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Peripheral blood and milk leukocytes subsets of lactating Sarda ewes

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Abstract

Leukocytes subpopulations in blood and milk of lactating Sarda ewes were investigated. Animals characterized by a SSC level <500×10^{3}cells/mL and a negative bacteriological examination were sampled in early, mid and late lactation. Milk differential cell count evidenced that macrophage represented the main population (42.8%±3.5) followed by lymphocytes (40.2%±3.4) and neutrophils (8,6%±2.1). Flow cytometry analysis showed that lymphocytes subsets in milk were quite different from blood. High CD8$^+$ and low CD4$^+$ lymphocytes percentages determined a CD4/CD8 ratio inversion in milk compared to blood (0.3%±0.03 *vs* 1.8%±0.08). CD8$^+$ decreased while, conversely, CD4$^+$ increased in late lactation. γδ T cells were more represented in milk (12.6%±1.3) than in blood (6.8%±0.3) and their proportions appeared similar throughout lactation in both compartments. IL-2 receptor was mainly expressed in milk on T cytotoxic lymphocytes. Data obtained in uninfected mammary glands could allow an early discrimination between physiological and pathological changes occurring in ewe milk. Further phenotypical and functional studies on milk leukocytes subsets might help to understand defense mechanisms of the ovine mammary gland against mastitis.

Introduction

Mammary gland secretions from different species contain a characteristic number and composition of cellular elements which reflects the animal's physiological, infectious and immunological status (Persson-Waller and Colditz, 1998; Harp *et al.,* 2004). Somatic cell count (SCC) is considered a widely used indicator of udder health and milk quality and its variations had already been described during lactation stages and infection in bovine, goat and sheep (Sheldrake *et al.,* 1983; Laevens *et al*., 1997). However, important information could also be obtained by milk differential cell counting and lymphocytes subsets identification being a useful tool for the understanding of the immunological mechanisms underlying mammary gland protection against intramammary infections (IMI). In healthy bovine uninfected mammary glands macrophages (M) represent the predominant cell type during lactation followed by lymphocytes (Ly) and polymorphonuclear neutrophilic leukocyte (PMN) (Sordillo *et al*., 1997; Månsson *et al*., 2006).

Limited data exist in literature for small ruminants, but it has been reported that milk differential cell count in ewes is similar to dairy cows (Cuccuru *et al*., 2001; Paape *et al*., 2007). In goat milk, conversely to what observed in bovine and ovine species, the proportions of PMN are higher than M whereas lymphocyte phenotype is less numerous than others (Paape *et al.,* 2001; Sousa *et al*., 2012). Generally, PMN are the predominant cell type during early inflammation representing more than 90% of infiltrating leukocytes found in milk. They are recruited by a variety of chemotactic factors to the site of infection where they exerted their fundamental phagocytic and killing activity (Paape *et al.* 1981; Sordillo *et al.* 1987). Macrophages are phagocytic cells that play an important role in both early non-specific defence and in generating a proper adaptive immunological response by means of antigen processing and presentation to lymphocytes. Lymphocytes, broadly categorized into B and T cell subsets, are responsible for specific recognition of antigen and activation of adaptive immune response. T lymphocytes are more represented than B lymphocytes in mammary gland secretions (Wirt *et al*., 1992) and they can be further subdivided into T lymphocytes, which include CD4$^+$ (T-helper) lymphocytes and CD8$^+$ (T cytotoxic) lymphocytes, and γδ T lymphocytes. Unlike other mammal species, there is a large number of γδ T cells in peripheral blood of ruminants, especially in young animals where they may represent 50% of all T cells (Evans *et al.,* 1994). Although its role is still not well established, these cells are considered a linkage between innate and adaptive immunity (Holtmeier *et al*., 2007) and are thought to be involved in the immune surveillance of epithelia (Janeway and Medzhitov, 1988). Depending on stage of lactation and tissue location, the percentages of lymphocytes subsets can vary significantly. Physiological changes in the proportions of milk cell phenotypes occur throughout lactation thus influencing susceptibility of mammary gland to microbial challenge. This is particularly evident during early non lactation and periparturient period when, especially in bovine, as it has been reported by several authors, animals experience a natural state of immunosuppression and a greater risk of developing mastitis (Yang *et al.,* 1997; Oviedo-Boyso *et al*., 2007). This might be due to various factors but there are indications that changes in the proportions of lymphocytes subpopulations may affect the functional capacity of the mammary gland immunity of the periparturient cow and it presumably may lead to an increased vulnerability to invading pathogens (Concha *et al*., 1995; Nonnecke *et al*., 2003).

