

Immune Cell Differentiation in Mammary Gland Tissues and Milk of Cows Chronically Infected with *Staphylococcus aureus*

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With 3 tables and 5 figures

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Summary

This study identifies and compares the distribution of mononuclear cells in the mammary gland tissues and milk of healthy and chronically infected with *Staphylococcus aureus* cows. Somatic cell counts (SCCs) during the 3 months before the study were $>1 \times 10^6$ cell/ml in the infected quarters and $<1 \times 10^5$ cell/ml in the infection-free quarters. Immediately after slaughter, samples from the tissues above the gland cistern and supra-mammary lymph node were collected. No histological differences were found between the supra-mammary lymph nodes of the healthy and infected udders, and both appeared normal. In the milk of the healthy infection-free mammary glands, SCC was $<50\,000$ cells/ml while epithelial cells were the predominant type. The percentage of CD18⁺ was low than 45%, of which over three-quarters were polymorphonuclear (PMN), and less than one-quarter were mononuclear cells. The later comprised CD4⁺ or CD8⁺ T-lymphocytes, macrophages (Mo) but not B-cells. In the tissues, there were few CD18⁺ leukocytes, and most of the cells were T-lymphocytes. The number of B-lymphocytes bearing CD21⁺ was similar to that of CD8⁺ and were localized in the connective tissue as clusters of 2–5 cells, mainly in areas with no alveoli, or as single cell having a dendritic like form. The number of Mos was negligible. In the milk of the infected glands, SCC exceeded 700 000 cells/ml, of which $>95\%$ were CD18⁺ positive. The distribution of the leukocytes had two patterns: one presented ($>80\%$) of PMN cells and a small number of mononuclear cells; the second had less than 50% PMN and many mononuclear cells. The CD8⁺ cells in these infected sections were observed throughout the mammary epithelial cells (MEC) around the alveoli and in the alveolar lumen (AL). The numbers and the location of CD21⁺ B-lymphocytes were similar to those in the infection-free mammary glands. The number of CD5⁺ positive cells was lower than T and B- cells combined and were located throughout the mammary epithelial cells, around the alveoli and within the connective tissue. Mo numbers were high in most of those infected quarters, and were localized around the connective tissue and within the AL.

Introduction

Staphylococcus aureus (*S. aureus*) udder infection in its sub-clinical form impairs alveolar function, reduces milk yield, and has a deleterious effect on milk composition, including increased milk Somatic cell counts (SCC) (Gudding et al.,

1984; Nickerson, 1989). In the mammary gland, the number and distribution of the leukocytes are important for success of the udder defenses against pathogens. Most studies address the somatic cells and their distribution in the milk, but the leukocytes in the udder tissue could differ from those in the milk and may play a more important role in mammary immunity than the latter. Moreover, their distribution in the different mammary gland compartments may have crucial roles in protection against and/or recovery from infection. In healthy, uninfected mammary glands the number of somatic cells in milk is low ($<10^5$ cells/ml), while the percentage somatic cells, which comprises leukocytes within the SCC range from below 60% (Schalm et al., 1971; Leitner et al., 2000a) up to 98% (Concha et al., 1978). The distribution of leukocyte types in milk also varies: some studies have found percentage of macrophages (Mo) up to 60% (Lee et al., 1980; Concha, 1986; Sordillo et al., 1988; Miller et al., 1991), in others Mo did not exceed 20% (Schalm et al., 1971; Park et al., 1992; Leitner et al., 2000a,b). The B-lymphocyte content is also 'controversial', ranging from 2 to 20% (Concha et al., 1980; Shafer-Weaver et al., 1996; Park et al., 1992; Leitner et al., 2000a,b). T-lymphocytes are predominantly CD8⁺ cells so that the CD4⁺ : CD8⁺ ratio is less than unity (<1) (Park et al., 1992; Leitner et al., 2000a,b). Although there are variations in the distribution of leukocytes and epithelial cells in the milk from non-infected glands, they probably arise from differences in methods, breeds, stages of lactation, sampling ect. In previous study (Leitner et al., 2000b), the distribution of SCC in the milk was determined by both light microscopy (LM) and flow cytometry. A high correlation was found between the two methods ($r = 0.96$, $P < 0.001$) for total lymphocytes and PMN cells, whereas a lower correlation ($r = 0.78$, $P < 0.03$) was found for macrophages and epithelial cells. This lower correlation was due to difficulty in distinguishing between Mos and epithelial cells by LM in some samples. In the flow cytometry method, epithelial cells were regarded as the negative cells (not labeled) by the CD11a/CD18 mAb. Moreover, no significant difference was found between the percentage of epithelial cells in milk from infection-free udder quarters identified by LM and that of CD11a/CD18 negative cells identified by flow cytometry (44 and 45% respectively).

