Effects of Milking Frequency on Phagocytosis and Oxidative Burst Activity of Phagocytes from Primiparous and Multiparous Dairy Cows During Early Lactation

S. Llamas Moya,* M. Alonso Gómez,† L. A. Boyle,* J. F. Mee,* B. O'Brien,* and S. Arkins†¹

*Teagasc, Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland

†Department of Life Sciences, University of Limerick, Limerick, Ireland

ABSTRACT

The objective of this study was to investigate the effect of milking frequency on the phagocytosis and respiratory burst activity of polymorphonuclear neutrophils (PMN) and monocytes of primiparous and multiparous cows under 2 nutritional management regimens during early lactation. At calving, 12 primiparous and 12 multiparous cows were randomly assigned to 1 of 4 treatments, in which animals were milked once (OAD) or twice a day at a high or low nutritional level. Blood samples were taken 1 to 7 d before calving (prepartum) and 1 to 7, 14 to 21, and 42 to 49 d postpartum. Phagocytic and oxidative burst activity of PMN and monocytes were determined in whole blood and analyzed separately by flow cytometry. Once-a-day milking reduced significantly the percentage of phagocytic PMN and tended to decrease the number of bacteria ingested by these cells. The percentage of oxidative burst positive cells and overall respiratory burst activity of monocytes also tended to be reduced by OAD milking. The reduction of oxidative burst activity of monocytes was more pronounced 1 to 7 d postpartum compared with the prepartum sample and other postpartum samples. Oxidative burst activity of PMN and monocytes of multiparous cows was impaired compared with primiparous cows. The percentage of oxidative burst positive monocytes from multiparous cows was reduced prepartum and also 1 to 7 d postpartum. Once-a-day milking reduced the mean respiratory burst activity of PMN from primiparous cows to levels similar to that of multiparous cows. Therefore, an OAD milking regimen reduces phagocytic activity of PMN and monocytes and would be detrimental for the immune system in high-yielding dairy cows during early lactation.

Key words: milking frequency, phagocytosis, oxidative burst, dairy cow

INTRODUCTION

Modern dairy cows are genetically selected for high milk production (Søndergaard et al., 2002; Coffey et al., 2004). Higher milk outputs, however, increase the metabolic load of dairy cows, which may affect negatively the health and welfare of these animals (Collard et al., 2000; Ingvarsen et al., 2003). Furthermore, high-yielding cows may have impaired reproductive performance and survival (Lucy, 2001) and experience an increased incidence of infectious diseases (Søndergaard et al., 2002; Ingvarsen et al., 2003; Meglia et al., 2005).

Milking frequency is an important factor influencing milk yield in high-producing dairy cows (Rémond et al., 2004; Patton et al., 2006). The metabolic stress associated with high-yielding animals in early lactation may be reduced by adopting once-a-day (OAD) milking, taking into consideration that this milking strategy is associated with decreased milk production (Davis et al., 1999; Keane, 2004; Patton et al., 2006). Research has shown that dairy cows on OAD milking have a better reproductive performance (O'Brien et al., 2005) and improved welfare, particularly in terms of hoof health (Boyle et al., 2005) and behavior (O'Driscoll et al., 2006). Once-a-day milking has given inconsistent effects on udder health, as indicated by SCC which seems to depend on the initial sanitary condition of the udder (Lacy-Hulbert et al., 1995; Rémond et al., 2004). An OAD milking regimen, however, increases the incidence of milk leakages and udder firmness scores, because of the longer time interval between milking sessions (Gleeson et al., 2007). Once-a-day milking of dairy cows also has relevant benefits for grass-based milk production systems, such as those existing in Ireland (Davis et al., 1999; Rémond and Pomiès, 2005). This milking strategy can improve the productivity of large or fragmented farms, as well as facilitate overcoming labor shortages, and enhance the producer's lifestyle because of fewer work constraints (Dalley and Bateup, 2004; Rémond and Pomiès, 2005). In addition, the reduced milk production and lactation length of OAD cows can be compensated for by adopting adequate nutritional

Received May 23, 2007.

Accepted November 3, 2007.

¹Corresponding author: sean.arkins@ul.ie

management strategies (Andersen et al., 2003). Increasing the energy content of the diet fed during lactation results in greater milk yield (Andersen et al., 2003; Patton et al., 2006). Furthermore, dietary energy density has been shown to affect the immunity of healthy and diseased cows (Stabel et al., 2003; Røntved et al., 2005).

