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Research paper

Effects of intra-mammary bacterial infection with coagulase negative staphylococci and stage of lactation on shedding of epithelial cells and infiltration of leukocytes into milk: Comparison among cows, goats and sheep

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ABSTRACT

The effects of mammary gland bacterial infection and stage of lactation on leukocyte infiltration into the mammary gland were compared among cows, goats and sheep. Animals were at two stages of lactation: mid or late. In mid-lactation animals, bacterial-free glands and coagulase negative *Staphylococcus* (CNS)-infected glands were compared. In late lactation only uninfected glands were studied. Of mid-lactation bacteria-free animals, goats had the highest number of leukocytes and % polymorphonuclears (PMNs), whereas sheep had the lowest and leukocytes number in cows were intermediate between sheep and goats. Based on %PMN, two cell clusters were found in sheep, which overlapped with the parallel cell clusters of cows and goats, but with a slightly higher number of leukocytes in each cell cluster. At late lactation, goats had higher values for %PMN and leukocyte numbers in comparison to cows, which had a similar cellular profile to sheep. The cellular immune response to CNS infection was similar for the three animal species, although the number of cells was different, while the basal cell level at mid-lactation and especially at the end of lactation was species specific.

1. Introduction

Somatic cells (SC) are normally found in milk of dairy animals. After the colostrum period of lactation, the number of SC in the milk of uninfected glands of cows is in the range of 1×10^4 to 1×10^5 cells mL^{-1}. These cells are normally composed of ~50% leukocytes, whereas ~50% are released glandular epithelial cells (Shoshani et al., 2000). In this situation the ratio of polymorphonuclear neutrophils (PMNs) to mononuclear (lymphocytes and macrophages) is usually ~1 (Dosogne et al., 2003; Mehne et al., 2010). The number of SC in the milk of bacteria free goats and sheep glands is typically higher than that of cows, up to 5×10^5 cells mL^{-1} (Leitner et al., 2011; Silanikove et al., 2010). However, in comparison to cows, much less is known regarding the normal composition of SC in goats during lactation and, to the best of our knowledge, virtually no information exists in relation to sheep.

Intra-mammary infection (IMI) is the major cause of increased SC in milk during lactation, causing infiltration of leukocytes from the blood, dramatically changing the proportion and distribution of leukocytes in milk. However,

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such a response to IMI is not uniform: the type of infecting bacteria species and duration of infection play a major role in defining the extent to which leukocytes are altered during IMI.

So far, research has focused mainly on the immune response during clinical IMI, such as that caused by *coliforms* infection, in which there is a massive migration of PMN from blood to milk. While clinical IMI is an important disease in dairy production, most of IMI are subclinical chronic infections caused by Gram positive bacteria: coagulase positive staphylococci (mainly *Staphylococcus aureus*), various coagulase negative staphylococci (CNS) species and streptococci. It is estimated that 5–50% of glands in bovine, caprine and ovine dairy herds are subclinically infected with bacteria (Parker et al., 2007; Taponen et al., 2006; Shwimmer et al., 2007). The somatic cell count (SCC) during CNS IMI is usually from 1×10^5 to 2×10^6 cells mL^{-1}, depending on the species of bacteria involved (Djabri et al., 2002; Schukken et al., 2003). These IMI tend to become chronic since the immune system fails to clear the infection so that a different "strategy" is adopted by the immune system, increasing the proportion of migrating mononuclear cells (Leitner et al., 2000). Again, compared to the information available for cows under such situations, much less is known regarding goats and sheep.

Involution is the process of functional reset of the mammary gland occurring in three stages: cessation of milk secretion, collapse of alveoli accompanied by apoptosis and re-growth of stromal adipose tissue (Alexander et al., 2001). During the natural cycle of lactation, demand of milk by the offspring decreases as time progresses, slowly changing the circulation of hormones, which leads to reduced milk production and negative cell renewal. While in modern farming, because the cycle of lactation is influenced by milking, nutrition and reproduction, many animals do not reach the end of the natural lactation. According to the literature, SCC increases toward the end of lactation, with PMN being the major leukocyte present in milk. Since assessment of the health status of the mammary gland is not mentioned in many studies, it is possible that many animals are also subclinically infected at the end of the lactation. Thus it is impossible to evaluate whether the immune response during this time is due to infection of the mammary gland or to the process of early, forced involution. Therefore, in the present study, animals selected were in late lactation, free of infection and with a notable decline in the curve of milk production. In addition, in contrast to natural conditions, in modern intensive dairy farming, most lactating animals are also pregnant and are induced into involution by cessation of milking (drying off) ~60 days before the next parturition. Recent evidence suggests that this sudden cessation of milking in high-producing animals requires an emergency response by the mammary gland to cope with milk accumulation in the gland and the possible intrusion of pathogens into the mammary gland during the drying off period and close to parturition (unpublished data). The lack of such a response in lactating animals induced into involution while producing small amounts of milk suggests that the pre-involution period is associated with an auto-defensive response that allows an orderly involution process with an effective antimicrobial immune response (Leitner et al., 2011; Silanikove et al., 2005).

The effects of mammary gland bacterial infection and stages of lactation on milk quality were compared among cows, goats and sheep in a previous research (Leitner et al., 2011). In this study, mid-lactation uninfected, mid-lactation subclinically chronically infected with CNS and late-lactation uninfected animals were studied in order to assess the cellular immune profiles in milk of these species. The specific aims of the present work were to study and compare leukocyte infiltration into the mammary gland of bacterial-free dairy cows, goats and sheep during lactation and in the three stages of natural involution, comparing it to glands with chronic IMI caused by CNS. Intra-mammary bacteria-free mid-lactation animals within each species served as a basis for comparison (co-variant), and natural CNS infections were assessed as a model for IMI comparison.