In the early stages of mammary infection, including that caused by *S. aureus*, SCC increases to $>10^6$ cells/ml with neutrophils being the predominant cell type amongst leukocytes in the milk (Paape et al., 1981; Sordillo et al., 1989;

Leitner et al., 2000b; Shoshani et al., 2000). In the chronic stage the percentage of CD8⁺ T-lymphocytes increases while that of neutrophils decrease (Leitner et al., 2000b). It is important therefore, to elucidate the role of the mononuclear cells in mammary gland immunity, and their distribution in tissues. Yamaguchi et al., (1999) working with mammary gland tissues of healthy cows, reported that CD8⁺ subpopulations were higher than those of CD4⁺ and that the CD4⁺ were primarily localized in the interalveolar connective tissues whereas the CD8⁺ cells were located in close proximity to the mammary epithelium. The present study aimed to determine the distribution of the mononuclear cells in the milk and mammary gland tissues, and to compare the distributions in healthy cows with those in animals chronically infected with *S. aureus*.

Materials and Methods

Animals

Six Israeli–Holstein cows in mid- period of their second or higher lactation were included in this study. Four of them were known to have chronic intramammary *S. aureus* infection in one or two quarters (1629/1,3; 1718/2; 1814/1; and 1826/1,3) and two (1665 and 1761) were free of udder infection in all quarters. SCCs during the 3 months before the study were $> 1 \times 10^6$ cell/ml in the infected quarters and $< 1 \times 10^5$ cell/ml in the infection-free quarters. The udder conditions were confirmed by three consecutive bacteriological examinations of weekly quarter milk samples, before the study began. The cows were milked three times daily.

Experimental design

Milk samples of the studied quarters (one quarter of each of the two infection-free cows and the six infected quarters of the four *S. aureus* infected cows) were taken three times during the last 3 weeks before slaughter, and were subjected to bacteriological examination, SCC and somatic cells differentiation counts. Results are exhibits as the mean and standard division (SD) of these three sampling. Immediately after slaughter, samples from the tissues above the gland cistern and supra-mammary lymph node were collected and transferred on ice to the laboratory. Of each sample, half was fixed into 10% neutral buffered formalin (NBF) and half frozen and stored in -80°C . The samples were embedded in parafine wax, sectioned at 3–4 μ and stained with hematoxylin and eosin (H + E) for histopathological examination. The frozen cutting sections were used for *in-situ* labeling by the different mAbs.

Bacteriological examinations

Duplicate quarter foremilk samples were taken aseptically according to the International Dairy Federation (IDF, 1985) procedures and submitted to the laboratory within 1 h. Bacteriological analysis was performed according to accepted standards (Hogan et al., 1999). A 0.01-ml of aliquot each milk sample was spread over blood-agar plates (Bacto-Agar; Difco Laboratory) containing 5% of sheep red blood cells. All plates were incubated at 37°C and examined for growth at 18 and 42 h. Colonies suspected as staphylococci were tested for coagulase [(tube test) Anilab, Rehovot, Israel] and strain

identification was performed using ID 32 Staph kit (Bio Merieux S.A., 69280 Marcy-l'Etoile, France).

Somatic cell counts

Somatic cell counts were performed with a Coulter[®] Counter (CC) Model Z1 (Coulter Electronics Ltd., Beds., England) according to the revised protocol of the A2B sub-group of the IDF [Mastitis Experts; IDF (1991)].

Antibodies and Conjugates

The monoclonal antibodies (mAbs) (VMRD Inc. Pullman, WA, USA) used for the detection of different leukocytes were: (i) leukocytes, anti-CD11a/CD18: BAT75A, (ii) lymphocyte; anti-CD4: CACT138A, anti-CD8: CACT80C, anti-CD21: GB25A, and anti-CD5: B29A, (iii) anti-CD14 (monocyte/macrophage); CAM36A, (iv) anti granulocyte; CH138A (G1). All mAbs were species-reactive with bovine cells.

The polyclonal antibodies used were goat F(ab')₂ anti-mouse immunoglobuline G (IgG) (H + L) conjugated with fluorescein isothiocyanate (FITC) that exhibited minimal cross-reaction to human-, bovine- and horse-serum proteins, (Jackson Immuno Research Lab., West Grove, PA, USA) and affinity isolated goat anti-mouse IgG-1 conjugated with TRI-COLOR, (CALTAG Laboratories, Burlingame, CA, USA).

Flow cytometry procedure

FACScan (Becton Dickinson Immunocytometry System, San Jose, CA, USA) was used. Milk samples were kept at room temperature and analysed not more than 3 h after collection. The number of somatic cells in each sample was determined by CC and the volume of milk containing approximately 1×10^6 cells was split into 15-ml test tubes, one tube for each mAb and the control. The tubes were centrifuged for 10 min (230 g, 4°C), the fat was removed by aspiration, the supernatant was discarded and the pellets were resuspended in phosphate-buffered saline (PBS) and washed by centrifugation once more for 5 min (200 g, 4°C). To each tube (cell pellets), 50- μ l of one of the mAbs or PBS (as a negative control) it was then, mixed gently and incubated for 1 h at 4°C. Cells were washed ($\times 3$) in PBS and 50- μ l of one (single) or two (double) of the conjugated antibodies were added, mixed gently and incubated for 30 min at 4°C. Following incubation, the cells were washed and resuspended in PBS to a final volume of 1 ml. To calculate the percentages of the different leukocytes, 10 000 events were read per sample.

