Neutrophil and Monocyte Function in Neonatal Dairy Calves Fed Fresh or Frozen Colostrum

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KEY WORDS: colostrum, monocyte, neutrophil, phagocytosis

ABSTRACT

A study was conducted to compare neutrophil and monocyte activation in neonatal dairy calves fed fresh or frozen colostrum. Fifteen male Holstein calves were fed either refrigerator-stored, non-maternal colostrum or frozen, non-maternal colostrum. Blood samples were collected at days 2 and 21 of life to determine phagocytic cell function using flow cytometry. Serum immunoglobulin concentration was also measured at day 2 of life. It was found that calves fed frozen colostrum had a higher percentage of neutrophils activated at 2 days of age when compared to calves fed non-frozen colostrum. Neutrophil phagocytosis of bacteria, measured as an increase in fluorescence of neutrophils, percent monocytes activated and monocyte phagocytosis of bacteria were not different between the two colostrum groups. At 21 days of age, no statistical significance was found in any of the cell activation indices measured between calves fed fresh or frozen colostrum. There was no association found between immunoglobulin levels and type of colostrum fed. These results suggest that the role of fresh colostral leukocytes, as a key determinant for optimum immune function in the neonate, may not be as large of a factor as once thought, and that products of cellular destruction such as cytokines and transfer factor may be important in early cellular immune function.

INTRODUCTION

The ingestion and absorption of colostrum is essential for establishing passive immunity in neonatal calves.[23] The epitheliochorial (syndesmochorial) placenta of the cow forms a syncytium between maternal endometrium and fetal trophectoderm separating the maternal and fetal blood supplies.[23] This placentation does not allow transfer of immunoglobulins in utero leaving calves virtually agammaglobulinemic at birth.[3] The failure of passive transfer of maternal Ig to the calf via colostrum has been widely investigated. Several studies report the various risk factors associated with failure of passive transfer[22,1] and its effect upon growth, disease incidence and mortality,[18,6] as well as the importance of assuring colostrum feeding to the calf in the first hours of life.[20,15]

The primary immunoglobulin found in

bovine colostrum is IgG1,[23] with smaller amounts of IgM and IgA.[19] IgG1, a gamma 1 heavy chain, is the most predominant subtype of IgG found in colostrum and serves as a source of antibodies to bind to a variety of pathogens. Colostrum contains high amounts of vitamins, trace elements and developmentally important hormones.[17,19] Additionally, colostrum also contains immunological components including cytokines, and significant numbers of maternal leukocytes.[17] The leukocyte component is primarily macrophages (40-50%), lymphocytes (22-25%) and neutrophils (25-37%).[17] Viable macrophages and thymus derived and bone marrow derived lymphocytes are present in colostrum and milk and are capable of responding to antigenic and mitogenic stimulation.[3] Bovine colostrum also contains interleukin (IL)1 beta, IL-6, tumor necrosis factor (TNF) alpha, and interferon (IFN) gamma, which have been suggested to promote the development of the immune system in young animals.[21]

Colostrum can be preserved for future use by brief refrigeration, freezing or storage at ambient temperatures using fermentation or chemical treatment. Cold storage facilities are common on dairy farms and use of frozen stored colostrum has increased.[8] Freezing results in virtually no loss of nutrients during storage but requires extra handling, and daily thawing. A comparison of methods of colostrum storage by Carlson and Muller showed that the composition of frozen colostrum was constant compared to a breakdown of nutrients in fermented and chemically treated colostrum.[2]