2. Materials and methods

2.1. Animals and study layout

Animals in the study were in two stages of lactation: mid-lactation (ML) and late-lactation (LL) and were further sub-typed into bacterial-free glands (ML-F) and infected ones (ML-I). In LL animals, only uninfected glands were studied. The health condition of the udder was evaluated by total SCC and bacterial culture and was followed during the last 2–3 months before sampling. ML-I and ML-F animals were tested twice during the 7 days before milk sampling, whereas LL animals were tested 2–3 days before drying-off (~60 days before the next parturition). All uninfected animals participating in the study had negative bacteria cultures while those chronically infected were found to have a positive culture with the same CNS spices in at least in 2–3 consecutive samplings.

2.1.1. Cows

Mid-lactating cows (MLC) producing >35 L milk d^{-1} and late-lactating cows (LLC), producing <14 L milk d^{-1} were included in the study. Of the MLC, 149 glands were free of bacteria (MLC-F) and 31 glands were subclinically infected with CNS, mainly *Staphylococcus chromogenes* (MLC-I). Of the LLC, 172 glands free of bacteria were tested. Cows were provided with 10 m^2 of confined shaded slatted floor and 10 m^2 of concrete-surfaced yard for each cow. Food composed of a high-quality total mixed ratio was offered ad lib. in mangers located in the sheds.

2.1.2. Goats

Lactating Alpine, Saanen and Shami crossbreed dairy goats producing >3 L milk d^{-1} at mid-lactation (MLG) and 0.3–1 L milk d^{-1} in late-lactation (LLG) were included in the study. Of the MLG, 37 glands were free of bacteria (MLG-F) and 43 glands were subclinically infected with CNS, mainly *Staphylococcus epidermidis* and *Staphylococcus simulans* (MLG-I). In LLG, 64 glands free of bacteria were tested. The goats were kept in 4 m^2 closed sheds with an extra 4 m^2 of open yard for each animal. Food composed of

high-quality total mixed ration was offered ad lib. in mangers located in free-stall barns.

2.1.3. Sheep

Lactating Assaf dairy sheep producing >2.5 L milk d^{-1} in mid-lactation (MLS) and 0.5–1 L milk d^{-1} in late-lactation (LLS) were included in the study. Of the MLS, 21 glands were free of bacteria (MLS-F) and 29 glands were sub-clinically infected with CNS, mainly *S. epidermidis* and *S. chromogenes* (MLS-I). In the LLS group, 11 glands free of bacteria were tested. Sheep were kept in 4 m^2 closed sheds with an extra 4 m^2 of open yard for each animal. High quality food was offered in mangers located in free-stall barns.

2.2. Samples analysis

Bacteriological analysis in pre-trial periods and on milk sampling days was performed according to accepted standards of microbiological procedures, as described by Oliver et al. (2004). Colonies suspected to be *Staphylococcus* were identified by means of the ID-32-API STAPH test (BioMerieux, Marcy-l'Etoile, France) and were considered to be CNS. The isolate identification was >98% with T >65%. In addition, for some isolates 16S rRNA was performed (Hy Laboratories, Rehovot, Israel). Milk was sampled from each quarter/half during the morning milking. For the bacteriological tests, the teats were cleaned and disinfected and 5 mL of milk were sampled. On the test day, an additional sample (100–300 mL) was taken from each gland for the following analysis: SCC was performed using Fossomatic 360 (Foss Electric, Hillerod, Denmark) and differentiation of cells by FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA) (Leitner et al., 2003) using anti-bovine (B), ovine (O) and caprine (goat) (G) monoclonal antibodies (VMRD Inc., Pullman, WA, USA). The flow cytometer identifies milk leukocytes and is referenced in many published works. In all the assays, we used CD18 or CD45 to identify leukocytes from non-immune cells. Accordingly, $CD18^-$ are considered epithelial cells that was tested earlier by comparing flow cytometry with light microscopy (Shoshani et al., 2000). The SCC consisted of the total number of cells in a milk sample, where the number of leukocytes was calculated as the percentage of $CD18^+$ results from 10,000 cells read by the flow cytometer and the number of epithelial cells as $CD18^-$. The other mAbs used were specific to PMN, Mo, B and T (CD4 and CD8) lymphocytes. Because a 3 channel flow cytometer was used, it was possible to read only three cell markers. Since not all the $CD18^f$ cells are marked with one of the mAb used, the results can be ±3–5%. For the non-specific fluorescence, only tubes with the secondary antibodies were used.

Monoclonal antibodies were: anti-CD18/11a – BAT 75A (IgG-1) (B,O,G), anti-CD4 – GC 50A1 138A (IgM) (B); GC 1A (C,G), anti-CD8 – CACT 80C (IgG-1) (B,O,G), anti-CD21 – BAQ 15A (IgM) (B,O,G), anti-CD14 – CAM 36A (IgG-1) (B,O,G), anti-polymorphonuclear (PMN) (G1) (IgM) (B); PG 68 A (O,G). All monoclonal antibodies used were species-reactive with bovine cells. The secondary polyclonal antibodies (CALTAG Laboratories, Burlingame, CA, USA) used were: goat anti-mouse IgG-1 conjugated with TRI-COLOR (TC) and goat anti-mouse IgM conjugated with FITC.

