

J. Dairy Sci. 85:3268–3276

© American Dairy Science Association, 2002.

Blood and Milk Neutrophil Chemiluminescence and Viability in Primiparous and Pluriparous Dairy Cows During Late Pregnancy, Around Parturition and Early Lactation

Jalil Mehrzad,* Luc Duchateau,* Satu Pyörälä†, and Christian Burvenich*

*Ghent University,

Faculty of Veterinary Medicine,

Department of Physiology, Biochemistry, and Biometrics,

Salisburylaan 133, B-9820 Merelbeke, Belgium; and

†University of Helsinki,

Faculty of Veterinary Medicine,

Department of Clinical Veterinary Sciences, Saari Unit,

Helsinki, Finland

ABSTRACT

Extensive studies have shown the polymorphonuclear leukocytes (PMN) dysfunction inextricably links to parturition. To investigate the effect of parity on PMN function, phorbol 12-myristate 13-acetate (PMA) stimulated luminol-amplified chemiluminescence (CL) and viability of blood and milk PMN were investigated in primiparous and pluriparous dairy cows during periparturient period. The CL kinetics of blood and milk PMN and hematological profiles were also assessed. Milk PMN CL was always lower than blood PMN CL. Blood and milk PMN CL and milk PMN viability were significantly higher in primiparous cows throughout the study. Blood PMN CL in pluriparous cows showed a sharper decrease. Both in pluriparous and in primiparous cows, minimal blood PMN CL appeared at periparturient day (PPD) 2. After PPD 7, blood PMN CL recovery rate was faster in primiparous cows. Milk PMN CL was minimal at PPD 2 in both groups. Whereas no changes were observed in blood PMN viability, the viability of milk PMN in primiparous cows was substantially higher than in pluriparous cows. The number of circulating eosinophils and immature neutrophils was substantially higher in primiparous cows throughout the study. The CL kinetics of blood PMN at PPD -2 and 2 and of milk PMN at PPD 2 exhibited different responses to PMA, with higher intensity and durability, peaking and subsiding more slowly in primiparous dairy cows. The pronounced reduction in PMN CL and viability in milk PMN of pluriparous cows may be involved in the underlying mechanisms that

make these animals more susceptible to periparturient infectious diseases.

(**Key words:** chemiluminescence, neutrophil, periparturient, pluriparous, primiparous)

Abbreviation key: AUC = area under the curve, CL = chemiluminescence, MPO = myeloperoxidase, PMA = phorbol 12-myristate 13-acetate, PMN = polymorphonuclear leukocyte, PPD = periparturient day, ROS = reactive oxygen species.

INTRODUCTION

Bovine blood and milk polymorphonuclear leukocytes (PMN) have the potential to produce reactive oxygen species (ROS) to eventually kill engulfed bacteria (Weber et al., 1983; Dulin et al., 1988; Kehrli et al., 1989; Mehrzad et al., 2001a; 2001b). It is widely accepted that PMN ROS production is an important defense mechanism against Gram-negative and -positive bacteria (Burvenich et al., 1994). Quantification of ROS can be measured following PMN stimulation with soluble agents, e.g., phorbol 12-myristate 13-acetate (PMA) or with particles, e.g., zymosan, bacteria, latex beads. The most widely used technique to quantify PMN ROS production is chemiluminescence (CL) assay (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999). The different CL responsiveness of blood and milk PMN to PMA stimulation during physiological conditions could result from differences in protein kinase C, NADPH-oxidase, and myeloperoxidase (MPO) activities (Webb et al., 1974; Babor, 1984). As these enzyme activities reflect intracellular and extracellular reactions, changes might offer some evidence about the cow's susceptibility to early lactation-related infectious diseases.

The transition from pregnancy to lactation causes stress in dairy cows. This transition is accompanied by a gradual decrease in PMN CL (Moreira da Silva et al., 1998; Hoeben et al., 2000). The decrease in PMN MPO

Received April 19, 2002.

Accepted July 18, 2002.

Corresponding author: C. Burvenich; e-mail: christian.burvenich@rug.ac.be.

activity (an index of PMN CL) has also been reported in mastectomized cows during the same period (Kimura et al., 1999). Furthermore, the immunocompromising effect of parturition is boosted by lactogenesis (Shuster et al., 1996, Goff and Horst, 1997, Mehrzad et al., 2001a). The severity of coliform mastitis has been reported to be less pronounced in animals with higher preinfection PMN ROS production (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). Increased severity of dairy cows to *Escherichia coli* mastitis has also been associated with increased parities (van Werven et al., 1997).

In this paper, the effect of parity on the PMN CL suppression that occurs around parturition was studied. CL was studied in both blood and milk PMN. PMN viability was also included because it was recently found to also be influenced by a lactation cycle (Mehrzad et al., 2001a). The kinetics of blood and milk PMN CL was further studied in detail.

MATERIALS AND METHODS

Animals and Experimental Procedures

A total of 24 Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) was selected. Cows were in their first to fifth lactation, clinically healthy, and showed no sign of typical periparturient diseases after calving. To confirm that cows had no mastitis pathogens, 10 and 50 ml quarter-foremilk samples were collected after parturition once weekly throughout the experiment and examined for bacteriological infection and SCC, respectively. The animals had no parasites, and there was no distinguishable allergy caused by ecto- and/or endoparasites. Only cows with a quarter SCC $<2 \times 10^5$ cells/ml and milk samples that cultured negative for major mastitis pathogens were considered clinically healthy. Parities were combined as follows: primiparous cows (first gestation, 2.3 ± 0.4 yr, $n = 12$) and pluriparous cows (fourth to fifth gestations, 5.7 ± 0.6 yr, $n = 12$). The mean milk production for primiparous and pluriparous cows was 17.2 ± 1.3 L/d and 23.7 ± 2.3 L/d, respectively. Before calving (from 32 ± 3 d before parturition), 80-ml blood samples were aseptically collected twice weekly from the external jugular vein into evacuated tubes (BD-Vacutainer System, Plymouth, UK) containing 125 IU heparin for further processing. After calving (until 32 ± 3 d after parturition), 1.5 L mixed cisternal quarter milk samples were aseptically collected in the morning twice weekly after cleaning and disinfection of the teats, using a sterile teat cannula (Vangroenweghe et al., 2001) and stored on ice for cell isolation and SCC determination. No additional stimulation of the mammary gland was used prior to milk collection. After milk sam-

pling, blood samples were collected for further processing using the same procedure as before calving. To compare blood and milk parameters between the primiparous and pluriparous cows, the two groups were classified at three different periods: 1) 3 d before parturition until parturition, 2) from parturition until 7 d after parturition, and 3) from more than 7 d until 5 wk after parturition.