Immunohistochemistry

Fresh frozen tissues (in 2-methylbutane, nitrogen – Sigma Aldrich, Milwaukee, WI, USA) were sectioned (6 μ), placed on poly-L-lysine-coted glass microscopic slides (0.1% solution in water) (Sigma Diagnostic, poly-prep[™] Slides, St Louis, MO, USA), fixed in acetone (5 min at -20°C) and stored (-80°C) until used. Endogenous peroxidase was inactivated by immersion of the sections in a solution of Perox-Block (Zymed, San-Francisco, CA, USA) for 45 s at room temperature (RT). Sections were then rinsed in PBS (0.01 M-sodium phosphate-0.138 M-NaCl)preincubated with blocking solution (Histo-stain[™] – SP Kits, Zymed) for 1 h at RT, rinsed again in PBS

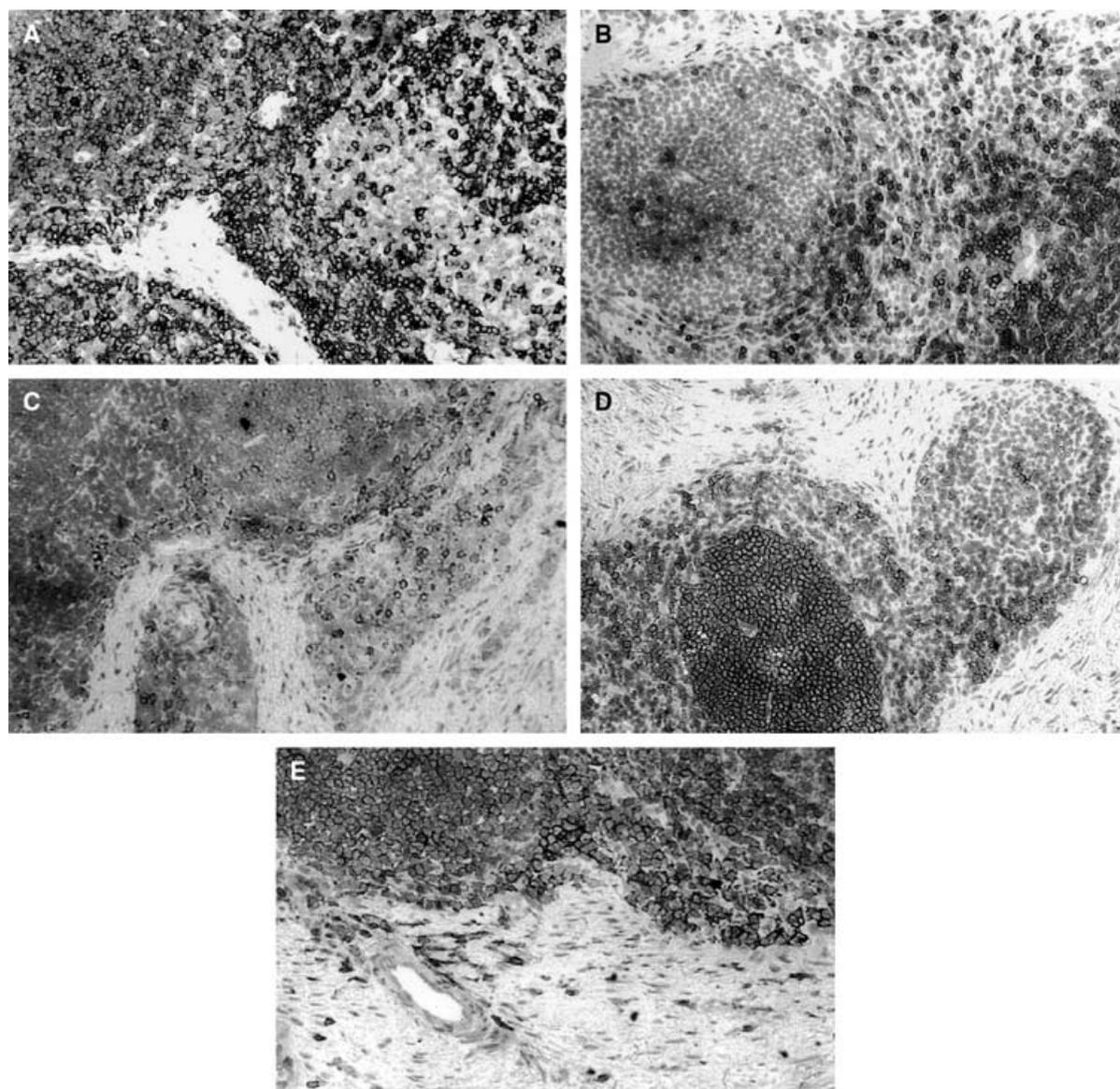


Fig. 1. Immunohistochemistry of bovine supra-mammary lymph node mononuclear cells. Panel A: Leukocytes labeled with anti-CD18, cells are scattered in the follicle cortex and paracortex with sporadic cells in the medulla sinus. Panel B: Lymphocytes labeled with anti-CD3, cells are located in the paracortex. Panel C: Lymphocytes labeled with anti-CD8, cells are located in the paracortex. Panel D: Lymphocytes labeled with anti-CD21, cells are located mainly in the secondary follicle. Panel E: Macrophages labeled with anti-CD14 are seen in the paracortex proximate to the medulla and in the connective tissues in the medulla close to a vein. All panels are magnificant (1×20).

