In Vitro Effects of Nonesterified Fatty Acids on Bovine Neutrophils Oxidative Burst and Viability[1]

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ABSTRACT

An in vitro study was conducted to examine the influence of nonesterified fatty acids (NEFA) on bovine polymorphonuclear leukocytes (PMN). Eight healthy, midlactating Holstein cows were used as blood donors. Blood PMN were isolated and incubated with a mixture of NEFA, reflecting composition of bovine plasma NEFA at concentrations that were intended to mimic those found in blood of cows undergoing high, moderate, or low lipomobilization intensity (2, 1, 0.5, 0.25, 0.125, and 0.0625 mM). Control samples were incubated in absence of NEFA. Phagocytosis and oxidative burst activities were assessed by a 2-color flow cytometric method, which was based on oxidation of intracellular dihydrorhodamine 123 to green fluorescent rhodamine 123. Oxidative burst products were generated by incubating PMN with *Staphylococcus aureus* labeled with propidium iodide. A flow cytometric technique was used to detect PMN viability, necrosis, and apoptosis using fluorescein isothiocyanate-labeled annexin-V and propidium iodide. Phagocytic activity was not affected by NEFA. The highest concentration of NEFA (2 mM) was associated with a dramatic increase of phagocytosis-associated oxidative burst activities with a reduction in cell viability (48.0 vs. 97.5% in control samples) and with a marked increase of necrosis (49.4 vs. 0.5% in control samples). Conversely, the mixture of NEFA did not affect the occurrence of apoptosis. Enhancement of the oxidative burst associated with the highest concentration of NEFA might explain the reduced viability and higher percentage of necrosis observed under the same conditions. This study demonstrated a substantial resistance of bovine PMN to an overload of fatty acids. However, observation that the highest concentration of NEFA regulated some PMN functions encourages the possibility of in vivo studies to assess the relationships between intensity of lipomobilization, plasma NEFA, and bovine PMN functions.
Key words: polymorphonuclear leukocyte, nonesterified fatty acids, phagocytosis, oxidative burst

INTRODUCTION

Periparturient dairy cows undergo a series of substantial hormonal and metabolic changes (Kehrli et al., 1998). The main periparturient neuroendocrine changes are related to parturition or onset of lactation and regard estrogens, progesterone, prolactin, growth hormone, endogenous opioids, and glycocorticoids (Kehrli et al., 1998). Metabolically, early lactating dairy cows require more energy than they are able to obtain through feed (Goff and Horst, 1997). As a consequence, they come into a temporary state of negative energy balance, characterized by hypoglycemia, lipomobilization, and increased NEFA that may eventually lead to ketotic states (Roberts et al., 1981). In particular, previous studies indicated that plasma NEFA of early lactating cows should be around 0.5 mM and that higher values are detected in cows with intense lipomobilization and suffering from subclinical or clinical ketosis (Veenhuizen et al., 1991; Busato et al., 2002).

Periparturient cows also experience a high incidence of infections, which has been ascribed to a temporary immunodepression. Different researchers ascribed the impairment of immune functions around calving to the above-reported neuroendocrine (Kehrli et al., 1998) or metabolic changes (Kaneene et al., 1997; Suriyasathaporn et al., 2000; Lacetera et al., 2004). Other researchers hypothesized that periparturient immunodepression may be associated with changes of subpopulations of blood or milk leukocytes (Zerbe et al., 2000).

It is well known that around parturition and during early lactation, cows become extremely sensitive to intramammary infections caused by gram-positive and gram-negative pathogens (Burvenich et al., 2003). Epidemiological studies demonstrated that risk of clinical mastitis was higher in ketotic cows (Erb and Grohn, 1988; Oltenacu and Ekesbo, 1994). To protect cows from mastitis and other infections, integrity of innate immunity is crucial (Burvenich et al., 1994; Suriyasathaporn

