Analysis of Bovine Mammary Gland Lymphocytes

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ABSTRACT

Each year farmers lose millions of dollars to mastitis in spoiled milk, culled animals, and antibiotic treatments. An ideal way to combat the mastitis problem would be to improve the availability of effective vaccines. However, much remains to be learned about the bovine mammary gland and its resident lymphocyte populations. Our work aims to establish information and methods to study bovine mammary gland lymphocytes that will be useful in assessing efficacy of vaccines developed for mastitis. Immunoperoxidase staining of frozen tissue sections was used to determine the location and relative density of lymphocytes in the mammary gland with respect to their specific location within different tissues and their location in the whole gland. CD4+ T lymphocytes were present in the loose connective tissue, and were more common near the lower region of the mammary gland, while CD8+ T lymphocytes were present in greater numbers than CD4+ T lymphocytes in the alveolar epithelium as well as loose connective tissue in the middle to upper region of the gland. Some $\gamma\delta$ T cells were in the loose connective tissue in the upper most region of the gland. B cells were found in smaller numbers than T cells and were located in the intralobular connective tissue in the upper to middle regions and in the interstitial connective tissue in the lower and teat region of the gland.

INTRODUCTION

Mastitis is one of the most costly bovine diseases. According to the University of Kentucky College of Agriculture the United States looses an estimated $1.7 billion annually to spoiled milk, treating the infection and culled animals (2). Mastitis is a bacterial infection caused by *Streptococccus agalactiae, Staphylococcus aureus*, coliforms, such as *Escherichia coli*, *Klebsiella* species, and *Enterobacter aerogenes*, and other environmental streptococci (2). It can be diagnosed during milking by hardening of the gland and the appearance of the milk, which is either watery looking, or chunky like cottage cheese. This is caused by macrophages, neutrophils and lymphocytes, which have entered the gland to fight the infection. Treatment usually consists of a 2-4 day antibiotic course (8). During this time, and for several days after, the milk cannot be used and must be thrown away. Vaccination would be an ideal way to combat mastitis. Not only would it prevent the bacterial infection, saving copious amounts of money, downtime for the cows, and milk; it would also help to limit the amount of antibiotics used. Limiting the amount of antibiotics used decreases the exposure of the bacteria to the antibiotics, which minimizes the possibility that the bacteria could develop a resistance to the antibiotic. This is critical concern since the advent of several antibiotic resistant strains of bacteria. Currently there is a vaccine on the market that targets *Escherichia coli*. However, it is only marginally effective. Any new information gathered about the immunology of the bovine mammary gland would greatly aid in the development of new and better vaccines.

Previously, research has focused on milk to learn more about the cell types in the mammary gland and their functions. There were two main types of T lymphocytes present, CD4+ and CD8+. CD4+ T cells, also know as T helper cells, release cytokines, which direct both antibody production by B cells and cell mediated immunity. CD8+ T lymphocytes, also known as cytotoxic T cells, kill self-altered cells, such as virus infected or tumor cells. In the milk, it was found that there were more CD8+ T lymphocytes than CD4+ T lymphocytes, which is opposite to the ratio of CD4+ to CD8+ T lymphocytes in peripheral blood. This suggests a migration of CD8+ T lymphocytes into milk. These CD8+ T lymphocytes also displayed high levels of the surface antigen CD2 and low levels of CD45R, which indicates that they are memory T cells, and implies that lymphocyte trafficking through the mammary gland is selective for memory lymphocytes (10). Memory lymphocytes are lymphocytes that remember a specific antigen, so when that antigen is encountered again the immune response can happen more quickly and efficiently. When the CD4+ to CD8+ ratio was examined in milk from healthy cows and cows with bacterial induced mastitis it was found that the CD8+ T lymphocytes were predominant in milk from healthy animals while CD4+ T lymphocytes were predominant in milk from animals with mastitis (9).

MATERIALS AND METHODS

Mammary gland specimens. Mammary gland specimens were collected from six cows through North Bend Processing, North Bend, WI. The specimens were collected from the right rear quadrant from recently slaughtered cows that did not have mastitis.

Frozen Tissue Sections. The right rear quarter was trimmed so that fourteen cube tissue sections (10 x 10 x 5 mm) could be taken from a single slice descending quadrant. The tissue cubes were placed in disposable specimen molds (Miles Inc. Elkhart, IN), and embedding medium (O.C.T. compound, Fisher Scientific, Pittsburgh, PA) was poured around the cube so that the meniscus was above the surface of the mold. Water soaked cork pieces were laid over the top to keep the tissue and embedding medium in place. The cubed sections were snap frozen by placing them in 2-methyl butane immersed in liquid nitrogen for 20 seconds. The cubes were degassed by placing them on dry ice for 10 minutes, and sealed in plastic bags and stored at –80°C. The cubes were cut into 6 µm sections with a cryostat at –20°C and placed on glass microscope slides.