Table 1. Somatic cell counts (SCC) \pm SD and cells proportion \pm SD of epithelial cells, leukocytes bearing CD18⁺, PMN, macrophage (Mo) and lymphocytes bearing CD4⁺ or CD8⁺, in milk from two quarters of infection-free cows (1665 and 1761) and six quarters of four chronically *Staphylococcus aureus*-infected cows (1629, 1718, 1814 and 1826)

Quarter	<i>S. aureus</i>	SCC $\times 10^3$	Epithel.	CD18 ⁺	PMN	Mo	CD4 ⁺	CD8 ⁺
1665/2	–	50 \pm 8	61 \pm 5	39 \pm 6	32 \pm 4	0	3 \pm 0	4 \pm 0
1761/4	–	51 \pm 11	59 \pm 7	41 \pm 6	28 \pm 5	3 \pm 0	3 \pm 1	7 \pm 3
1629/1	+	> 5000	6 \pm 2	94 \pm 1	85 \pm 3	1 \pm 1	3 \pm 0	5 \pm 1
1629/3	+	> 5000	3 \pm 1	97 \pm 2	83 \pm 1	3 \pm 3	3 \pm 2	8 \pm 2
1718/2	+	2400 \pm 455	11 \pm 2	89 \pm 2	42 \pm 4	11 \pm 3	9 \pm 2	27 \pm 3
1814/1	+	977 \pm 97	3 \pm 0	97 \pm 1	47 \pm 5	14 \pm 3	13 \pm 3	23 \pm 3
1826/1	+	954 \pm 120	4 \pm 1	96 \pm 2	93 \pm 2	1 \pm 0	1 \pm 1	1 \pm 0
1826/3	+	750 \pm 35	3 \pm 0	97 \pm 3	94 \pm 2	2 \pm 0	1 \pm 0	1 \pm 0