Blood and Milk PMN Preparation and Enumeration

All materials and reagents used for the isolation of blood and milk PMN were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes (Carlson and Kaneko, 1973). The isolation procedure of PMN from blood yielded $>98\%$ of granulocytes (PMN + eosinophils) with predominantly PMN ($>86\%$) and a viability of $>98\%$ in both groups. After counting the cells using an electronic programmable particle counter (Coulter counter Z2; Coulter Electronics Ltd., Luton, UK) and determining the viability and percentage of PMN, the cell suspension was adjusted to a concentration of 5×10^6 cells/ml in Dulbecco's PBS (DPBS; Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with gelatin (0.5 mg/ml; Merck, Darmstadt, Germany). Initial volumes of 1.5 L milk were processed using a high-capacity centrifuge (RC-3BP; Sorvall, Newtown, CT) after 60% vol/vol dilution with cold PBS (0.01 M phosphate buffer (KH_2PO_4 - Na_2HPO_4) - 0.15 M NaCl, pH = 7.2). Isolation of PMN from milk was performed using three centrifugation steps as previously described (Mehrzad et al., 2001a), yielding $>60\%$ PMN with different viability throughout the experiment. The isolation procedure "time" for milk PMN was similar to that of blood PMN (54 ± 3 min). To quantify the yield of milk cell isolation, the SCC in whole milk was determined with a fluoro-opto electronic method (Fossomatic 400 cell counter; Foss Electric, Hillerød, Denmark). Milk cell recovery rate was calculated using $\text{RR} = (\text{N}_i \cdot \text{V}_i) / (\text{SCC} \cdot \text{V}_m)$, in which RR = recovery rate, N_i = concentration of cells in isolated cell suspension, V_i = volume of isolated cell suspension (ml), SCC = somatic cell count in whole milk, and V_m = volume of milk for isolation (ml). The overall recovery rate throughout the study yielded $35\% \pm 3.6$ and $33\% \pm 4.2$ in primiparous and pluriparous cows, respectively. The total number of leukocytes and isolated blood and milk cells was determined using an electronic particle counter (Mehrzad et al., 2001a, 2001b).

The total number of different circulating leukocytes was determined using smear preparation of blood sample (Mehrzad et al., 2001a, 2001b). Similar whole-blood staining procedure was performed to isolate blood and milk cells.

Viability and Identification of Blood and Milk PMN

The viability of isolated PMN was determined in duplicate by means of flow cytometry (FACSScan; Becton Dickinson Immunocytometry Systems, San José, CA), using propidium iodide exclusion (Mehrzad et al., 2001a).

Using light microscopy, differential cell counts on the isolates were performed on eosin-Giemsa-stained smears. Identification of the cells on isolates and whole blood was based on morphological characteristics as described by Hayhoe and Flemans (1969). Isolated milk cell differentiation was based on morphological characteristics (McDonald and Anderson, 1981), with some modification (Mehrzad et al., 2001a). Overall, macrophages were large, had a vacuolated nucleus, and contained whitish globules in their cytoplasm. Large lymphocytes had regular and dark-bluish-stained nuclei; however, monocytes had less condensed and irregular nuclei and always higher cytoplasm-to-nucleus ratio. Epithelial cells, though less than 3% (negligible) in milk "isolates," were identified as large, polygonal, and uniform stained-light-bluish cells.

To quantify percentages of each cell type in the samples, PMN (mature and immature), monocytes/macrophages, lymphocytes, eosinophils, and epithelial cells (only in milk) were identified on 200 cells per slide and expressed as percentage of particular cells in respective samples.

Chemiluminescence Assay

Luminol-enhanced PMA-stimulated cellular CL was used to quantify the ROS production of PMN isolated from blood and milk (Mehrzad et al., 2001a). The area under the curve (AUC) was calculated over a period of 30 min. The CL response was corrected for the actual number of viable PMN in each sample. As we previously demonstrated that the contribution of milk macrophages to luminol-dependent CL is negligible (Mehrzad et al., 2001a), the CL response was expressed per 10^3 viable PMN. For milk PMN CL assay, the formula $CL_{PMN} = 10^3 \times Cl_{isolated\ cells} / (4 \cdot 10^5 \times \% PMN \times \%V)$ was used to perform the corrections, where Cl = mean RLU (relative light unit)/s, $4 \cdot 10^5$ = total number of cells per well, $\%PMN$ = total percentage of PMN in isolated cells, $\%V$ = percentage of viable PMN. The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and Hoeben et al. (2000) for interference of eosinophils.

In addition, the kinetics of ROS production of blood PMN was compared between primiparous and pluriparous cows at periparturient day (PPD) -2 and 2; and for milk PMN this comparison was performed at PPD 2.

Statistical Analyses

The difference between primiparous and pluriparous cows for blood PMN CL and viability was assessed and tested at three different periods: 1) 3 d before parturition until parturition, 2) from parturition until 7 d after parturition and 3) from more than 7 d until 5 wk after parturition. The first two analyses were based on a mixed model with cow as random effect and the actual day of measurement and parity as fixed effects. The third analysis was based on a mixed model with cow as random effect and parity as fixed effect, but time was now introduced as a continuous fixed effect, and further the interaction between time and parity was added.

