Short communication

Lymphocytes from one side of the bovine mammary gland migrate to the contra lateral gland and lymph node tissue

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Received 2 March 2005; received in revised form 9 May 2005; accepted 20 May 2005

Abstract

The four quarters of bovine mammary glands are completely separated and two quarters on each side (right or left) are connected to ipsi lateral supra mammary lymph nodes. It is not clear whether cells infused into the cistern of the mammary gland are capable of migrating to lymph nodes or the general circulation. To examine cell migration, a prescapular lymph node was removed from each of two lactating and three non-lactating dairy cows, and isolated lymphocytes were stained with Hoechst 33342. Autologous stained cells were infused into the mammary gland and then activated by intramammary infusion of zymosan-stimulated serum (source of C5a). After 17 h, \textit{Escherichia coli} J5 bacterin was infused into the contra lateral mammary gland to mimic infection. After 43 h cows were euthanized and tissue samples (mammary quarters, right and left supra mammary, mesenteric, ileocecal and prescapular lymph nodes, liver and spleen) were collected for microscopic examination as well as flow cytometric analysis. Hoechst stained cells were detected not only in infused quarters, but also in contra lateral quarters as well as in both supra mammary lymph nodes. This indicates that cells infused into the mammary gland migrate to contra lateral tissues and supra mammary lymph nodes.

Keywords: Bovine; Lymphocyte migration; Mammary gland; Mastitis

In cattle, the four quarters of the mammary gland are completely separated anatomically and the two quarters on each side (right or left) are connected to the ipsi lateral supra mammary lymph node by lymph ducts. Naive lymphocytes migrate through blood circulation, lymph node, lymphatic system, then back to blood circulation (von Andrian and Mempel, 2003). When cows have mastitis (inflammation due to bacterial infection of mammary glands), lymphocytes in the ipsi lateral supra mammary lymph nodes are activated and proliferate. They migrate into the mammary gland to fight bacterial infection (Solity and Quinn, 1999; Kehrli and Harp, 2001).
lymphocytes in mammary glands are activated (effector/memory) cells (Park et al., 1992; Taylor et al., 1994). Effector/memory cells preferentially migrate to peripheral tissue rather than lymph nodes (Masopust et al., 2004; Weninger et al., 2002). However, we do not know whether lymphocytes in bovine mammary gland also migrate to other tissues especially the other side of the mammary gland, blood circulation or supra mammary lymph nodes. Some cells migrate into mammary gland secretion and are removed by milking. It is not known whether lymphocytes in mammary gland secretion can migrate back into mammary gland, supra mammary lymph node or other tissues. Understanding cell migration is important to prevent/treat mastitis, which is the biggest economic problem to the dairy industry (Nonnecke and Harp, 1989; Sordillo et al., 1997; Wang et al., 2002). We wondered: (1) whether intramammary infused and activated lymphocytes would migrate to various tissues and blood circulation and if so (2) what is the route of migration. In order to address these questions, we isolated cells from prescapular lymph node and stained them with Hoechst 33342. These stained cells were infused into the left side of the mammary gland and activated by zymosan-stimulated serum to mimic the cell condition in mammary gland, which contains mostly activated cells. Zymosan-stimulated serum is a source of complement factor C5a, which is known to activate not only neutrophils but also antigen presenting cells and T cells (Morgan et al., 1983; Nielsen and Leslie, 2002; Tsuji et al., 1997). In order to induce cell migration, *Escherichia coli* J5 vaccine was infused into the contra lateral mammary gland. Blood and mammary gland secretions were collected at intervals and several tissues and lymph nodes were obtained when cows were euthanized. The presence of Hoechst stained cells in tissues was determined by both flow cytometry and microscopy. Cells in blood and mammary gland secretion were examined by flow cytometry.