2.3. Statistical analysis

All statistical analyses were carried out with JMP software (SAS Institute, 2002). The main parameters analyzed were: milk yield (L d^{-1}), SCC (×1000), log SCC, and SCC differentiation. For each animal species, the effects of Group (i.e., mid-lactation, infection-free, ML-F; mid-lactation, infected, ML-I; and late-lactation, LL) and Lactation number i.e., 1st lactation, 2nd lactation, and 3rd on the analyzed parameters were determined by two-way ANOVA in a random design.

The statistical model (1) was:

$$Yijk = \mu + \alpha i + \beta j + eijk \quad (1)$$

where μ is the mean of all data, αi is the difference between the mean of Group i and the trial mean (fixed effect), βj is the difference between the mean of lactation j and the trial mean (fixed effect), and $eijk$ is the residual variance between measurements (random error). Multiple comparisons between groups were made applying the Tukey–Kramer HSD t test.

The effects of group (i.e., ML-F, ML-I, or LL) and animal species (i.e., cow, goat, or sheep) on the analyzed parameters were determined with a three-way ANOVA model, in a random design.

The statistical model (2) was:

$$Yijk = \mu + \alpha i + \beta j + \alpha\beta ij + eijk \quad (2)$$

where μ is the mean of all data, αi is the difference between the mean of group i and the trial mean (fixed effect), βj is the difference between the mean of animal j and the trial mean (fixed effect), $\alpha\beta ij$ is the interaction between group and animal (fixed effect), and $eijk$ is the residual variance between measurements (random error).

3. Results

3.1. Cows

MLC-I and MLC-F studied were 155 days in milk (DIM), whereas LLC were 426 DIM. Milk yield measured on the cow level (all four glands) was similar in MLC-I and MLC-F groups (~37 L d^{-1}), but significantly lower in LLC (13.3 L d^{-1}) (Table 1). Considering MLC-F as the baseline for comparison, end of lactation was characterized by increased SCC, of which ~65% were leukocytes. CD4 and CD8 T-lymphocytes and monocytes were evenly distributed (~10% each), higher than in MLC-F, whereas PMN remained unchanged in LLC and were ~25% of the leukocyte population in milk. In contrast, infection in mid-lactation was characterized by almost a × 2 increase in SCC than in late lactation. The great majority of cells in MLC-I were also leukocytes, with a significant increase of PMN. CD4 and CD8 T lymphocytes and monocytes were evenly distributed (6–9% each). Very few B-lymphocytes were detected in the milk, and only in late lactation. The main differences between MLC-I and LLC were SCC, the percentage of PMN (higher in MLC-I) and CD4 T-lymphocytes

Table 1
Days in milk (DIM), milk yield, SCC and cell differentiation (percent and number) in milk according to groups of cows' milk for mid-lactation, bacteria-free (MLC-F), mid-lactating, infected (MLC-I), and late-lactation, bacteria-free (LLC).[a]

No. of quarters	MLC-F 149	MLC-I 31	LLC 172	*P* [*F*]
DIM	154 ± 4.1 b	155 ± 12.4 b	426 ± 6.8 a	<0.001
Milk (L d^{-1})	37.1 ± 1.75 a	36.2 ± 1.51 a	13.3 ± 0.19 b	<0.001
SCC (×10^3)	49 ± 3 c	850 ± 126 a	441 ± 35 b	<0.001
Log SCC	4.57 ± 0.02 c	5.69 ± 0.09 a	5.49 ± 0.02 b	<0.001
[b]CD18$^+$ %	58.7 ± 0.9 c	80.9 ± 2.66 a	64.4 ± 1.44 b	<0.001
CD18$^+$ # (×10^3)	25.2 ± 2 c	717 ± 118 a	322 ± 31 b	<0.001
PMN %	35.6 ± 1.45 b	54.9 ± 3.03 a	24.5 ± 1.11 b	<0.05
PMN # (×10^3)	18.3 ± 1 c	485 ± 96 a	113 ± 1.11 b	<0.001
CD4$^+$ %	4.29 ± 0.31 b	6.6 ± 0.7 b	11.8 ± 0.56 a	<0.001
CD4$^+$ # (×10^3)	2.3 ± 0.1 b	62 ± 14 a	64 ± 8.56 a	<0.05
CD8$^+$ %	10.2 ± 0.6	9.9 ± 1.16	12.7 ± 0.81	NS
CD8$^+$ # (×10^3)	4.5 ± 0.4 b	89 ± 29 a	64 ± 7.7 a	<0.05
CD21$^+$ %	0	0	2.1 ± 0.3 a	<0.001
CD21$^+$ # (×10^3)	0	0	12.9 ± 3.7 a	<0.05
CD14$^+$ %	5.27 ± 0.31 b	9.8 ± 0.9 a	10.3 ± 1.74 a	<0.001
CD14$^+$ # (×10^3)	3.1 ± 0.3 b	48 ± 9 a	56 ± 8.3 a	<0.05

[#] Number of cells.
[a] Results are presented as mean ± SE; values within rows with no common letters differ significantly ($P < 0.05$).
[b] Total leukocytes.

(higher in LLC). Log SCC and % leukocytes were plotted in Fig. 1. Although MLC-F and LLC have an equally large distribution regarding % leukocytes, LLC show higher log SCC. MLC-I are somewhat clustered at high % leukocyte and high log SCC. In Fig. 2, a plot of log PMN and log mononuclear cells is shown. Here, MLC-F clustered at low log PMN and log mononuclear cells. Although both the other two groups revealed higher log of PMN and mononuclear cells, a shift toward mononuclear cells can be seen in LLC, whereas a shift toward PMN can be seen in MLC-I.