The ingestion of colostrum containing maternal leukocytes immediately after birth has been shown to stimulate the development of the neonatal immune system.[17] In a study by LaMotte and Eberhart, it was shown that there is an increase in neutrophil numbers between 6 and 12 hours after birth in colostrum fed but not colostrum deprived calves as a result of colostrum ingestion.[14] This study also concluded that phagocytosis was more efficient in colostrum fed versus colostrum deprived calves. Cell containing colostrum and cell-free colostrum have also been compared for the ability to protect calves from enteropathic E. coli. Calves receiving colostral cells excreted fewer bacteria than calves receiving cell-free colostrum. Calves receiving colostral cells had better responses to some mitogens and foreign antigens but the mechanisms were unclear.[21] The immunoglobulin content of colostrum has been shown to remain unaffected by the freezing process, however the influence of freezing on the leukocyte component and its effect on further leukocyte activation has not been demonstrated. The hypothesis of the present study is that calves fed non-frozen colostrum will have a stronger early immune response to bacterial stimuli, measured as higher activation of neutrophils and monocytes and higher proportion of these cells phagocytizing bacteria compared to those fed frozen colostrum. The goal of this study was to explore the association between colostrum source (fresh or frozen) and neutrophil and monocyte activation during the neonatal period.

MATERIALS AND METHODS

Animals

Fifteen male Holstein calves were selected to conduct this study. To be included in this study, bull calves had to be born via normal parturition (calving difficulty of 1 or 2 on a 1 to 5 scale; that is, little or no assistance was provided at birth). Calves that were excluded in this study included weak bull calves at time of first bleeding, calf size 1 and 5 (on a scale from 1 to 5; 1 being calves that are markedly smaller than normal and 5 being calves that are much larger than normal size); premature calves (calves born > 10 days before expected birthdate), calves resulting from induced parturition or calves with treatments for diarrhea or dehydration by day 2 of life. To determine if a calf was prematurely born, the date of birth was compared with the expected date of parturition of the dam. All breeding and pregnancy diagnosis data were extracted from on-farm computerized dairy management software

PCDART Software (Dairy Record Management Systems, Raleigh, NC). Calving difficulty scores and calf size were recorded by trained farm personnel shortly after birth.

Animal management

Calves were managed according to the farm's written standard operating procedures (SOP). Briefly, liveborn calves were fed 1.9 L (2 quarts) of high quality, refrigerator-stored colostrum and the umbilical stump was disinfected with 7% iodine solution within 1 hr of birth. Calves were then placed individually into a clean 1m x 1.5m covered hutch for the first 21 days of life. Calves received another 1.9 L of good quality colostrum at their second feeding (within 6 hrs of birth). The colostrum that the calf received was not from its dam. The majority of colostrum fed to calves was from cows that calved within 96 hrs of the calf's birth, and was stored at 4 ºC until feeding. When the demand of colostrum was higher than the storage of it, frozen colostrum was thawed at 37 ºC to feed the calves. Calves were fed 3 L of high quality (20% crude protein, 20% fat) milk replacer twice daily through 21 days of age. From day 3 of life, calves were offered fresh, high quality starter grain ad libitum. For the purposes of this study, calves that received one feeding of fresh, stored colostrum between the two feedings would be placed in the fresh calf group. Assignment of calves to the fresh and frozen groups was based on availability of colostrum at the time of the calves' birth.

Sampling Protocol and Processing Methods

At 2 and 21 (±1) days of age, blood samples were taken to determine phagocytic cell function (using flow cytometry). All blood samples were collected in the afternoon, before the calves received their second daily feeding of milk. Calves were bled via jugular venipuncture using one 10 cc blood collection tube without anticoagulant and another 10 cc blood collection tube with lithium heparin (BD Vacutainer®). Blood was collected and handled with care to avoid hemolysis.

Blood processing for chemical analysis

Blood samples for IgG1 determination were collected in tubes without anticoagulant, centrifuged at 1800 rpm for 15 minutes within 2 hours of collection and stored at -20 ºC until laboratory processing of the sample. Serum total protein concentration (measured in g/dL) using a refractometer, and IgG (mg/dL) using a single radial immunodiffusion kit (VMRD Inc., Pullman, WA) were measured on day 2 samples only.

