Leukocytic Infiltration of Bovine Mammary Parenchymal Tissue in Response to Corynebacterium bovis Colonization

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ABSTRACT

Morphological changes and local leukocyte response to prolonged Corynebacterium bovis colonization were studied in lactating bovine mammary glands. Morphometric analysis of parenchymal tissue demonstrated no adverse effects of colonization on the synthetic and secretory activity of mammary epithelium. Numbers of macrophages, lymphocytes, and plasma cells were higher in tissue from C. bovis-colonized quarters. However, there were no differences in numbers of neutrophils between colonized and uninfected quarters. Results suggest persistent C. bovis colonization may elicit effector cell populations in lactating mammary tissue where leukocyte concentrations tend to be lower.

INTRODUCTION

Corynebacterium bovis was the organism isolated most frequently in milk samples when routine teat disinfection and antibiotic therapy were not practiced (3). Previous studies revealed that C. bovis was extremely contagious during lactation, and colonization persisted for long periods (4, 15). Corynebacterium bovis was considered a minor mammary gland pathogen because it provoked only a mild inflammatory response (4) and did not affect adversely milk yield or secretion composition (11). Several studies examined the possible role of these commensal organisms in the control of intramammary infections caused by more pathogenic mastitis-causing organisms. Results from field trials suggested that quarters infected naturally with C. bovis may be more resistant to some major mastitis pathogens than uninfected quarters (3). Others demonstrated that quarters colonized with C. bovis were more resistant to experimental Staphylococcus aureus and Streptococcus dysgalactiae infection (12, 16) but not resistant to experimental Strep. agalactiae infection (5, 16).

Several natural biological control mechanisms were hypothesized to explain enhanced resistance of C. bovis colonized quarters to pathogenic mastitis-causing organisms. Previous studies speculated that increased resistance resulted from competitive growth inhibition, bacterial antagonism, or induced leukocytosis (2, 4, 5, 12). Increased immunity of the host was offered as another mechanism of resistance, but without extensive detail (2). Sordillo et al. (18) reported the effects of C. bovis colonization on the mammary gland humoral immune response. It was suggested that enhanced numbers of Ig-producing plasma cells in the teat end mucosa of C. bovis-colonized quarters may be a source of resistance during bacterial invasion (18). Objectives of this study were to examine the local leukocytic response of bovine mammary glands to prolonged C. bovis colonization and to clarify effects on mammary parenchymal tissue.

MATERIALS AND METHODS

Experimental Design

Eight lactating Holstein-Friesian cows were used. Duplicate quarter foremilk samples were collected at 48 and 24 h prior to slaughter for microbiological analyses. Mammary tissue from five C. bovis-colonized quarters and five
uninfected quarters was obtained at slaughter and processed for histological and ultrastructural examination. Data were analyzed by least squares analysis of variance using the General Linear Model procedure (SAS Institute, Cary, NC) to determine effect of infection status on morphological parameters.

**Microbiological Procedures**

Procedures for the isolation and identification of mastitis pathogens from milk were as described by the National Mastitis Council (6). Briefly, a .01-ml aliquot of milk sample was plated onto brain-heart infusion (BHI) agar supplemented with 5% defibrinated sheep blood, 1% yeast extract, and 1% horse serum. Plates were incubated for 48 h at 37°C and examined for bacterial growth. Isolates were counted and identified based on growth characteristics, colony morphology, hemolytic characteristics, and biochemical tests. *Corynebacterium bovis* isolates were identified initially as gram-positive, catalase-positive, and urea-positive organisms. Subcultures of these organisms onto BHI and BHI Tween 80 agar (Difco Laboratories, MI) differentiated *C. bovis* from other *Corynebacterium*. *Corynebacterium bovis* exhibited enhanced growth on BHI Tween 80 and was oxidase-positive at 48 h of incubation. Quarters were considered colonized when both foremilk samples collected at 48 and 24 h before slaughter contained *C. bovis*. Previous bacteriologic history of quarters showed that *C. bovis* colonization persisted from the previous lactation or occurred earlier in the present lactation (112 to 150 d in milk).

**Tissue Preparation**

Tissue samples for histological and cytological examination were obtained at necropsy and prepared for microscopy. Approximately 3 mm³ of mammary parenchymal tissue was removed from above the gland cistern and immersed in 2.5% glutaraldehyde in .1 M cacodylate buffer (pH 7 at 37°C) for 2 h. Tissue was then postfixed in .1 M cacodylate-buffered osmium tetroxide (pH 7 at 5°C) for 2 h, dehydrated in a graded series of ethanol, and embedded in epoxy resins. Thick sections (.5 to 1 μm) were stained with toluidine blue for light microscopy. Ultrathin sections approximately 60-nm thick were stained with 5% uranyl acetate in 50% methanol for 20 min followed by .4% lead citrate for 20 min and examined using a Philips 100 transmission electron microscope (Philips Export, Netherlands) at 60 kV.