The same analyses were done for milk PMN CL and viability, but obviously only for periods 2 and 3.

All other blood and milk parameters were analyzed in the same way but are considered to be exploratory analyses. Therefore, the results are only summarized in terms of differences (with their 95% confidence interval) between primiparous and pluriparous cows for the relevant periods.

RESULTS

Chemiluminescence of Blood and Milk PMN

The overall PMA-simulated CL of blood PMN was lower in pluriparous than in primiparous cows in the three periods studied ($P < 0.0001$) (Figure 1a,b; Table 1). The relative magnitude of the blood PMN CL differences in pluriparous cows during the periods 1, 2, and 3 was 59, 32, and 62% of those of primiparous cows, respectively. Blood PMN CL showed a sharp decrease during periparturient period both in the pluriparous and in primiparous cows. The decline in blood PMN CL was largest in the second study period, immediately after parturition, and went below AUC of 2,000 for all pluriparous cows, whereas it stayed above this level for all primiparous cows (Figure 1a). In the third period, blood PMN CL recovery rate was significantly faster in primiparous cows than in pluriparous cows (Table 1).

Throughout the whole study period, milk PMN CL was always lower than that of blood PMN both in primiparous and pluriparous cows. Additionally, the milk PMN CL was consistently higher in primiparous than in pluriparous cows in both periods studied ($P < 0.01$; Figure 1b). The relative magnitudes of the milk PMN CL differences in pluriparous cows during periods 2 and 3 were 62 and 78% of those of primiparous cows, respectively.

Figure 2 shows the kinetics of CL after PMA stimulation in blood and milk PMN of five primiparous cows and five pluriparous cows during periods 1 and 2. Dur-

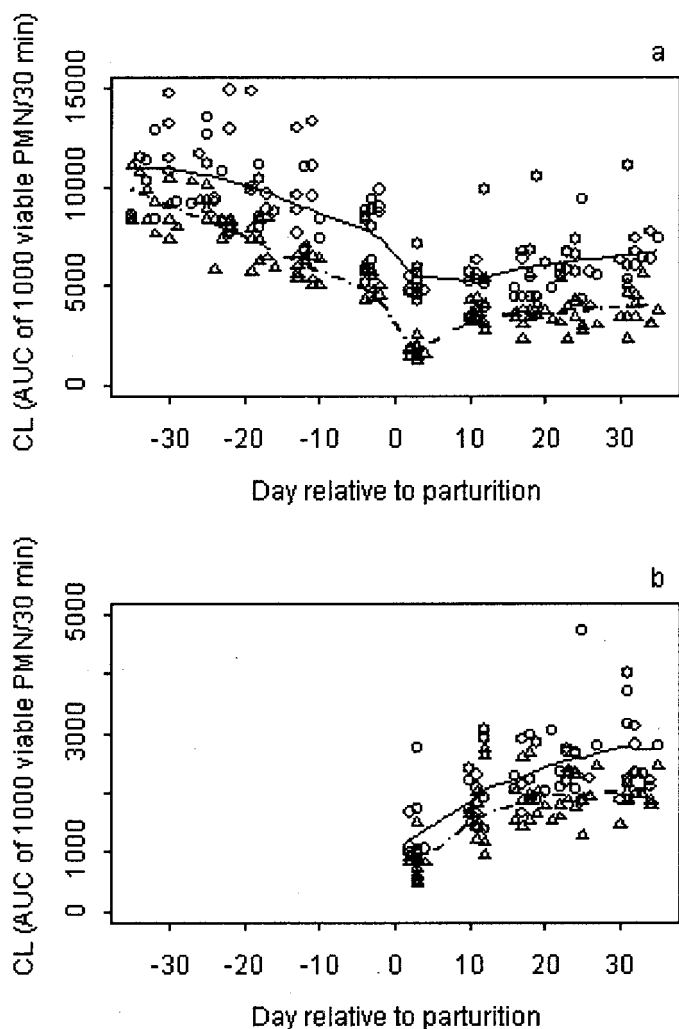


Figure 1. Chemiluminescence (CL) of polymorphonuclear leukocyte (PMA) stimulated blood (a) and milk (b) PMN in primiparous ($n = 12$, solid line and circles) and pluriparous ($n = 12$, dashed line and triangles) dairy cows during periparturient period (0 indicates the day of parturition). Data are expressed as the area under the curve (AUC) of continuously light emission of 10^3 viable PMN for 30 min.

ing these periods the lowest CL values were observed. Although blood PMN CL in primiparous cows at PPD -2 was higher than the CL in pluriparous cows, the RLU_{max} peaked faster in pluriparous cows. Similarly, at PPD 2 the CL peaked faster in pluriparous cows. Furthermore, the CL response by primiparous PMN decreased more slowly than the pluriparous PMN CL response. Beyond the slightly lower milk PMN CL intensity in pluriparous cows at PPD 2, the CL peak in milk PMN appeared faster in pluriparous cows than those of primiparous cows.

Viability of Blood and Milk PMN

No differences were observed for blood PMN viability of primiparous and pluriparous cows in the three study periods (Figure 3a), and blood PMN viability was always high. The viability of milk PMN was lower in pluriparous cows compared with primiparous cows in the two periods studied ($P < 0.001$; Figure 3b). In contrast, on PPD 2, 3, and 4, SCC in pluriparous cows was higher than in heifers (39,000 cells/ml). From PPD 7 onward, no differences in SCC could be detected between both groups (Table 1).

Enumeration of Leukocytes and Leukogram

The largest difference in total white blood cell count and number of mature neutrophils between primiparous and pluriparous cows was observed in period 1, before parturition, with the difference equal to $3719/\mu\text{l}$ for white blood cells and $2604/\mu\text{l}$ for mature neutrophils. In the two following study periods, the difference was much smaller. The largest difference in band neutrophils in favor of the primiparous cows was observed immediately after parturition, and a substantial difference was also noted in the first study period, immediately before parturition (Table 1). Finally, the number of metamyelocytes and myelocytes and eosinophils in primiparous cows was higher than in pluriparous cows in any of the three study periods.