### 1. Materials and methods

Two Jersey (8–9-year-old) and three Holstein cows (5–6-year-old) were used for this study. Two cows were in lactation and three were in the early stage of non-lactation and used for the studies following involution of the udder. Animal-related procedures were approved by the Institutional Animal Care and Use Committee of the USDA-ARS-National Animal Disease Center. Cows were anesthetized and placed into lateral recumbency. The left prescapular lymph node was surgically removed using aseptic technique, and cows were allowed to recover.

The prescapular lymph node was put into a sterilized beaker on ice and transferred to the lab immediately. Cells were isolated by dissociation of the lymph node using a metal mesh screen. Isolated cells were collected into 50 ml conical tube. After suspension in medium (RPMI 1640 + 25 mM HEPES buffer), cells were filtered through sterilized 50-μm mesh nylon screen into another 50 ml conical tube. Cell number was adjusted to 1 x 10^8 cells/ml in medium. Cells were incubated with 32 μg/ml of Hoechst 33342 (Sigma, St. Louis, MO) in medium for 30 min at 39 °C with gentle mixing. Labeled cells were washed twice with FBS to remove excess dye, and resuspended in medium (4–5 x 10^9 cells/25 ml). Cells were examined using flow cytometry (excited by UV and detected at 400 nm) to obtain mean fluorescent intensity of Hoechst staining which was more than 100 times higher than that of unstained cells. Cell viability was also examined using trypan blue exclusion and found to be >95%.

Six hours after surgery, preinfusion samples of blood and mammary gland secretions were collected and 12.5 ml/quarter of autologous Hoechst stained cells were infused into left front and left rear quarters. Immediately after, 12.5 ml/quarter of zymosan-stimulated serum (Roth and Kaeberle, 1981) was infused into the same quarters in order to activate the labeled cells. Seventeen hours after infusion of zymosan activated serum, mammary gland secretions were obtained and 2.5 ml of J5 vaccine (*E. coli* bacterin: Upjohn, Kalamazoo, MI) diluted with 10 ml physiological saline/quarter was infused into right front and right rear quarters. All infusions were done using a 20 ml syringe fitted with a disposable syringe nozzle (Genesis Industries Inc., Spring Valley, WI) through the teat canal to infuse contents of syringe into a cistern of mammary gland. After infusion infused quarters were massaged so that infused fluid would spread throughout the entire cistern well.
Blood (collected into sterile tubes containing ACD) and mammary gland secretions (collected into 50 ml centrifuge tubes) were obtained at 0 (just prior to infusion), 17 (just prior to J5 vaccine infusion), 24 and 41 h after infusion of stained cells. At 43 h, cows were euthanized and the following tissues were collected and put immediately onto ice: mammary gland tissue from all four quarters (dorsal portion, close to the abdominal attachment, about 5 cm³), right and left supra mammary lymph nodes (center section including cortex and medulla), mesenteric, ileocecal and right prescapular lymph nodes, liver and spleen. All samples were brought to the lab immediately after sampling and processed as below.

Red blood cells were removed from blood samples by hypotonic lysis (Kimura et al., 2002). After washing with PBS, cells were resuspended with PBS. Mammary gland secretions were centrifuged at 550 g for 10 min, then the top layer was aspirated down to 5–10 ml to get rid of as much of the cream layer as possible. Then PBS was added to a total volume of 50 ml and mixed vigorously to wash cells. This suspension was recentrifuged at 500 g for 5 min and the supernatant was aspirated off. Cell pellets were resuspended with PBS to a volume appropriate to pellet size. Cells from lymph nodes, spleen and liver were isolated by disassociation on a metal screen. Red cells were removed from spleen and liver suspensions by hypotonic lysis. Cells were then washed once with medium (RPMI 1640 + 25 mM HEPES, pH 7.2) and resuspended with medium. Cells from lymph nodes were suspended in medium. Mammary gland tissues were rinsed thoroughly with 0.01 M PBS (pH 7.4) and cut into small pieces using a scalpel blade. After two washings with chilled extraction buffer (HEPES-buffered Hank’s balanced salt solution containing 1 mM CaCl₂ and 10 mM dithiothreitol, pH 7.4), tissue pieces were dispersed at 4 °C in extraction buffer containing 0.5 mg/ml of Bacillus thermoproteolyticus thermolysin (Sigma), under continuous stirring. After 30 min, extracted cells were isolated by filtering the suspension through a 150-μm mesh nylon screen. Remaining fragments were further extracted under continuous stirring at 39 °C for 30 min with collagenase type I (Sigma) diluted to 1 mg/ml in RPMI 1640 supplemented with 20% FBS. Isolated cells were pooled and centrifuged through a cushion of FBS and pellets were obtained. Pellets were washed twice with medium, and incubated for 20 min at 39 °C with 2 mg/ml of DNase (type IV, Sigma) in RPMI 1640 with 20% FBS, and filtered through a 51-μm mesh nylon screen (adapted from Quiding et al., 1991). Cells isolated from tissues were suspended in an appropriate volume of medium prior to flow cytometric analysis.