**Morphometric Analysis**

Quantitative morphologic analysis was used to determine percentage tissue area composed of stroma, epithelia, and lumen. For each tissue sample, 10 replications of 100 contact points were counted per slide at a magnification of 600 x. A reference grid in the microscope ocular provided fixed points used in the counting process. Alveolar epithelium was characterized further as 1) inactive, 2) moderately active, or 3) fully active. For each tissue sample, cells were classified in alveolar cross-sections of 10 different fields at 400 x. Tissue specimens of mammary parenchyma also were examined for the presence of macrophages, lymphocytes, neutrophils, mast cells, and plasma cells. Prevalence of these cell populations was quantified morphometrically at 600 x in 10 randomly selected microscopic fields per sample.

**Ultrastructural Examination**

Epithelial cells were examined ultrastructurally for the presence of organelles including Golgi apparatus, rough endoplasmic reticulum (RER), mitochondria, and nuclei. Cytoplasmic areas also were examined for presence of degenerating junctional complexes, microvilli at apical surfaces, and accumulation of secretory vesicles and fat droplets.

**RESULTS**

Histological analysis of *C. bovis*-colonized and uninfected bovine mammary tissue is summarized in Tables 1 and 2. Tissue from *C. bovis*-colonized quarters exhibited similar morphology as uninfected control quarters. Although tissue from colonized quarters tended to have higher percentages of interalveolar stroma and more inactive secretory epithelium when compared with control quarters, these differences were not significant.

Ultrastructural analysis of mammary secretory epithelium from *C. bovis* colonized and uninfected control mammary glands is summa-
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**TABLE 1.** Histological analysis of lactating bovine mammary tissue from *Corynebacterium bovis* colonized and uninfected control quarters.

<table>
<thead>
<tr>
<th>Tissue classification</th>
<th>Infection status</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Epithelium</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Lumen</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Stroma</th>
<th>Corynebacterium bovis</th>
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<td>X</td>
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<td>X</td>
<td>SE</td>
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<td>X</td>
<td>SE</td>
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<td>38.6</td>
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<td>37.0</td>
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<td></td>
<td></td>
<td>41.6</td>
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<td>36.0</td>
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<tr>
<td>Lumen</td>
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<td>41.6</td>
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<td>36.0</td>
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<td></td>
<td></td>
<td>19.8</td>
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<td>27.0</td>
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<tr>
<td>Stroma</td>
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<td>19.8</td>
<td>2.4</td>
<td>27.0</td>
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</table>

*Data are expressed as mean percent ± SEM of total tissue area.*

**TABLE 2.** Cytological analysis of bovine mammary secretory epithelium from *Corynebacterium bovis* colonized and uninfected control quarters.

<table>
<thead>
<tr>
<th>Epithelial classification</th>
<th>Infection status</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Nonactive</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Moderately active</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Fully active</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
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<td>SE</td>
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<td>SE</td>
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<tr>
<td>Nonactive</td>
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<td>8</td>
<td>.8</td>
<td>12.0</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Moderately active</td>
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<td>42</td>
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<td>44.5</td>
<td>4.5</td>
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<td></td>
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<tr>
<td>Fully active</td>
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<td>55</td>
<td>5.3</td>
<td>44.4</td>
<td>5.3</td>
<td></td>
<td></td>
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</table>

*Data are expressed as mean percent ± SEM of total epithelial area.*

**TABLE 3.** Ultrastructural analysis of bovine mammary secretory epithelium from *Corynebacterium bovis* colonized and uninfected control quarters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Infection status</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Epithelium</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Lumen</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
<th>Stroma</th>
<th>Corynebacterium bovis</th>
<th>Control</th>
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<td>SE</td>
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<td>X</td>
<td>SE</td>
<td></td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>Nucleus</td>
<td></td>
<td>16.8</td>
<td>1.0</td>
<td>18.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td>22.4</td>
<td>2.4</td>
<td>23.4</td>
<td>2.4</td>
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<tr>
<td>Unoccupied cytoplasm</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Rough endoplasm reticulum</td>
<td></td>
<td>18.8</td>
<td>7.7</td>
<td>17.0</td>
<td>7.7</td>
<td></td>
<td></td>
<td>16.0</td>
<td>1.0</td>
<td>17.4</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td></td>
<td>9.4</td>
<td>.5</td>
<td>10.4</td>
<td>.5</td>
<td></td>
<td></td>
<td>7.6</td>
<td>.6</td>
<td>9.4</td>
<td>.6</td>
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<tr>
<td>Mitochondria</td>
<td></td>
<td>9.0</td>
<td>.7</td>
<td>7.4</td>
<td>.7</td>
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<td></td>
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</table>

