

Location of Lymphocytes in the Bovine Mammary Gland

Sarah D. Steenlage and Lisa M. Tepp

Faculty Sponsor: Bernadette C. Taylor, Departments of
Biology/Microbiology

ABSTRACT

Mastitis is an inflammation of the mammary gland, often due to bacterial infection. Farmers lose millions of dollars every year due to mastitis in cattle. Because of this problem, we are interested in the immunology of the bovine mammary gland. This study entails generating a broad picture of the location of the cells thought to be responsible for the specific immune response to foreign antigens in the mammary gland. It focuses on CD4, CD8 and $\gamma\delta$ T cells and B cells. To identify the location of these cells in the bovine mammary gland, tissue samples were collected from the right rear quarter of the mammary glands of six cattle. The tissue samples were taken from several sites vertically distributed from the teat end, along the teat canal and up into the upper gland and from several sites distributed laterally around the junction of the upper teat and the gland. Tissue blocks were snap frozen.

Monoclonal antibodies directed against CD4, CD8, $\gamma\delta$ T cell receptor and CD21 and immunohistochemical techniques were used to determine the distribution of T cell populations and B cells. Analysis of upper gland locations indicated a predominance of CD8 T cells, with fewer CD4 T cells. The teat area contained more B cells than the upper gland. Gamma delta T cells were rare throughout the gland. A monoclonal antibody specific for MHC class II stained many cells throughout the gland.

INTRODUCTION

Mastitis is one of the leading diseases in dairy cattle today. Mastitis is an inflammation of the mammary gland, which can cause abnormalities in the milk. A cow with mastitis can be identified when it is being milked, because the milk will either be chunky like cottage cheese or watery looking. In many cases the quarter that has mastitis will be swollen and hard. Mastitis is the result of bacteria infecting the mammary gland and the abnormality in the milk is due to the influx of macrophages, neutrophils and lymphocytes that are trying to kill off the bacteria. The National Mastitis Council reports that one of every three dairy cows contracts mastitis. The best way to treat mastitis is to give the cow antibiotics for 2-4 days. During the treatment and for several days after, the milk cannot be sold and is thrown away. In 1994, US farmers lost over \$2 billion due to mastitis (1).

The natural defense of the bovine mammary gland involves a variety of immune mechanisms with both non-specific and specific purposes. Lymphocytes are the cells responsible for the specific immune response to foreign antigens. These cells are responsible for the immunological memory that is necessary for a faster, stronger immune response when exposed to the same antigen at a later time. Vaccines target this part of the immune response by producing immunologic memory without causing an individual to have the full-blown dis-

ease. Vaccines are administered in a variety of ways and forms. For example, polio vaccine is given orally because it is known to generate memory lymphocytes in the area surrounding the gut. For a mastitis vaccine, it would be useful information to know what types of lymphocytes are present in the gland, and their distribution.

Milk has been used in studying the immunology of the mammary gland because it has been postulated that the cells in milk reflect the cells in the gland tissue. Out of the total population of cells in a cow's milk, 1-2% of them are lymphocytes indicating the presence of specific immune activity in the mammary gland (2). Even though milk has been valuable in studying the types of lymphocytes, which enter the mammary gland, it is very difficult to work with cells derived from milk in tissue culture due to the presence of bacteria and debris. Furthermore, milk may not reflect what is happening in the tissue. Therefore, it would be more accurate to study the cells in tissue samples using enzyme digestion to release the lymphocytes from the tissue. Because it is not clear exactly where lymphocytes are predominantly located in the mammary gland it would be valuable to determine the distribution of the lymphocytes in the mammary gland. Once the distribution of lymphocytes in the gland is known, areas rich in lymphocytes could be targeted for studies using enzyme digestion to release lymphocytes for further in vitro studies.

MATERIALS AND METHODS

Mammary glands. Cows were accessed through North Bend Processing, North Bend WI. Mammary glands from a total of six different cows were collected. History of the dairy cow was taken and cows were selected that did not have mastitis. The right rear quarter was taken from freshly slaughtered cows.