DISCUSSION

Marked suppression of blood PMN CL was observed around parturition and during early lactation in primiparous and pluriparous dairy cows. Although we did not study any cause-and-effect relationship between periparturition and PMN dysfunction, such an association with increased cow's susceptibility to mastitis pathogens was nonetheless reported elsewhere (Kehrli et al., 1989; Kremer et al., 1993; Shuster et al., 1996). This relationship might contribute to the severity of coliform mastitis during early lactation that links to particularly "parity" (van Werven et al., 1997). Our results also indicate that the impairment of blood PMN CL in primiparous and pluriparous dairy cows during periparturition is confined to the period between periparturient week -1 and 2. This is in accordance with previous reports (Moreira da Silva et al., 1998; Kimura et al., 1999; Hoeben et al., 2000), indicating that the transitional period from parturition to lactation is immunosuppressive. This can, in part, lead to an overall impaired bactericidal capacity of blood PMN during early lactation reported in vivo (Shuster et al., 1996) and in vitro (Mehrzhad et al., 2001a). While the role of ROS in PMN bactericidal activity is still the subject of

Table 1. Mean differences (with 95% confidence interval) between primiparous and pluriparous cows during three different study periods: 1) PPD-3,-2: at 2 or 3 d before parturition, 2) PPD2,3,4: at 2, 3, or 4 d after parturition, and 3) PPD+7: from 7 to 35 d after parturition. For the third study period, intercept is related to the mean difference at d 20 after parturition, whereas the slope is related to the difference in the change of the parameter over time.¹

Dependent variables	Primiparous vs. pluriparous			
	PPD-2,-3	PPD 2,3,4	PPD +7 (intercept)	PPD +7 (slope)
Blood PMN CL ²	3,407 (2,398; 4,417)	3,468 (2,950; 3,987)	2,234 (799; 3,667)	69 (41; 96)
Milk PMN CL ²	—	469 (100; 839)	514 (207; 822)	16 (-6; 37)
Blood PMN viability (%)	1.0 (0.0; 1.9)	0.5 (-0.2; 1.2)	0.6 (0.1; 1.0)	0.0 (-0.05; 0.03)
Milk PMN viability (%)	—	33 (28; 38)	22 (16; 27)	-0.2 (-0.6; 0.1)
SCC (×1,000/ml)	—	-38.8 (-13.6; -63.9)	-38.6 (-18.0; -59.2)	-0.98 (-0.63; -1.33)
WBC/ μ l	3,719 (2,377; 5,061)	412 (-1,112; 1,937)	-57 (-980; 866)	-3 (-61; 55)
Mature neutrophils/ μ l	2,604 (2,072; 3,137)	25.2 (-500; 551)	-454 (-91; 999)	25 (0.4; 49)
Band cells/ μ l	113 (78; 147)	336 (183; 489)	68 (-19; 156)	2.3 (-3; 7.6)
Meta + Myelo/ μ l	604 (363; 845)	551 (253; 850)	603 (496; 710)	-12 (-20.6; -4.5)
Eosinophils/ μ l	613.6 (456; 771)	202 (105; 301)	252 (159; 344)	12.1 (6.1; 18)

¹PMN = polymorphonuclear leukocyte, PPD = periparturient day, CL = chemiluminescence.

²Area under the curve (AUC) of 1,000 viable PMN.

debate (Reeves et al., 2002), it is generally agreed that PMN ROS is pivotal for bactericidal activity (Burvenich et al., 1994). Indeed, PMA-stimulated PMN CL was the central part of the current study. A previous study unequivocally revealed a positive correlation between PMA-stimulated PMN CL and their bactericidal activity as well as with latex-stimulated PMN CL (Mehrzaad et al., 2001b). Substantial evidence also revealed that PMN phagocytosis and CL are interrelated, which conclusively gives PMN bactericidal activity (Allen et al., 1972; Grebner et al., 1977).

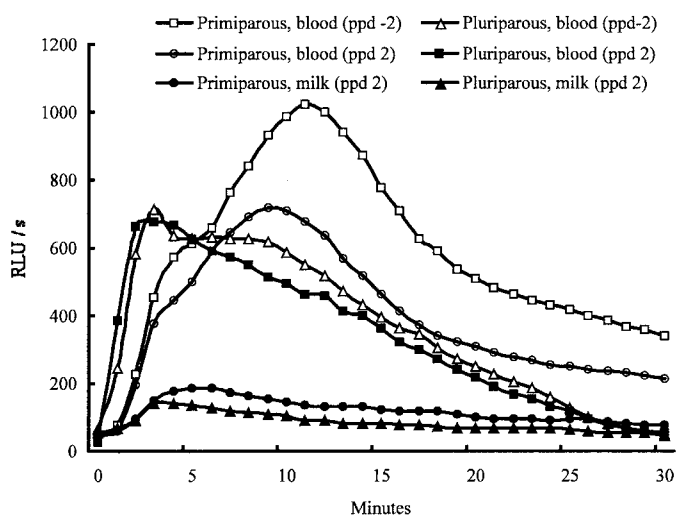


Figure 2. Blood and milk polymorphonuclear leukocytes (PMN) chemiluminescence profiles of primiparous and pluriparous dairy cows at d 2 before and d 2 after calving. The generation of CL was monitored continuously for 30 min after addition of 100 ng/ml PMA and 0.3 mM luminol to the 4.10^6 isolated cells suspended in 200 μ l. The data shown are means of five primiparous and five pluriparous dairy cows. PPD = periparturient day.