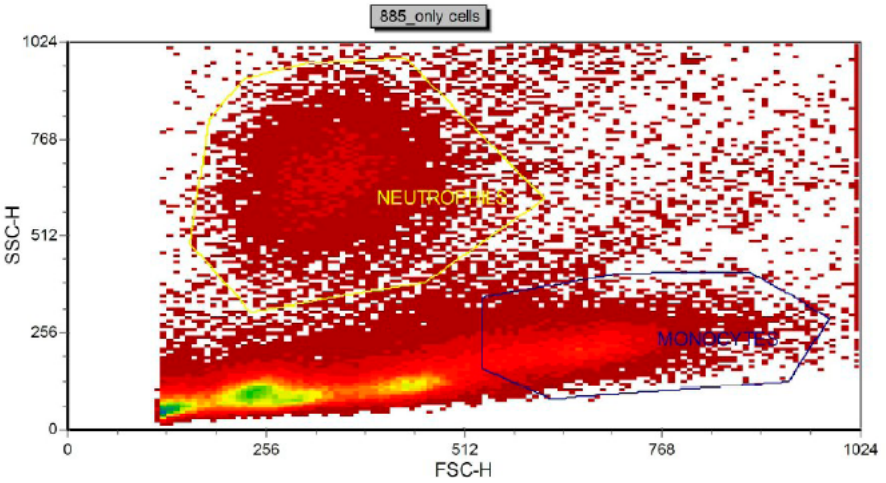
Blood processing for flow cytometry

Blood collected in heparinized tubes was gently agitated and left at room temperature in a horizontal position during transportation from the farm to the laboratory. Blood samples were processed within 2 hours of collection. Activation of phagocytic cells was measured using pHrodo™E.coli BioParticles ® Conjugate for phagocytosis (Molecular Probes ™, Invitrogen ™). A sample of the heparinized blood (100 µL) was incubated with pHrodo™E.coli BioParticles ® Conjugate (20 µL of reconstituted product) at 38 ºC in continuous agitation for 2 h. A control sample for each animal was used following the same process as described above but without using pHrodo™E.coli BioParticles ® Conjugate. After incubation, phagocytosis was stopped by placing the blood samples on crushed ice. To eliminate the background that red blood cells (RBCs) produce in the flow cytometry, RBCs were lysed using a commercial lysing solution (BD Lysing Buffer ™). The process consisted of adding 2 ml of the lysing solution to the samples, vortexing and waiting for 5 minutes to produce the lysis of RBCs. Samples were then washed twice by adding 2 ml of DPBS to the tubes and centrifuging for 5 minutes at 2000 rpm to eliminate the lysing buffer. Supernatant was removed by inverting the tubes. Tubes were then placed briefly on crushed ice to be taken to the laboratory to perform the flow cytometry.

Flow Cytometry

Neutrophils and monocytes were discriminated and quantified by combined measures of forward scatter (FS) which is related to

Figure 1 *- Flow cytogram of SS (side scatter) against FS (forward scatter) of blood leukocytes. Monocytes and neutrophils populations are gated based on their size (FS) and granularity (SS).*



the size of the cells, and side scatter (SS) that is related to the granularity of the cells (Figure 1). Neutrophils and monocytes were gated to FS against fluorescence cytograms, and analyzed for target fluorescence. The fluorescence emitted by the pHrod™ dye-labeled E. coli bacteria has its maxima at pH = 4 and decreases as pH increases. In the flow cytometer, the fluorescence emitted by the phagocytosing cells, when they had ingested the bacteria, was collected with the FL2 channel (fluorescence emitted at 600 nm). Control blood samples were used as baseline. The proportion of phagocytosing cells was defined as the percentage of gated cells with target fluorescence which were located in region 2 of the cytogram. In control samples this region was set with a percentage of 0.30%±0.03 to obtain the same baseline values between animal samples (Figure 2). To calculate the response of neutrophils and monocytes to the bacteria added in the sample, the initial percentage of phagocytosing cells was subtracted from the percentage of phagocytosing cells in the samples exposed to bacteria. The same procedure was performed to calculate the mean of fluorescence emitted by phagocytic neutrophils and monocytes.