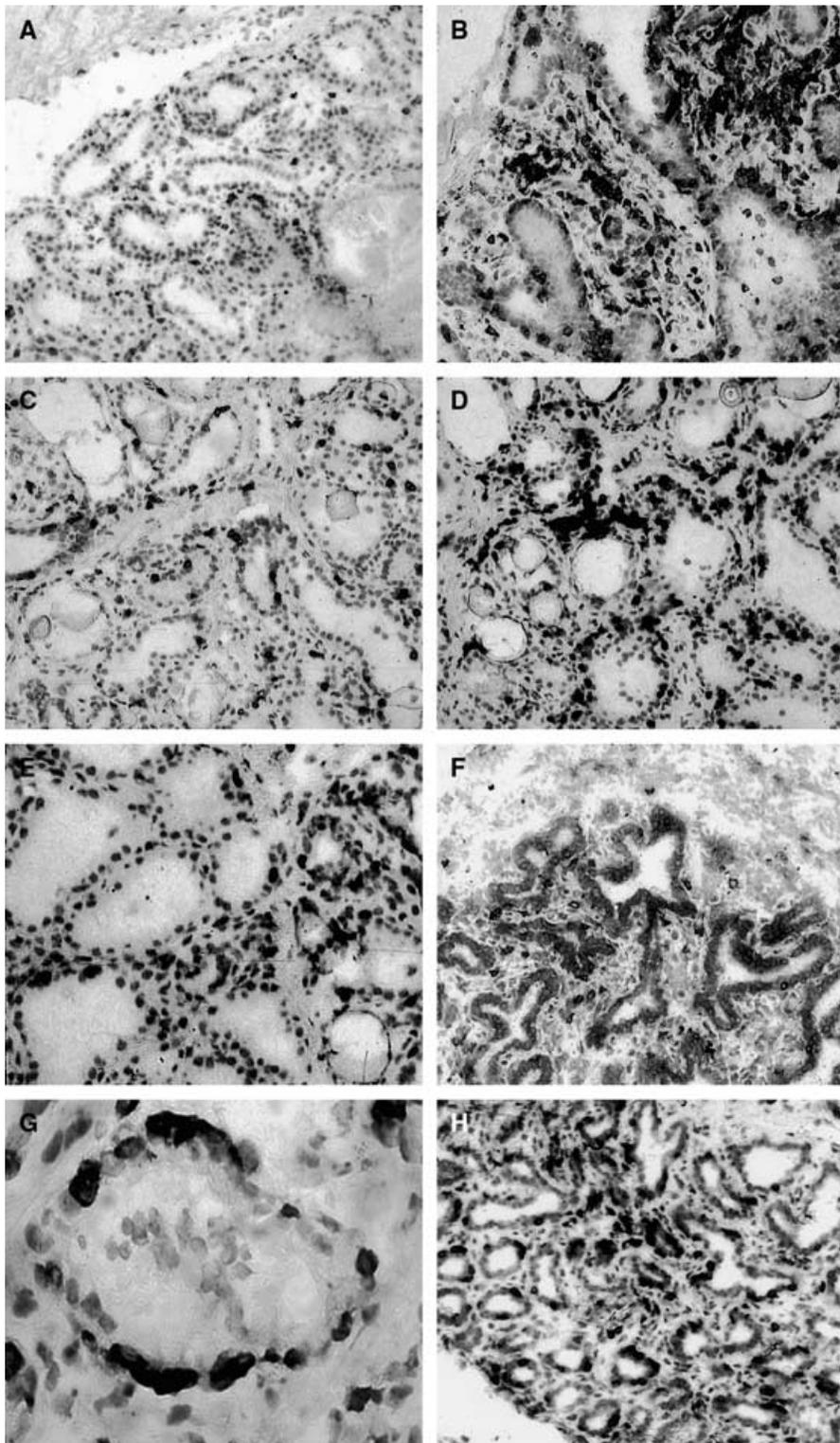


Fig. 2. Immunohistochemistry of *Staphylococcus aureus*-infected (B,D,F and H) and infection-free (A,C,E and G) bovine mammary gland. Panel A and B: Leukocytes labeled with anti-CD18, Panel C and D: Lymphocytes labeled with anti-CD3. Panel E and F: CD4⁺ T-lymphocytes scattered in the interalveolar connective tissues. Panel G and H: CD8⁺ T-lymphocytes were found throughout the mammary epithelial cells around the alveoli. Panels A–F and H, are magnificent (1 × 20) and panel G is magnificent 1 × 40.

and incubated with 1/50 or 1/100 dilutions of one of the primary antibodies (see above: Antibodies and Conjugates) for 1 h at RT. Sections were washed four times in PBS with 0.05% Tween 20 (PBST) for 5 min each, incubated with biotinylated goat anti-mouse IgG for 1 h at RT. After incubation, sections were rinsed four times in PBST and incubated with streptavidin-horseradish peroxidase conjugate PO (Zymed) at RT. After

washing in PBST, all sections were treated with substrate-chromogen mixture (3-amino-9-ethyl carbazole, Zymed) for 10–20 min at RT, rinsed with distilled water, counterstained with hematoxylin (1.5 min), rinsed again in tap water, dehydrated and mounted with Solution GVA Mount 95-8543, (Zymed). As an assay control, one of the tissue sections was tested each time and compared with a standard. The number

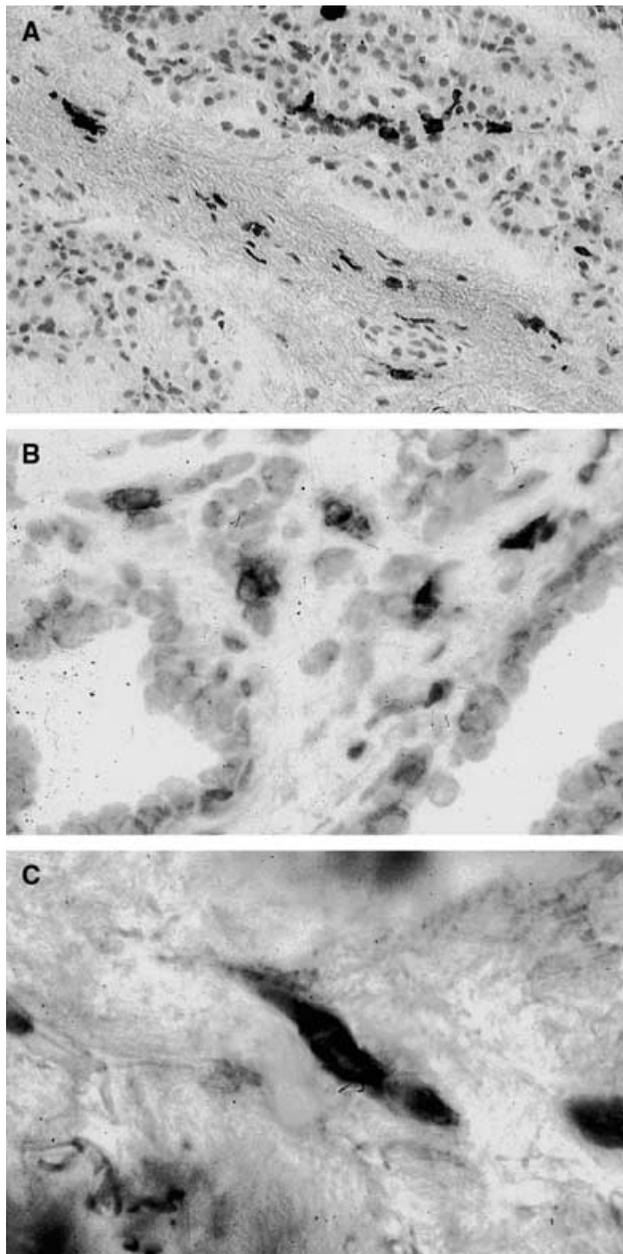


Fig. 3. Immunohistochemistry of CD21⁺ bearing bovine mammary gland lymphocytes. Panel A: Clusters of 2–5 cells mainly in area of interalveolar connective tissues magnificent (1 × 20). Panel B: Magnification (1 × 40) of the cells in the interalveolar connective tissues. Panel C: Single cell with dendritic like form (1 × 100).

of positive cells was taken as the average of counts in four similar areas, and the numbers were replaced with ratings of low (+), moderate (++) and high (+++).