The peripartum period is the most challenging time for dairy cows. Metabolic disturbances linked to negative energy balance are concurrent with the alteration of hormonal patterns around parturition. Hence, the peripartum period is generally associated with immunosuppression (Van Kampen and Mallard, 1997; Mallard et al., 1998). Polymorphonuclear neutrophils (PMN) and monocytes are characterized by their phagocytic activity, which is pivotal for defense against bacterial infections. Polymorphonuclear neutrophils and monocytes are responsible for the secretion of cytokines, thereby amplifying the inflammatory response (Baumann and Gauldie, 1994). In dairy cattle, pregnancy and lactation are associated with impairment of PMN and lymphocyte function (Kehrli et al., 1989a,b; Saad et al., 1989). Furthermore, it has been shown that periparturient PMN function is affected to a greater extent in cows in the fourth or greater lactation than in younger cows (Gilbert et al., 1993; Mehrzad et al., 2002). Polymorphonuclear neutrophils and monocytes are actively involved in the ingestion and intracellular killing of invading microorganisms mainly via intracellular reactive oxygen species (ROS) production (Mehrzad et al., 2004, 2005). Hence, the measurement of phagocytic and respiratory burst activity can give valuable information on functional activity of these immune cells (Menge et al., 1998; Kampen et al., 2004). The objective of this study was to investigate the effect of OAD milking on the functionality of PMN and monocytes, on the basis that this milking strategy would not negatively affect the phagocytosis and respiratory burst activity of primiparous and multiparous cows under 2 nutritional management regimens during early lactation.

MATERIALS AND METHODS

Animals and Management

A total of 24 spring-calving (mean calving date: March 11) Holstein-Friesian primiparous ($n = 12$) and multiparous cows ($n = 12$; parity group range 3 to 8; parity mean average 4.5) from the Moorepark Dairy Production Research Centre herd were used. Cows were assigned randomly at calving to 1 of 4 treatments in a 2×2 factorial design for the full lactation, consisting of OAD or twice-a-day (TAD) milking at a high (H) or low (L) nutritional level. Cows were housed in cubicles

Table 1. Estimated chemical composition of concentrate supplement

Chemical composition	
DM (g/kg)	870
CP (g/kg of DM)	214
Crude fiber (g/kg of DM)	89
Fat (g/kg of DM)	43
Ash (g/kg of DM)	68
Starch (g/kg of DM)	124
PDIE ¹ (g/kg of DM)	138
PDIN ² (g/kg of DM)	152
Net energy (UFL/kg) ³	1.16

¹PDIE = protein digested in the intestine when rumen fermentable energy is limiting.

²PDIN = protein digested in the intestine when rumen fermentable nitrogen is limiting.

³Estimated based on net energy value for ingredients; UFL = unité fourragère lait.

until March 22. During this time, H cows received silage ad libitum and 7 kg/cow per d of concentrates, whereas L cows received grass silage ad libitum and 4 kg/cow per d of concentrates (Table 1). Grazing management was based on a rotational grazing system. The first grazing cycle took place between March 22 and April 17. During this period, H cows received 4 kg/d of concentrate and L cows received 1 kg/d of concentrate. During the main grazing period, the difference in feeding regimens was dictated by postgrazing height. The H cows grazed to a height of 8.3 cm, whereas the L cows grazed to a height of 6.7 cm. Stocking density was based on herbage allowance (kg of DM/cow) calculated by taking account of the pregrazing herbage yield (kg of DM/ha), size of paddock (ha), and number of cows. The OAD cows were milked at 0730 h, and the TAD cows were milked at 0730 and 1530 h.

All animal procedures were carried out under licence in accordance with the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations 2002.

Blood Sampling

Blood was collected from all cows by coccygeal venipuncture into lithium-heparinized tubes (Vacutainer, Unitech Ltd., Dublin, Ireland). Blood samples were taken once in each period from all experimental animals during the following intervals: 1 to 7 d before calving (prepartum sample), and 1 to 7, 14 to 21, and 42 to 49 d postpartum. Blood samples were stored at ambient temperature for less than 5 h before the flow cytometry assay was performed.

Whole-Blood Flow Cytometry Assays

Flow cytometric analysis was performed on a BD LSR system (Becton Dickinson Biosciences, San José, CA)

equipped with a 488-nm argon laser and standard filter configuration (525 nm bandpass, 630 nm longpass). Data were analyzed with CellQuest software provided by the vendor. During data acquisition of phagocytic and oxidative burst assays, a live gate was set in the 630 nm (dark red) fluorescence histogram for those events having at least the same DNA content as diploid cells to exclude cell debris and bacterial aggregates. For each sample, the optical features of 10,000 phagocytes were acquired. The PMN and monocytes were analyzed separately for their 525 nm (green) fluorescence intensity. Forward and side scatter measurements were obtained from control samples of PMN and monocytes that were used for the oxidative burst measurements. Forward and side scatter were used for preliminary identification of cells. Low-angle forward scatter is roughly proportional to the diameter of the cell, whereas orthogonal, 90°, or side scatter is proportional to membrane irregularity. The control samples corresponded to PMN and monocytes that had been incubated without stimulus. Percentage fluorescence-positive events and mean fluorescence intensity (MFI), the latter being correlated with the mean number of bacteria ingested by single phagocytes or the mean oxidative burst activity of single phagocytes, were recorded (Menge et al., 1998). An index of overall phagocytic or oxidative burst activity was also calculated by multiplying the percentage of responding cells by the corresponding MFI: [index = (% positive cells) $\times$ (MFI) / 100] (Clapperton et al., 2005). Electronic gates were set according to the negative control included in each test defining less than 2% of the cells as positive.