Undoubtedly, when bacterial invasion occurs, especially with Gram-negatives, ROS production will facilitate pathogen elimination. When ROS is produced adequately, cell and tissue damage will be far less than when the pathogen is not eliminated. Uncontrolled generation of ROS is harmful for many cell systems, e.g., T-cell hyporesponsiveness and lymphocyte proliferation inhibition caused by ROS (Nonnecke and Harp, 1988; Cemerski et al., 2002). On the other hand, Nonnecke and Harp (1988) observed that blood and milk PMN by phagocytosis of *Staphylococcus aureus* inhibits lymphocyte cytotoxicity and enhances mononuclear cell viability. ROS also differently enhances natural killer cell and T-cell activity (Suthanthiran et al., 1984; Cemerski et al., 2002), indicating that PMN ROS may not only damage the cells and tissues but may also accelerate recovery of inflammation, e.g., mastitis. It is still debatable that the modulation caused by PMN might be somewhat different in heifers and pluriparous cows around parturition.

Other blood PMN functions such as chemotaxis (Kremer et al., 1993) and diapedesis (Hill et al., 1979; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996) were compromised during early lactation. The suppression in PMN CL has been found to be associated with the sudden changes in concentrations of ketone bodies (Moreira da Silva et al., 1998; Suriyasathaporn et al., 1999), glucocorticosteroids (Guidry et al., 1976), and pregnancy and lactation-associated molecules (Dossogne et al., 1999; Hoeben et al., 2000). Moreover, PMN CL in milk was lower than in blood in both primiparous and pluriparous dairy cows. The lower milk PMN CL after stimulation with PMA was also seen in other studies (Weber et al., 1983; Dulin et al., 1988; Mehrzaad et al., 2001a). This may be explained by their exhausted

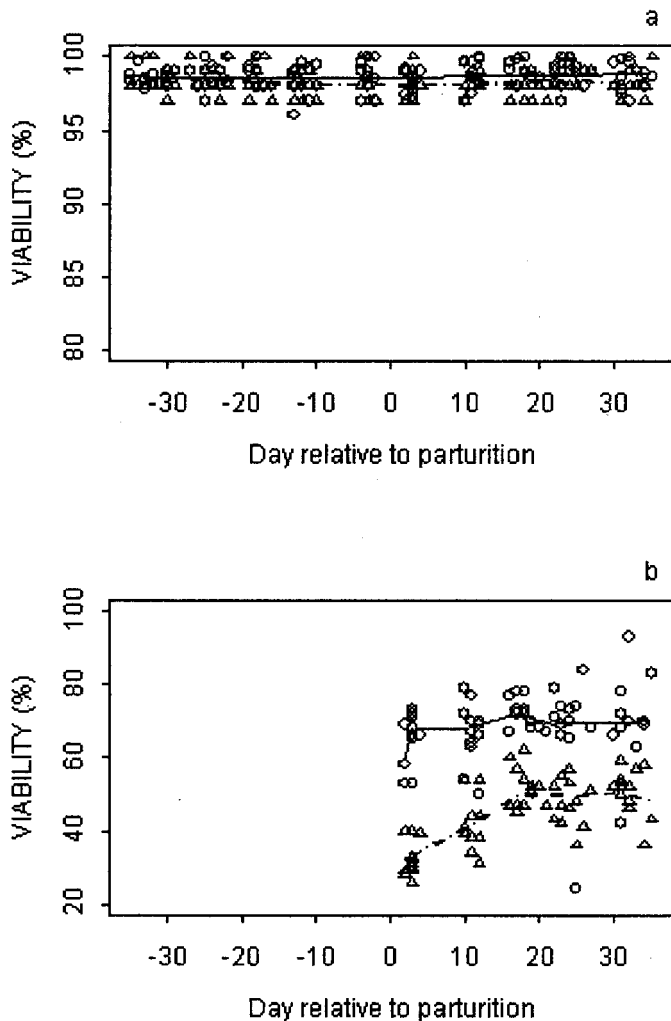


Figure 3. Viability of blood (a) and milk (b) polymorphonuclear leukocytes (PMN) in primiparous ($n = 12$, solid line and circles) and pluriparous ($n = 12$, dashed line and triangles) dairy cows during periparturient period (0 indicates the day of parturition). Values are expressed as percentage of viable PMN.

state through ingestion of fat globules and casein micelles (Russell and Reiter, 1975; Paape and Guidry, 1977) and/or by the effect of diapedesis through the blood/milk barrier (Smits et al., 1999). Diapedesis also caused apoptosis (Van Oostveldt et al., 2002), which lowered milk PMN CL.

The current study indicates that parity of the dairy cow influences blood PMN CL. As far as PMN ROS production capacity is concerned, the well-known alteration of PMN function seems to be more depressed in pluriparous cows. Because of the involvement of both superoxide anion and MPO- H_2O_2 system in luminol dependent CL (Rosen and Klebanoff, 1976; DeChatelet et al., 1982), the MPO-catalyzed bactericidal activity of blood PMN is likely to be more active in primiparous

dairy cows. Other blood PMN (e.g., immature neutrophils) were observed in isolated blood cells with a higher frequency in primiparous cows. This could have further lowered blood PMN CL in primiparous cows as they produce less ROS than mature neutrophils (Glasser and Fiederlein, 1987). But blood PMN CL was higher in primiparous cows around parturition and during early lactation, potentially revealing even higher immature neutrophil CL in primiparous cows than in pluriparous cows. This observation further supports our hypothesis that oxidation-reduction reactions in primiparous neutrophils could be more functional than those of pluriparous.

Our results also indicated that the milk PMN CL was minimal at periparturient wk 1 both in primiparous and pluriparous cows. This is in agreement with previous results (Mehrzhad et al., 2001a). What was unpredictable was the higher milk PMN CL in primiparous dairy cows during early lactation. Indeed, following milk PMN stimulation with PMA, the decreased ROS production capacity resulted mainly from previously ingested fat and casein in the milk compartment (Russell and Reiter, 1975; Paape and Guidry, 1977). This indicates that, in fact, milk PMN behave as prestimulated cells, rendering them less responsive toward PMA, which possibly implies an "exhausted state" for milk PMN CL. As the current result revealed, this potential exhausted state would be more peculiar for pluriparous cows, of which influencing factors remain to be clarified. According to Zecconi et al. (1994) and current observation, it can be concluded that the higher probability of developing clinical mastitis in pluriparous cows would result, at least in part, from lower milk PMN CL.

