Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands

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ABSTRACT

Somatic cell counts (SCC) are generally used as an indicator of udder health. In Germany, a cutoff value of 100,000 cells/mL is currently used to differentiate between healthy and diseased mammary glands. In addition to SCC, differential cell counts (DCC) can be applied for a more detailed evaluation of the udder health status. The aim of this study was to differentiate immune cells in milk of udder quarters classified as healthy based on SCC values of <100,000 cells/mL. Twenty cows were selected and 65 healthy udder quarters were compared with a control group of 15 diseased udder quarters (SCC > 100,000 cells/mL). Cells were isolated from milk of all quarters to measure simultaneously percentages of lymphocytes, macrophages, and polymorphonuclear neutrophilic leukocytes (PMNL) by flow cytometric analysis. The bacteriological status of all 80 quarters was also determined. Differential cell count patterns of milk samples (n = 15) with extreme low SCC values of ≤6,250 cells/mL revealed high lymphocyte proportions of up to 88%. Milk cell populations in samples (n = 42) with SCC values from >6,250 to ≤25,000 cells/mL were also dominated by lymphocytes, whereas DCC patterns of 6 out of 41 milk samples with SCC values from ≥9,000 to ≤46,000 cells/mL indicated already inflammatory reactions based on the predominance of PMNL (56–75%). In 13 of 15 milk samples of the diseased udder quarters (SCC >100,000 cells/mL), PMNL were categorically found as dominant cell population with proportions of ≥49%. Macrophages were the second predominant cell population in almost all samples tested in relation to lymphocytes and PMNL. Further analysis of the data demonstrated significant differences of the cellular components between udder

quarters infected by major pathogens (e.g., *Staphylococcus aureus*; n = 5) and culture-negative udder quarters (n = 56). Even the percentages of immune cells in milk from quarters infected by minor pathogens (e.g., coagulase-negative staphylococci; n = 19) differed significantly from those in milk of culture-negative quarters. Our flow cytometric analysis of immune cells in milk of udder quarters classified as healthy by SCC <100,000 cells/mL revealed inflammatory reactions based on DCC.

Key words: differential cell count, subclinical mastitis, udder health, low somatic cell count

INTRODUCTION

For mastitis diagnosis, traditional and well-established tests including SCC and microbial culture-based methods are standard (Viguier et al., 2009). According to current definitions of udder health in Germany, SCC ≤100,000 cells/mL in quarter foremilk samples are in the physiological range (DVG, 2002). It is accepted that the crossover of normal cellular defense in the mammary gland into an inflammatory reaction starts at a level of >100,000 cells/mL (Harmon, 1994; DVG, 2002). However, SCC vary with the status of lactation, age, stress of the animals, time and frequency of milking, and season, but primarily in response to udder infection (Dohoo and Meek, 1982; Harmon, 1994). Somatic cell count is a robust quantitative measurement, but does not divide the cells present in milk into different cell types (Kehrli and Shuster, 1994; Rivas et al., 2001a).

In the mammary gland, number and distribution of leukocytes are important for the success of udder defenses against invading pathogens (Leitner et al., 2003). Lymphocytes, macrophages, and PMNL play an important role in inflammatory responses within the mammary gland (Paape et al., 1979; Sordillo and Nickerson, 1988). Induction and suppression of immune responses

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are regulated by lymphocytes (Nickerson, 1989). They recognize antigens through membrane receptors specific for invading pathogens (Sordillo et al., 1997). Macrophages are active phagocytic cells in the mammary gland and capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson, 1988). Milk or tissue macrophages recognize the invading pathogens and initiate an immune response by the release of chemoattractants inducing the rapid recruitment of PMNL into the mammary gland (Paape et al., 2002; Oviedo-Boyso et al., 2007). The main task of PMNL is to defend against invading bacteria at the beginning of an acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007). Not only does the number of PMNL increase enormously but their defensive responses also increase (Targowski, 1983; Paape et al., 2003).

The distribution of leukocyte types varies in normal milk without any symptoms of mastitis. Some recent studies found lymphocyte proportions between 14 and 80%, macrophage proportions between 12 and 46%, and those of PMNL between 6 and 50% (Rivas et al., 2001a; Merle et al., 2007; Koess and Hamann, 2008). In milk from cows with mastitis, the proportions of PMNL can reach 95% (Paape et al., 1979; Kehrl and Shuster, 1994). During various phases of inflammation, both overall SCC and differential cell count (DCC) change (Nickerson, 1989). Therefore, in addition to SCC, determination of different types of immune cells present in milk is beneficial for describing the udder health status (Pillai et al., 2001; Rivas et al., 2001a). So far, however, little knowledge exists on DCC and the qualitative role of milk leukocytes in udders classified as healthy because DCC in low-SCC milk are difficult to perform (Dosogne et al., 2003).