Effect of Milking Frequency on Phagocytosis

In vitro phagocytic activity was determined using the Phagotest kit (Orpegen Pharma, Heidelberg, Germany). After addition of fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* to whole blood, bacteria ingested by phagocytes and generating a green fluorescence signal were quantified. The Phagotest kit contained bacterial suspensions and several buffers such as the quenching, washing, lysing, and DNA-staining solutions mentioned below. The test was performed according to the manufacturer's instructions with minor modifications. Briefly, 20 μ L of stabilized and opsonized FITC-labeled *E. coli* suspension (1×10^9 bacteria/mL) was added to 1 of 2 whole-blood samples (100 μ L) in test tubes (Falcon, Becton Dickinson). The other sample served as a control and remained unstimulated. After vortexing all samples, the test tubes were incubated at 39°C in a water bath for 10 min (Menge et al., 1998); a control tube remained on ice. Phagocytosis was stopped by putting the tubes back into ice. To eliminate

the fluorescence of nonphagocytosed bacteria, 100 μ L of quenching solution was added. The cells were washed twice with 3 mL of washing solution and pelleted by centrifugation ($250 \times g$ for 5 min, 4°C). Cells were resuspended and incubated in 2 mL of lysing solution for lysis of erythrocytes and fixation of phagocytes for 20 min at room temperature. After centrifugation, cells were recovered by resuspending in 3 mL of washing solution, followed by a final centrifugation step. The cells were resuspended in 100 μ L of DNA-staining solution, and analyzed by flow cytometry as described subsequently.

Effect of Milking Frequency on Respiratory Burst Activity

The oxidative burst activity was determined using Bursttest (Phagoburst) kit (Orpegen Pharma). In this assay, formation of ROS by the membrane-bound NADPH oxidase was measured intracellularly by the oxidation of the fluorogenic dihydrorhodamine 123 to the membrane-adherent fluorescent rhodamine 123. The Bursttest assay was performed according to the instructions of the manufacturer with some modifications. Several stimuli were used separately to induce oxidative burst: stabilized and opsonized but nonlabeled *E. coli* suspension (1×10^9 bacteria/mL), the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as the high control, and the chemotactic peptide *N*-formyl-MetLuePhe (fMLP) as the low physiological stimulus. Twenty microliters of each stimulus was added to 100- μ L whole-blood samples. A sample without stimulus (20 μ L of washing solution) served as negative background control. All tubes were vortexed and placed in a 39°C water bath for 10 min (Menge et al., 1998). Phagocytosis was stopped by putting the tubes back into ice. The samples were supplemented with 20 μ L of substrate solution, vortexed, and incubated for 10 min at 39°C. Cells were lysed, washed, and fixed as described for the phagocytosis assay.

Statistical Analysis

Data were analyzed statistically using PROC MIXED of SAS (V9.1, 2002; SAS Institute Inc., Cary, NC). The model included fixed effects of parity group (primiparous or multiparous), milking frequency (TAD or OAD), nutritional levels (H or L), and sampling time (prepartum, 1 to 7 d, 14 to 21 d, or 42 to 49 d postpartum), and all possible 2-way interactions. The repeated statement was used to take into account repeated measures for each individual animal. When significant effects were found, Tukey's test was used to establish pairwise differences. Statistical differences were considered sig-

Table 2. Effect of lactation number (primiparous or multiparous) and milking frequency (once or twice a day) on percentage of phagocytic polymorphonuclear neutrophils (PMN) and monocytes, mean fluorescence intensity (MFI), and phagocytic index following stimulation with fluorescein isothiocyanate-labeled *Escherichia coli*¹

	Primiparous	Multiparous	SE	P-value	Once a day	Twice a day	SE	P-value
PMN								
% phagocytic cells	43.0	43.7	2.2	0.82	39.9	46.9	2.1	0.03
MFI	746.1	641.6	46.8	0.10	694.2	693.5	44.2	0.99
Phagocytic index ²	302.7	287.5	20.7	0.60	257.5	332.8	19.9	0.01
Monocytes								
% phagocytic cells	34.3	33.8	1.9	0.87	32.7	35.4	1.8	0.27
MFI	268.4	298.3	25.5	0.40	293.1	273.7	24.3	0.57
Phagocytic index	73.0	85.2	19.1	0.64	89.3	68.9	18.1	0.43

¹Twelve primiparous and 12 multiparous cows were equally distributed to the treatments. Data are presented as mean $\pm$ SE.