3.2. Goats

MLG-I and MLG-F studied were ~125 DIM, whereas LLG were at 253 DIM as shown in Table 2. As in cows, milk yield was similar in MLG-I and MLG-F groups, but lower in LLG. In contrast to cows, when MLG-F was considered as the baseline for comparison, LLG exhibited more than a × 2 increase SSC in comparison to MLG-I. In both cases leukocytes were the majority of SC, but were higher in MLG-I than in LLG. As in cows, %PMN increased in MLG-I but not in LLG; however, the absolute number of PMN increased in LLG because of the absolute increase in total SCC in that period. In comparison to cows, lower percentages of mononuclear cells were observed in MLG-I and LLG. Also in contrast to cows, %CD4 did not increase in LLG. Log SCC and % leukocytes were plotted in Fig. 3. LLG and MLG-I both revealed high % of leukocytes, but most LLG are clustered at a higher SCC. In Fig. 4, a plot of log PMN and log mononuclear cells is shown. Although overlapping is high, MLG-F clustered

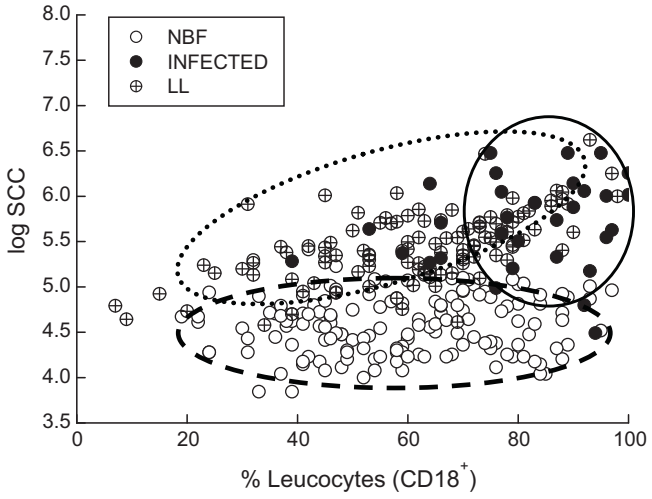

Fig. 1. Percent leukocytes (CD18$^+$) vs. log SCC in milk according to groups of cows: mid-lactation, bacteria-free (MLC-F) (encircled dashed); mid-lactating, infected (MLC-I) (encircled solid); and late-lactation, bacteria-free (LLC) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

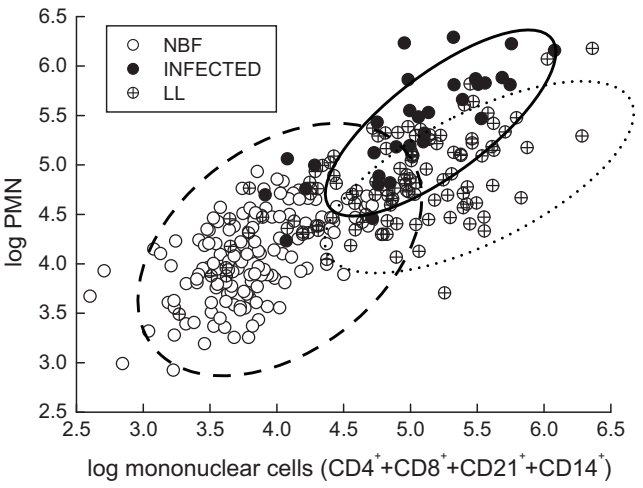

Fig. 2. Log mononuclear cells (CD4$^+$CD8$^+$CD21$^+$CD24) vs. log PMN in milk according to groups of cows: mid-lactation, bacteria-free (MLC-F) (encircled dashed); mid-lactating, infected (MLC-I) (encircled solid); and late-lactation, bacteria-free (LLC) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

Table 2
Days in milk (DIM), milk yield, SCC and cell differentiation (percent and number) in milk according to groups of goats' milk for mid-lactation, bacteria-free (MLG-F), mid-lactating, infected (MLG-I), and late-lactation, bacteria-free (LLG).[a]

No. of quarters	MLG-F 37	MLG-I 43	LLG 64	*P* [*F*]
DIM	129 ± 15.9 b	121 ± 6.3 b	253 ± 4.8 a	<0.001
Milk (L d^{-1})	3.1 ± 0.1 a	2.87 ± 0.14 a	0.99 ± 0.17 b	<0.001
SCC (×10^3)	464 ± 74 c	2808 ± 601 b	7694 ± 1090 a	<0.001
Log SCC	5.67 ± 0.93 b	6.15 ± 0.08 a	6.49 ± 0.09 a	<0.001
[b]CD18$^+$ %	57.4 ± 3.5 c	86.3 ± 2.15 a	74.4 ± 3.2 b	<0.001
CD18$^+$ # (×10^3)	291 ± 58.8 c	2651 ± 606 b	6479 ± 962 a	<0.001
PMN %	49.3 ± 3.26 b	81.0 ± 1.98 a	[c]52.5 ± 3.3 b	<0.001
PMN # (×10^3)	256 ± 56.5 b	2537 ± 586 a	3113 ± 431 a	<0.001
CD4$^+$ %	0.82 ± 0.23	0.51 ± 0.15	1.61 ± 0.09	NS
CD4$^+$ # (×10^3)	3 ± 1 b	11 ± 3.4 b	25 ± 6.3 a	<0.05
CD8$^+$ %	1.37 ± 0.24	0.86 ± 0.17	0.73 ± 0.12	NS
CD8$^+$ # (×10^3)	5 ± 1 c	12 ± 3.2 b	28 ± 6.75 a	<0.05
[c]CD14$^+$ %	2.52 ± 0.39	2.11 ± 0.26	2.5 ± 0.23	NS
CD14$^+$ # (×10^3)	11 ± 2 c	77 ± 24 b	128 ± 21 a	<0.05

[#]Number of cells.
[a] Results are presented as mean ± SE; values within rows with no common letters differ significantly ($P < 0.05$).
[b] Total leukocytes.
[c] Only macrophages (most PMN were also CD14+; 52.8 ± 2.7 + 18.8 ± 2.9).

at low log PMN and log mononuclear cells compared to the other groups, but at higher levels than those for cows, due to higher SCC. Also, in contrast to cows, a shift toward mononuclear cells can be seen in MLG-I, whereas a shift toward PMN can be seen in LLG.