Frozen tissue sections. Within 4 hours of slaughter, the right rear quarter was trimmed to resemble a single slice up the center of the quarter. Fourteen cubes of tissue (10 x 10 x 5 mm) were taken throughout the slice (see figure 1), but mainly concentrating around the upper teat sphincter. The tissue cubes were placed in disposable specimen molds (Miles Inc. Elkhart, IN). Embedding medium (O.C.T. compound, Fisher Scientific, Pittsburg, PA) was put onto the tissue cube until the meniscus was above the surface of the mold. Water soaked cork pieces were placed onto mold to keep tissue section and medium in place. Sections were then snap frozen by placing them in 2-methyl butane immersed in liquid nitrogen for 20 seconds. The sections were then de-gassed by placing the sections on dry ice for 10 minutes. The sections were placed in sealed plastic bags and stored at -80°F. A cryostat was used for slicing the frozen tissue cubes into sections 6µm thick. Sections were collected onto glass microscope slides.

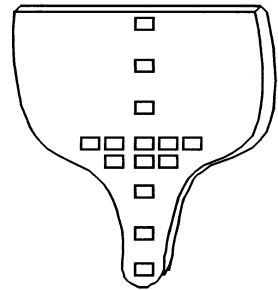


Figure 1. Section of right rear quarter of bovine mammary gland showing sites from which tissue cubes were collected

Staining. Slides with tissue sections were fixed in acetone for five minutes. After drying, a PAP pen was used to draw a wax circle around the tissue section to contain staining liquids. The slides were then incubated for 30 minutes with 100µl of 0.3% H₂O₂ at room temp to inhibit endogenous peroxidase activity. After rinsing for 5 minutes in PO₄ saline PH. 7.2

(Becton Dickinson Microbiology Systems Cockeysville, MD.) on a rocker, the slides were blocked with 100 μ l of 10% goat serum in saline and allowed to stand for 30 minutes. Excess serum was drained off and 100 μ l of primary antibody was added and incubated for 30 minutes. Table 1 lists the primary antibodies used to identify T and B cells. Another primary antibody, the monoclonal antibody UC-H9 was also used to analyze MHC class II expression in all tissue sections. After removing excess antibody, the slides were rinsed three times in PO₄ saline on a rocker for five minutes. Biotin-conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) diluted 1:1000 in 1% bovine serum in PO₄ saline was placed on the slides for 30 minutes. Slides were rinsed three times in saline on a rocker for five minutes. Horseradish peroxidase conjugated streptavidin (Zymed) diluted 1:400 in PO₄ saline was added and incubated for 30 minutes then rinsed three times in PO₄ saline for five minutes. Amino ethyl carbazole (AEC) working solution prepared from 1ml AEC stock, 9ml of Acetate buffer (pH 5.2) and 5 μ l of 30% H₂O₂ was filtered using a 0.22 micron syringe filter. The AEC working solution was added to slides and allowed to develop. Immersing the slides in distilled water stopped development. Slides were counterstained by dipping into Mayer hematoxylin for 45 to 60 seconds, rinsed in distilled water, then dipped in Scott solution for six seconds and rinsed in distilled water. The stained tissue sections were mounted in gel/mount (Biomedica Corp., Foster City, CA) medium and sealed with clear nail polish.

RESULTS

Tissue cubes were taken from 14 locations in the mammary gland as shown in figure 1. Sections of mammary gland tissue from each of the cubes from four cows were analyzed for tissue location and cell density of CD4+ T cells, CD8+ T cells, B cells and $\gamma\delta$ T cells and for expression of MHC class II.

The gland is arranged in a highly branched compound structure of tubuloalveoli lined with simple cuboidal epithelium. Groups of tubuloalveoli secretory units form lobules separated by intralobular connective tissue. Among the alveoli, loose connective tissue underlies the cuboidal epithelium. It was noticed that some of the cell types studied tended to localize in certain types of tissue (for example loose connective tissue versus intralobular connective tissue). It was also noticed that certain cell types were more numerous, depending on the general location in the mammary gland.

The CD4+ T cells were present, but in relatively few in numbers in the loose connective tissue, and were rare in the intralobular connective tissue throughout the gland. Very few CD4+ T cells were found in the epithelium surrounding alveoli.

The CD8+ T cell distribution was more varied, depending on the region of the gland. The dorsal and middle region of the gland contained some CD8+ T cells among the cuboidal epithelial cells lining of the alveoli. The loose connective tissue in these areas contained relatively high numbers of CD8+ T cells. Only a few CD8+ T cells were found in the intralobular connective tissue. The ventral or teat region of the gland contained small numbers of the CD8+ T cells scattered throughout the epidermis and grouped in pockets of loose connective tissue.