Tissue section staining. Tissue sections 2, 6, 12, and 14 from cows one, two, four and five were immunohistochemically stained. Briefly, the sections were fixed in acetone for five minutes. When dry, a wax circle was drawn around each section to create a mini-well for the staining solutions with a PAP pen (The Binding Site, San Diego, CA). The sections were incubated with 100 µl of 0.6% H_2O_2 and 0.2% NaN_3 at room temperature for 15 minutes, and washed for five minutes in phosphate buffered saline (PBS) buffered to pH 7.2. The sections were blocked with 100 µl of 10% goat serum in PBS for 15 minutes at room temperature. The excess serum was drained off and blotted around the edges before 100 µl of primary antibody (Table 1) diluted 1:1000 in PBS was added for 15 minutes at room temperature. The sections were washed for five minutes in PBS three times. The secondary antibody, 100 µl of biotin conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) diluted 1:500 in 1% bovine serum and PBS, was added and incubated for 15 min at room temperature. The sections were washed for five min in PBS three times. Following the secondary antibody, 100 µl of horseradish peroxidase conjugated strepavidin (Zymed) diluted 1:200 in PBS was added and incubated for 15 min at room temperature. The sections were washed for five min-

utes in PBS three times. The sections were developed by adding 100 μl of freshly prepared amino ethyl carbazole (AEC) working solution and watching the color development under a microscope. Development was stopped by immersing the section in deionized water. The AEC working solution consisted of 1 ml of AEC stock, 9 ml of acetate buffer, pH 5.2, and 5 μl of 30% H_2O_2 which was filtered before use with a 0.22 μm syringe filter. The AEC stock consisted of 120 mg of AEC (Sigma) and 15 ml of dimethylformamide stored in the refrigerator in the dark. The acetate buffer consisted of 7.3 ml of 0.2 M sodium acetate, and 2.7 ml of 0.2M acetic acid. The sections were counterstained by dipping the section into Mayers Hematoxylin (Fisher Scientific) for 40 seconds, rinsing with running DI water, followed by 15 seconds in Scotts Solution and a rinsing with running DI water.

RESULTS

Sections from the upper, middle, lower and teat regions from four normal, healthy cows were immunohistochemically stained with the panel of monoclonal antibodies previously stated.

The upper gland. Small numbers of CD4+ T cells were seen in tight clusters in the intralobular connective tissue, while small numbers of CD8+ T cells were found in loose clusters in the intralobular connective tissue. A couple of gamma delta T cells were seen scattered in the intralobular connective tissue. No B cells were observed.

The middle gland. Similarly, small numbers of CD4+ T cells were seen in tight clusters in the intralobular connective tissue. Moderate numbers of CD8+ T cells were found in loose clusters in the intralobular connective tissue and small numbers were observed in the alveolar epithelium. A couple of gamma delta T cells were seen scattered in the intralobular connective tissue and a couple of B cells were found scattered in the interlobular connective tissue.

The lower gland. Small numbers of CD4+ T cells were seen in tight clusters in the intralobular connective tissue. Moderate numbers of CD8+ T cells were found in loose connective tissue and small numbers were observed in the alveolar epithelium. No gamma delta T cells were observed. A couple of B cells were seen in very loose clusters in the loose connective tissue.

The teat region. No CD4+, CD8+, or gamma delta T cells were seen, while moderate numbers of B cells were observed scattered throughout the fibrous and loose connective tissue.

DISCUSSION

Sections from four healthy cows were examined to characterize the lymphocyte populations in the mammary gland based on their location in the gland and their location in the tissue layers. In healthy cows there are more CD8+ than CD4+ T cells, and the CD8+ T cells are located mainly in the middle to lower parts of the gland in the intralobular connective tissue and the alveolar epithelium, while the CD4+ T cells are clustered just in the intralobular connective tissue. Some B cells are present mainly in the fibrous connective tissue. Previously, Sarah Steenlage and Lisa Tepp had characterized the lymphocyte populations with similar results; however this study was able to obtain better quality tissue sections, which allowed for a more accurate and extensive characterization of the mammary gland lymphocyte populations. A research team in Japan has also reported this general distribution of lymphocytes (11).

These results are consistent with previous milk studies, which have shown more CD8+ than CD4+ T cells, and similar numbers of gamma delta T cells and B cells (10). The

CD8+:CD4+ ratio found in the milk study could be explained by the presence of CD8+ T cells in the alveolar epithelium. Since these cells are present in healthy animals they could have a role in maintaining the epithelium, and are secreted out more easily than the CD4+ T cells.

Similar findings regarding mammary lymphocyte populations have been reported in other species of mammals. A study conducted in 1989 on the mammary gland tissue of pregnant versus non-pregnant sheep found the majority of lymphocytes in the ductal and alveolar epithelium were CD8+ T cells, while B cells and CD4+ T cells were mainly located in the periductal and intralobular connective tissue (7). An increase in CD8+ T cells near the epithelium has also been reported in sows (1), and similar findings have been reported in goats (5).

Recent reports have indicated that some intraepithelial lymphocytes (IEL) in the mucosal tissues are CD8αα homodimer rather than the typical CD8αβ heterodimer (6). These CD8αα IEL originate in the thymus, but acquire their expression of CD8αα homodimer in the intestinal microenvironment (4). It would be interesting to determine if the CD8+ T cells on the mammary tissue expressed the CD8αα homodimer, since mammary tissue is considered mucosal tissue.

Using the information from this tissue work, enzymatic digestions have been started to release live lymphocytes from the mammary tissue for further characterization through flow cytometry. However, optimizing a protocol has proven to be slow and very challenging.

Mastitis is a costly infection that specifically plagues the dairy industry. Vaccines would be an ideal method to prevent mastitis; however only a semi-effective vaccine against *E. coli* has been developed. Any new information learned about the immune regulation and response in the mammary gland would aid in improved vaccine development.

ACKNOWLEDGEMENTS

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Table 1. Monoclonal antibodies used in immunoperoxidase staining of frozen tissue sections

Monoclonal antibody	Antigen identified	Cells identified
IL-A12	BoCD4	T, helper
IL-A17	BoCD8	T cytotoxic
86 D	$\gamma\delta$ T cell receptor	$\gamma\delta$ T
CC21	CD21	B
MMIA	CD3	T