Little information is still available on composition of leukocytes subsets in dairy ewe's milk. Phenotypic analysis of sheep milk cells might help in understanding immunological reactions able to protect the ovine mammary gland from mastitis onset, a disease considered to cause consistent economic damages and to negatively affect dairy farms profitability. Relying on such assumptions, the aim of this investigation was to evaluate leukocytes subsets changes throughout an entire lactation in ovine milk and blood.

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Materials and methods

Animals and experimental design

The present study was performed on a flock of multiparous Sarda ewes reared at Experimental Zooprophylactic Institute (IZS) of Sardegna region farm located in Surigheddu (40°35'49"N, 8°22'47"E). All ewes were fed a diet of green forage or hay, depending on the period, *ad libitum* and supplemented with a commercial concentrate. They were allowed to graze during the day and kept indoor during the night. Only ewes (n=28) whose udder health could be recognized by a low SCC (<500×10^{3}cells/mL) and negative milk bacteriological analysis were considered for this trial. IZS Ethics Committee approved the experimental design and all handling of the animals.

Blood and milk sampling and analysis

Blood and milk samples were obtained from lactating ewes at early (<70 days), middle (from 120 to 130 days) and late lactation stage (more than 160 days). Whole blood was collected by jugular venipuncture in EDTA vials for flow cytometry analysis. One hundred mL samples of composite milk (representing each half udder) were aseptically collected during the morning milking and submitted to the lab within 2 hours.

Thirty-five mL of each milk sample were immediately assayed for SCC and differential cell count while 1 mL aliquots were stored at -20°C pending bacteriological examination. SCC was determined by fluoro-opto-electronic method (Fossomatic, Foss, Denmark) (ISO, 2006). Milk differential cell count was carried out by microscopic method (ISO, 2008). Bacteriological analysis was performed according to accepted standards (International Dairy Federation, 1981). For milk lymphocytes subsets identification, Fifty ml of each sample were skimmed by centrifugation at 1000 g at 10°C for 20 min. After removal of fat layer and supernatant, cell pellets at the bottom of each tube were resuspended in Dulbecco's Phosphate Buffered Saline (GIBCO, Life Technologies, Paisley, UK) transferred in a clean tube and adequately washed. Cells were counted in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA, USA) and concentration adjusted at 10^6 cells/mL.

Immunostaining and flow cytometry

Monoclonal antibodies (mAbs) listed in Table 1 were used to perform three- and four-colour immunostaining protocols. Two direct three-colour immunofluorescence (WC1/CD8/CD4; CD25/CD8/CD4) *lyse and wash* staining procedures were carried out for blood samples. Briefly, 50 µL of blood were incubated for 20 min at 4°C in the dark with 5 µL (IgG concentration 0.1 mg/mL) of each conjugated mAbs. Following a red blood cells lyses by addition of 450 µL of hypotonic solution (FACS Lysing Solution, BD Biosciences, Franklin Lakes, NJ, USA) and incubation for 10 min at room temperature in the dark, cells were washed and resuspended with 500 µL of sheat fluid (FACS Flow, BD Biosciences.

Two four-colour staining protocols (WC1/CD8/CD45/CD4; CD25/CD8/CD45/CD4), combining direct and indirect immunofluorescence, were set up to identify milk lymphocytes subsets. Briefly, 100 µL of milk derived cell suspension were incubated with 10 µL (IgG concentration 0.1 mg/L biotilinated anti CD45 mAb for 20 min at 4°C in the dark. Following a washing step, samples were incubated, as previously described, with 10 µL (IgG concentration 0.1 mg/mL) of each directly conjugated mAbs and 2 µL of Streptavidin-Pe/Cy5 (protein concentration 0.02 mg/mL). Stained cells were analyzed on a flow cytometer (FACS Calibur, BD Biosciences).