Results

The supra-mammary lymph nodes of both the healthy and infected udders appeared to be normal. Immunohistochemistry findings of the supra-mammary lymph node of one of the free-infected cows (no1665) are shown in Fig. 1. The majority of cells in the follicle, cortex and paracortex were CD18⁺ with scattered cells in the medullary sinus (A). CD3⁺ (B) or CD8⁺ (C) T-lymphocytes were located in the paracortex. Anti-CD21-labeled B-lymphocytes were located mainly in the secondary follicle (D), while anti-CD14 Mo, (E) were seen in the paracortex proximate to the medulla and in the connective tissues in the medulla close to a vein. In the milk of the healthy uninfected mammary glands (cows 1665/2 and 1761/4), SCC was low (50 000 cells/ml) and epithelial cells were the predominant type (>55%), fewer than 45% were leukocytes (CD18⁺), and of those more than three-quarters were PMN and under one-quarter were mononuclear (Table 1). The histological section of both glands showed fully lactating alveoli with no inflammatory infiltration (data not shown). The number of CD18⁺ was low (Fig. 2A), most of which were T-lymphocytes (CD3⁺ Fig. 2C) and of those CD8⁺ (Fig. 2G) were higher than CD4⁺ (Fig. 2E). The number of B-lymphocytes bearing CD21⁺ (Fig. 3) was similar to that of CD8⁺. The CD8⁺ cells were found throughout the MEc around the alveoli whereas the CD4⁺ were scattered in the interalveolar connective tissues. The CD21⁺ cells were localized in the connective tissue (Fig. 3B) with clusters of two to five cells, mainly in areas with no alveoli (Fig. 3A) or as single cells with dendritic-like cells (Fig. 3C). The number of Mo was negligible.

The SCC in milk of the *S. aureus* infected mammary glands were greater than 700 000 cells/ml, of which >95% were CD18⁺ (Table 1). The leukocytes exhibited two distribution patterns, in cows 1629/1,3 and 1826/1,3 more than 80% PMNs and there was a small number of mononuclear cells, in cows 1718/2 and 1814/1, fewer than 50% leukocytes were PMNs and there were many mononuclear cells (Table 1). B-lymphocytes could not be detected in any of these milk samples. The histological section of all six glands showed fully lactating alveoli, and only 1629/3 exhibited an inflammatory infiltration (data not shown). All of *S. aureus* infected glands (tissues) contained large numbers of mononuclear cells regardless of the

Table 2. Population rating: low (+), moderate (++) and high (+++) of leukocytes (CD18⁺), macrophage (Mo) and lymphocytes bearing CD21⁺ (B cells) or CD4⁺ or CD8⁺ (T cells) in tissue from two quarters of infection-free cows (1665 and 1761) and six quarters of four chronically *Staphylococcus aureus*-infected cows (1629, 1718, 1814 and 1826)

Quarter	<i>S. aureus</i>	CD18 ⁺	Mo	CD21 ⁺	CD3 ⁺	CD4 ⁺	CD8 ⁺
1665/2	–	+	–	+	+	–	+
1761/4	–	+	–	+	+	–	+
1629/1	+	++	++	+	+	–	+
1629/3	+	+++	+	+	++	+	++
1718/2	+	+++	++	+	+++	++	+++
1814/1	+	+++	++	–	+++	+	+++
1826/1	+	+++	+	–	+++	+	++
1826/3	+	+++	–	++	+++	++	+++

Table 3. Somatic cell counts (SCC), proportion (%) and number of mononuclear cells in the milk and population rating: low (+), moderate (++) and high (+++) in tissue from two quarters of infection-free cows (1665 and 1761) and six quarters of four chronically *S. aureus*-infected cows (1629, 1718, 1814 and 1826)

Quarter	<i>S. aureus</i>	SCC × 10 ³	Mononuclear cells (CD18 ⁺)		
			Milk		Tissue
			%	Number (× 10 ³)	
1665/2	–	50	7	3.5	+
1761/4	–	51	13	6.6	+
1629/1	+	> 5000	9	450	++
1629/3	+	> 5000	14	700	+++
1718/2	+	2400	47	1128	+++
1814/1	+	977	50	488	+++
1826/1	+	954	3	28.6	+++
1826/3	+	750	3	22.5	+++