Data of a previous study (Schwarz et al., 2010) indicated a high standard of udder health in a representative part of the dairy cow population in the German federal state Hesse and confirmed the threshold of 100,000 cells/mL differentiating between healthy and diseased mammary glands. However, unexpectedly high numbers of mastitis pathogens were found in the SCC range $\leq 100,000$ cells/mL. In some cases, pathogens were detected in milk samples from some cows with an SCC of 1,000 cells/mL. Based on these data, we suspected inflammatory processes even in the SCC range of mammary glands classified as healthy according to the current definitions. Therefore, the objective of this study was a detailed evaluation of the health status in udder quarters with SCC clearly below 100,000 cells/mL based on a statistical analysis of DCC. Leukocytes were isolated from quarter foremilk samples and differentiated simultaneously into lymphocytes, macrophages, and PMNL using flow cytometry.

MATERIALS AND METHODS

Animals and Farms

Twenty Holstein-Frisian cows in good health status were selected from 3 German dairy farms (A–C) for detailed analysis of their udder health status based on DCC in quarter foremilk samples. The animals were in different lactations (1 to 6) and stages of the lactation. In total, 80 foremilk samples were selected for DCC analysis. Sixty-five samples were obtained from udder quarters of 18 cows classified as healthy ($\text{SCC} \leq 100,000$ cells/mL). Fifteen foremilk samples were chosen as the control group. Seven of those samples with SCC from 100,000 to 624,000 cells/mL were derived from 7 individual cows. A further 8 quarter foremilk samples with SCC ranging from 100,000 to 1,394,000 cells/mL were collected from 2 cows that were clinically diagnosed with mastitis. Symptoms included flecks in the milk, swelling, or redness of the involved quarters.

In farms A to C, 50 to 160 dairy cows were housed in pen barns and milked twice per day in milking parlors. The milking operations were similar in all farms. After forestripping into a foremilk cup, the milkers used damp cotton tissues for udder cleaning. Teats were dipped after milking with iodine solution. In all of the dairy farms, animals were fed with a TMR consisting of grass and maize silage, rape grist, and cereals. Water was available ad libitum. All farms were conventional milk producers and the average herd milk yields ranged between 8,000 and 10,000 kg/yr (305-d milk yield: 7,393 to 9,385 kg/yr).

Milk Sampling

Quarter foremilk samples were obtained according to German Veterinary Society (DVG, 2000) standards. Before milking, teat ends were scrubbed with 70% ethanol and the first 2 squirts of milk were discarded. Aliquots of 110 mL of milk per udder quarter were collected aseptically in sterile 14-mL plastic sample tubes and 2 sterile 50-mL plastic tubes (Sarstedt AG & Co., Nümbrecht, Germany). Ten milliliters were saved for SCC and bacterial measurements and 100 mL for DCC analysis. Quarter foremilk samples were taken in all farms during morning milking. Further processing occurred within 7 h.

SCC and Bacteriological Examinations

Somatic cell counts were determined using a Fossomatic 5000 (Foss Electric, Hillerød, Denmark). The cytobacteriological analysis of all quarter foremilk samples was performed according to the IDF (1981)

Table 1. Monoclonal antibodies used for differentiation and labeling of bovine milk cells applying flow cytometry analysis

Type	Description	Name	Isotype	Specificity	Host	Company
α -CD11b	PMNL, macrophages, and monocytes	MM10A	IgG2b	Bovine	Mouse	VMRD ¹
α -CD14	Macrophages and monocytes	MM61A	IgG1	Bovine	Mouse	VMRD
α -IgG1	APC ² marker	550874	IgG1, κ	Murine	Rat	BD ³
α -IgG2b	FITC ⁴ marker	553395	IgG2a, κ	Murine	Rat	BD

¹VMRD Inc., Pullman, WA.²Allophycocyanin³Becton, Dickinson & Co., Heidelberg, Germany.⁴Fluorescein isothiocyanate.

standards. Promptly after collection, quarter foremilk samples were cooled in a cold box and transported to the laboratory. Ten microliters milk were streaked onto a quadrant of a 7% bovine blood agar plate containing 0.05% esculin (Merck KGaA, Darmstadt, Germany), incubated for 48 h at 37°C, and examined.