Fifty microliters/well of cells prepared as above were dispensed into appropriate wells in 96 well microtiter plates which contained 50 µl each of the following primary antibodies (mAbs obtained from VMRD, Pullman, WA). CD4 (GC50A, 14 µg/ml), CD8 (BAQ111A, 14 µg/ml) and CD45 (CACTB51A, 7 µg/ml). Cells were incubated for 15 min at room temperature and centrifuged at 1000 x g for 2 min and supernatant was discarded. Cells were washed with 200 µl/well of PBS, centrifuged and supernatant was discarded. Fifty microliters of each rat anti-mouse IgG2a + b conjugated with PerCP, and goat anti-mouse IgM conjugated with APC (both from BD Biosciences/Pharmingen, San Diego, CA, 1:10) were added and incubated for 7.5 min at room temperature in the dark. Cells were washed once with PBS and resuspended with PBS. Data were acquired on a flow cytometer (LSR, BD Biosciences) and analyzed using Cell Quest software (BD Biosciences). In order to determine the presence of Hoechst stained cells in samples, at least 50,000 cells/sample were examined.

Small sections of tissue were embedded in OCT compound (Sakura Finetechnical Co. Ltd., Tokyo) and frozen in liquid nitrogen immediately. Tissue was kept at −70 °C until cutting. Ten micrometer sections were cut with a cryotome, and fixed in 1% glutaraldehyde for 1 h. Sections were examined at 400× magnification for the presence of labeled cells under ultraviolet illumination. Four representative fields were examined and scored as: none, no stained cells in all fields; low, 0–2 stained cells per field; medium, 3–5 stained cells per field; medium high, 6–25 per field; high, 26 + per field.

2. Results and discussion

Since there was no significant difference between lactating and dry cows, data were combined together. As shown in Table 1, more than 50% of the mononuclear cells in mammary secretions from left
side (left front and left rear) quarters were Hoechst stained as detected by flow cytometry 17, 24 and 41 h after cell infusion. Mononuclear cells in secretions from right side (right front and right rear) quarters showed increased percentages of Hoechst stained cells 17, 24 and 41 h after infusion compared with time 0, but the differences were not statistically significant. Blood samples did not show any significant changes in Hoechst 33342 stained cells at any time (Table 1).

The percentages of Hoechst 33342 stained mononuclear cells in each tissue detected by flow cytometry are summarized in Table 2. All four quarters, right and left supra mammary lymph nodes, mesenteric lymph nodes and liver contained more than 2% Hoechst stained cells (four S.D. above the mean value for control samples obtained from four non-treated dry cows). The Hoechst-labeled prescapular lymphocyte suspensions used to infuse the cows contained a mean of 33.3% CD4+ cells and 20.1% CD8+ cells. In contrast, the labeled cells found in the left side mammary gland (left front and left rear quarters) had a mean of 4.1% CD4+ and 10.9% CD8+. A representative histogram is shown in Fig. 1. Data from microscopic examination of tissue sections are summarized in Table 3 and representative micrographs are shown in Fig. 2. Hoechst stained cells were observed in all tissues except mesenteric lymph nodes, ileocecal lymph nodes and liver. Five of five cows had labeled cells in the infused (left front and left rear) quarters and the ipsi lateral supra mammary lymph nodes. Four of five cows had labeled cells in the contra lateral (right front and right rear, non-infused) quarters and supra mammary lymph nodes.