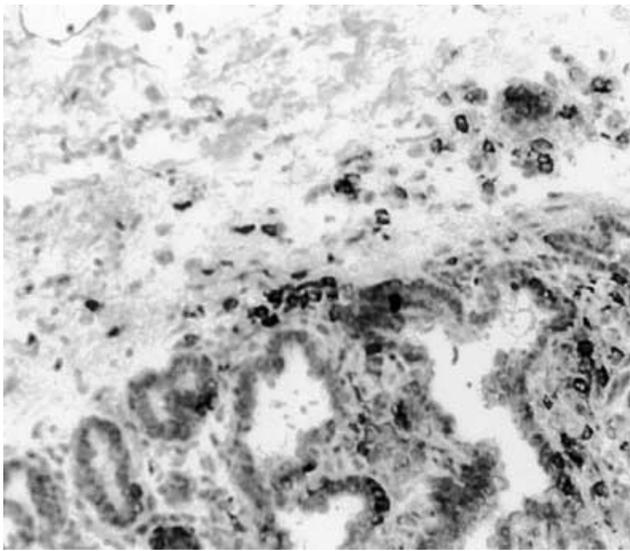


Fig. 4. Immunohistochemistry of CD5⁺ bearing bovine mammary gland lymphocytes. Cells are located throughout the mammary epithelial cells around the alveoli and in the connective tissue.

percentage and number of these cells in the milk (Table 3). There were many CD3⁺ T-lymphocytes (Fig. 2D), and there were two to three times as many CD8⁺ (Fig. 2H) as CD4⁺ (Fig. 2F) in all of these infected quarters except one (1629/1) (Table 2). The CD8⁺ in these infected sections, were observed throughout the MEC around the alveoli and in the AL. The numbers and location of CD21⁺ B-lymphocytes were similar to those in the infection-free mammary glands. There were fewer CD5⁺ cells than T and B cells all together and were located throughout the MEC cells around the alveoli and in the connective tissue (Fig. 4). There were more CD5⁺ in the infection quarters than in the infection-free mammary glands and the ones were mainly around the MEC and within the alveoli. Examination of following sections of the same infected gland, labeled with anti-CD21 or anti-CD5 revealed that the cells were in the same area of the connective tissue. No numbers were high in most of those infected quarters; they were localized in the connective tissue around the AL (Fig. 5A) and in the AL (Fig. 5B, C) of the gland.

Discussion

The milk and tissue samples from both healthy quarters contained few mononuclear. CD21⁺ – bearing B-cells and cells with dendritic-like cells were detected only in the tissue and not in the milk, and were in the same abundance as CD8⁺ T-lymphocytes. These results suggest that information taken from milk samples in regarding B-lymphocytes represents their distribution in the healthy gland to only a limited extent. There are two subsets of B cells designated B-2 and B-1 (reviewed in ref. Hardy et al., 1994). The B-2 cells have high diverse of Ig repertoire, and are predominantly localized in blood, and in the spleen and lymph nodes, which participate in T cell-dependent response; and B-1 cells with a more limited Ig repertoire which are restricted to peritoneum and other body cavities. The B-1 cells have an extensive capacity for self-renewal, expressed as the pan-T-cell glycoprotein Ly-1 in mice (CD5 in humans), and are localized mainly in the gut lamina propria and peritoneal cavity (Kroese et al., 1993) but not in Peyer's patches and they form a common pool (Murakami et al., 1994). These cells produce Abs against polysaccharides of gram-positive bacteria and against lipopolysaccharide of gram-negative bacteria (Allison and Nawata, 1992), preferentially of the IgM and IgA antibody classes (reviewed in ref. Murakami and Honjo, 1995). We suggest that some of the CD21⁺ cells in the udder are also CD5⁺ and could be some of the B-1 like cells. Moreover, the dendritic-like CD21⁺ cells may be dendritic cells that are important as local antigen-presenting cells (APC). Their presence might be crucial to the immune response, especially in view of lack of macrophages in the tissues of those udders. Those APCs may be the cells that presenting antigens to the mammary gland leukocytes including the B-1-like cells which synthesize and secrete IgA antibodies locally, with no involvement of the systemic immune system. This could explain the presence of specific to *S. aureus* IgA antibodies only in the milk of *S. aureus* positive infected quarters, and not in any of the uninfected quarters of the same cow. (Leitner et al., 2000c).