DCC

Isolation of Milk Cells. Milk samples were centrifuged for 15 min at $200 \times g$ and 4°C before cream layers and supernatants were discarded. To wash the cells, cell pellets were resuspended in PBS and then centrifuged again for 15 min at $200 \times g$ and 4°C. Washed pellets were resuspended at a final dilution of 1×10^6 cells/100 μ L based on predetermined SCC values for each milk sample.

Antibody Staining of Milk Cells. For DCC analysis, 100 μ L of the cell suspension was transferred into a 5-mL BD Falcon tube (Becton, Dickinson and Co., Heidelberg, Germany). For identification of PMNL and macrophages, nonconjugated monoclonal antibodies against CD11b- and CD14-molecules were used (Table 1). Volumes of 5 μ L of anti-CD11b (IgG2b) and 10 μ L of anti-CD14 (IgG1), both diluted 1:10 in PBS, were added and the tubes incubated on ice for 30 min. After adding 2 mL of FACS Lysing Solution (Becton, Dickinson and Co.), a further incubation step of 15 min followed. Cells were centrifuged (5 min at $250 \times g$) then resuspended in 3 mL of PBS and allowed to stand for 5 min. After a second wash step, cells were resuspended in 50 μ L of PBS and then incubated with secondary antibodies. Specifically, 10 μ L of rat anti-mouse-IgG2b diluted 1:10 in PBS and 5 μ L of nondiluted rat anti-mouse-IgG1 were applied (Table 1). After incubation for 30 min, cells were washed twice again and finally resuspended in 500 μ L of PBS. Cells were kept on ice during all procedures, centrifuged at 4°C, and incubated in the dark.

Flow Cytometry Analysis. Stained samples were analyzed with a FACSCalibur flow cytometer (Becton,

Dickinson and Co.) with standard optical equipment using an argon ion laser and a red diode laser with excitation wavelengths of 488 and 635 nm, respectively. The software CellQuest Pro (Becton, Dickinson and Co.) was used for data collection and analysis. Five thousand cells from each sample were differentiated into lymphocytes, macrophages, and PMNL. Lymphocytes were identified by size (forward light scatter, FSC-H) and granularity (side light scatter, SSC-H). The PMNL were measured as CD11b⁺ cells, whereas macrophages were defined as both CD11b⁺ and CD14⁺ cells. Gates enclosing the antibody-positive cells were placed outside the upper limit of background fluorescence. Cells with no antibody labeling served as a negative control and were regarded as a measure for background fluorescence. In addition, isotype control antibodies (rat-IgG1, κ isotype control, 554686 and rat-IgG2a, κ isotype control, 554688; Becton, Dickinson and Co.) were used to allow measurement of background staining.

Statistical Analysis

Associations between the individual cell populations and SCC, as well as between the individual cell populations and the bacteriological status of the udder quarters were analyzed by applying linear mixed models with the SAS program (version 9.1; SAS Institute Inc., Cary, NC). The statistical model included the fixed effects of herd, lactation number, position of the udder quarter, as well as a regression on SCC up to the third polynomial degree, to fit regression curves. The nonsignificant regression coefficients of different polynomial structures were removed from the model by using F-statistics sum of square type I tests at $P < 0.05$ instead of likelihood ratio tests. Based on type I sums of squares at $P < 0.05$, a sequential analysis approach is appropriate for polynomial formulated models (Littell et al., 1998). Variance analysis of SCC groups was done by modeling a fixed SCC group effect and removing SCC covariates from the statistical model.

RESULTS

SCC and Bacteriological Status of Quarter Foremilk Samples

Quarter foremilk samples were taken from 20 cows housed in 3 German dairy farms (A to C) to determine the bacteriological status and SCC. The 80 udder quarters selected showed an SCC mean value of 85,780 cells/mL with a standard deviation of 233,040 cells/mL (Table 2). Bacteria were identified in 24 of the 80 quarter foremilk samples. Thirteen of the 15 control quarters (SCC >100,000 cells/mL) were culture positive. In one quarter with an SCC value of 454,000 cells/mL, *Staphylococcus aureus* was isolated. In 3 quarters (SCC 181,000 to 1,394,000 cells/mL), *Streptococcus uberis* was detected. A double infection with *Strep. uberis* and *Streptococcus dysgalactiae* was diagnosed in an udder quarter with an SCC of 139,000 cells/mL. In 5 further quarters (SCC 104,000 to 624,000 cells/mL), CNS were identified. *Corynebacterium* spp. could be isolated from 3 quarters with SCC from 116,000 to 587,000 cells/mL. In 11 of the 65 udder quarters with SCC <100,000 cells/mL, bacteria were found, which indicated a latent mastitis in these quarters according to DVG (2002) definitions. Interestingly, all of those bacteria were CNS and detected in the SCC range from 7,000 to 59,000 cells/mL.