²Phagocytic index = [(% phagocytic cells) $\times$ (MFI)]/100.

nificant at $P < 0.05$. Tendencies toward significance ($0.05 < P < 0.1$) are also presented. Data are presented as least squares means $\pm$ standard errors.

RESULTS

Effect of Milking Frequency on Phagocytosis

There was no effect of parity group on the phagocytic activity of PMN or monocytes. The TAD animals had a greater percentage of phagocytosing PMN than did OAD cows ($P < 0.05$; Table 2). This effect of milking frequency was also reflected in the phagocytic index of PMN ($P < 0.05$). No effect of parity group or milking frequency was observed on the phagocytic activity of monocytes. Nutritional management did not affect the phagocytic activity of PMN or monocytes. The phagocytic activity of both PMN and monocytes was affected by sampling time ($P < 0.001$; Table 3). The percentage of phagocytosing PMN and monocytes was the lowest at 1 wk prepartum and the greatest during 42 to 49 d postpartum.

Effect of Milking Frequency on Respiratory Burst Activity

Both inactivated bacteria and PMA induced an oxidative burst in PMN and monocytes ($P < 0.001$). Results showed that the percentage of PMN and monocytes presenting oxidative burst activity following stimulation with fMLP did not differ from that of the control samples (washing buffer). Therefore, samples corresponding to activation by fMLP are not included in this section.

Flow cytometry analysis showed that parity group had an effect on the percentage of PMN ($P = 0.09$; Table 4) and monocytes ($P < 0.01$; Table 5) presenting oxidative burst activity after stimulation with *E. coli*. The PMN and monocytes from primiparous cows had greater oxidative burst activity than did those of multiparous cows. The PMN of primiparous cows had greater MFI than those of multiparous cows after *E. coli* stimulation ($P < 0.05$; Table 4). The opposite effect of parity group, however, was observed on the MFI of monocytes when stimulated with bacteria but not with

Table 3. Effect of sampling time (1 to 7 d prepartum, 1 to 7, 14 to 21 or 42 to 49 d postpartum) on percentage of phagocytic polymorphonuclear neutrophils (PMN) and monocytes, mean fluorescence intensity (MFI) and phagocytic index following stimulation with fluorescein isothiocyanate-labeled *Escherichia coli*¹

	Prepartum	1 to 7 d	14 to 21 d	42 to 49 d	SE	P-value
PMN						
% phagocytic cells	34.1 ^a	42.1 ^{ab}	42.9 ^b	54.4 ^c	2.9	0.001
MFI	615.7	704.8	815.5	639.6	75.0	0.14
Phagocytic index ²	212.1 ^a	309.1 ^b	309.3 ^b	350.1 ^{bc}	30.3	0.007
Monocytes						
% phagocytic cells	27.5 ^a	31.8 ^a	31.1 ^a	45.9 ^b	2.7	0.001
MFI	311.4	257.4	263.8	300.8	38.9	0.62
Phagocytic index	93.6	49.7	47.3	125.8	30.2	0.10

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Twelve primiparous and 12 multiparous cows were equally distributed to the treatments. Data are presented as mean $\pm$ SE.

²Phagocytic index = [(% phagocytic cells) $\times$ (MFI)]/100.

Table 4. Effect of lactation number (primiparous or multiparous) and milking frequency (once or twice a day) on percentage of oxidative burst positive polymorphonuclear neutrophils (PMN), mean fluorescence intensity (MFI), and oxidative burst index following stimulation with nonlabeled *Escherichia coli* or phorbol 12-myristate 13-acetate (PMA)¹

	Primiparous	Multiparous	SE	P-value	Once a day	Twice a day	SE	P-value
PMN								
% oxidative burst positive cells								
<i>E. coli</i>	16.6	11.9	2.0	0.08	14.6	13.9	1.9	0.78
PMA	16.7	16.4	4.3	0.96	17.6	15.5	4.1	0.72
MFI								
<i>E. coli</i>	321.2	261.2	18.1	0.02	275.4	307.0	17.3	0.20
PMA	592.4	357.2	36.5	0.001	428.3	521.3	35.0	0.06
Oxidative burst index ²								
<i>E. coli</i>	67.4	31.7	11.2	0.03	46.6	52.6	10.7	0.69
PMA	111.4	103.7	33.4	0.86	115.0	100.2	32.0	0.74

¹Twelve primiparous and 12 multiparous cows were equally distributed to the treatments. Data are presented as mean $\pm$ SE.

²Oxidative burst index = [(% oxidative burst positive cells) $\times$ (MFI)]/100.

PMA ($P = 0.05$; Table 5). Mean oxidative burst activity of both PMN and monocytes followed the same trend, with cells of primiparous cows having a greater oxidative burst index than multiparous cows after stimulation with bacteria ($P < 0.05$). Following stimulation with PMA, the PMN and monocytes of primiparous cows had greater MFI than those of multiparous cows ($P < 0.05$).