The kinetics of cellular CL of blood and milk PMN showed further disparities between the primiparous and pluriparous cows. In addition to a longer onset time for primiparous blood PMN CL at PPD -2 and 2, the PMA-stimulated CL at PPD -2 cumulatively maintained for at least 13 min in primiparous cows, whereas it is only maintained for less than 5 min in pluriparous cows. This shorter time of CL response toward stimuli potentially results in a less effectiveness of the oxygen-dependent intracellular bactericidal mechanism of blood PMN around parturition in pluriparous dairy cows. Furthermore, the PMA-stimulated CL at PPD 2 was also, of course, less intense than those of PPD -2 and increased for at least 11 min in primiparous cows, while in pluriparous it only peaked around 4 min and then subsided. This also demonstrates that there is less intracellular bactericidal efficiency in pluriparous dairy cows at PPD 2. Although several intracellular bactericidal mechanisms have been described, evidence exists that the production of ROS is one of the most important killing mechanisms, especially for Gram-negatives.

Moreover, the shape of the kinetic events during PMA-induced luminol-dependent CL reveals some details on the location of the ROS that are produced intra- and extracellularly (Rosen and Klebanoff, 1976; DeChatelet et al., 1982). The luminol-dependent CL kinetics after 3 to 4 min are considered to be the result of intracellular events (DeChatelet et al., 1982; Edwards et al., 1986; Faulkner and Fridovich, 1993). As the luminol-dependent CL requires hydrogen peroxide (Lind et al., 1983; Edwards and Swan, 1986; Faulkner and Fridovich, 1993), likely, the intracellular hydrogen peroxide production is higher in primiparous blood PMN than in pluriparous ones. Subsequently, impairment of intracellular reactions of MPO-H₂O₂ system is more pronounced in pluriparous dairy cows. The decreased intracellular events of ROS production could be the main cause of the significant decrease of CL activity immediately before and after parturition both in primiparous and pluriparous cows. The slightly higher intensity and peak in primiparous milk PMN CL suggest that the diminished oxygen-dependent intracellular killing of milk PMN toward pathogens is more pronounced in pluriparous cows. As previously reported (Mehrzahl et al., 2001a), it is unlikely that in the current study other milk cells contributed significantly to this CL shape.

Analogous with CL, the milk PMN viability was substantially higher in primiparous than in pluriparous dairy cows during early lactation. This observation contrasts with the viability of blood PMN, which was consistently very high (~100%). The minimal milk PMN viability was also most pronounced within the first week postpartum. This is in accordance with our previous study (Mehrzahl et al., 2001a). In a recent study (Piccinini et al., 1999), though no differentiation between lactation number is made, the viability values of PMN from uninfected quarters also range from 30 to 70%. Another comparative study (Van Oostveldt et al., 2001) revealed also lower viability of milk PMN during early lactation.

The exact mechanism for higher survival of milk PMN in primiparous dairy cows and/or their more pronounced impairment in pluriparous cows remains unknown. There are, however, some possible explanations for this discrepancy. According to studies on human and bovine PMN (Mayer et al., 1989; Jankowski et al., 2002), PMN NADPH-oxidase activity (an index of PMA-induced PMN CL) and their viability are interrelated. PMA is a potent NADPH-oxidase and protein kinase C agonist (Karlsson et al., 2000). As the NADPH-oxidase is a trigger of PMN respiratory burst and proton transportation into the PMN phagosomal and cytosolic space, the enzyme's activity is regulated by MPO as well (Edwards and Swan, 1986). The lower milk PMN CL in pluriparous cows suggests NADPH-oxidase and

MPO activity impairment. The contribution of neutrophil NADPH-oxidase activity is pivotal for their viability (Mayer et al., 1989; Jankowski et al., 2002). Furthermore, NADPH-oxidase activity contributes to phagosomal and cytosolic pH homeostasis (Reeves et al., 2002), maintaining PMN stoichiometry and electroneutrality (Takanaka and O'Brien, 1988). This phenomenon helps prevent rapid cytosolic acidification and necrosis, and thus might have had an effect on PMN survival in our study. This preliminary evidence supports the assumption of rapid cytosolic milk PMN acidification in pluriparous cows, which could result in a faster PMN necrosis. However, there could also be other physiological factors, such as recruitment of younger neutrophils in the milk compartment of primiparous cows, which might involve the delay of apoptosis and the increase of viability. The consistently higher milk PMN viability in primiparous cows suggests that primiparous cows might be a better source of milk PMN. Similarly, the higher quality of blood PMN would make primiparous cows better PMN donors for *in vitro* test.

Number of circulating leukocytes and hematological profiles of primiparous cows differed from those of pluriparous cows, particularly immediately before and after parturition. The elevated leukocytes and neutrophil counts during these periods may reflect a response to the inevitable higher and short-lasting circulating levels of cortisol, a known marginal pool enhancer (Boggs et al., 1964), in periparturient dairy cows in both groups (Guidry et al., 1976; Peter and Bosu, 1987). Comparatively, the considerably higher circulating immature PMN in primiparous cows reveals that the overall hematopoiesis is more functional in primiparous dairy cows. This could explain the existence of more juvenile neutrophils in circulation, consequently boosting PMN viability and extending their functional lifespan in the milk compartment. The remarkably higher circulating eosinophils in primiparous cows during periparturition strongly suggest more active eosinophilopoiesis in primiparous cows. Though not as crucial as PMN in mastitis, the way in which compound I of eosinophil peroxidase reacts with H₂O₂ is similar to that of MPO, but with substrates like CL⁻, however, it is far higher (Arnhold et al., 2001), which yields the strongest bactericidal substance: HOCL. Moreover, micromolar of eosinophil major basic protein activates neutrophil in a noncytotoxic manner, substantially boosting PMN CL (Moy et al., 1990). The involvement of eosinophil in bactericidal activity of bovine blood PMN especially during periparturient period still remains to be documented.