3.3. Sheep

MLS-I and MLS-F were assessed between 60 and 90 DIM, and LLS were assessed at 172 DIM as shown in Table 3. Contrary to cows, MLS-I had decreased milk yield, which was even lower in LLS. The increase in SCC in LLS compared to MLS-F was milder than in the other two species, whereas in MLS-I, this increase was the highest (almost 40 times the values in MLS-F). The majority of SC in MLS-I were leukocytes, of which PMN represented the main cell type. In contrast to the other two species, %PMN also increased significantly in LLS. Also, in contrast to cow and goats, a significant increase in CD14 was observed in LLS, but not in MLS-I. Log SCC and % leukocytes were plotted in Fig. 5. In sheep, MLS-I are completely separated from MLS-F and LLS by high log SCC and % leukocytes, whereas MLS-F and LLS overlap. In Fig. 6, a plot of log PMN and log mononuclear cells is shown. As in Fig. 5, overlapping of MLS-F and LLS was high, while MLG-I could be completely differentiated by high log PMN and high log mononuclear cells.

3.4. Combined species analysis

In order to simultaneously compare the leukocyte response in the three studied dairy species, log total leukocytes was plotted against %PMN separately for the three tested conditions (Fig. 7). In bacterial free animals at mid-lactation, goats had the highest number of leukocytes and

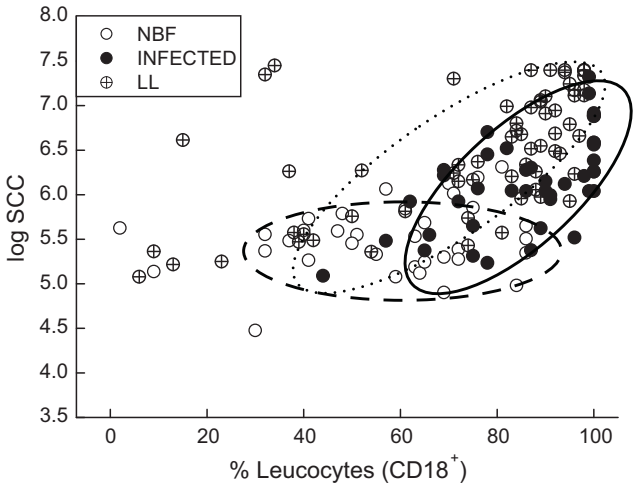

Fig. 3. Percent leukocytes (CD18$^+$) vs. log SCC in milk according to groups of goat: mid-lactation, bacteria-free (MLG-F) (encircled dashed); mid-lactating, infected (MLG-I) (encircled solid); and late-lactation, bacteria-free (LLG) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

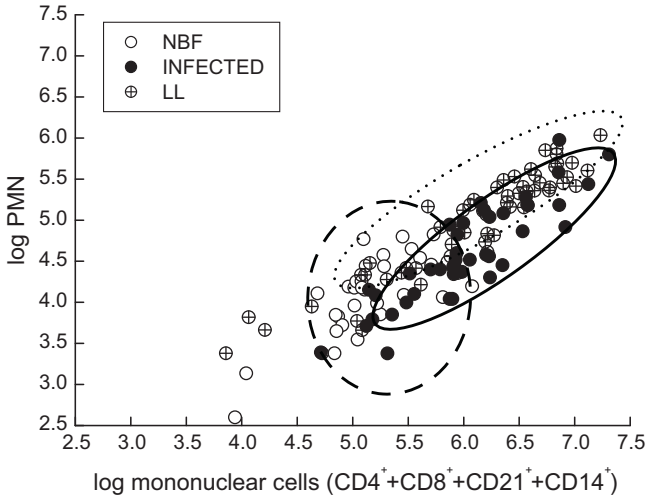

Fig. 4. Log mononuclear cells (CD4$^+$CD8$^+$CD21$^+$CD24) vs. log PMN in milk according to groups of goats: mid-lactation, bacteria-free (MLG-F) (encircled dashed); mid-lactating, infected (MLG-I) (encircled solid); and late-lactation, bacteria-free (LLG) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

Table 3
Days in milk (DIM), milk yield, SCC and cell differentiation (percent and number) in milk according to groups of sheep milk for mid-lactation, bacteria-free (MLS-F), mid-lactating, infected (MLS-I), and late-lactation, bacteria-free (LLS).[a]

