered to these animals until the end of the experimental period. The method of fixation of the bacteria (heat or formalin inactivated) did not affect the level of protection seen (data not shown).

**Somatic cell counts in milk following experimental bacterial challenge.** The mean somatic cell count in milk from challenged quarters of nonvaccinated control animals showed a logarithmic increase up to the fourth milking, after which therapy was administered (Fig. 1). In contrast, the challenged...
quarters which had received intramammary vaccinations showed only a small elevation in mean cell count. The challenged quarters of animals which were vaccinated subcutaneously all showed a rapid elevation in mean cell count which was greater than or equal to that in the control group for the two milkings immediately after challenge (Fig. 1).

**Effect of vaccination of *S. uberis*-specific antibody titers.** The mean levels of specific antibody in the serum and milk of the control animals were low prior to experimental challenge. In contrast, despite the considerable cow-to-cow variation, mean levels of specific IgG1, IgG2, and IgM were significantly elevated in the milk from quarters given intramammary vaccination as determined prior to challenge (Fig. 2A) compared with the levels before vaccination (*P* < 0.028) or with levels in the control cows (*P* < 0.004). Only *S. uberis*-specific IgG1 and IgG2 levels were significantly elevated in milk following subcutaneous vaccination (Fig. 2B) when compared with prevaccination levels (*P* < 0.05) or levels in the control animals (*P* < 0.01). The only antibody response which appeared to show any correlation with protection was the small but significant increase in *S. uberis*-specific IgM level present in milk following intramammary vaccination. Mean levels of specific antibody of all three isotypes in serum increased following intramammary or subcutaneous vaccination (Fig. 3), but the considerable cow-to-cow variation resulted in these increases not being statistically significant from prevaccination levels or from those of control animals (*P* > 0.06).

**Opsonic activity in milk and serum.** Despite the increased levels of specific antibody, especially IgG2, induced in both milk and serum by intramammary and subcutaneous administration of killed bacteria, no increase in opsonic activity was detected in any of the sera or quarter milk samples tested (data not shown). This observation supports the clinical data suggesting that the protection seen following intramammary vaccination did not appear to be achieved by increased phagocytic uptake promoted by enhanced opsonic activity.

**Effect of vaccination on the proliferative response of lymphocytes.** Both mammary gland lymphocytes (MGLs) and peripheral blood lymphocytes (PBLs) showed strong proliferative responses to *S. uberis* in vitro (Table 1). Intramammary vaccination induced positive responses in MGLs but not in PBLs. Following subcutaneous vaccination, positive responses were obtained from both MGLs and PBLs. These responses were detectable at a final concentration of 10^2 bacteria per ml, were maximal at 10^6/ml, and were not a result of nonspecific mitogenic stimulation as indicated in duplicate 3-day cultures (data not shown). By comparison, MGLs from both groups of animals were hyporesponsive to stimulation by phytohemagglutinin when compared with PBLs, and these results demonstrate that, although being hyporesponsive to conventional mitogenic stimulation, MGLs are capable of responding to specific antigens and that vaccination with *S. uberis* via the intramammary or subcutaneous route is capable of inducing primed lymphocyte populations within the dry gland. Control animals had not been assigned at this time; therefore, no data on lymphocyte response were available.

**DISCUSSION**

The data presented in this communication clearly show that protection of the bovine mammary gland against infection by *S. uberis* was achieved by multiple intramammary vaccinations with whole killed bacterial cells. Bacteria were never isolated from the milk from these quarters, and no clinical signs were observed. In contrast, clinical mastitis did develop in challenged quarters of animals vaccinated subcutaneously, but the mean bacterial recoveries from these quarters were approxi-
isotypes known to capable of opsonizing *Escherichia coli* (1, 7) and *Staphylococcus aureus* (3) for phagocytosis by bovine neutrophils, there was no evidence of any change in this property following vaccination with *S. uberis* by either route. These data support earlier findings that live bacteria administered subcutaneously modified the pathogenesis of mastitis caused by *S. uberis* in the absence of enhanced opsonic activity (5). Subsequent experiments also demonstrated that *S. uberis* can resist the bactericidal activity of neutrophils despite the presence of Ig bound to the surface of the bacteria (12) and suggested that, unlike the situation for other mastitis-causing pathogens, the protection against mastitis caused by *S. uberis* is not achieved simply by the elevation of specific antibacterial antibody. The potential role of locally produced IgA in protection against *S. uberis* following local vaccination is not known, since we have been unable to detect specific IgA in milk by using anti-isotypic reagents that are available. The potential involvement of primed MGLs in the protection against *S. uberis* cannot be ruled out. Positive responses were seen in cells collected at the mid-dry period, at a time when the cell populations in the gland are stable and present in sufficient numbers to isolate and assay. However, the inability to obtain cells from secretions of control, nonvaccinated animals means that the contribution of these cells and the possible protective role of IgM in the absence of opsonic activity require further investigation.