Milking frequency tended to have an effect on MFI of PMN stimulated with PMA, with TAD cows having greater MFI than OAD animals ($P = 0.06$; Table 4). Furthermore, a parity group by milking frequency interaction showed that PMN of TAD primiparous cows had the greatest MFI in response to PMA stimulation ($P < 0.01$; Figure 1). Additionally, the percentage of oxidative burst-positive monocytes after bacterial stimulation tended to be greater in TAD cows compared with OAD animals ($P = 0.05$; Table 5). A similar trend was also detected on the mean oxidative burst activity of monocytes following *E. coli* stimulation ($P = 0.09$).

Flow cytometry analysis of PMA-stimulated samples showed a milking frequency by nutritional level interaction in the MFI levels of monocytes ($P < 0.01$; Figure

2). Monocytes from TAD cows on the low nutritional level had greater MFI than those from TAD animals on the high nutritional level after PMA stimulation ($P < 0.05$).

A parity group by sample time interaction was found in the percentage of oxidative burst positive monocytes following stimulation with *E. coli* ($P < 0.05$; Figure 3A). This interaction indicated that the percentage of monocytes presenting oxidative burst was smaller in multiparous cows within 1 wk pre- and postpartum ($P < 0.05$). The interaction between milking frequency and sampling time in the percentage of activated monocytes tended to be significant ($P = 0.09$; Figure 3B). This indicated that a larger percentage of monocytes from TAD cows presented oxidative burst activity 1 to 7 d postpartum compared with monocytes from OAD cows ($P < 0.05$).

PMN and Monocyte Characteristics

The PMN from multiparous cows differed in size (i.e., forward scatter; primiparous vs. multiparous: 470.2 vs.

Table 5. Effect of lactation number (primiparous or multiparous) and milking frequency (once or twice a day) on percentage of oxidative burst positive monocytes, mean fluorescence intensity (MFI) and oxidative burst index following stimulation with nonlabeled *Escherichia coli* or phorbol 12-myristate 13-acetate (PMA)

	Primiparous	Multiparous	SE	P-value	Once a day	Twice a day	SE	P-value
Monocytes								
% oxidative burst positive cells								
<i>E. coli</i>	7.9	1.7	1.3	0.001	3.1	6.4	1.2	0.06
PMA	6.0	7.3	2.1	0.64	7.0	6.3	2.0	0.80
MFI								
<i>E. coli</i>	87.8	126.7	14.0	0.05	105.6	108.8	13.6	0.87
PMA	308.0	177.6	36.2	0.02	227.8	257.8	35.9	0.55
Oxidative burst index ²								
<i>E. coli</i>	7.2	1.9	1.3	0.007	3.0	6.1	1.2	0.09
PMA	27.2	30.9	10.2	0.79	25.4	32.7	10.2	0.61

¹Twelve primiparous and 12 multiparous cows were equally distributed to the treatments. Data are presented as mean $\pm$ SE.

²Oxidative burst index = [(% oxidative burst positive cells) $\times$ (MFI)]/100.

Figure 1. Effect of lactation number (primiparous or multiparous) and milking frequency (once or twice a day) on mean fluorescence intensity of oxidative burst positive polymorphonuclear neutrophils following stimulation with phorbol 12-myristate 13-acetate. ^{a,b}Different letters indicate significant differences ($P < 0.05$). Data are presented as mean $\pm$ SEM of 6 cows.

Figure 2. Effect of milking frequency (once or twice a day) and nutritional level (high or low) on mean fluorescence intensity (MFI) of oxidative burst positive monocytes following stimulation with phorbol 12-myristate 13-acetate. ^{a,b}Different letters indicate significant differences ($P < 0.05$). Data are presented as mean $\pm$ SEM of 6 cows.

515.1; $P = 0.04$) and membrane irregularity (i.e., side scatter; primiparous vs. multiparous: 576.0 vs. 616.4; $P = 0.06$) from those of primiparous cows. No parity group effects were found for the physical characteristics of monocytes. Milking frequency and nutritional level did not affect the measurements of forward scatter or side scatter of PMN and monocytes. A sampling time effect showed that the size and membrane irregularity of both PMN and monocytes changed before and after parturition ($P < 0.05$; data not shown).

DISCUSSION

In this study, the effect of milking frequency on the functional capacity of blood phagocytes was examined during early lactation of dairy cows. Results showed that the phagocytosis of PMN was impaired in dairy cows on the OAD treatment. In addition, the mean respiratory burst activity by individual PMN following stimulation with PMA tended to be reduced in OAD cows. Furthermore, the percentage of monocytes producing ROS after bacterial stimulation was significantly reduced in OAD animals, in particular 1 wk after calving. Therefore, reducing the frequency of milking decreased significantly the percentage of PMN and monocytes activated when stimulated with bacteria. It has previously been reported that OAD milking results in neutrophilia for up to 2 wk after parturition (Keane,

2004; Gleeson et al., 2007). Thus, it seems that although the number of trafficking blood PMN may increase in response to OAD milking (Keane, 2004; Gleeson et al., 2007), their functional capabilities in terms of phagocytosis are reduced. Furthermore, neutrophilia has been shown to be indicative of stressful events, such as surgical procedures or transport (Ting et al., 2003; Yagi et al., 2004).