There were no $\gamma\delta$ T cells in the middle, and ventral or teat regions of the gland, but occasionally a few were found in the loose connective tissue in the most dorsal region of the gland.

The B cell numbers varied depending on the type of tissue and the region of the gland. Relatively few B cells were found in the loose connective tissue or epithelium surrounding

alveoli. The dorsal and middle regions contained numerous B cells in the intralobular connective tissue, while the ventral and teat regions were highly populated with B cells in the fibrous interstitial connective tissue.

All regions of the gland contained many MHC class II expressing cells both in the loose connective and in the intralobular connective tissue. MHC class II appeared to be expressed by blood vessel endothelial cells in the loose connective tissue throughout the gland.

DISCUSSION

The bovine mammary gland is a large highly structured organ composed of alveoli surrounded by secretory epithelium, supported by connective tissue. The cuboidal secretory epithelial cells secrete milk into the alveoli. The alveoli converge to ducts, which lead to a main duct opening through the teat. Bacteria are able to enter the gland through the teat canal causing mastitis.

Two T Lymphocyte populations in the mammary gland were studied: CD8+ and CD4+ T lymphocytes. CD4+ T lymphocytes, also known as T helper cells, release cytokines and detect antigens associated with MHC class II proteins where they present antigens brought into cells from the outside. This study showed that CD4+ T cells are present in uninfected mammary glands, although in fewer numbers compared to CD8+ T cells. This study also showed that MHC class II proteins are found throughout the gland. High expression of MHC II would allow for high levels of antigen presentation to T helper cells. Studies have shown that CD4+ T lymphocytes are increased in the milk after an infection of the mammary gland and become a greater percentage than the CD8+ T lymphocytes (6). This suggests that CD4+ T lymphocytes do play a role in the immune response to bacterial induced mastitis.

CD8+ T lymphocytes, also known as cytotoxic T cells, release cytokines and detect antigens associated with MHC class I proteins. MHC class I proteins can only present antigens produced in the cytoplasm of infected cells. T cytotoxic cells kill virus-infected cells and tumor cells. Our results indicated a greater concentration of CD8+ T lymphocytes than CD4+ cells in the upper portion of the uninfected mammary gland. This reflects the finding that CD8+ T cells outnumber CD4+ T cells in milk from healthy cows (6). This substantial population of resident CD8+ T cell may have some protective or regulatory function in the normal gland. Some reports suggest that the CD4+ T lymphocytes are inhibited by active CD8+ Lymphocytes (4). The greater number of CD8+ T lymphocytes in the mammary gland could perhaps decrease inflammation induced by T helper cells after a bacterial infection.

It has been shown that antibodies found in milk are produced locally within the mammary gland as well as in regional lymph nodes. This study confirmed the presence of resident B cells in the gland tissue (5). Since the bacteria enter the gland through the teat canal, it is speculated that a greater number of B cells will be found near the point source of infection, around the sphincter at the end of the teat canal. This was supported by our results of the higher concentration of B cells in the ventral portion of the gland.

Functions of the $\gamma\delta$ T lymphocytes are not established, but they are found in relatively high numbers underlying the epithelium in the intestinal tract and underlying the skin. Our results did not show a significant concentration of $\gamma\delta$ cells anywhere in the gland mammary, suggesting that these cells do not play a major role in local protection of the mammary gland.

ACKNOWLEDGMENTS

Sara Gilbertson

Tom Volk, Ph.D.

North Bend Processing

Annie Voyles, M.S.

Rod Mowbrey, Ph.D.