In short, the more pronounced PMN CL and milk PMN viability suppression during periparturient period in pluriparous cows can reflect PMN bactericidal inefficiency during the early phase of bacterial invasion,

potentially boosting pluriparous cows' susceptibility to mastitis. Whether the primiparous dairy cows' udders are more protected against invading pathogens remains to be tested.

ACKNOWLEDGMENTS

Ministry of Agriculture and Small Enterprises of Belgium (S/6014 and S/5871), Ministry of Science, Research and Technology of Iran, and the Research Fund of Ghent University (BOF-project; Grant 011D4001) provided financial support of the study. We also thank K. Demeyere and E. Vander Elstraten for technical assistance.

REFERENCES

- Allen, R. C., R. L. Stjernholm, and R. H. Steele. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.* 47:679–684.
- Arnhold, J., P. G. Furtmüller, G. Regelsberger, and C. Obinger. 2001. Redox properties of the couple compound I/native enzyme of myeloperoxidase and eosinophil peroxidase. *Eur. J. Biochem.* 268:5142–5148.
- Babior, B. 1984. The respiratory burst of phagocytes. *J. Clin. Invest.* 73:599–601.
- Boggs, D. R., J. W. Athens, G. E. Cartwright, and M. M. Wintrobe. 1964. The effect of adrenal glucocorticosteroids upon the cellular composition of inflammatory exudates. *Am. J. Pathol.* 44:763–770.
- Burvenich, C., M. J. Paape, A. W. Hill, A. J. Guidry, R. H. Miller, R. Heyneman, W. D. J. Kremer, and A. Brand. 1994. Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. *Vet. Quart.* 16:45–50.
- Carlson, G., and J. J. Kaneko. 1973. Isolation of leukocytes from bovine peripheral blood. *Proc. Soc. Exp. Biol. Med.* 142:853–856.
- Cemurski, S., A. Cantagrel, J. P. M. van Meerwijk, and P. Romagnoli. 2002. Reactive oxygen species differently affect T cell receptor signaling pathways. *J. Biol. Chem.* 277: 19585–19593.
- DeChatelet, L. R., G. D. Long, P. S. Shirley, D. A. Bass, M. J. Thomas, F. W. Henderson, and M. S. Cohen. 1982. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. *J. Immunol.* 129:1589–1593.
- Dosogne, H., C. Burvenich, A. E. Freeman, M. E. Kehrl Jr., J. C. Detilleux, J.-F. Beckers, and D. Hoeben. 1999. Pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early post-partum dairy cows. *Vet. Immunol. Immunopathol.* 67:47–54.
- Dulin, A. M., M. J. Paape, and S. C. Nickerson. 1988. Comparison of phagocytosis and chemiluminescence by blood and mammary gland neutrophils from multiparous and nulliparous cows. *Am. J. Vet. Res.* 49:172–177.
- Edwards, S. W., and T. F. Swan. 1986. Regulation of superoxide generation by myeloperoxidase during the respiratory burst of human neutrophils. *Biochem. J.* 237:601–604.
- Faulkner, K., and I. Fridovich. 1993. Luminol and lucigenin as detectors for O_2^- . *Free radical Biol. Med.* 15:447–451.
- Glasser, L., and R. L. Fiederlein. 1987. Functional differentiation of normal human neutrophils. *Blood.* 69:937–944.
- Goff, J. P., and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. *J. Dairy Sci.* 80:1260–1268.
- Grebner J. V., E. L. Mills, B. H. Gray, and P. G. Quie. 1977. Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. *J. Lab. Clin. Med.* 89:153–159.
- Guidry A. J., M. J. Paape, and R. E. Pearson. 1976. Effect of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. *Am. J. Vet. Res.* 37:1195–1200.
- Hayhoe, F. G. J., and R. J. Flemans. 1969. An Atlas of Haematological Cytology. Wolfe Publishing Ltd. G. Barry Carruthers, M. D., London.
- Heyneman, R., C. Burvenich, and R. Vercauteren. 1990. Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. *J. Dairy Sci.* 73:985–994.
- Hill, A. W., A. L. Shears, and K. G. Hibbitt. 1979. The pathogenesis of experimental *Escherichia coli* mastitis in newly calved dairy cows. *Res. Vet. Sci.* 26:97–101.
- Hoeben, D., E. Monfardini, G. Opsomer, H. Dosogne, A. De Kruif, J.-F. Beckers, and C. Burvenich. 2000. Chemiluminescence of bovine polymorphonuclear leukocytes during the periparturient period and relation with metabolic parameters and bovine pregnancy-associated glycoprotein. *J. Dairy Res.* 67:249–259.
- Jankowski, A., C. C. Scott, and S. Grinstein. 2002. Determinants of phagosomal pH in neutrophils. *J. Biol. Chem.* 277:6059–6066.
- Karlsson, A., J. B. Nixon, and L. C. McPhail. 2000. Phorbol myristate acetate induces neutrophils NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase. *J. Leukocyte Biol.* 67:396–404.
- Kehrli, M. E. Jr., B. J. Nonnecke, and J. A. Roth. 1989. Alterations in bovine peripheral blood neutrophil function during the periparturient period. *Am. J. Vet. Res.* 50:207–214.
- Kimura, K., J. P. Goff, and M. E. Jr. Kehrl. 1999. Effect of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. *J. Dairy Sci.* 82:2385–2396.
- Kremer, W. D. J., E. N. Noordhuizen-Stassen, F. J. Grommers, A. J. J. M. Daemen, P. A. J. Henricks, A. Brand, and C. Burvenich. 1993. Preinfection chemotactic response of blood polymorphonuclear leukocytes to predict severity of *Escherichia coli* mastitis. *J. Dairy Sci.* 76:1568–1574.
- Lind, J., G. Merenyi, and T. E. Eriksen. 1983. Chemiluminescence mechanism of cyclic hydrazides such as luminol in aqueous solutions. *J. Am. Chem. Soc.* 105:7655–7661.
- Mayer, S. J., P. M. Keen, N. Craven, and F. J. Bourn. 1989. Regulation of phagolysosome pH in bovine and human neutrophils: the role of NADPH oxidase activity and an Na^+/H^+ antiporter. *J. Leukocyte Biol.* 45:239–248.
- McDonald, J. S., and A. J. Anderson. 1981. Total and differential somatic cell counts in secretions from noninfected bovine mammary glands: the peripartum period. *Am. J. Vet. Res.* 42:1366–1368.
- Mehrzad, J., H. Dosogne, E. Meyer, R. Heyneman, and C. Burvenich. 2001a. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. *J. Dairy Res.* 68:399–415.
- Mehrzad, J., H. Dosogne, F. Vangroenweghe, and C. Burvenich. 2001b. A comparative study of bovine blood and milk neutrophil functions with luminol-dependent chemiluminescence. *Luminescence.* 16:343–356.
- Moreira da Silva, S., C. Burvenich, A. Massart-León, and L. Brosse. 1998. Assessment of blood neutrophil oxidative burst activity in dairy cows during the period of parturition. *Anim. Sci.* 67:421–426.
- Moy, J. M., G. J. Gleich, and L. L. Thomas. 1990. Noncytotoxic activation of neutrophils by eosinophil granule major basic protein: effect on superoxide anion generation and lysosomal enzyme release. *J. Immunol.* 145:2626–2632.
- Nonnecke, B., and J. A. Harp. 1988. Effect of *Staphylococcus aureus* on bovine mononuclear leukocyte proliferation and viability: modulation by phagocytic leukocytes. *J. Dairy Sci.* 71:835–842.
- Paape, M. J., and A. J. Guidry. 1977. Effect of fat and casein on intracellular killing of *Staphylococcus aureus* by milk leukocytes. *Proc. Soc. Exper. Biol. Med.* 155:588–593.

