J. Dairy Sci. 98:1–7
http://dx.doi.org/10.3168/jds.2015-9573

Evolution of phagocytic function in monocytes and neutrophils blood cells of healthy calves

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ABSTRACT

The immune system of newborn calves is immature and must mature gradually. Understanding how this immunity is established may define different profiles. Twelve healthy calves were monitored during 8 time periods to assess the innate immune system during the first trimester of life. Blood samples were collected, and the blood phagocytes, identified by the expression of CD14 and CH138 surface molecules, were evaluated for phagocytic functionality (*Staphylococcus aureus* and *Escherichia coli* stained with propidium iodide) and the intracellular production of reactive oxygen species (2,7′-dichlorofluorescin diacetate oxidation). Functional changes in the CD14+ and CH138+ cells occurred at 40 d of age, with sporadic increases in phagocytosis intensity and reactive oxygen species production, and decreased phagocytosis occurred at 60 d of age. Therefore, fewer phagocytes were active from 40 d of age, although those that were active performed their roles with greater efficacy. That change presumably occurred because the calf phagocytes began to support the immune response without the influence of passive immunity. The animals failed to reach the stability needed to complete the maturation of the innate immune response by 90 d of age. These data are applicable for healthy calves only.

Key words: phagocytosis, oxidative metabolism, flow cytometry, innate immunity, newborn calf

INTRODUCTION

Great advances have been made in the field of veterinary medicine, with a better understanding of diseases, new diagnostic methods, and new drugs. However, the calf-rearing stage continues to show high rates of morbidity and mortality, and septicemia, diarrhea, and pneumonia, as well as bovine babesiosis and anaplasmosis, remain the leading causes of mortality despite advances (Windeyer et al., 2014).

The hormonal changes in late pregnancy, including increased maternal and fetal cortisol, affect the innate immune response of newborn calves, causing a decrease in phagocytosis and in the bactericidal activity of neutrophils and macrophages. Furthermore, neonates show low levels of complement factors and a decrease in their activity, a decreased functional activity of natural killer cells, and a lower ratio of dendritic cells (Barrington and Parish, 2001; Morein et al., 2002; Chase et al., 2008).

At birth, naïve newborns are transferred from a sterile uterine environment to a septic environment. The immaturity of protective mechanisms and the time required to develop humoral and cell-mediated immune responses may account for their inability to initiate the immune response at birth (Barrington and Parish, 2001). At this stage, their immune response is primary and characterized by low concentrations of immunoglobulins (Cortese, 2009).

Few studies have evaluated the maturation of the cellular immune system in healthy calves from birth to puberty. Kampen et al. (2006) assessed lymphocyte subpopulations in calf blood from birth to 6 mo of age using flow cytometry. However, relatively little information has been reported regarding the characterization or roles of blood phagocytes. In a preliminary study, Batista et al. (2012) evaluated the local phagocytes in the calf respiratory system, showing the existence of variation in the functionality of these cells over time. To understand the development of cellular immunity in calves, the present study examined the immunophenotype and the function of blood phagocytes over the first 3 mo of calf life to identify periods of increased disease susceptibility.

Received March 14, 2015.
Accepted August 29, 2015.
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MATERIALS AND METHODS

The present study was approved by the Animal Ethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo; FMVZ-USP). Twelve male Holstein calves, born of normal delivery, introduced into the study at 1 to 5 d of age were used. The total protein and γ-globulin of all the animals were ≥50 and 10 g/L, respectively, which is indicative of the successful acquisition of passive immunity and animal health (Feitosa et al., 2001). The calves were allocated to the experimental neonatology center of the Clinic for Cattle and Small Ruminants at FMVZ-USP in individual iron cages suspended, measuring 1.40 × 1.47 × 0.72 m, and after completing 30 d of life they were transferred to stalls measuring 1.375 × 2 m with 2 calves per stall. During the day, the animals were released in a collective cemented place with incidence of sunlight. The animals were monitored daily to ensure their health during the study evaluated by blood count and by clinical examination. No abnormalities were found on clinical examination or in the blood count of these animals using reference parameters described by Peixoto et al. (2002). Nutritional management consisted of whole milk powder intake. They were given 4 L of milk per day, divided into 2 feedings. The animals were fed with pelleted feed for calves 2 times a day and ad libitum hay, water, and mineral salt. The serum immune function of these animals was evaluated during 8 intervals within the first 3 mo of life: first week of life (**M1**), 8 to 14 d old (**M2**), 15 to 22 d old (**M3**), 23 to 30 d old (**M4**), 31 to 40 d old (**M5**), 41 to 50 d old (**M6**), 51 to 60 d old (**M7**), and 90 d old (**M8**). The blood samples were collected by jugular venipuncture with vacuum method used in tubes with heparin.