No. of quarters	MLS-F 21	MLS-I 29	LLS 11	*P* [*F*]
DIM	62 ± 12.6 b	89 ± 11.6 b	172 ± 7.7 a	<0.001
Milk (L d^{-1})	3.13 ± 0.16 a	2.00 ± 0.17 b	0.90 ± 0.13 c	<0.001
SCC (10^3)	99 ± 19 b	3907 ± 482 a	235 ± 93 b	<0.001
Log SCC	4.91 ± 0.05 b	6.51 ± 0.05 a	5.15 ± 0.17 b	<0.05
[b]CD18$^+$ %	17.2 ± 3.1 b	87.3 ± 1.6 a	26.4 ± 5.2 b	<0.001
CD18$^+$ # ($\times 10^3$)	28 ± 15 b	3498 ± 467 a	92 ± 44 b	<0.001
PMN %	6.2 ± 1.0 c	72.7 ± 3.2 a	17.7 ± 3.6 b	<0.05
PMN # ($\times 10^3$)	9 ± 4 c	2869 ± 418 a	58 ± 15 b	<0.001
CD4$^+$ %	1.0 ± 0.16	2.3 ± 0.62	2.3 ± 0.9	NS
CD4$^+$ # ($\times 10^3$)	1 ± 1 b	89 ± 21 a	8 ± 3.4 b	<0.05
CD8$^+$ %	3.5 ± 0.47	2.75 ± 0.5	2.8 ± 0.7	NS
CD8$^+$ # ($\times 10^3$)	4 ± 1 b	102 ± 22 a	10 ± 7 b	<0.001
CD14$^+$ %	0.7 ± 0.5 b	3.9 ± 0.4 b	11.9 ± 3.4 a	<0.05
CD14$^+$ # ($\times 10^3$)	1 ± 0.4 c	139 ± 20 a	17 ± 3 b	<0.05

#Number of cells.
[a] Results are presented as mean ± SE; values within rows with no common letters differ significantly ($P < 0.05$).
[b] Total leukocytes.

%PMN, sheep had the lowest and cows were intermediate between sheep and goats (Fig. 7A). In infected animals, goats and cows exhibited separate profiles that could be differentiated by %PMN, which was higher in goats (Fig. 7B). In sheep, two clusters were observed based on %PMN, which overlapped with the parallel clusters in cows and goats, apart from having a slightly higher number of leukocytes in each cluster. At late lactation (Fig. 7C), goats had higher values for %PMN and leukocyte numbers in comparison to cows, which had a similar cellular profile to sheep.

4. Discussion

4.1. Uninfected glands

The mammary gland defense system can be divided into two categories: anatomical and physiological. Together they prevent pathogens from entering the mammary gland via the teat canal and immune response, which can be divided further into innate and specific immunity. The higher SCC in uninfected glands of goats and sheep than in cows is a well-known phenomenon (Haenlein, 2002; Silanikove et al., 2010). However, it may simply be explained by the fact that modern dairy cows are intensively selected for high milk yield and thus the lower SC content results from its dilution in the milk. In fact, multiplying milk yield by SC content indicates that cows secrete more SC into the milk than goats and sheep.

The number of leukocytes (CD18$^+$) in milk from uninfected glands is relatively low compared to that in the blood and the proportion of the leukocytes is different. However, the anatomical location of the different leukocytes depends on the cell type, where PMNs are found mainly in the milk and the mononuclear cells, lymphocytes and

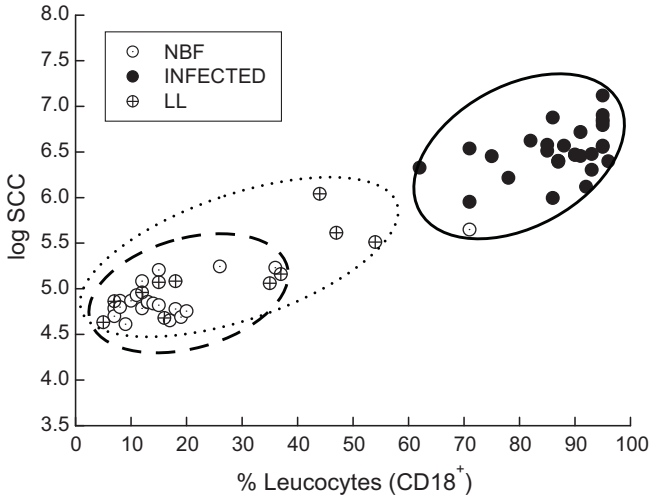

Fig. 5. Percent leukocytes (CD18$^+$) vs. log SCC in milk according to groups of sheep: mid-lactation, bacteria-free (MLS-F) (encircled dashed); mid-lactating, infected (MLS-I) (encircled solid); and late-lactation, bacteria-free (LLS) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

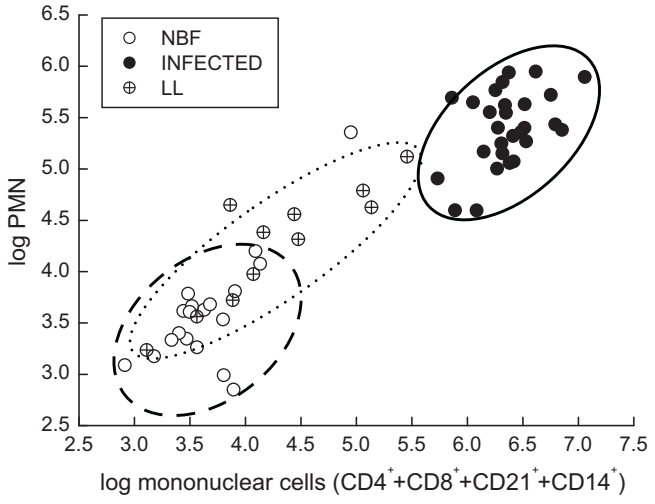

Fig. 6. Log mononuclear cells (CD4$^+$CD8$^+$CD21$^+$CD24) vs. log PMN in milk according to groups of sheep: mid-lactation, bacteria-free (MLS-F) (encircled dashed); mid-lactating, infected (MLS-I) (encircled solid); and late-lactation, bacteria-free (LLS) (encircled doted). NBF, no bacterial finding; INFECTED, infected glands; LL, late lactation.