Ten thousand events were acquired from blood samples and fifty thousand events from milk samples. Analysis of data was performed by Cell Quest Pro software (BD Biosciences). Lymphocytes were gated out from other blood cell populations based upon forward and side scatter characteristics (FSC *vs* SSC). Instead, for milk samples, a two dimensional dot plots was generated (SSC *vs* FL3) and lymphocyte gate was set using side scatter characteristics and CD45/RPE-Cy5 fluorescence. Results obtained for single positive CD4$^+$, CD8$^+$ and WC1$^+$ and double positive CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ were recalculated to 100% of the lymphocyte gate cells.

Statistical analysis

Data were checked for normality by Anderson-Darling test and logarithmic transformations were applied for non-normally distributed parameters. SAS Proc Mixed with sampling time treatments as fixed effects and animal as a random effect was performed on normally distributed and log transformed data. One way ANOVA was used to compare milk and blood samples for the entire lactation. The results were presented as means ± standard error (SE). Differences were considered significant if P was <0.05.

Results and discussion

SSC arithmetic means during lactation are shown in Table 2. There was no statistically significant difference for SCC level of early, middle, and late lactation stage. SCC arithmetic mean of the entire lactation without considering each lactation stage, was 122.4×10^3 cells/mL whereas the geometric mean was 99.7×10^3 cells/mL. Milk differential cell count evidenced how the majority of milk cells was represented by Mϕ, followed by Ly and PMN (Table 2). No significant differences were evidenced between the different sampling times.

Flow cytometry revealed how milk lymphocyte subpopulations differed from those analysed in blood (Table 2). Lymphocyte subsets identification showed that there were no demonstrable changes in CD8$^+$, WC1$^+$ and CD25$^+$ blood counts between the different time points apart from a steady increment of CD4$^+$ T-cells. Similar changes in CD4$^+$ lymphocytes were found in milk, while CD8$^+$ decreased significantly, throughout lactation causing a significative CD4/CD8 ratio increment.

Although SCC trend seemed to increase from early till late lactation stage, we found no significant differences between the three sampling times. This is in accordance with other authors who reported no influence of stage of lactation on SCC levels. (Othmane *et al*., 2002; Paape *et al.,* 2007). The geometric mean of composite milk samples (99.7×10^3 cells/mL) was similar to that found by others in uninfect-

Table 1. Conjugated monoclonal antibodies used in flow cytometry.

Antibody	Target	Clone	Conjugated with	Manufacturer
CD45	All leukocytes	1.11.32	Biotin	AbD Serotec
CD4	T helper lymphocytes	44.38	Alexa 647	AbD Serotec
CD8	T cytotoxic lymphocytes	38.65	RPE	AbD Serotec
WC1	γδ T-lymphocytes	CC15	FITC	AbD Serotec
CD25	IL-2 Receptor	9.14	FITC	AbD Serotec

ed udder of dairy cows (Sheldrake *et al*., 1983; Dohoo and Donald, 1988) and ewes (Gonzalez-Rodriguez *et al*., 1995; Pengov, 2001). Pengov (2001) suggested the value of 250×10^3 cells/mL as an appropriate threshold for discriminating ewe mammary gland positive for IMI while Gonzalez-Rodriguez *et al.* (1995) indicated the value of 300×10^3 cells/mL. However, these latter, pointing out that a suitable threshold has to take in consideration differences in SCC content related to ovine breeds, proposed distinct values for Assaf and Castellana (400×10^3 cells/mL) and for Churra (200×10^3 cells/mL). Moreover, Berthelot *et al.* (2006) proposed a dynamic approach based on the evaluation of a set of individual SCC scores and on the use of two thresholds able to discriminate healthy (SCC<500×10^3 cells/mL on each sample) from infected (SCC>1-1.2×10^6 cells/mL on at least two milk samples) ewes. The small number of animals included in this study did not allow us to indicate any SCC threshold for Sarda breed but we did notice, in a previous study (Dore *et al*., 2011), that trespassing the 500×10^3cells/ml limit PMN became the predominant cell phenotype in milk (>50%). Several authors documented that differential cell count of milk from uninfected ewes is similar to that observed in cows (Morgante *et al.,* 1996; Albenzio *et al*., 2004, 2011; Souza *et al*., 2012). We found a mean percentage of PMN (8.6%) within the range (2-40%) reported by Sousa *et al.* (2012) and earlier by Paape *et al.* (2001) (2-28%). Albenzio *et al.* (2011) reported higher PMN proportions (42.34%) but it has to be noticed that their average SCC (225.1×10^3 cells/mL) was nearly twice of that we found in this study (122.4×10^3 cells/mL). In agreement with others, macrophages were the predominant subpopulation in milk with percentages (42.8%) similar to those indicated by Cuccuru *et al.* (1997) in Sarda ewes and by Morgante *et al.* (1996) in Comisana breed. Lymphocytes were the second most representative subpopulation (40.2%) in ewe milk in accordance with Albenzio *et al*. (2004), but we found higher values respect to Paape *et al.* (2007). As it can be easily notice, there is a large variability of published results on milk differential cell counts in healthy mammary glands. Schröder *et al.* (2005) speculated that a possible explanation of such variation might be ascribed to several technical factors including sampling vial material, smear preparation technique and subjective differences in microscopic observation and interpretation skills.