DCC of Quarter Foremilk Samples Depending on SCC

For a more detailed evaluation of the udder health status, 5,000 cells per quarter foremilk sample were differentiated into lymphocytes, macrophages, and PMNL by flow cytometry. Over all samples ($n = 80$), the proportions of lymphocytes ranged between 2.37 and 87.75%, with a mean of 48.50% and a standard deviation of 23.39% (Table 2). Macrophages ranged between 2.37 and 55.71%, with a mean of 21.74% and a standard deviation of 12.07%. Polymorphonuclear neutrophilic leukocytes proportions varied between 4.48 and 85.27%, with a mean of 29.76% and a standard deviation of 22.44%.

Because of the wide variations found within the cell populations, particularly in the case of lymphocytes and PMNL, DCC data were tested statistically for correlation with SCC.

The proportion of lymphocytes decreased from >60% at SCC values <10,000 cells/mL to 18.67% at an amount of 1,394,000 cells/mL (Figure 1). However, lymphocytes were the predominant cell population in 75% of the 65 healthy mammary glands (SCC ≤100,000 cells/mL), as clearly demonstrated for a single udder quarter (Figure 2A and B). The milk leukocytes secreted from this healthy quarter (SCC of 10,000 cells/mL) consisted of 58.20% lymphocytes, 23.06% macrophages, and 18.74% PMNL (Figure 2A and B). The statistical analysis indicated a significant ($P < 0.001$) negative correlation between percentages of lymphocytes and SCC (Table 3).

As indicated by the calculated trend lines, percentages of PMNL (Figure 3) and lymphocytes (Figure 1) emerged in contrary directions as SCC increased. Polymorphonuclear neutrophilic leukocytes increased from <30% within the SCC range of <10,000 cells/mL to 63.65% at SCC of 1,394,000 cells/mL. Apparent inflammatory reactions (e.g., increased PMNL proportion of 74.43%) could be detected in samples with SCC values ≥9,000 cells/mL. This relationship was observed in 6 udder quarters (SCC from 9,000 to 46,000 cells/mL) of 2 different cows housed in farm C. However, PMNL were the predominant cell population in 86% of the 15 diseased mammary glands (SCC >100,000 cells/mL), as demonstrated for a single udder quarter (Figure 2C and D). Although 85.27% of milk leukocytes of this quarter were PMNL, percentages of macrophages and lymphocytes of 12.35 and 2.38%, respectively, were low (Figure 2C and D). This quarter showed an SCC value of 116,000 cells/mL and the bacteriological examination revealed *Corynebacterium* spp. The statistical analysis indicated a significant ($P < 0.001$) positive correlation between percentages of PMNL and SCC (Table 3).

Within an SCC range of 8,000 to 100,000 cells/mL, the proportions of macrophages ranged between 7.51 and 55.71% (Figure 4). At SCC <8,000 cells/mL and in samples with >100,000 cells/mL, the proportions of

Table 2. General overview of SCC and differential cell counts (DCC) of the 80 quarter foremilk samples analyzed using flow cytometry

Item	SCC (× 1,000 cells/mL)	DCC (%)		
		Lymphocytes	Macrophages	PMNL
Mean	85.78	48.50	21.74	29.76
SD	233.04	23.39	12.07	22.44
Minimum	2	2.37	2.37	4.48
Maximum	1,394	87.75	55.71	85.27

Figure 1. Differential cell counts depending on SCC: Proportions of lymphocytes ($\square$ LYM), pictured in combination with a calculated potential trend line; each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

macrophages were $<40\%$. The statistical analysis revealed no significant correlation between percentages of macrophages and SCC (Table 3). In addition, the percentages of the individual cell populations were significantly influenced by lactation number and the farm but not by the position of the udder quarter (Table 3).

Statistical analysis revealed a significant negative correlation between lymphocyte percentages and SCC as well as a significant positive correlation between PMNL percentages and SCC. However, to test whether the immunological status differed statistically within the SCC range $\leq 100,000$ cells/mL, all udder quarters analyzed were classified into SCC groups I to IV, as defined in a previous study (Schwarz et al., 2010). Group IV represented the control quarters. Fifteen (18.75%) of the 80 samples analyzed belonged to group I according to their SCC values $\leq 6,250$ cells/mL. Forty-two samples (52.5%) showed SCC values between $>6,250$ and $\leq 25,000$ cells/mL (group II). Eight samples (10%) with SCC from $>25,000$ cells/mL to $\leq 100,000$ cells/mL were categorized into group III. Fifteen other samples (18.75%) with SCC $>100,000$ cells/mL were assorted into group IV.