In this study, we examined the migration of cells infused into the mammary gland cistern to the mammary gland tissue, and the supra mammary lymph nodes. We determined that infused, activated cells can migrate not only into dorsal mammary gland tissue but also into ipsi lateral and contra lateral supra mammary lymph nodes. These findings were supported by data from both flow cytometric and microscopic analysis. Although migrated Hoechst stained cells were very small in number except in cell-infused quarters, this was statistically significant compared to control tissues obtained from four non-treated dry cows. This is the first report that cells infused into the mammary gland cistern can migrate not only into tissues on the infused side of the gland, but to the contra lateral side as well. Small numbers of migrating cells may help fight off bacteria causing mastitis in addition to the cells already present in the infected mammary glands. In addition, direct activation of cells in mammary gland by infusion may be an alternative pathway of vaccine administration to activate/recruit cells into mammary glands.

We chose the prescapular lymph node as a source of autologous cells for labeling and reinfusion into donor cows. This was done due to the superficial location of this node, and relative ease of removal, and because it has been demonstrated that in cattle, the mammary

Table 1
Percentage of Hoechst 33342 stained mononuclear cells by flow cytometry (mean ± S.E.M.) after infusion of stained cells into left front and left rear quarters

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0 h (preinfusion)</th>
<th>17 h</th>
<th>24 h</th>
<th>41 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.6 ± 0.6</td>
<td>0.4 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Milk right</td>
<td>0.8 ± 0.3</td>
<td>2.7 ± 2.1</td>
<td>8.8 ± 8.3</td>
<td>4.7 ± 2.1</td>
</tr>
<tr>
<td>Right rear</td>
<td>0.6 ± 0.3</td>
<td>2.3 ± 1.2</td>
<td>4.6 ± 4.1</td>
<td>3.9 ± 3.1</td>
</tr>
<tr>
<td>Left front</td>
<td>0.7 ± 0.2</td>
<td>78.2 ± 12.8</td>
<td>54.4 ± 20.5</td>
<td>65.3 ± 14.6</td>
</tr>
<tr>
<td>Left rear</td>
<td>0.5 ± 0.2</td>
<td>81.2 ± 10.3</td>
<td>54.4 ± 27.8</td>
<td>69.9 ± 12.6</td>
</tr>
</tbody>
</table>

Changes were not statistically significant for blood and right mammary gland secretion (p > 0.05).

Table 2
Percentage of Hoechst 33342 stained mononuclear cells by flow cytometry (43 h after infusion, mean ± S.E.M.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RF*</th>
<th>RR*</th>
<th>LF*</th>
<th>LR*</th>
<th>Right supra</th>
<th>Left supra</th>
<th>Ileocecal</th>
<th>Mesenteric</th>
<th>Liver</th>
<th>Spleen</th>
<th>Prescapular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.2</td>
<td>3.0</td>
<td>38.9</td>
<td>47.4</td>
<td>2.9</td>
<td>9.9</td>
<td>9.9</td>
<td>0.9</td>
<td>4.3</td>
<td>3.2</td>
<td>1.5</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>1.0</td>
<td>2.0</td>
<td>14.7</td>
<td>16.1</td>
<td>0.7</td>
<td>7.3</td>
<td>0.7</td>
<td>2.6</td>
<td>1.8</td>
<td>1.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Mammary gland quarter: RF, right front; RR, right rear; LF, left front; LR, left rear.
gland is closely linked to the peripheral immune system. Prescapular lymph node cells preferentially migrate to mammary nodes and tissue (Harp et al., 1988). Interestingly, we observed that while the infused population of labeled cells from the prescapular lymph node was 33.3% CD4+ and 20.1% CD8+, the percentage of cells recovered from the mammary gland tissue that were double-labeled with Hoechst and CD4 or CD8 were predominantly CD8+ (10.9% CD8+ versus 4.1% CD4+). It has been consistently reported in the literature that lymphocytes in mammary gland secretions and tissues are predominantly CD8+ during lactation and in the early dry off (Asai et al., 1998; Yang et al., 1997). The data from the present study support the notion that there is a selective mechanism for recruitment of lymphocytes into the mammary gland tissue that maintains this predominance of CD8+ cells. Further study of receptors on both lymphocyte subsets and endothelial tissues of the mammary gland may help to define this mechanism.