Jeffery Degenhardt, MD., Franciscan Skemp Hospital

REFERENCES

1. Mazzola, V. 1994. *Agricultural Research*, 42:18.
2. Hibbitt, K.G., N. Craven, and E. H Batten. 1992. *Bovine Medicine: Diseases and Husbandry of Cattle*. A. H. Andrews, Ed. Blackwell Scientific Pub. Cambridge MA. P 285.
3. Sordillo, L. M., K. Shafer-Weaver, and D. DeRose. 1997. Immunobiology of the Mammary Gland. *J. Dairy Sci.* 80:1851-1865.
4. Park, Y. H., L. K. Fox, M. J. Hamilton and W. C. Davis. 1993. Suppression of proliferative response of BoCD4+ T lymphocytes by activated BoCD8+ T lymphocytes in the mammary gland of cows with *Staphylococcus aureus* mastitis. *Vet. Immunol. Immunopathol.*, 36: 137-151.
5. Butler, J. E., 1997. *Immunoglobulins and Immune Cells in Animals' Milks* p 21.
6. Taylor, B.C., R.G. Keefe, J.D. Dellinger, Y. Nakamura, J.C. Cullor and J.L. Stott. 1997. T cell populations and cytokine expression in milk derived from normal and bacteria-infected bovine mammary glands. *Cellular Immunology*, 182: 68-76.

Table 1: Monoclonal antibodies used as primary antibodies to stain tissue sections for identification of T cells and B cells.

Cells	Function	Identifying monoclonal antibody
CD4+	Also known as T helper cells. Release cytokines. . Only detect antigens associated with MHC II	IL-A11
CD8+	Also known as T cytotoxic cells. Release cytokines. Detect antigens associated with MHC I.	CC63
B cells	Secrete antibodies.	CC21
$\gamma\delta$ cells	Unknown function.	86D

Legends

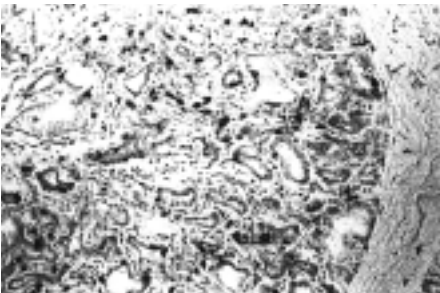


Figure 2. Mid section of bovine mammary gland stained with monoclonal antibody specific for CD8+ T cells. Darkly stained CD8+ T cells are numerous in the loose connective tissue (40X magnification).

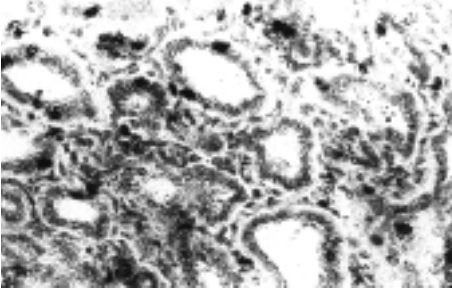


Figure 3. Mid section of bovine mammary gland showing stained CD8+ T cells in the cuboidal epithelium surrounding alveoli and in loose connective tissue (80X magnification).

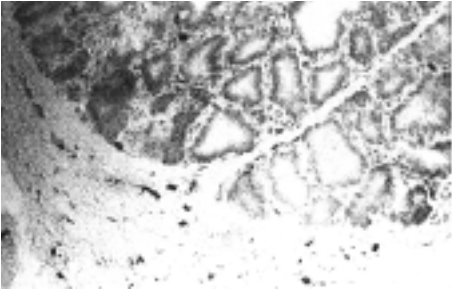


Figure 4. Mid section of bovine mammary gland showing stained B cells in mainly in the intralobular connective tissue (40X magnification).

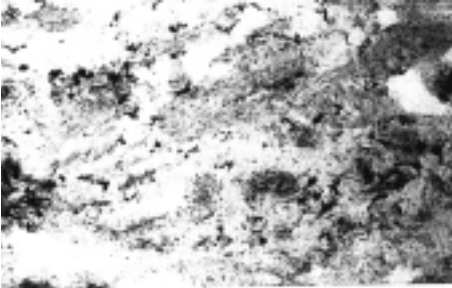


Figure 5. Teat section of bovine mammary gland showing darkly stained B cells in the fibrous interstitial connective tissue (40X magnification).

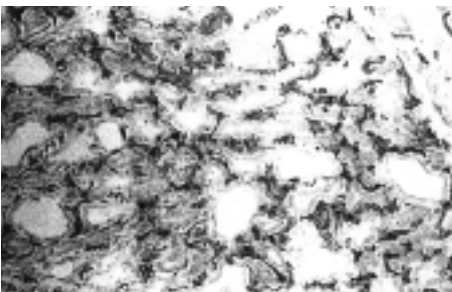


Figure 6. Mid section of bovine mammary gland showing MHC class II expression on numerous cell types (40X magnification).