et al., 2000). Negative effects of ketones on functional activity of polymorphonuclear leukocytes (**PMN**) have already been reported for both sheep and dairy cows (Hoeben et al., 1997, 1999, 2000; Sartorelli et al., 1999, 2000). Hoeben et al. (2000) has described negative relationships between chemiluminescence response of PMN and plasma concentrations of BHBA and NEFA. Recently, Mehrzad et al. (2004, 2005) also demonstrated that chemiluminescence and viability of resident milk PMN (static innate immunity) play a crucial role in the severity of subsequently induced *Escherichia coli* mastitis. Finally, Van Oostveldt et al. (2001) reported that apoptosis of circulatory and milk PMN was higher in early lactating than in midlactating cows; those researchers suggested that apoptotic and necrotic processes might explain the downregulation of PMN functions shortly after calving.

With regard to other infections, Kaneene et al. (1997) indicated that NEFA concentrations in serum have potential as diagnostic indicators of disease risk in dairy cows in problem herds with high levels of peripartum disease.

In a series of in vitro studies carried out in dairy sheep and cows with NEFA concentrations mimicking those occurring in conditions of intense lipomobilization, we reported that NEFA dramatically impaired lymphocyte functions (Lacetera et al., 2002a,b, 2004).

The present in vitro study was carried out to assess whether high concentrations of NEFA could alter phagocytosis, oxidative burst, viability, apoptosis, and necrosis of circulatory PMN of lactating cows. In other words, the study was aimed to evaluate PMN functions under conditions simulating those of intense lipomobilization, which characterize the periparturient period.

MATERIALS AND METHODS

Animals and Blood Sample Collection

Eight clinically healthy Holstein-Friesian cows in their second or third lactation and at approximately 100 DIM were used as blood donors. Midlactating cows were preferred to early lactating cows to avoid possible interference caused by isolation of PMN from animals with high plasma NEFA consequent to intense lipomobilization. Furthermore, midlactation is a period during which most of the concerned cell functions are optimal in comparison with their status at early lactation (Van Oostveldt et al., 2001). Cows were selected from the dairy herd at Gent University (Biocentrum Agri-Vet, Melle, Belgium) and at the time of the study, average milk yield was approximately 23 L/d.

Blood was collected aseptically by jugular venipuncture using tubes containing different anticoagulants depending on the PMN isolation procedure used (described subsequently).

Further tubes without anticoagulant were used for preparation of sera pool. The sera pool was filter-sterilized and stored at −80°C until use.

Isolation of PMN

Two different procedures were used to isolate PMN. The first procedure was used in the phagocytosis and oxidative burst study. In this case, PMN were isolated following the method described by Carlson and Kaneko (1973). The second procedure was used to study viability, apoptosis, and necrosis. Isolation of PMN was carried out according to the method described by Roets et al. (1999).

Use of the 2 isolation procedures was due to results of a previous study (Van Oostveldt et al., 1999). That study indicated that the isolation procedure suggested by Carlson and Kaneko (1973) was responsible for a high percentage of false-positive cells when used to assess apoptosis in polymorphonuclear cells by cytofluorimetric procedure.

In both cases, after isolation, PMN pellets were resuspended in Dulbecco's PBS (**DPBS**; Sigma, St. Louis, MO) supplemented with 2% BSA (A grade, endotoxin-tested, Sigma). Afterward, the PMN were counted with an automatic cell counter (Coulter Counter ZF, Coulter Electronics Ltd., Luton, UK), and their concentration was adjusted to 1×10^7 cells/mL.

The viability of PMN was assessed immediately after the isolation and before incubation with NEFA by propidium iodide (**PI**; Sigma) exclusion (1 µg of PI/mL, final concentration) using flow cytometry (**FCT**). The analysis was performed after the cells were incubated for 10 min in the dark at room temperature.

Incubation of PMN with NEFA

The NEFA mixture used in the study was a combination of C16:0 (30%), C16:1 (5%), C18:0 (15%), C18:1 (45%), and C18:2 (5%) and reflected the composition of bovine plasma NEFA (Noble et al., 1971). The mixture was prepared as described elsewhere (Lacetera et al., 2002a) using the method of Strang et al. (1998) with minor modifications.