The clinical disease in the animals vaccinated subcutaneously appears to be the result of a rapid and intense inflammatory response as indicated by large numbers of somatic cells in the milk. It is possible that this reflects the immune status of a quarter in which vaccination resulted in an acquired mammary hypersensitivity. Increased speed of neutrophil recruitment into the mammary gland following vaccination of ruminants with *E. coli* (14) and staphylococci (1) has been reported previously. In addition, milk has been found to contain antibodies which are opsonic for *E. coli* (6, 7) and *Staphylococcus aureus* (16); therefore, the enhanced inflammatory response was considered to be a benefit of such a vaccination regime. In contrast, the data presented in this report suggest that inflammation is a disadvantage when vaccinating cattle against *S. uberis* mastitis. This effect may have been mediated by local lymphocyte populations since both subcutaneous and intramammary administration resulted in *S. uberis*-specific sensitization of these cells; however, the identity of the lymphocyte subpopulations present in these cultures is not known. Alternatively, the effect could be mediated by the presence of antigen-antibody complexes within the quarter following infec-

![Figure 3](http://iai.asm.org/)  
**FIG. 3.** Effect of route of administration of killed bacteria on the mean levels of *S. uberis*-specific IgG1, IgG2, and IgM in serum for four cows given intramammary vaccination and three cows given subcutaneous vaccination. Symbols: ☐, prevaccination; ■, postvaccination.

mately 10,000-fold lower than the corresponding samples from control (nonvaccinated) animals.

The reduction in bacterial numbers isolated from milk following intramammary or subcutaneous vaccination did not appear to correlate with increased levels of *S. uberis*-specific IgG1 and IgG2 in milk, since these were induced to a similar extent by both routes of vaccination. However, the level of *S. uberis*-specific IgM was significantly elevated in milk from quarters given intramammary vaccination and was not raised by subcutaneous vaccination. Despite the increased levels of IgG and IgM, these data support the hypothesis that *S. uberis* can resist bactericidal activity of neutrophils despite the presence of Ig bound to the surface of the bacteria (12) and suggested that, unlike the situation for other mastitis-causing pathogens, the protection against mastitis caused by *S. uberis* is not achieved simply by the elevation of specific antibacterial antibody. The potential role of locally produced IgA in protection against *S. uberis* following local vaccination is not known, since we have been unable to detect specific IgA in milk by using anti-isotypic reagents that are available. The potential involvement of primed MGLs in the protection against *S. uberis* cannot be ruled out. Positive responses were seen in cells collected at the mid-dry period, at a time when the cell populations in the gland are stable and present in sufficient numbers to isolate and assay. However, the inability to obtain cells from secretions of control, nonvaccinated animals means that the contribution of these cells and the possible protective role of IgM in the absence of opsonic activity require further investigation.

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### TABLE 1. Mean proliferative response of PBLs and MGLs to *S. uberis* antigen in vitro, following vaccination by the intramammary or subcutaneous route

<table>
<thead>
<tr>
<th>Route</th>
<th>Stimulus</th>
<th>PBL1 (Mean, SD)</th>
<th>PBL2 (Mean, SD)</th>
<th>MGLs (Mean, SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intramammary</td>
<td>Control</td>
<td>315 (158)</td>
<td>248 (133)</td>
<td>1,882 (1,751)</td>
</tr>
<tr>
<td></td>
<td><em>S. uberis</em></td>
<td>1,450 (960)</td>
<td>1,330 (1,139)</td>
<td>34,702 (15,567)</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>77,011 (52,177)</td>
<td>103,688 (79,402)</td>
<td>59,378 (60,751)</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>Control</td>
<td>1,113 (916)</td>
<td>1,348 (1,610)</td>
<td>1,517 (1,751)</td>
</tr>
<tr>
<td></td>
<td><em>S. uberis</em></td>
<td>3,292 (989)</td>
<td>23,817 (20,801)</td>
<td>40,969 (19,969)</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>291,978 (3,573)</td>
<td>162,863 (99,492)</td>
<td>17,405 (15,259)</td>
</tr>
</tbody>
</table>

* PBL1 were collected prior to vaccination, and PBL2 and MGLs were collected during the mid-dry period, immediately before the third vaccination. Killed *S. uberis* cells were added to cultures to give a final concentration of 10⁶/ml.

* Values represent the mean counts per minute for triplicate cultures from the four animals in each group.

* PHA, phytohemagglutinin.

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tion. However, this enhanced inflammatory response did not occur in the fully protected quarters, from which bacteria were never reisolated, suggesting that bacterial growth within the gland may be required to generate sufficient antigen to initiate the response, which may then be maintained by the degranulation of inflammatory cells (9).

In conclusion, multiple intramammary vaccinations with whole killed S. uberis cells resulted in the complete protection of the bovine mammary gland from experimental infection with the same organism. Bacteria were never reisolated from these quarters, even at the first milking postchallenge, and protection was achieved in the absence of a marked neutrophil response or elevated levels of opsonic antibody. It is therefore tempting to speculate that the protective effect was as a result of the inhibition of the growth of S. uberis in vivo, and this is currently being investigated.

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REFERENCES