Once-a-day milking can reduce the magnitude and duration of negative energy balance during early lactation (Keane, 2004). Results from the current study, however, show that it causes impairment of the phagocytic activity of PMN and monocytes. According to Keane (2004), results from this study indicate that reducing milking frequency to OAD causes immune stress, despite the likely reduction of metabolic load as a result of the reduced milk production. Reducing the frequency of milking to OAD may constitute an important stressor, because it is likely to increase cow discomfort due to udder enlargement (Osterman and Redbo, 2000; Gleeson et al., 2007). Gleeson et al. (2007) also showed that OAD dairy cows have poorer locomotion scores, which is correlated with increases in udder firmness. Thus, OAD milking may cause stress because of the physical discomfort that results from engorgement of the udder. This stress seems to counterbalance any beneficial effects of a reduction in negative energy balance at the start of the lactation period.

Results from this study also showed differences in the functionality of immune cells of primiparous and

Figure 3. A) Effect of lactation number (primiparous or multiparous) by sampling time (1 to 7 d prepartum, and 1 to 7, 14 to 21 d, or 42 to 49 d postpartum) interaction; and B) milking frequency (once or twice a day) by sampling time interaction on the percentage of oxidative burst positive monocytes following stimulation with nonlabeled *Escherichia coli* (* $P < 0.05$). Data are presented as mean $\pm$ SEM of 12 cows.

multiparous dairy cows, in particular in terms of oxidative burst activity. Several publications have reported impaired immune function in multiparous cows compared with primiparous cows (Gilbert et al., 1993; Mehrzad et al., 2002). In agreement with Mehrzad et al. (2002), a greater percentage of PMN and monocytes from primiparous cows presented oxidative burst activity following stimulation with *E. coli*, compared with

those of multiparous cows. Additionally, the mean oxidative burst activity was also greater in primiparous cows. Results from this study also showed that the size and irregularity of PMN had larger values in multiparous cows. Lessard et al. (2004) found that monocyte functions of multiparous cows, in terms of tumor necrosis factor- α , prostaglandin E_2 , and nitric oxide production, were also impaired compared with those of primiparous cows. This may account for the greater incidence of infectious diseases in multiparous cows, in particular during the peripartum period. For example, van Werven et al. (1997) reported an increased severity of *E. coli*-induced mastitis associated with increasing parity number. Furthermore, results from this study showed that the percentage of activated monocytes from multiparous cows was lesser than those of primiparous cows 1 wk before and 1 wk after calving. This also supports the reported suppression of immune function during this period, which could increase the incidence of peripartum diseases such as mastitis and metritis. Ingvarstsen et al. (2003) reported a significant positive correlation between the occurrence of mastitis and milk yield. Multiparous cows have greater milk production and greater negative energy balance compared with primiparous cows (Mehrzad et al., 2002; Coffey et al., 2004). Thus, it is likely that primiparous cows are subjected to a lower metabolic load during the peripartum period. In addition, because milk production is reduced, it is also possible that udder discomfort is reduced. Nonetheless, our study showed that reducing the frequency of milking to OAD in primiparous cows reduced the mean oxidative burst activity by individual PMN after PMA stimulation to levels similar to that of multiparous cows in both milking frequency regimens. Thus, this indicates that the stress associated with OAD milking precluded the likely enhanced immune status of primiparous dairy cows.

Nutrition management can reduce the risk of metabolic disturbances during the peripartum period (Meglia et al., 2005). Increasing the amount of concentrates fed leads to greater milk yield and therefore may counteract the reduced milk production associated with OAD milking (Andersen et al., 2003; O'Brien et al., 2005; Patton et al., 2006). Results from this study showed that nutritional management had a minimal impact on the functional capabilities of immune cells. This lack of influence of nutritional level agrees with previous reports, in which no effect of feeding intensity during the dry period was found on PMN phagocytosis or oxidative burst activity (Meglia et al., 2005). In addition, Røntved et al. (2005) found no effect of diet energy density on tumor necrosis factor- α responsiveness in early lactation. In the present study, results indicated that the mean respiratory burst activity by individual

mononuclear cells was greater in TAD cows on the low nutritional level compared with animals on the same milking frequency group but on the higher nutrient level. The metabolic load to which these animals may be subjected is likely increased not only because of the greater milk production associated with the increased milking frequency, but also because of the improved nutritional status.