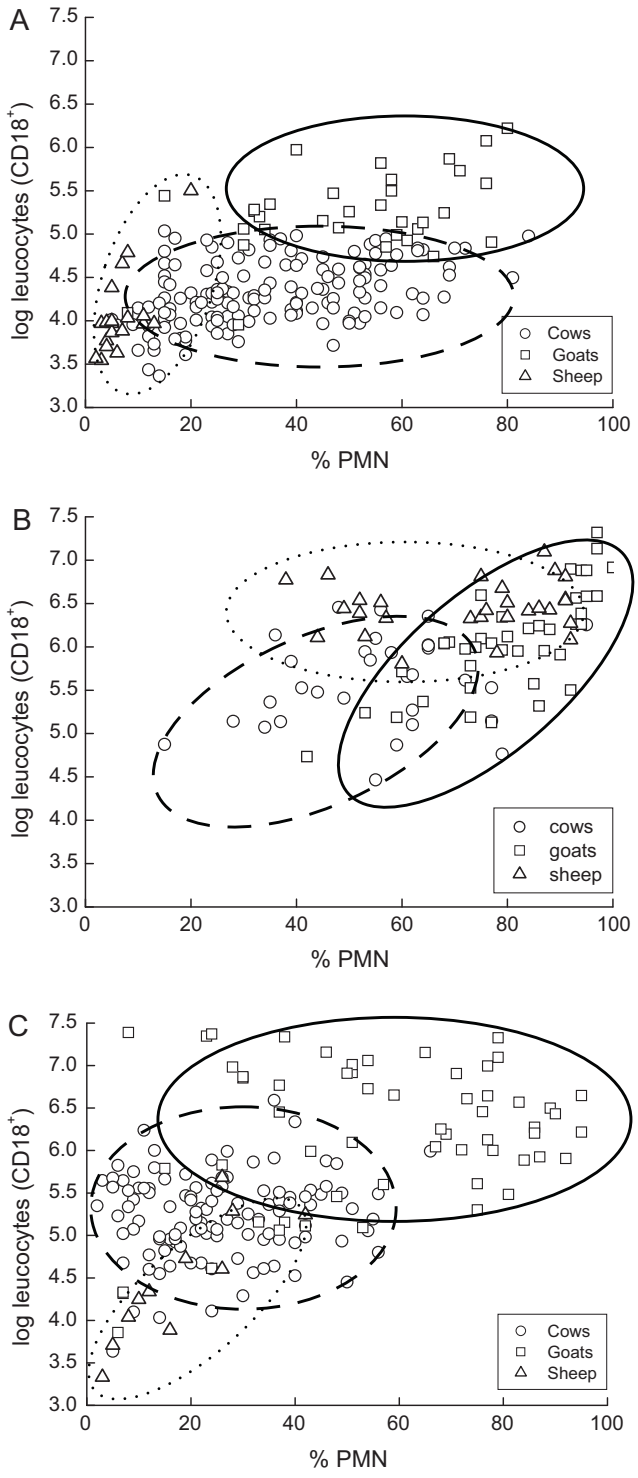

Fig. 7. Percent PMN vs. log leucocytes of cows (encircled dashed) sheep (encircled doted) and goats (encircled solid): with no bacterial findings (A), infected (B), and at late-lactation (C).

macrophages are mostly found in the mammary tissues (Leitner et al., 2003). Thus, it is possible that the actual numbers of mononuclear cells, lymphocytes and macrophages involved in immunological processes in the mammary is higher in the gland tissue than that measured in milk. Although it would be expected that this phenomenon is similar in the three animal species studied, we cannot estimate how it contributed to the differences found in leukocyte proportions in our study. With these limitations taken into account, it appears that SC population in uninfected mid-lactation milk of goats was similar to cows. In particular, epithelial cells accounted for ~50% of the SCC in goats and cows, whereas in sheep for ~80% of the cells. In certain studies the percent of epithelial cells of normal healthy mammary glands was lower than the results in this work (Concha, 1986; Lee et al., 1980; Leitner et al., 2003). These differences may be explained by different methods of identification and/or definition of uninfected glands and cell types (Mehne et al., 2010; Rainard and Riollet, 2006). In the method and definitions used in the present study, epithelial cells were actually identified as "negative" cells, that is, cells not labeled by CD18 mAb (non-leukocytes SC). We assume that these cells are passively shed into milk as a result of normal tissue renewal in the gland epithelia.

There are two possible explanations regarding the higher epithelial cells in sheep: first, it may be that the antibodies used for quantifying PMN underestimate their content in sheep, leading to overestimation of epithelial cells. However, the detection of a dramatic increase of PMN in the milk of infected glands in sheep overrules this possibility. Thus, our results support a second possibility that sheep shed more epithelial cells into milk in comparison to cows and goats, probably because these cells play an important role in the immune response. Our study also highlighted the fact that the basal concentration of leukocytes in uninfected mid-lactation milk in sheep was much lower in comparison to cows and goats. It is possible that in sheep the strategy of preventing bacterial entry into the mammary gland normally relies more on non-cellular or anatomical factors.

Sheep also differ from cows and goats because of the higher proportion of epithelial cells shedding. Thus, epithelial cell shedding in sheep might play a major role in activating the immune system, whereas in cows and goats this task is shared among macrophage and epithelial cells. Our notion is based on recent findings which show that the mammary epithelial cells have the capacity to phagocyte and secrete cytokines, or in other words to behave like macrophage (Atabai et al., 2007; Monks et al., 2002; Monks and Henson, 2009). B-lymphocytes were not found in the milk of most animals studied although some had ~1%. These results are controversial because in certain studies B-lymphocytes were found in the milk although not found in others (Grönlund et al., 2006).