*Data are expressed as mean percent ± SEM of total secretory cell area.*

Quantitative microscopic comparison of leukocytes infiltrating *C. bovis*-colonized and uninfected mammary glands is summarized in Table 4. Macrophages and lymphocytes were the most prevalent cell types observed within the epithelial lining. Numbers of leukocytes within interepithelial spaces were similar regardless of infection status of quarters. However, colonized quarters tended to have more lymphocytes infiltrating the alveolar epithelium compared with controls (Figure 1).

Significantly more macrophages were enumerated within the alveolar lumen of *C. bovis*-colonized quarters compared with controls. There were no significant differences in numbers of neutrophils between colonized and uninfected quarters. Lymphocytes were not observed in luminal areas, regardless of infection status.

Subepithelial stromal areas were the most common site of leukocyte infiltration. Significantly more macrophages, lymphocytes, and plasma cells were enumerated in *C. bovis*-colonized quarters than in controls (Figure 2). Numbers of neutrophils were similar in all quarters whereas mast cell numbers tended to be lower.
TABLE 4. Enumeration\(^1\) of infiltrating leukocytes into bovine mammary tissue from *Corynebacterium bovis* colonized and uninfected control quarters.

<table>
<thead>
<tr>
<th>Tissue area</th>
<th>Cell type</th>
<th>Control ((%))</th>
<th>(\overline{X})</th>
<th>SE</th>
<th>(Corynebacterium bovis) ((%))</th>
<th>(\overline{X})</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium</td>
<td>Macrophage 7.8</td>
<td>1.3</td>
<td>9.0</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocyte 13.2</td>
<td>1.8</td>
<td>18.6</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophil .2</td>
<td>.2</td>
<td>.4</td>
<td>.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>Macrophage .4</td>
<td>.8</td>
<td>2.8</td>
<td>.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophil .6</td>
<td>.4</td>
<td>.8</td>
<td>.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroma</td>
<td>Macrophage 5.6</td>
<td>1.7</td>
<td>13.8</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocyte 6.6</td>
<td>1.6</td>
<td>12.8</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma cells 5.6</td>
<td>1.6</td>
<td>11.4</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mast cell 9.2</td>
<td>.9</td>
<td>6.8</td>
<td>.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophil .8</td>
<td>.4</td>
<td>1.4</td>
<td>.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data are expressed as mean percent \(\pm\) SEM of total tissue area.

in colonized quarters when compared with those of uninfected controls.

DISCUSSION

Persistant colonization of bovine mammary glands with *C. bovis* did not bring about major histopathological changes in mammary parenchymal tissue. Histological and ultrastructural comparison of tissue samples from *C. bovis*-colonized quarters revealed only minor reductions in secretory activity. This was indicated by the small, but nonsignificant, increase in stromal areas and inactive secretory epithelium, which contained fewer cytoplasmic organelles associated with milk synthesis and secretion. Coryneforms were never observed attached or proximal to mammary parenchymal tissue, which suggest *C. bovis* primarily colonizes the streak canal area (2, 8, 16). In contrast, Anderson et al. (1) observed colonization and extensive pathologic alterations of mouse mammary tissue following experimentally induced mastitis with a virulent strain of *C. bovis*. They reported histopathological changes ranging from small areas of epithelial hyperplasia 1 d after *C. bovis* was inoculated by intramammary route to coagulative necrosis and extensive abscess formation 8 d after inoculation (1). However, morphological evidence provided in this study corroborates previous reports (4, 11), which found no significant effect of intramammary *C. bovis* infection on milk yield or secretion composition in bovine mammary glands. Some isolated areas of mammary tissue were occupied by involuted epithelium, but a compensatory hypertrophy of remaining lactating tissue may explain the lack of substantial decreases in milk production from severe infections.
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Figure 2. Mammary parenchymal tissue from *Corynebacterium bovis*-colonized quarters demonstrating: a) plasma cell (P) located in the subepithelial stroma (S) in close proximity to a lymphocyte (LY) and b) two plasma cells with abundant rough endoplasmic reticulum (R) involved in clasmatisation (arrows). E = Epithelium.

quarters colonized with *C. bovis*. Although some strains of *C. bovis* may cause clinical mastitis in cows (7) and be more pathogenic in mouse mammary glands when inoculated by intramammary route in large numbers (1), a preponderance of evidence suggests that *C. bovis* is generally a minor mammary gland pathogen (3, 4, 5, 11, 12) having little or no detrimental effect on bovine mammary gland structure and function.