The mean percentages of lymphocytes were significantly ($P < 0.001$) higher in groups I to III (50.61–62.99%) than in group IV (19.17%; Figure 5). In addition, mean percentages in group I were significantly ($P < 0.05$) higher than those in group III (Figure 5).

Significant differences were not detected between the mean percentages of macrophages within the 4 SCC groups (21.15–27.13%; Figure 5). Mean percentages of PMNL differed significantly ($P < 0.001$) between groups I to III (15.35–22–26%) and IV (59.68%; Figure 5). However, significant differences could not be calculated between the mean percentages of PMNL within groups I to III.

DCC of Quarter Foremilk Samples Depending on the Bacteriological Status

Significant correlations were identified between the individual cell populations, in particular with respect to lymphocytes or PMNL, and SCC. To analyze and compare DCC data depending on the mastitis pathogens detected, all 80 mammary glands analyzed were classified into 3 groups (no, minor, and major pathogens) according to Reneau (1986). In 56 (70%) of the 80 quarters, no pathogens could be detected. Minor pathogens were detectable in 19 samples (23.75%), whereas major pathogens were isolated in 5 samples (6.25%).

The mean lymphocyte percentage in milk of culture-negative udder quarters (58.48%) was significantly ($P < 0.01$) higher than in milk of culture-positive quarters (24.91–37.63%; Figure 6). In case of macrophages the mean percentages (19.87–24.18%) indicated no signifi-

Table 3. Results of variance analysis for the percentage of the individual cell populations in 80 quarter foremilk samples analyzed using flow cytometry¹

Cell population	Effect			
	SCC	Quarter position	Lactation number	Farm
Lymphocytes	***	NS	*	*
Macrophages	NS	NS	*	NS
PMNL	***	NS	NS	*

¹Analyzed factors were SCC, quarter positions (front right, rear right, front left, and rear left), lactation number (1, 2, and ≥ 3), and farm (A to C).

*** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

cant differences depending on the bacteriological status (Figure 6). Polymorphonuclear neutrophilic leukocytes showed significantly ($P < 0.01$) higher mean percentages in culture-positive udder quarters (38.90–55.96%) than in culture-negative quarters (17.23%; Figure 6).

DISCUSSION

Together with SCC, determination of DCC in milk is an important tool characterizing udder health (Pillai et al., 2001). Clear SCC cutoffs exist defining an udder as healthy or not. But even in healthy udders, inflammations can be suspected under special circumstances

Figure 2. Dot plots of the flow cytometric analysis of milk leukocytes: forward scatter (FSC-H) versus side scatter (SSC-H) plots (a and c) show the size (FSC-H) and granularity (SSC-H) of the leukocytes. The CD11b FITC versus CD14 APC plots (b and d) show the results of the fluorescence analysis of the leukocytes. Lymphocytes (R1) were recognized based on physical parameters. Macrophages (R2) were identified as CD11b⁺ and CD14⁺ cells, whereas PMNL (R3) were defined as CD11b⁺ cells. Data show representative FACS profiles of a healthy mammary gland with SCC of 10,000 cells/mL (a and b) and a diseased mammary gland with SCC of 116,000 cells/mL and detection of *Corynebacterium* spp. (c and d). Color version available in the online PDF.

Figure 3. Differential cell counts depending on SCC: proportions of PMNL (■), pictured in combination with a calculated logarithmic trend line; each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

(Schwarz et al., 2010). The immunological status of mammary glands classified as healthy based on DCC is poorly investigated. Reviewing the literature, Medzhitov (2007) reported that a lack of knowledge on host

defense in asymptomatic infections might exist because almost all studies performed so far concentrated on symptomatic infections. In this study, we differentiated leukocytes purified from quarter foremilk samples

Figure 4. Differential cell counts depending on SCC: proportions of macrophages (○ MAC); each symbol represents the result of 1 udder quarter analyzed, but overlapping is possible.

Figure 5. Comparison of differential cell counts within the SCC range of healthy mammary glands ($\leq 100,000$ cells/mL). All 80 udder quarters analyzed were classified into SCC groups I to IV (group I, empty bars, SCC $\leq 6,250$ cells/mL, $n = 15$; group II, light gray bars, SCC $> 6,250$ to $\leq 25,000$ cells/mL, $n = 42$; group III, dark gray bars, SCC $> 25,000$ to $\leq 100,000$ cells/mL, $n = 8$; group IV, black bars, SCC $> 100,000$ cells/mL, $n = 15$). Group IV represents the control quarters. Data are expressed as mean $\pm$ standard error of the means for percentages of the individual cell populations in the 4 SCC groups defined. *** $P < 0.001$; * $P < 0.05$; NS = $P > 0.05$.

to improve knowledge of the immunological status of clinically healthy and subclinically infected bovine mammary glands. Although SCC of $> 100,000$ cells/mL are normally related to inflammatory processes inside the mammary gland, a SCC of $\leq 100,000$ cells/mL is generally accepted as normal or physiological (Harmon, 1994; DVG, 2002), but can also be related to latent mastitis in the presence of pathogens (Schwarz et al., 2010).