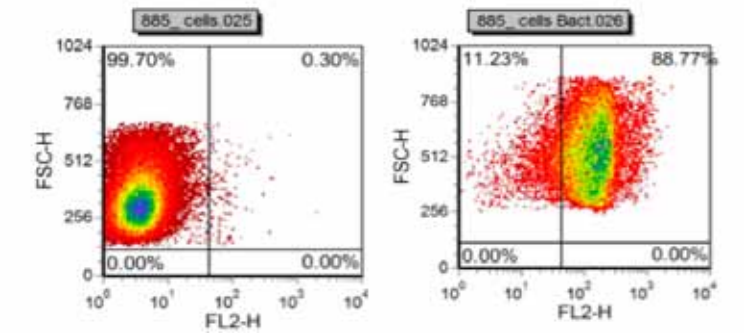
Statistical Analyses

All statistical analyses were performed using SAS ver 9.2 (SAS Institute). Because results were not normally distributed, the NPAR1WAY Procedure was used. Values were calculated and compared using the Wilcoxon Two-Sample Test, and Kruskal Wallis Test. Statistical significance was stated at a p-value of less than 0.05 and a trend was defined as having p-value between 0.05 and 0.10. In the event that there is an association between serum immunoglobulin concentration and neutrophil and monocyte function, two separate models were developed; one with all calves enrolled in the study and one in which the calves with failure of passive transfer of immunity, as defined by IgG <1000 mg/dL, were not included.

RESULTS

Of the fifteen calves enrolled in the study, results from 2 calves were removed because the day 2 sample used to determine serum IgG concentration was lost (one from each colostrum group). Results of the models with and without calves with failure of passive transfer of immunity were similar (data not shown), therefore data from the remaining thirteen calves were used in all analyses presented. 5 calves were in the frozen

Figure 2 - *Forward scatter versus fluorescence cytogram of gated neutrophils without (left) and with (right) bacteria. Most of the neutrophils without bacteria are in region 1, where the emitted fluorescence is low. With the addition of bacteria there has been a shift of the neutrophil population towards region 2, where the emitted fluorescence by neutrophils is greater than in region 1.*



colostrum group, and 8 calves were in the fresh colostrum group.

Data for blood samples collected at 2 days of age are summarized in Table 1. Calves fed frozen colostrum tended to have a higher percentage of neutrophils activated after stimulation (p= 0.079). Mean percent neutrophils for calves fed fresh colostrum was 88.97% while calves fed frozen colostrum had a mean average of 94.75 % neutrophil activation. Fluorescence of neutrophils, percent monocytes activated and fluorescence of monocytes was not different between the two colostrum feeding groups. At 21 days of age, no statistical significance was found in cell activation between calves fed fresh or frozen colostrum (data not shown).

DISCUSSION

In this study, calves were placed into treatment groups (fed fresh or frozen colostrum) according to the availability of colostrum at the time of the calf's birth. Therefore the calves were not randomly assigned to treatment. All calves also received high quality colostrum leading to a possible bias in that calves from dams that produce poor quality colostrum may be immunocompromised or some other way 'different immunologically' when compared to calves born from cows with high quality colostrum. However this information has not been previously reported. Another possible limitation is that the calves did not receive maternal colostrum so no association could be made in this study comparing the benefit of maternal co-

Table 1. *Measures of humoral and cellular immune function at 2 and 21 days of age in dairy calves fed fresh or frozen colostrum. Values are LSM increase in stimulation or fluorescence of immune cells after exposure to E coli bacteria for 8 and 5 calves in the fresh and frozen colostrum treatments, respectively.*

		Colostrum Source		
	Immune variable	**Fresh**	**Frozen**	**P value**
Day 2 Sample	Serum IgG (mg/dL)	1526	1650	0.76
Day 2 Sample	Increased % neutrophils stimulated	88.97	94.75	0.08*
	Increased neutrophil fluorescence	215.20	253.14	0.38
	Increased % monocytes stimulated	83.99	84.84	0.66
	Increased monocyte fluorescence	333.97	300.16	0.83
Day 21 Sample	Increased % neutrophils stimulated	81.76	72.89	0.55
	Increased neutrophil fluorescence	104.10	168.11	0.46
	Increased % monocytes stimulated	61.31	55.94	0.95
	Increased monocyte fluorescence	220.57	278.90	0.16

lostrum versus stored or pooled colostrum. In another study performed by our lab, no morbidity, mortality or growth differences were observed in calves fed maternal versus non-maternal colostrum.[7]