Nickerson and Heald (13) described changes in the local cellular immune responses of bovine mammary glands from the initial to later stages of experimental *S. aureus* mastitis. They found as inflammation progressed up through d 10, numbers of macrophages, lymphocytes, and plasma cells increased whereas number of neutrophils decreased in infected quarters (13). A similar pattern of leukocyte infiltration was observed in the present study after prolonged *C. bovis* colonization, but it was not of the same magnitude. Macrophages were a predominant infiltrating cell type within parenchymal tissue of *C. bovis*-colonized quarters. Macrophages phagocytize milk constituents, cocci, and degenerated neutrophils, which may explain the significant increase in debris-laden cells within the alveolar lumina of colonized tissues. The major function of these cells appears to be removal of foreign material, but they also play an important role in antigen processing and in regulating the magnitude of lymphocyte response in the bovine mammary gland (14). Significantly more macrophages in stromal and luminal areas may have had better access to antigens and caused the local enhancement of lymphocyte and plasma cell populations in *C. bovis*-colonized glands (18).

Significant increases in lymphocyte populations within the stroma of *C. bovis*-colonized quarters suggest that those cells accumulated in tissues as a result of antigenic stimulation (13). Lymphocytes enumerated within the epithelial lining tended to be higher in colonized quarters, but no lymphocytes were observed in luminal areas irrespective of infection status. The increased population of lymphocytes appeared to remain localized within the stromal and...
interepithelial areas. A primary function of mammary lymphocytes is their role as plasma cell precursors (14). Preferential infiltration of lymphocytes within parenchymal tissue of C. bovis-colonized glands coincides with significant increases in plasma cell populations when numbers are normally low. These findings corroborate a previous report (18) that suggested persistent colonization of bovine mammary glands with C. bovis enhanced recruitment of sensitized precursor B-lymphocyte populations and caused subsequent proliferations into Ig-producing plasma cells.

Mast cell numbers decreased slightly in tissue from C. bovis-colonized quarters compared with those of controls. A similar pattern in mast cell numbers was observed by others (13) as mammary infection progressed. Mast cells in connective tissue of other organ systems were reported to release heparin and histamine under pathological conditions, resulting in increased vascular permeability (17). Degranulation of mast cells may have occurred in colonized quarters and contributed to the significant increase of infiltrating leukocytes observed in parenchymal tissue areas.

The ability of mammary gland neutrophils to phagocytize mammary pathogens was critical to the outcome of intramammary infections (15). Several studies found that quarters shedding C. bovis had significantly higher SCC when compared with bacteriologically negative quarters (4, 10, 11, 16). Significant protection against some mastitis-causing organisms also was reported in quarters experimentally infected with C. bovis (5, 12, 16). Preexisting neutrophil populations in milk from experimentally colonized quarters was implicated was implicated in providing protection by inhibiting teat end colonization by more pathogenic organisms (3, 12). However, there is evidence suggesting that naturally infected C. bovis quarters are not as resistant to subsequent intramammary infection (9). In this study, numbers of neutrophils infiltrating the epithelium, lumen, and stroma of parenchymal tissue from naturally colonized glands were not significantly higher when compared to uninfected glands. Prolonged exposure of bovine mammary glands to natural C. bovis colonization appears ineffective in maintaining a permanent elevation of neutrophils in mammary tissue of sufficient magnitude to prevent new infection. Studies are in progress to evaluate the relevance of local cellular responses of teat end tissues to prolonged C. bovis colonization in preventing infection by major mastitis-causing pathogens.

In conclusion, persistent colonization of bovine mammary glands with C. bovis was not detrimental to delicate secretory tissue. Enhancement of nonspecific resistance through elevated neutrophil populations does not appear to be the mechanism by which C. bovis protects the mammary gland from major mastitis-causing pathogens once they breach the streak canal. However, significantly more macrophages, lymphocytes, and plasma cells associated with colonized quarters may enhance effector cell populations and augment specific immunity against pathogenic organisms.

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REFERENCES

LEUKOCYTIC RESPONSE TO CORYNEBACTERIUM BOVIS