In conclusion, results from this study showed that milking frequency affects the functionality of immune cells. Once-a-day milking impaired phagocytic activity of PMN. Oxidative burst activity of monocytes was also impaired by this milking frequency regimen. This effect was more pronounced in the week following parturition. Results from the current study indicate that udder discomfort, rather than a possible lessening of the metabolic load by OAD milking, is likely responsible for the impaired phagocytic functionality. Thus, OAD milking may have a detrimental impact on the immune status of dairy cows during early lactation that warrants further research.

CONCLUSIONS

The peripartum period is associated with depression of the immune response, which can be further impaired by the metabolic stress suffered by high-yielding dairy cows. The reduction in the frequency of milking to OAD is a valid management alternative to lessen the metabolic load during early lactation. Nonetheless, results from this study showed that OAD milking had a negative impact on the functionality of immune cells. This was indicated by the reduced phagocytosis and decreased production of ROS by PMN and monocytes from OAD-milked cows. Most likely, the stress associated with the physical discomfort caused by the engorgement of the udder from cows on the OAD milking strategy may have altered the functionality of immune cells. This suggests that limiting the milk production of high-yielding animals during early lactation has an important detrimental effect on immunity.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Tommy Condon and the Moorepark Dairy Production Farm staff. Special thanks go to Ultan Cronin for his invaluable assistance during the flow cytometric analysis.

REFERENCES

- Andersen, J. B., N. C. Friggens, K. Sejrsen, M. T. Sørensen, L. Munksgaard, and K. L. Ingvarsen. 2003. The effects of low vs. high concentrate level in the diet on performance in cows milked two or three times daily in early lactation. *Livest. Prod. Sci.* 81:119–128.
- Baumann, H., and J. Gauldie. 1994. The acute phase response. *Immunol. Today* 15:74–80.
- Boyle, L., B. O'Brien, and D. Gleeson. 2005. Effect of milking frequency and nutrition on cow welfare. Page 348 in *Book of Abstracts of the 56th Annual Meeting of the European Association for Animal Production*. Uppsala, Sweden. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Clapperton, M., S. C. Bishop, and E. J. Glass. 2005. Innate immune traits differ between Meishan and Large White pigs. *Vet. Immunol. Immunopathol.* 104:131–144.
- Coffey, M. P., G. Simm, J. D. Oldham, W. G. Hill, and S. Brotherstone. 2004. Genotype and diet effects on energy balance in the first three lactations of dairy cows. *J. Dairy Sci.* 87:4318–4326.
- Collard, B. L., P. J. Boettcher, J. C. M. Dekkers, D. Petitclerc, and L. R. Schaeffer. 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. *J. Dairy Sci.* 83:2683–2690.
- Dalley, D., and N. Bateup. 2004. Once a day: Is it opportunity knocking? Pages 1–6 in *Proc. Dairy 3: The Combined Massey, Dexcel, Dairy Farmer Event*. Rotorua, New Zealand.
- Davis, S. R., V. C. Farr, and K. Stelwagen. 1999. Regulation of yield loss and milk composition during once-daily milking: A review. *Livest. Prod. Sci.* 59:77–94.
- Gilbert, R. O., Y. T. Grohn, P. M. Miller, and D. J. Hoffman. 1993. Effect of parity on periparturient neutrophil function in dairy cows. *Vet. Immunol. Immunopathol.* 36:75–82.
- Gleeson, D., B. O'Brien, L. A. Boyle, and B. Earley. 2007. Effect of milking frequency and nutritional level on aspects of the health and welfare of dairy cows. *Animal* 1:125–132.
- Ingvarsen, K. L., R. J. Dewhurst, and N. C. Friggens. 2003. On the relationship between lactational performance and health: Is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. *Livest. Prod. Sci.* 83:277–308.
- Kampen, A. H., T. Tollersrud, S. Larsen, J. A. Roth, D. E. Frank, and A. Lund. 2004. Repeatability of flow cytometric and classical measurement of phagocytosis and respiratory burst in bovine polymorphonuclear leukocytes. *Vet. Immunol. Immunopathol.* 97:105–114.
- Keane, C. J. 2004. An investigation into the effect of metabolic status on the health and welfare of dairy cows. PhD Thesis. University College Dublin, Ireland.
- Kehrli, M. E., B. J. Nonnecke, and J. A. Roth. 1989a. Alterations in bovine neutrophil function during the periparturient period. *Am. J. Vet. Res.* 50:207–214.
- Kehrli, M. E., B. J. Nonnecke, and J. A. Roth. 1989b. Alteration in bovine lymphocyte function during the periparturient period. *J. Vet. Res.* 50:215–220.
- Lacy-Hulbert, S. J., M. W. Woolford, and A. M. Bryant. 1995. Influence of once daily milking and restricted feeding on milk characteristics in late lactation. *Proc. N.Z. Soc. Anim. Prod.* 55:85–87.
- Lessard, M., N. Gagnon, D. L. Godson, and H. V. Petit. 2004. Influence of parturition and diets enriched in n-3 or n-6 polyunsaturated fatty acids on immune response of dairy cows during the transition period. *J. Dairy Sci.* 87:2197–2210.
- Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? *J. Dairy Sci.* 84:1277–1293.
- Mallard, B. A., J. C. Deffers, M. J. Ireland, K. E. Leslie, S. Sharif, C. Lacey VanKampen, L. Wagter, and B. N. Wilkie. 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. *J. Dairy Sci.* 81:585–595.
- Meglia, G. E., A. Johannisson, S. Agenäs, K. Holtenius, and K. Persson Waller. 2005. Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. *Vet. J.* 169:376–384.
- Mehrzed, J., L. Duchateau, and C. Burvenich. 2004. Viability of milk neutrophils and severity of bovine coliform mastitis. *J. Dairy Sci.* 87:4150–4162.
- Mehrzed, J., L. Duchateau, and C. Burvenich. 2005. High milk neutrophil chemiluminescence limits the severity of bovine coliform mastitis. *Vet. Res.* 36:101–116.

