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Flow cytometric analysis: Interdependence of healthy and infected udder quarters

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ABSTRACT

An important question about intramammary infections that is still debated in the literature is the independence or interdependence of the quarters of dairy cows. The present study sought to explore milk neutrophil function and the milk lymphocyte profile of uninfected quarters from uninfected and infected (one infected quarter per cow) udders to evaluate interdependence of the quarters. Thus, 32 (8 cows) and 18 (6 cows) uninfected quarters from uninfected and infected udders were used, respectively. Using flow cytometry, we evaluated the percentage of milk neutrophils and their expression of adhesion molecules L-selectin (CD62L), β_2-integrin (CD11b), and an endothelial-selectin ligand (CD44); levels of intracellular reactive oxygen species (ROS); phagocytosis of *Staphylococcus aureus* by milk neutrophils; and neutrophil viability. Furthermore, we assessed the percentage of B-cell ($CD21^+$) and T-lymphocyte subsets ($CD3^+/CD4^+/CD8^-$, $CD3^+/CD8^+/CD4^-$, $CD3^+/CD4^+/CD25^-$, $CD3^+/CD4^+/CD25^+$, and $CD3^+/CD4^-/CD25^-$) using flow cytometry with monoclonal antibodies. The infected quarter did not affect somatic cell count or the percentage of neutrophils in the neighboring uninfected quarters. Furthermore, the infected quarter did not influence neutrophil viability, intracellular reactive oxygen species production, or phagocytosis of *S. aureus* by milk neutrophils. Conversely, the expression of adhesion molecules CD11b, CD62L, and CD44 by milk neutrophils differed between uninfected quarters from infected versus uninfected udders. The lymphocyte subsets did not differ between groups, except for a higher percentage of B cells in uninfected quarters from infected udders than in those from uninfected udders. Thus, our study strongly supports the hypothesis of interdependence of quarters based on the influence of infection on both the percentage of B cells and the expression of adhesion molecules by milk neutrophils in the neighboring uninfected quarters.
Key words: mastitis, somatic cell count, lymphocyte, neutrophil, dairy cow

INTRODUCTION

Several previous studies have evaluated aspects of IMI based on the assumption that quarters within a cow are independent of each other (Berry and Meaney, 2006; Jensen et al., 2013) because of the anatomical construction of the udder, which implies that the infection of one quarter does not influence the immune status of the neighboring quarters (Merle et al., 2007; Jensen et al., 2013). An important question about IMI that is still debated in the literature is the independence or interdependence of the quarters of dairy cows (Schwarz et al., 2011). For instance, does a local inflammatory response against invading bacteria in an infected quarter influence the immune response of neighboring uninfected quarters? If it does, the local cross talk between udder quarters could be responsible for priming the neighboring uninfected quarters and therefore may influence the immune response against new infections. Some studies have evaluated the interdependence of quarters by determining the probability of an infection spreading from an infected to an uninfected quarter (Barkema et al., 1997; Sol et al., 2000; Berry and Meaney, 2006) or by evaluating milk SCC (Wever and Emanuelson, 1989), percentage of immune cells, or certain immune response parameters (Merle et al., 2007; Schwarz et al., 2011). The reasons that uninfected quarters from infected udders are more likely to be contaminated than quarters from uninfected udders may include the animal's susceptibility to mastitis, the transmission patterns of infection, or inherent immune competency (Barkema et al., 1997; Berry and Meaney, 2006). The transmission pattern of IMI in particular,

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should be considered, given the contagious behavior of pathogens and the fact that transmission occurs not only between cows but also between quarters within a cow (Barkema et al., 1997).

The aim of this study was to better elucidate the interdependence of quarters, exploring milk neutrophil function and milk lymphocyte profiles in uninfected quarters from infected and uninfected udders using flow cytometry analysis. To our knowledge, this is the first study to perform a broad evaluation of milk neutrophil function and milk lymphocyte subsets in uninfected quarters from udders with different infectious statuses. We believe that this new knowledge may improve the understanding of the interdependence of quarters, which may influence the probability of new infections in uninfected quarters.

MATERIALS AND METHODS

Animals

The present study used 50 mammary quarters from 14 Holstein dairy cows, which were collected at different lactation stages (DIM) and parities from a commercial dairy herd in São Paulo State, Brazil. The milk samples were divided into groups 1 and 2 as follows: group 1 included culture-negative milk samples from quarters of uninfected udders with no abnormal secretions in the strip cup test (n = 32 quarters; 8 dairy cows); and group 2 included culture-negative milk samples with SCC $<1 \times 10^5$ cells/mL from quarters from infected udders (only 1 quarter was considered as infected) with no abnormal secretions in the strip cup test (n = 18 quarters; 6 dairy cows). All cows with more than one infected quarter were excluded from this study.

Sample Collection

First, the strip cup test was performed to identify the presence of clots, flakes, or otherwise obviously abnormal secretions. Predipping was then performed, with one towel used for each teat. After discarding the first 3 milk streams, teat ends were scrubbed with cotton soaked in 70% ethanol, and a single milk sample from each mammary quarter was aseptically collected into sterile vials for bacteriological analysis. Finally, milk samples were collected for evaluation of SCC (40 mL), neutrophil function, and milk lymphocyte profiles (1 L of milk). The samples were kept at 4°C until arrival at the laboratory. The milk samples for the bacteriological analysis were collected once on the same day as those for analysis of immune parameters and were stored at −20°C for up to 30 d until the analysis.

Bacteriological Analysis

Bacteriological analysis was performed by culturing 0.01 mL of each milk sample on 5% sheep blood agar plates. The plates were incubated for 72 h at 37°C, followed by Gram staining, observation of colony morphologies, and biochemical testing (Oliver et al., 2004). A milk sample was considered culture-positive when the growth of ≥3 colonies was detected, except for animals with *Staphylococcus aureus* or *Streptococcus agalactiae* infections in their quarters, which were considered culture-positive when the growth of ≥1 colony was detected (Piepers et al., 2007; Piepers and De Vliegher, 2013).

Determination of SCC

Milk samples for SCC determination were collected in 40-mL vials containing microtablets of the preservative bronopol (2-bromo-2-nitropane-1,3-diol). Subsequently, the SCC analysis was performed using a Somacount 300 automated somatic cell counter (Bentley Instruments, Chaska, MN).

Definition of IMI Status

A quarter was considered uninfected when it was culture-negative, with no abnormal secretions in the strip cup test, and had a milk SCC $\leq 1 \times 10^5$ cells/mL, as the threshold for an uninfected quarter proposed by Bansal et al. (2005). A quarter was considered infected when any mastitis pathogen was isolated from a single milk sample or it had SCC $>2 \times 10^5$ cells/mL, as the threshold for an uninfected quarter as proposed by Schepers et al. (1997) and Schukken et al. (2003).

Separation of Milk Cells

The separation of the milk cells was performed as described by Koess and Hamann (2008). Briefly, 1 L of milk was diluted with 1 L of PBS (pH 7.4; 1.06 m*M* Na_2HPO_4, 155.17 m*M* NaCl, and 2.97 m*M* $Na_2HPO_4 \cdot 7H_2O$). After centrifugation at 1,000 × *g* for 15 min, the cream layer and supernatant were discarded. The cell pellet was then washed once using 30 mL of PBS and centrifuged at 400 × *g* for 10 min. The cells were resuspended in 1 mL of RPMI-1640 nutritional medium (R7638, Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and counted using a Neubauer chamber. Cell viability was evaluated using trypan blue exclusion. The milk cells were then diluted with nutritional medium containing 10% fetal bovine serum to a concentration of 2×10^6 viable cells/mL.