PMN, M and Ly did not differ throughout the lactation in contrast to other authors who found that macrophages had a constant increase (Morgante *et al,.* 1996) and lymphocytes slightly decreased from early till late lactation (Cuccuru *et al.,* 1997). Previous studies documented leukocytes subpopulation changes in dairy cows particularly occurs during mammary involution, close to dry period, and at calving (Sordillo *et al*., 2002; Rainard and Riollet, 2006). Presumably, our experimental design did not allow us to evidence any changes in the differential cell count because samples collection was limited to lactation not comprising peripartum and drying off period. Gating strategy used in four-colour flow cytometry demonstrates its efficacy especially when a high cellularity occurs in milk samples. We observed a dramatic change in scatter properties when milk samples with SCC higher than 500×10^3 cells/mL were acquired due to prevailing PMN proportions on other cell phenotypes. Being well acknowledged that the consistent increase in the number of leukocytes in the mammary gland and their secretion caused by IMI is mostly to be referred to a massive migration of PMN, flow cytometry could be of important use to early detect differential cell count dynamics in a fast and easy way.

Our results, in accordance with previous findings reported in cattle, goats and sheep (Park *et al*., 1992; Guiguen *et al*., 1996; Persson-Waller and Colditz, 1998), evidenced that milk lymphocytes have a pattern of phenotypes quite distinct from homologous blood cells (Table 2). A comparison between milk and blood lymphocytes subsets showed that the CD4/CD8 ratio is inverted (0.3%±0.03 *vs* 1.8%±0.08) throughout lactation owing to prevailing $CD8^+$ proportions in milk. These data

Table 2. Somatic cell count levels, differential cell count and lymphocytes subsets in ewes milk and blood during lactation.

Milk	SCC, $\times10^3$ cells/mL	Mϕ, %	Ly, %	PMN, %	$CD4^+$, %	$CD8^+$, %	CD4/CD8, %	$WC1^+$, %	$CD4^+CD25^+$, %	$CD8^+CD25^+$ %
Lactation stage										
Early (n=28)	108.8±13.10	49.8%±6.01	34.5%±5.45	12.2%±4.44	10.6±1.23[a]	65.7±3.18[a]	0.2±0.04[a]	15.3±2.16	38.2±2.19[a]	12.9±1.12
Middle (n=28)	102.5±18.29	33.6%±5.50	47.5%±6.29	4.7%±2.34	12.4±1.40[a]	68.3±3.15[a]	0.2±0.03[a]	9.5±1.97	22.9±2.51[b]	9.5±0.89
Late (n=28)	155.9±17.63	45%±6.32	38.7%±6.09	9.1%±3.61	19.5±1.93[b]	54.7±3.75[b]	0.4±0.07[b]	13.0± .48	35.8±2.61[a]	12.4±1.33
P between lactation stages	0.0600	0.1509	0.0972	0.2825	0.0009	0.0195	0.0014	0.201	0.0006	0.108
Entire lactation° (n=84)	122.4±9.77	42.8±3.48	40.2%±3.45	8.6%±2.06	14.1±0.98*	62.9±2.03*	0.3±0.03*	11.6±1.3*	32.4±1.58	11.6±0.66*