It is of interest to speculate on the route of migration. If infused cells migrated from the cistern to the alveoli of the infused side of the mammary gland

Table 3
Presence of Hoechst 33342 stained cells in tissue by microscopic examination (four fields)

<table>
<thead>
<tr>
<th>Cow I.D.</th>
<th>Right side mammary gland</th>
<th>Left side mammary gland</th>
<th>Right supra mammary node</th>
<th>Left supra mammary node</th>
<th>Mesenteric lymph node</th>
<th>Ileocecal lymph node</th>
<th>Prescapular lymph node</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idaline</td>
<td>Low #</td>
<td>High #</td>
<td>Low #</td>
<td>Medium #</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>#13</td>
<td>None</td>
<td>High #</td>
<td>Low #</td>
<td>Medium #</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>#2</td>
<td>Low #</td>
<td>High #</td>
<td>None</td>
<td>Medium high #</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Medium #</td>
</tr>
<tr>
<td>#1734</td>
<td>Low #</td>
<td>High #</td>
<td>Low #</td>
<td>Medium high #</td>
<td>None</td>
<td>None</td>
<td>Low #</td>
<td>None</td>
<td>Medium #</td>
</tr>
<tr>
<td>Autumn</td>
<td>Low #</td>
<td>High #</td>
<td>Low #</td>
<td>High #</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Total</td>
<td>Low 4/5</td>
<td>High 5/5</td>
<td>Low 4/5</td>
<td>Medium # 2/5</td>
<td>Medium high # 2/5</td>
<td>0/5</td>
<td>0/5</td>
<td>Low 1/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Idaline and #13 were lactating and others were dried; none: no stained cells in all fields; low #: 0–2 stained cells per field; medium #: 3–5 stained cells per field; medium high #: 6–25 per field; high #: 26 + per field.
and then to the ipsilateral supra mammary lymph node via afferent lymphatic ducts, they would then likely exit through efferent ducts and travel to the thoracic duct where they would enter into the blood circulation. The infusion of J5 vaccine into the contralateral mammary gland would have created an inflammatory focus that would facilitate the migration of the activated, labeled cells from the blood into the inflamed tissue. Although we found no labeled cells in the blood, most of them may have already migrated back into tissue by the time of the first sampling. In addition, the dilution factor due to the large volume of blood may have made them difficult to find.

Alternatively, the labeled cells infused into the mammary cistern may have migrated into the alveolar tissue, to the ipsilateral supra mammary lymph node and then directly to the contralateral lymph node and into the contra lateral mammary tissue. There is a
report in the literature that this pathway exists, at least in some cows (El Hagri, 1945). The data in this study are not sufficient to discount the possibility that some of the labeled cells may have migrated via this pathway.

In summary, we found that autologous lymphocytes infused into bovine mammary gland can migrate not only up into the infused gland tissue but also into contra lateral mammary glands in addition to both ipsilateral and contra lateral supra mammary lymph nodes.

This information may lead to improved strategies for the prevention and treatment of bovine mastitis.

References