- Mehrzad, J., L. Duchateau, S. Pyörälä, and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and multiparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.* 85:3266–3276.
- Menge, Ch., B. Neufeld, W. Hirt, N. Schmeer, R. Bauerfeind, G. Baljer, and L. H. Wieler. 1998. Compensation of preliminary blood phagocyte immaturity in the newborn calf. *Vet. Immunol. Immunopathol.* 62:309–321.
- O'Brien, B., D. Gleeson, and J. F. Mee. 2005. Effect of milking frequency and nutritional level on milk production characteristics and reproductive performance of dairy cows. Page 348 in *Book of Abstracts of the 56th Annual Meeting of the European Association for Animal Production*. Uppsala, Sweden. Wageningen Academic Publishers, Wageningen, the Netherlands.
- O'Driscoll, K. K. M., L. Boyle, B. O'Brien, D. Gleeson, and A. Hanlon. 2006. Effect of milking frequency and nutritional level on grazing and lying behaviour of dairy cows. Page 117 in *Proc. 40th Int. Soc. Appl. Ethol.*, Bristol, UK. ISAE Scientific Committee 2006, UK.
- Osterman, S., and I. I. Redbo. 2000. Effects of milking frequency on lying down and getting up behaviour in dairy cows. *Appl. Anim. Behav. Sci.* 71:167–176.
- Patton, J., D. A. Kenny, J. F. Mee, F. P. O'Mara, D. C. Wathes, M. Cook, and J. J. Murphy. 2006. Effect of milking frequency and diet on milk production, energy balance, and reproduction in dairy cows. *J. Dairy Sci.* 89:1478–1487.
- Rémond, B., and D. Pomiès. 2005. Once-daily milking of dairy cows: A review of recent French experiments. *Anim. Res.* 54:427–442.
- Rémond, B., D. Pomiès, D. Dupont, and Y. Chilliard. 2004. Once-a-day milking of multiparous Holstein cows throughout the entire lactation: Milk yield and composition, and nutritional status. *Anim. Res.* 53:201–212.
- Røntved, C. M., J. B. Andersen, J. Dernfalk, and K. L. Ingvarsen. 2005. Effects of diet energy and milking frequency in early lactation on tumor necrosis factor- α responsiveness in dairy cows. *Vet. Immunol. Immunopathol.* 104:171–181.
- Saad, A. M., C. Concha, and G. Astrom. 1989. Alterations in neutrophil phagocytosis and lymphocyte blastogenesis in dairy cows around parturition. *Zentralbl. Veterinarmed. B.* 36:337–345.
- Søndergaard, E., M. K. Sorensen, I. L. Mao, and J. Jensen. 2002. Genetic parameters of production, feed intake, body weight, body composition and udder health in lactating dairy cows. *Livest. Prod. Sci.* 77:23–34.
- Stabel, J. R., J. P. Godd, and K. Kimura. 2003. Effects of supplemental energy on metabolic and immune measurements in periparturient dairy cows with Johne's disease. *J. Dairy Sci.* 86:3527–3535.
- Ting, S. T. L., B. Earley, and M. A. Crowe. 2003. Effect of repeated ketoprofen administration during surgical castration of bulls on cortisol, immunological function, feed intake, growth, and behaviour. *J. Anim. Sci.* 81:1253–1264.
- Van Kampen, C., and B. A. Mallard. 1997. Effects of peripartum stress and health on circulating bovine lymphocyte subsets. *Vet. Immunol. Immunopathol.* 59:79–91.
- van Werven, T., E. N. Noordhuizen-Stassen, 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.
- Yagi, Y., H. Shiono, Y. Chikayama, A. Ohnuma, I. Nakamura, and K. Yayou. 2004. Transport stress increases somatic cell counts in milk, and enhances the migration capacity of peripheral blood neutrophils of dairy cows. *J. Vet. Med. Sci.* 66:381–387.