Assays for population quantification, for the assessment of the intracellular production of reactive oxygen species (**ROS**) and for phagocytosis by blood leukocytes were performed using flow cytometry (Hasui et al., 1989; Kampen et al., 2004a,b). The blood cells were incubated with *Staphylococcus aureus* (ATCC 25923) or *Escherichia coli* (O98: H28) stained with propidium iodide (**PI**; catalog no. P4170, Sigma-Aldrich, St. Louis, MO). The labeling of bacteria was performed as established by Hasui et al. (1989), with certain modifications (Souza et al., 2012).

The assays were performed in polypropylene tubes suitable for flow cytometry. To assess the basal intracellular ROS production, 100 μL of blood from each animal were added to each tube and incubated at 37°C for 30 min with 200 μL of 0.3 m*M* 2,7′-dichlorofluorescin diacetate (**DCFH-DA**). To conduct the phagocytosis tests, 100 μL of *S. aureus* or *E. coli* stained with PI were added to those tubes in ratios bacteria to phagocytes of up to 25:1 [multiplicity of infection (**MOI**) = 25].

The test to identify the blood phagocytes was performed together with the functional assessment. After centrifuging (250 × *g* for 8 min at 4°C) and discarding the supernatant, 1 μL of mouse IgG subscript 1 antibovine CD14 monoclonal antibody (catalog no. MM61A, VMRD Inc., Pullman, WA) or 1 μL of mouse IgM antibovine CH138 monoclonal antibody (catalog no. CH138A, VMRD Inc.) was added to the tubes according to the population to be identified, and the tubes were left standing at room temperature for 30 min. After the incubation period, 1,000 μL of ice-cold PBS were added, and then a new centrifugation (250 × *g* for 8 min at 4°C) was performed. The supernatant was discarded, 1 μL of goat antimouse IgG subscript 1 monoclonal secondary antibody conjugated to the fluorochrome allophycocyanin (catalog no. A10541, Invitrogen Corp., Carlsbad, CA) was added to the tubes to label the monocytes, and goat antimouse IgM monoclonal secondary antibody conjugated to allophycocyanin (catalog no. M31505, Invitrogen Corp.) was added to the tubes to identify granulocytes according to the manufacturer's recommendations. The tubes were kept at room temperature in the dark for 30 min. An additional washing was performed and the samples were resuspended in 400 μL of ice-cold PBS + BSA (0.1%) and kept in the dark until they were read in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA). The cytometer was connected to a computer with the software CellQuest (Becton Dickinson Immunocytometry Systems). The cells labeled with CD14 and CH138 were analyzed by fluorescence light (**FL**) 4, the bacteria stained with PI by FL3 and the ROS production by FL1, 100,000 events were acquired from each tube (total events), and the recorded data were analyzed using the software Flow Jo version 7.6.1 for Windows (Tree Star Inc., Ashland, OR).

Statistical analysis was performed using the statistical software GraphPad InStat version 3.01 (GraphPad Software Inc., San Diego, CA). The normality of the data distributions was assessed using the Anderson-Darling test. The tests to assess the differences between the data means were performed using ANOVA for the data with a normal distribution or the Mann-Whitney U test for the data without a normal distribution. The differences were considered statistically significant when their probability was <0.05.

RESULTS AND DISCUSSION

The motivations for understanding the defense mechanisms of calves are numerous and usually centered on