Our results indicated that lymphocytes were the predominant cell population in healthy mammary glands. Milk samples with an extreme low SCC value of $\leq 6,250$ cells/mL revealed high lymphocyte proportions of up to 88% (mean value: 63%). In an SCC range from $> 6,250$ to $\leq 25,000$ cells/mL, a high mean proportion of lymphocytes (58%) was determined, too. To our knowledge, information about DCC in milk samples with that low SCC is not available in the literature. Flow cytometry studies measuring lymphocytes based on a combination of physical parameters and nucleic acid staining revealed mean proportions of 5% for ud-

der quarters with SCC from 25,000 to 100,000 cells/mL (Östensson et al., 1988) and 57% for udder quarters with SCC $< 200,000$ cells/mL (Dosogne et al., 2003). The clearly higher proportions of lymphocytes measured in our study are explainable by the analysis of samples with clearly lower SCC and the predominance of lymphocytes in those samples. The proportions of 2 to 42% of lymphocytes in milk of diseased udder quarters were clearly lower compared with those in healthy udder quarters. Similar observations were described before (Östensson, 1993; Rivas et al., 2001a). In view of the flow cytometric method, considering and identifying preferably all lymphocytes, we measured them based on physical characteristics according to previous studies (Riollet et al., 2001; Rivas et al., 2001b). Because lymphocytes isolated from blood were almost indistinguishable from those isolated from milk (Redelman et al., 1988), the definition of our lymphocyte gate resulted from the analysis of blood and milk cells. However, other recent studies (Rivas et al., 2001a; Koess and Hamann, 2008) used an antibody against

Figure 6. Differential cell counts depending on the bacteriological status of the mammary glands. The 80 udder quarters analyzed were classified into no pathogens (dark gray bars, $n = 56$), minor pathogens (e.g., CNS, black bars, $n = 19$), or major pathogens (e.g., *Staphylococcus aureus*, empty bars, $n = 5$). Data are expressed as mean $\pm$ standard error of the means for percentages of the individual cell populations in the 3 groups defined. *** $P < 0.001$; ** $P < 0.01$; NS = $P > 0.05$.

CD3-molecules for the identification of lymphocytes and detected CD3⁺ lymphocytes with wide variations from 11 to 88% in non-mastitic milk.

In mammary glands classified as healthy (SCC $\leq 100,000$ cells/mL), the proportion of PMNL ranged from 4 to 74%. Our data showed that PMNL in milk samples with SCC values $\leq 6,250$ cells/mL were rare (mean proportion: 15%). At an SCC level of $> 6,250$ to $\leq 25,000$ cells/mL, the mean proportion of PMNL of 17% was also low. Comparable data for such low SCC values are not available from the literature. However, some authors (Koess and Hamann, 2008) measured PMNL with a mean proportion of 42% in milk of healthy udder quarters with SCC $< 100,000$ cells/mL, applying an antibody against CD11b-molecules. In an experimental study, CD11b⁺ PMNL were measured preinoculation with proportions between 11 and 36% in milk of quarters with SCC $< 200,000$ cells/mL (Rivas et al., 2001a). However, inflammation is generally defined as an increase of leukocytes, especially PMNL, in tissues or body fluids infected by pathogens. An increased transfer of PMNL from blood into the mammary gland at the beginning of an inflammation could be detected (Burvenich et al., 1994; Kehrl and Shuster, 1994; Paape et al., 2002, 2003). Therefore, a high PMNL percentage in milk is accepted as an important indicator of inflammatory reactions (Pillai et al., 2001; Paape et al., 2002). Polymorphonuclear neutrophilic leukocytes have

previously been reported as predominant cell population in secretions of diseased mammary glands (Paape et al., 1979; Kehrl and Shuster, 1994). We made the unexpected observation that, in milk of mammary glands classified as healthy, PMNL dominated at SCC $\geq 9,000$ cells/mL. This finding suggests that inflammatory processes can appear within an SCC range that is clearly below the cutoff value of 100,000 cells/mL. Factors that might have triggered the elevated proportion of PMNL might be manifold. A dairy cow is under constant pressure from udder pathogenic microorganisms of the environment. The elevated proportion of PMNL could be evidence for the initial phase of an inflammation. In this regard, it is also possible that PMNL are able to defend pathogens successfully and prevent mastitis. However, although we could not isolate any pathogens in such quarters, they might be infected nonetheless. Negative bacteriological results could depend on intermittent pathogen shedding (Sears et al., 1990), presence of antimicrobials, or other inhibitors in milk (Reiter, 1978). At the time of examination pathogens could also be ingested by phagocytes or survive intracellularly in the host (Newbould and Neave, 1965; Hill et al., 1978). Shedding of too low amounts of pathogens or ceased growth may be further reasons for negative bacteriological results (Sears et al., 1990).