Blood	$CD4^+$	$CD8^+$	CD4/CD8	$WC1^+$	$CD4^+CD25^+$	$CD8^+CD25^+$
Lactation stage						
Early (n=28)	23.3±1.27[a]	14.7±0.72	1.7±0.11	6.6±0.58	30.5±2.38	3.4±0.46
Middle (n=28)	25.7±1.2 a	15.9±0.83	1.8±0.15	7.3±0.50	30.9±1.38	2.2±0.40
Late (n=28)	29.5±1.21[b]	15.6±0.73	2±0.14	6.4±0.57	31.7±1.78	3.7±0.93
P between lactation stages	0.0054	0.5919	0.1964	0.5465	0.9142	0.2518
Entire lactation° (n=84)	26.2±0.76*	15.4±0.44*	1.8±0.08*	6.8±0.32*	31.0±1.08	3.1±0.37*

SCC, somatic cell count; M , macrophages; Ly, lymphocytes; PMN, polymorphonuclear neutrophilic leukocytes.*Significant difference between milk and blood when data are referred to whole lactation (P<0.001). °Calculated as the average of values from the three time points. Data are shown as means ± SE.

are consistent with other authors who observed that αβ T lymphocytes are more numerous than γδT lymphocytes in milk and, conversely to peripheral blood, predominantly express the $CD8^+$ phenotype in human, porcine, bovine, caprine and ovine species (Shafer-Weaver *et al*., 1996; Wagstrom *et al*., 2000). We also found that γδ WC1+ T-cells are more represented in milk (12.6%±1.3) than in blood (6.8%±0.3). The percentages of $CD4^+$, $CD8^+$ and $WC1^+$ lymphocytes were more similar to those reported by Guiguen *et al.* (1996) in goat milk than to what observed by Persson-Waller and Colditz (1998) in ewe milk. These discrepancies could be due to phenotypical differences related to ovine breeds, since the previous work was performed on Merino ewes. Furthermore, Persson-Waller and Colditz (1998) used different clones of monoclonal antibodies and, particularly for $WC1^+$ detection, we preferred the mAb clone CC15 to the clone 19-19, having previously tested its performance in flow cytometry, because it guarantees a clearer definition of the positive cell population. The predominance of cytotoxic T-cells compared to helper T-lymphocytes in ovine milk suggested, as other author have already noticed in bovine (Taylor *et al.,* 1994), the existence of a selective migration determined by differential adhesion molecules expression (Harp, 2004; Van Kampen, 1999). Activated $CD8^+$ lymphocytes, coexpressing CD25, IL-2 receptor, were higher in milk respect to blood; the same cannot be said about $CD4^+CD25^+$ whose percentages were similar between the two compartments. Taylor *at al.* (1994) found no $IL\text{-}R^+$ lymphocytes in bovine milk while Persson-Waller and Colditz (1998) observed a higher proportion of activated lymphocytes in ewes dry secretions respect to milk. In agreement with other findings, (Taylor *et al.,* 1994; Asai *et al.,* 1998) the proportions of $CD4^+$ increased conversely to $CD8^+$ that progressively and significantly decreased during late lactation. Asai *et al.* (1998) reported that a higher CD4/CD8 in cow milk during dry period respect to lactating period could be related to an increase of $CD4^+$ lymphocytes. In accordance with such observation, we had noticed (data not published) significant differences between percentages of CD4+ (28.2%±2.7) and CD8+(46.8%±1.9) milk lymphocytes during drying off compared to those found in previous late lactation (CD4+: 10.5%±1.7; CD8+: 70.4%±2.7) and following early lactation (CD4+: 10.6%±1.8; CD8+: 70.7%±3.4), suggesting pronounced lymphocytes subsets variations during dry period.

Conclusions

Macrophages represented the main population detected in uninfected mammary glands of Sarda ewes followed by Ly and PMN. T cytotoxic prevailed on T helper lymphocytes phenotype in milk throughout lactation determining a CD4/CD8 ratio inversion respect to blood. Marked differences were evidenced between lymphocytes subsets of blood and milk compartment.

With the present research we intended to study the physiological changes of leukocytes subpopulations during lactation in ewes. Our results could provide a useful baseline to early detect pathological changes attributable to IMI. A better understanding of mammary gland immunity would certainly prevent economic losses resulting from mastitis, thus helping to increase profits of dairy sheep farms, a sector of economic importance for italian food industry, producing almost 500.000 tons of ovine milk and 67.000 tons of pecorino cheese (Eurostat, 2011).

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