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Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation

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ABSTRACT

To examine the effect of parity on polymorphonuclear neutrophils (PMN) function, phagocytic and bactericidal activity of the PMN isolated from blood and milk against *Staphylococcus aureus* was compared between groups of 6 primiparous and 6 multiparous healthy dairy cows during early lactation using bacteriological and PMN–pathogen interaction assays. Latex-stimulated luminol-amplified chemiluminescence (CL) and viability of these PMN were also investigated. The phagocytosis and killing of *S. aureus* by blood were remarkably higher than those of milk PMN. Similarly, the CL and viability in blood PMN were markedly higher than in milk PMN. Both in blood and in milk the phagocytosis of *S. aureus* by PMN in primiparous cows was substantially higher than in multiparous cows. The killing activity of blood PMN against *S. aureus* was 42.3 ± 3.4% and 23.2 ± 1.7% in primiparous and multiparous, respectively. Milk PMN killed only 20.7 ± 2% *S. aureus* in primiparous and 10.2 ± 1.3% in multiparous cows. Blood and milk PMN CL and milk PMN viability were significantly higher in primiparous cows. The pronounced reduction in phagocytic and bactericidal activity in blood and milk-resident PMN from multiparous cows, in part, resulted from the pronounced decrease of PMN viability and free radicals production capacity; this suggests that heifers' udders could be more protected against *S. aureus*, which remains to be tested in the field.

1. Introduction

Mastitis is a very common disease that causes major economic losses to the dairy industry worldwide. This inflammatory reaction develops most frequently in response to an intramammary bacterial infection (Smith et al., 1985; Paape et al., 2002; Burvenich et al., 2003). *Staphylococcus aureus* is one of the most virulent organisms with low cure rates involved in bovine mastitis (Miles et al., 1992; Burvenich et al., 2003; Smith et al., 2005). To establish intramammary infection, *S. aureus* has to overcome several host defence barriers (phagocytosis, recruitment of polymorphonuclear neutrophils (PMN), etc.). *S. aureus* possesses factors such as extracellular polysaccharide layer (exopolysaccharide or slime) and capsule polysaccharides that boost bacterial evasion by, e.g., impairing complement- and antibody-mediated opsonisation and phagocytosis (Wilkinson et al., 1979; Xu et al., 1992). This evasion mechanism enhances the virulence capacity of *S. aureus* (Quie and Belani, 1987; Baselga et al., 1994).

Several antimicrobial systems exist in the mammary gland (Paape et al., 2002; Burvenich et al., 2003). It is, however, the massive influx of PMN that will resolve the infection. These cells have enormous potential to eventually kill phagocytosed bacteria (Paape et al., 2002;

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Burvenich et al., 2003; Mehrzad et al., 2004, 2005). The phagocytic and bactericidal activity of PMN in dairy cows is one of the major protective mechanisms against invading pathogens in the mammary gland (Mehrzad et al., 2001a; Paape et al., 2002; Burvenich et al., 2003). When PMN engulf invading bacteria free radicals are rapidly produced inside the phagosome and phagolysosome. These free radicals react very broadly against Gram negative and positive bacteria, fungi and protozoa (Heyneman et al., 1990; Hampton et al., 1996; Chapman et al., 2002; Reeves et al., 2002; Burvenich et al., 2003). Free radicals production capacity of PMN can be measured following PMN stimulation with particles, e.g., zymosan, bacteria, latex beads. The most widely used technique to quantify free radicals production of PMN is the chemiluminescence (CL) assay (Piccinini et al., 1999; Hoeben et al., 2000; Mehrzad et al., 2001a, 2001b, 2005). The CL of PMN reflects intracellular and extracellular events of the PMN–pathogen interactions, which are very critical for the mechanisms of bacterial killing. The increased incidence of severe mastitis during early lactation is correlated with a decreased function of blood and milk PMN (Burvenich et al., 2003; Mehrzad et al., 2004, 2005), further emphasizing the pivotal role of PMN in the defence against intramammary pathogens. Despite their importance, little study has been conducted on the issue of PMN–*S. aureus* interaction in the mammary gland.