The interdependence of infections, inflammatory processes, and immune responses in individual udder

quarters is discussed controversially in the literature. Some authors suggested that individual udder quarters within a cow can be influenced by infections of neighboring quarters (Merle et al., 2007), whereas others did not find any evidence for an interdependence of udder quarters (Wever and Emanuelson, 1989) because they did not find DCC to be affected by the bacteriological status of adjacent quarters. Our data indicated immunological interdependence as well as independence between the 4 udder quarters at low and high SCC levels. In the 6 udder quarters of 2 different cows with SCC from 9,000 to 46,000 cells/mL in foremilk samples, elevated PMNL proportions between 56.21 and 74.43% were determined. In the remaining 2 quarters of these cows (control group), clearly higher SCC values of 104,000 and 587,000 cells/mL, PMNL proportions of 74.56 and 77.56%, and bacterial infections with CNS or *Corynebacterium* spp. were detected. However, no interactions between the individual quarters were observed in 5 of the 7 cows of the control group with 3 healthy and 1 diseased udder quarter. Each of these cows showed SCC values >100,000 cells/mL, PMNL proportions of 72.08 to 85.27%, and an infection with CNS or *Corynebacterium* spp. in 1 udder quarter. In the remaining 3 quarters, SCC values <100,000 cells/mL and PMNL proportions between 5.12 and 27.79%, respectively, were detected.

Another factor that might have triggered the elevated proportion of PMNL is stress (Davis et al., 2008). Although we did not measure parameters considering stress, such as corticosterone in plasma, the influence of stress in our study might be minimal because the animals analyzed were kept under optimal conditions and according to national guidelines. No obvious symptoms of stress (i.e., kicking during pre-milking preparation of the udder or during taking the quarter foremilk samples) could be observed.

The antidromic trend of lymphocyte and granulocyte percentages at increasing SCC is caused by the composition of milk leukocytes. Because they primarily consist of lymphocytes, macrophages, and PMNL (Sordillo and Nickerson, 1988), the increase of the percentage of one cell population implies the decrease of at least one of the other cell populations. However, our statistical analysis indicated significant effects of the farms on percentages of both PMNL and lymphocytes. These effects might be due to a non-randomized selection of the cows within the farms and the different numbers of cows selected per farm. Whereas cows with healthy mammary glands were predominantly selected from farms A and C, samples from cows with diseased quarters were predominantly collected in farm B. In addition, a significant effect of the lactation number on percentages of lymphocytes and macrophages was

found. This phenomenon might be explainable due to the exposure of the mammary glands during the lactation. The milking process by milking machines and the constant contact with the environment could be responsible for both increasing SCC values (Harmon, 1994) and increasing percentages of PMNL (Vangroenweghe et al., 2001) detected in the course of the lactation period and in milk of cows with higher lactation numbers. Vice versa, the mammary glands of cows in their first lactation period, in particular at the beginning of the lactation, were only influenced slightly by milking machines and the environment.

Beside PMNL, macrophages possess phagocytic functions, too. We detected proportions of macrophages in healthy mammary glands (SCC $\leq$ 100,000 cells/mL) between 2 and 56%. Milk samples with extreme low SCC values of $\leq$ 6,250 cells/mL showed a mean proportion of macrophages of 22%. Within the SCC range from >6,250 to $\leq$ 25,000 cells/mL, the mean macrophage proportion was 24%. Due to the expression of CD14 (Berthon and Hopkins, 1996; Sopp et al., 1996) and CD11b (Splitter and Morrison, 1991; Howard and Naessens, 1993) on monocytes and macrophages, we defined macrophages as both CD11b⁺ and CD14⁺ cells. To our knowledge, this is the first study measuring DCC in milk with such a definition of macrophages. A mean value of 34% of macrophages identified with an antibody against CD14-molecules was reported for the SCC range <100,000 cells/mL (Koess and Hamann, 2008). Rivas et al. (2001a) measured macrophages with an antibody against CD11b-molecules and detected proportions between 1 and 20% in quarters with SCC of <200,000 cells/mL. In case of diseased udder quarters (SCC >100,000 cells/mL), we measured macrophages with proportions of 9 to 42% (mean proportion: 21%). These results were similar to the mean proportion of 16% (Koess and Hamann, 2008) and proportions of macrophages between 21 and 48% (Rivas et al., 2001a). Although we found macrophage percentages with wide variations in both healthy and diseased mammary glands, they had been reported to be the predominant cell population in milk of healthy mammary glands (Lee et al., 1980; Östensson et al., 1988). This difference might be explainable by different definitions of healthy mammary glands. In our study, we focused on the analysis of immune cells in milk with very low SCC values. However, Lee et al. (1980) defined mammary glands as healthy based on negative bacteriological examinations, but did not present any SCC values. Östensson et al. (1988) analyzed foremilk samples of bacteriologically negative quarters with an SCC mean value of 89,000 cells/mL.