The proportion of mononuclear cells among the leukocytes in milk samples from the *S. aureus* infected glands varied among the cows. However, the absolute number was significantly higher than that of the uninfected milk samples because of the overall increase in somatic cells. This phenomenon was also found in the tissues where, as in all infected samples, the

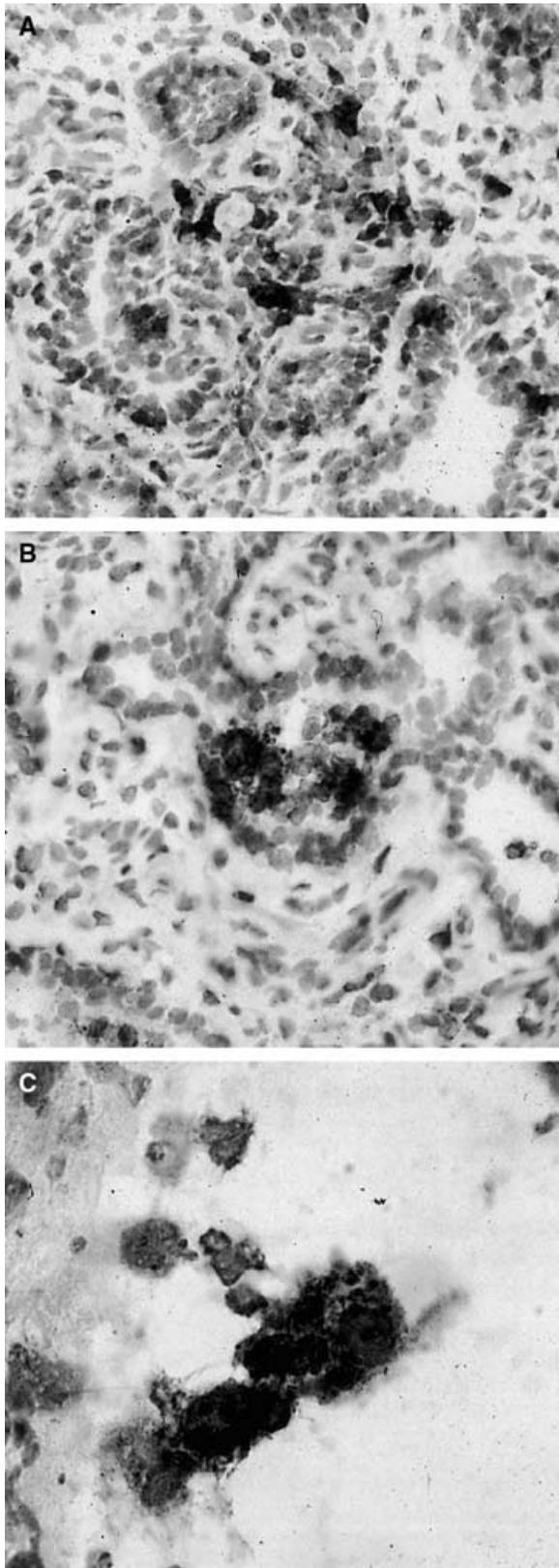


Fig. 5. Immunohistochemistry of CD14⁺ bearing bovine mammary gland macrophages. Panel A: Mo are mainly around the mammary epithelial cells and within the alveoli (1 × 20). Panel B: Magnification (1 × 40) of the cells within the alveoli. Panel C: Magnification (1 × 100) of two cells within the alveoli.

number of the mononuclear cells increased. The main increase was in the CD8⁺ T-lymphocytes and Mo although this increase was not always correlated with that found in the milk of the same quarter. The ratio of CD4⁺ to CD8⁺ in milk from healthy udders has been found to be less than unity (Asai et al., 1998; Leitner et al., 2000a; Soltys and Quinn 1999). However, in some studies (Taylor et al., 1994) the time within the lactation period was found to be important, as the number of CD4⁺ increased as the lactation progressed. In the present study in all tissues tested, regardless of whether they were healthy or infected, the CD4 : CD8 ratio was lower than unity. However, in the *S. aureus*-infected tissues, CD8⁺ cells were observed only among the MEc cells around the alveoli and in the AL, whereas in the healthy section they were found among all the MEc entire. CD8⁺ T-Lymphocytes have cytotoxic functions important in disrupting and killing target cells, which carry an intracellular pathogenic agent. One-way in which *S. aureus* could avoid phagocytoses by Mo and neutrophil is by penetrating into cells of the mammary gland (Vesga et al., 1996; Bayles et al., 1998). Thus, the cytotoxic CD8⁺ lymphocytes may be the predominant immune cells acting in this situation. It has been suggested (Yamaguchi et al., 1999) that some subpopulations of CD8⁺ in mammary glands of healthy udder are extrathymic in origin as they express $\gamma\delta$ T cells receptor (TCR) as in the case of mice intestinal mucosa. This suggestion needs further study because the percentage of CD8⁺ and TCR $\gamma\delta$ T-lymphocytes in blood and milk of healthy bovines are high, and even higher levels are presents in milk derived from *S. aureus* infected cows (Park et al., 1994; Soltys & Quinn, 1999). We suggest that the majority of CD8⁺ in the milk are thymic in origin, transferred from the blood because of the *S. aureus* infection, whereas the majority of these cells in the tissue are extrathymic in origin; however this assumption needs further clarification. The number of cells identified as Mo by the mAb anti-CD14 was small, but they were found in the milk of the healthy udder as well as in the milk of the infected quarters, and were located mainly in the AL of the gland. It seems therefore, that the mononuclear cells in the milk represent mainly the cells transferred from the blood especially at the time of udder contamination, whereas in the mammary tissues at least some of the mononuclear cells appear to be long lived and to induce a local immune response. This assumption should be further investigated.

Acknowledgements

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