It is widely accepted that clinical mastitis in heifers is predominantly local and to a much lesser extent systemic (Burvenich et al., 2003; Vangroenweghe et al., 2004) compared to multiparous dairy cows. It also appears that the transition from pregnancy to parturition is accompanied by a gradual decrease in PMN bactericidal capacity (Hoeben et al., 2000; Mehrzad et al., 2001a, 2002). The severity of clinical mastitis has been reported to be less pronounced in dairy cows with higher pre-infection PMN free radicals production (Heyneman et al., 1990; Mehrzad et al., 2004, 2005). Increased severity of mastitis in dairy cows has also been associated with increased parities (Van Werven et al., 1997). All of these changes might be due to the fact that blood and milk PMN in heifers react faster than in multiparous cows. To the best of our knowledge, the influence of parity on overall killing capacity of blood and milk PMN has never been assessed.

Since *S. aureus* is a common cause of contagious mastitis in dairy cows, blood and milk PMN bactericidal activity against this bacterium was investigated during relatively immune suppression, early lactation (Dosogne et al., 2001; Mehrzad et al., 2001a, 2002; Burvenich et al., 2003). The main objective of this study was, therefore, to compare the phagocytic and bactericidal activity of blood and milk-resident PMN against *S. aureus* in heifers and older cows to get a better understanding of the bactericidal activity of the PMN.

2. Materials and methods

2.1. Animals and experimental procedures

In total, 12 Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected. Primiparous cows (first gestation, 2.4 ± 0.5 yr, $n = 6$) and multiparous cows (fourth to fifth gestation, 5.8 ± 0.7 yr, $n = 6$) were used. After calving (until 30 ± 5 days (mean $\pm$ S.D.) after parturition for both groups), 1.5 L mixed cisternal 4-quarter-morning milk samples and 80 ml blood samples were aseptically collected (Mehrzad et al., 2001a, 2002, 2004, 2005). The cows were clinically healthy and showed no signs of periparturient diseases after calving. To confirm that cows had no mastitis pathogens, 10 and 50 ml quarter-foremilk samples were collected after parturition and the day before the experiment and examined for bacteriological infection and somatic cell count (SCC), respectively. Only cows with a quarter SCC $< 2 \times 10^5$ cells/ml and milk samples that cultured negative for major mastitis pathogens were considered as clinically healthy; to do this, the quarter-milk samples were aseptically cultured onto Columbia sheep blood agar (Biokar Diagnostics, Beauvois, France) for 18 h at 37 °C. Blood samples were aseptically collected from the external jugular vein into evacuated tubes (BD-Vacutainer System, Plymouth; UK) containing 125 IU heparin for further processing.

2.2. Blood and milk PMN preparation, enumeration and identification

Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes (Carlson and Kaneko, 1973; Mehrzad et al., 2001a, 2001b, 2002). 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 as described below and percentage of PMN, the suspension was adjusted to a concentration of 5×10^6 viable blood PMN/ml with Hank's Balanced Salt Solution (HBSS) containing Ca^{2+} and Mg^{2+} (Gibco BRL, Life Technologies Inc., MD, Gaithersburg) supplemented with 1 g bovine serum albumin/l (BSA; Sigma Chemicals, St. Louis MO USA) and 25 mM-HEPES buffer (HBSS-BSA; Sigma) for PMN functional assays. The milk samples were maintained on smashed ice following sampling and during the isolation procedure. Initial volumes of 1.5 L milk were processed using a high-capacity centrifuge (RC – 3BP, Sorvall, Newtown, CT, USA was carefully) after 60%, v/v, dilution with cold phosphate buffered saline (PBS; 0.01 M phosphate buffer (KH_2 PO_4–Na_2HPO_4) −0.15 M NaCl, pH 7.2). Fat was carefully removed after the first centrifugation ($600 \times g$, 15 min, 4 °C) and the pellet was washed twice at $300 \times g$ for 10 min and $200 \times g$ for 15 min at 4 °C, in the cold PBS. After counting the isolated milk cells using Coulter counter and determination of the viability, the cells were finally resuspended to a concentration of 5×10^6 viable milk cells/ml with HBSS-BSA for further PMN functional assays. Isolation of cells from milk was performed using three centrifugation steps as previously described (Mehrzad et al., 2001a, 2002), yielding ~60% PMN with different viability throughout the experiment. The isolation procedure time for milk PMN was similar to that of blood PMN (55 ± 4 min). Milk somatic cell count (SCC) in whole milk was determined with a fluoro-opto electronic method (Fossomatic 400 cell