Phagocytic leukocytes, neutrophils and monocytes, were present and functional at both 2 and 21 days of age in the calf. Neutrophils and monocytes were activated when blood was incubated with bacteria for 2 h. This activation produced an increase in the proportion of phagocytizing monocytes and neutrophils and an increase in respiratory burst after bacterial ingestion, measured as mean fluorescence emitted. In this experiment there was a greater cellular immune response in terms of neutrophil and monocyte activation as well as fluorescence emitted in calves two days old than in calves at 21 days of age. The trend of lower proportion neutrophils activated at 2 days of age in the 8 calves fed fresh colostrum was mostly explained by the wide variation in response in calves in this colostrum group. One possible explanation for the higher neutrophil activation in calves fed frozen colostrum is the role of cytokines and/or transfer factor and products of cellular destruction of leukocytes. While not well defined it has been shown that transfer factor is released from lymphocytes when frozen becoming available to stimulate the cellular immune response.[9] The calves fed fresh colostrum did activate a sufficient amount of leukocytes and demonstrated cellular response to bacteria. At 21 days of age, the results from calves receiving fresh and frozen colostrum were similar, showing no statistically significant trends or results. The present study had a small sample size of 13 calves. Future studies could include more animals in an attempt to repeat the results as well as include heifer calves to compare cell function between sexes.

A study by Reber et al.[17] demonstrated that ingestion of maternal colostral leukocytes by calves immediately after birth stimulated the development of the neonatal immune system. A second group of calves in the study was fed cell-free colostrum and was shown to not effectively stimulate a mixed leukocyte response until 2 or 3 weeks after birth. In contrast to their study, the present study demonstrated that calves fed fresh colostral leukocytes stimulated an immune response but not to the level of calves fed frozen colostrum suggesting that there is another colostral or cellular component that leads to optimum immune stimulation in the neonate. Similar to the study by Reber et al.[17], the present study also showed that calves at roughly 3 weeks of age had similar cellular activation regardless of the type of colostrum fed suggesting that there was development of the neonatal immune system without dependence on the effects of colostral leukocytes. The colostrum in the present study was non maternal, limiting the ability to demonstrate an association with cell function and maternal colostrum. Future studies could be designed to compare fresh or frozen maternal colostrum to determine if there is an association between live maternal leukocytes and an increased cell function when compared to frozen colostrum containing products of cellular destruction.

In the present study, the bacteria used to stimulate leukocytes was the k-12 strain of E. coli, a non-pathogenic strain against which the cow might not produce antigen specific immunoglobulins. This may explain why no correlation was found between colostrum source and phagocytosis in most of the variables measured. Results of the study by Donovan et al.[5] suggest that specific responsiveness against antigens to which the dam has mounted an immune response can be transferred to a neonatal calf by live maternal cells in colostrum. In this study, a significant in vitro response to BVDV was observed 1 day after ingestion of whole colostrum that contained live maternal cells compared to cell-free colostrum. In contrast, the responses to PPD preparations, antigens to which the cows were naïve, were unaffected by the transfer of live maternal cells.[5]

The importance of colostrum and

adequate antibody transfer in the development of the immune system of the neonate is well documented. However, the role of fresh colostral leukocytes as a key determinant for optimum immune function in the neonate may not be as large of a factor as once thought. The present study has shown that calves fed frozen colostrum without the benefit of live colostral leukocytes tended to have superior neutrophil activation when compared to calves fed fresh colostral leukocytes. Products of cellular destruction such as cytokines and transfer factor need to be further examined for their role in neonatal immune stimulation.

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