4.2. Effect of CNS infection at mid-lactation

The teat-end is normally populated with different bacteria of the animal environment and natural skin bacterial microbiota. The number of bacteria attempting to penetrate into the udder at any time is unknown, but supposedly high. However, milk secreted under normal healthy mammary conditions does not contain contaminating pathogenic microorganisms. Once bacteria penetrate the anatomical barrier of the teat canal, a variety of defense mechanisms are activated in the mammary gland, which can be separated into two distinct categories: innate immunity (PMN, macrophages) and specific immunity including T and B lymphocytes.

If the encounter is with PMN or macrophages, the event can end successfully: removal or killing of the intruder with no sign of infection. If the former event is unsuccessful, bacteria multiply and may attempt to colonize the mammary gland releasing exo- and endo-secreted products, many of which are chemoattractants for leukocytes. With certain bacteria such as coliforms, the infection is acute and in the majority of cases the bacteria are removed, although in many cases the gland does not fully recover. Most IMI are due to Gram positive bacteria, which become chronic with the failure of the immune system to clear the intruder, resulting in colonization of the gland with a constant high leukocyte count. Macrophages, in addition to their capacity to phagocyte bacteria, play a key role because following their encounter with pathogens they activate the immune system by secreting a wide range of cytokines. Secretion of cytokines, such as interleukin- (IL-) 1 beta, IL-6, IL-8, tumor necrosis factor-(TNF-) alpha and interferon- (IFN-) gamma by macrophages are known to elicit the acute phase response and allow the accumulation of leukocytes composed mainly of PMN at the establishment of infection of the mammary gland (Rainard and Riollet, 2006). In the present study, all mid-lactation-infected animals had a significant elevation of SCC and a change in the proportion of leukocytes/epithelial cells; of which more than 80% were leukocytes. The main difference among the species studied is the elevation of SCC: in sheep and goats, SCC increased approximately ×3 as in cows during CNS infection; however, the increase in goats from their baseline was lower than in sheep. In addition, sheep showed the highest relative increase in PMN proportion in response to CNS infection, five times the relative increase observed in cows and goats. These differences, as suggested previously (Leitner et al., 2004, 2011), may evolve from a larger decrease in milk secretion in the infected glands and/or from an over-response to the infecting bacteria in sheep.

Of the leukocytes, PMNs were the major cell type with some significant differences in T-lymphocytes bearing CD8 and macrophages among the animal species. However, these differences could be related to the stage of the chronic infection rather than to the animal species. In previous studies (Leitner et al., 2003), it was reported that as chronic infection progressed the number of PMNs decreased and the number of macrophages and lymphocytes, mainly CD8, increased. All in all, the immune response in the mammary gland to the various CNS bacteria was similar in the three animal species: an increase in leukocyte count that failed in clearing the intruder.

4.3. Immune response in late lactation

Involution of a mammary gland can be induced at any stage of lactation by cessation of emptying the gland. There are two types of involution in natural conditions: abrupt involution is induced when the dam produces copious amounts of milk and milk stasis is induced unexpectedly due to infant loss or sudden weaning; natural involution occurs when the offspring no longer depends on its mother's milk as its main source of food, the mother produces a small amount of milk and the cessation of the dam–offspring interrelationship is predicted. This study was done toward the end of lactation when milk yield in each of the tested species was considerably lower than at peak lactation and free of bacterial infection. Thus, in agreement with a recent study in cows (unpublished data), the response of the animals in the three studied species resembled the response to natural weaning. In previous studies (Leitner et al., 2011), it was shown that the period preceding natural involution involves an adaptive response that has features of an auto-defensive metabolic mechanism, which cause changes in milk composition accompanied by infiltration of white blood cells that "clean" dead epithelial cells without damaging the epithelial tissue. This response appears to be important for executing an orderly involution process and effective antimicrobial immune response after cessation of gland evacuation, when washing of bacteria out of the mammary gland no longer takes place (i.e., induction of involution). It was demonstrated in the present study that late lactation was associated with an immunological response, which may contribute to effective involution. This response was considerably different from the acute phase response that is characterized by inflow of PMN, which is found in IMI or when involution is induced abruptly (Lynch et al., 2010). However, the three dairy species exhibited different immune responses: the milder response toward expected involution in terms of increase of SCC was observed in sheep, whereas goats showed the highest elevation in SCC. Goats and cows responded differently in comparison to infection at mid-lactation: SCC in cows increased approximately 17 times in the infected glands and ∼eight times in non-infected glands at the end of lactation, whereas in goats, SCC increased ∼six times during infection and ∼17 times at the end of lactation.

The question needs to be addressed due to the vast infiltration of PMN into the milk of uninfected glands in goats through the end of the lactation. PMNs infiltration is a normal chemotactic response to penetration of microorganisms. However, macrophages are the cells most important in cleaning the dead epithelial cells throughout the end of the lactation. One explanation for this phenomenon is the different milk secretion, exocrine in cows and sheep, and apocrine in goats. Another explanation may be related to the CD14 receptors found on the goats' PMN at this stage of lactation, which may suggest that PMN in goats serve similar functions as macrophages in cows and sheep. However, these assumptions should be further investigated.

5. Conclusions

The present study highlighted basic differences among cows, sheep and goats regarding the basal leukocyte populations at mid-lactation (uninfected state), which were amplified toward the expected involutionary period at the end of lactation. The differences among species most likely reflect diverged evolutional adaptations of the mammary gland immune system to different stages of the lactation cycle. On the other hand, the pattern of the response to CNS IMI infection was basically similar, and the qualitative differences among the 3 species most likely reflected the different basal conditions and the type of invading bacteria.

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