Beside leukocytes, epithelial cells can also be found in milk. Although some researchers (Lee et al., 1980;

Koess and Hamann, 2008) described low proportions of 1 to 3%, others reported proportions of epithelial cells of 10 to 19% (Miller et al., 1991) or even 44% (Leitner et al., 2000). However, proportions of $\geq 10\%$ should be discussed critically. Miller et al. (1991) analyzed milk of primiparous cows during the first 75 d of lactation, whereas Leitner et al. (2000) measured epithelial cells based on nonspecific identification using flow cytometry. Because cows selected in our study were in different lactations and at least 70 d in lactation, we presume that percentages of epithelial cells in their milk were low. Therefore, and because their immunological properties are not well understood, we did not consider epithelial cells. They were completely disregarded in other flow cytometric DCC studies (Rivas et al., 2001a; Koess and Hamann, 2008), too.

Variations in the distribution of leukocytes in milk from non-infected mammary glands, as shown in other studies, were probably dependent on differences in methods, sampling, investigators (Schröder and Hamann, 2005), breeds (Leitner et al., 2003), stages of lactation (Vangroenweghe et al., 2001; Dosogne et al., 2003), and variable SCC. In contrast to other authors (Schröder and Hamann, 2005), we did not observe influences of the composition of the sample tubes (glass or plastic) used on differences in phagocytic cell percentages (data not shown). However, due to the rapid characterization of a large number of cells as well as the definite identification of individual cell populations, using specific-antibody flow cytometric analysis gives more accurate results compared with microscopic analysis (Loken and Stall, 1982; Rivas et al., 2001a; Dosogne et al., 2003; Koess and Hamann, 2008).

In our study, bacteriological examinations revealed udder pathogenic microorganisms in 24 of the 80 udder quarters analyzed (11 of the 65 healthy quarters with SCC $< 100,000$ cells/mL and 13 of the 15 control quarters with SCC $> 100,000$ cells/mL). Statistical analysis of DCC data revealed significant differences of cellular components in milk between culture-positive and culture-negative udder quarters. Interestingly, these results also indicated that, even in case of the presence of minor pathogens, the percentages of lymphocytes and PMNL differed significantly from those in culture-negative quarters. However, DCC patterns of udder quarters with SCC $\leq 10,000$ cells/mL and culture positive for minor pathogens did not indicate inflammatory reactions. Therefore, it can be speculated that the pathogens detected in these samples originated from contaminations of the skin, teat canal, or environment. Nevertheless, in milk samples with $> 10,000$ to 25,000 cells/mL containing minor pathogens, the proportions of PMNL were already twice as high compared with bacteriological negative quarters and reached

25%. This observation suggested that the pathogens originated from mammary gland tissue and that, even at such low cell numbers, PMNL were recruited from blood into milk to phagocyte bacteria. Differential cell count results of quarters infected with major pathogens indicated an IMI and were in agreement with previous observations (Piccinini et al., 1999).

CONCLUSIONS

Somatic cell count is an undisputed and well-established criterion for the evaluation of udder health and milk quality. However, in addition to SCC, DCC can be used for a more detailed analysis of the udder health status. Analyzing DCC of udder quarters classified as healthy by having SCC $< 100,000$ cells/mL, inflammatory reactions were detectable at an SCC level of $\geq 9,000$ cells/mL due to predominating PMNL proportions in foremilk samples of the corresponding quarters. Our study indicated inflammatory reactions in udder quarters with SCC that were clearly below the current threshold of 100,000 cells/mL. Further research in this field should concentrate on longitudinal examinations of immune cells in milk of udder quarters classified as healthy that reveal high percentages of PMNL.

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