- Peter, A. T., and W. T. K. Bosu. 1987. Periparturient endocrine changes associated with retained placenta in dairy cows. *Theriogenology*. 28:383–394.
- Piccinini, R., V. Bronzo, P. Moroni, P. Luzzago, and A. Zecconi. 1999. Study on the relationship between milk immune factors and *Staphylococcus aureus* intramammary infections in dairy cows. *J. Dairy Res.* 66:501–510.
- Reeves, E. P., H. Lu, H. L. Jacobs, C. G. M. Messina, S. Bolsover, G. Gabella, E. O. Potma, A. Warley, J. Roes, and A. W. Segal. 2002. Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature (Lond.)* 416:291–297.
- Rosen, H., and S. J. Klebanoff. 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. *J. Clin. Invest.* 58:50–60.
- Russell, M. W., and B. Rieter. 1975. Phagocytic deficiency of bovine milk leukocytes: an effect of casein. *J. Reticuloendothelial Soc.* 18:1–13.
- Shuster, D. E., E. K. Lee, and M. E. Jr. Kehrl. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving compared with cows at mid lactation. *Am. J. Vet. Res.* 57:1569–1576.
- Smits, E., C. Burvenich, A. J. Guidry, R. Heyneman, and A. Massart-Leën. 1999. Diapedesis across mammary epithelium reduces phagocytotic and oxidative burst of bovine neutrophils. *Vet. Immunol. Immunopathol.* 68:169–176.
- Suriyasathaporn, W., A. J. J. M. Daemen, E. N. Noordhuizen-Stassen, M. Nielen, S. J. Dieleman, and Y. H. Schukken. 1999. β -hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. *Vet. Immunol. Immunopathol.* 68:177–186.
- Suthanthiran, M., S. D. Solomon, P. S. Williams, A. L. Rabin, A. Novogrodsky, and K. H. Stenzel. 1984. Hydroxyl radical scavengers inhibit human natural killer cell activity. *Nature (Lond.)* 307:276–278.
- Takanaka, K., and P. J. O'Brien. 1988. Proton release associated with respiratory burst of polymorphonuclear leukocytes. *J. Biochem.* 103:656–660.
- Van Oostveldt, K., F. Vangroenweghe, H. Dosogne, and C. Burvenich. 2001. Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early- and mid-lactating healthy cows. *Vet. Res.* 32:617–622.
- Van Oostveldt, K., M. J. Paape, and C. Burvenich. 2002. Apoptosis of bovine neutrophils following diapedesis through a monolayer of endothelial and mammary epithelial cells. *J. Dairy Sci.* 85:139–147.
- van Werven, T., E. N. Noordhuizen-Stassen, A. J. J. M. Daemen, Y. H. Schukken, A. Brand, and C. Burvenich. 1997. Preinfection in vitro chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. *J. Dairy Sci.* 80:67–74.
- Vandeputte-Van Messom, G., C. Burvenich, E. Roets, A. Massart-Leën, R. Heyneman, W. D. J. Kremer, and A. Brand. 1993. Classification of newly calved cows into moderate and severe responders to experimentally induced *Escherichia coli* mastitis. *J. Dairy Res.* 60:19–29.
- Vangroenweghe, F., H. Dosogne, J. Mehrzad, and C. Burvenich. 2001. Effect of milk sampling techniques on milk composition, bacterial contamination, viability and functions of resident cells in milk. *Vet. Res.* 32:565–579.
- Webb, L. S., B. B. Keele, and R. B. Jr. Johnston. 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. *Infect. Immun.* 9:1051–1056.
- Weber, L., E. Peterhans, and R. Wyler. 1983. The chemiluminescent response of bovine polymorphonuclear leukocytes isolated from milk and blood. *Vet. Immunol. Immunopathol.* 4:397–412.
- Zecconi, A., V. Bronzo, R. Piccinini, G. Spreafico, and G. Roffo. 1994. Phagocytic activity of bovine polymorphonuclear leukocytes. *J. Dairy Res.* 61: 271–279.