The stock solution of NEFA (88.8 m*M*) was diluted to 20 m*M* in DPBS supplemented with 2% BSA, adjusted to pH 7.4, and sterilized by passage through a filter. Then, this solution was serially diluted with DPBS supplemented with 2% BSA to reach the final concentrations in PMN suspensions of 2, 1, 0.5, 0.250, 0.125, and 0.0625 m*M*. Concentrations tested were intended to mimic those found in blood of cows undergoing

Table 1. Effects of NEFA on phagocytosis of polymorphonuclear leukocytes (PMN)[1]

Concentration of NEFA	Phagocytosis	MFI[2]
	(% fluorescence[3])	
0 mmol/L	56.6 ± 5.3	7.6 ± 1.9
0.0625 mmol/L	60.0 ± 5.3	9.4 ± 1.9
0.125 mmol/L	58.4 ± 5.4	8.4 ± 1.9
0.250 mmol/L	58.6 ± 5.3	8.9 ± 1.9
0.5 mmol/L	54.4 ± 5.3	8.6 ± 1.9
1 mmol/L	63.5 ± 5.3	12.4 ± 1.9
2 mmol/L	49.6 ± 5.8	12.9 ± 2.0

[1]Data were collected from 10,000 cells per sample in the gate set for PMN. Values reported are means ± SEM of 8 cows. Treatment differences were not significant.

[2]Mean fluorescence intensity.

[3]Percentage of positive cells.

high (2 and 1 m*M*), moderate (0.5 and 0.25 m*M*), or low (0.125 and 0.0625 m*M*) lipomobilization intensity (Roberts et al., 1981; Veenhuizen et al., 1991; Busato et al., 2002). Control samples were incubated in the absence of NEFA (0 m*M*).

Labeling of Staphylococcus aureus with PI

Staphylococcus aureus Newbold 305 was grown overnight in tryptic soy broth (Sigma) at 37°C. Tryptic soy broth was prepared by dissolving 30 g of the product in 1 L of sterile double-distilled water followed by autoclaving at 120°C for 30 min. The day after, bacteria were treated for labeling as already described elsewhere (Smits et al., 1997).

Phagocytosis and Oxidative Burst Study

In this study, the concentration of PMN suspensions was 4.5×10^5 cells per tube. Instead of NEFA, control

Table 2. Effects of NEFA on phagocytosis-induced oxidative burst of polymorphonuclear leukocytes (PMN)[1]

Concentration of NEFA	Ox burst	MFI[2]
	(% fluorescence[3])	
0 mmol/L	94.5 ± 3.3[a]	18.0 ± 3.3[a]
0.0625 mmol/L	85.2 ± 3.3[b]	17.2 ± 3.3[a]
0.125 mmol/L	84.8 ± 3.3[b]	15.6 ± 3.4[a]
0.250 mmol/L	85.5 ± 3.3[b]	18.2 ± 3.3[a]
0.5 mmol/L	85.7 ± 3.3[b]	18.0 ± 3.3[a]
1 mmol/L	94.3 ± 3.3[a]	24.2 ± 3.3[a]
2 mmol/L	92.1 ± 3.5[a]	43.5 ± 3.5[b]

[a,b]Within columns, means with different letters differ significantly ($P \leq 0.05$) for effect of concentration of NEFA.

[1]Data were collected from 5,000 cells per sample in the gate set for PMN. Values reported are mean ± SEM of 8 cows.

[2]Mean fluorescence intensity.

[3]Percentage of positive cells.

tubes contained PMN with either DPBS or staurosporine (Sigma) (0.107 µ*M*, final concentration) or its solvent [dimethyl sulfoxide (**DMSO**), 0.05%]. Staurosporine was dissolved in DMSO to obtain a stock solution (0.214 m*M*), and aliquots were stored at –80°C. Before use, staurosporine was diluted in DPBS, reaching the final concentration of 0.107 µ*M* after the addition to cell suspensions. Staurosporine was used, in the light of its effects as inhibitors of bovine PMN oxidative burst (Smits et al., 1997). The PMN were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C for 4 h.

After the incubation with NEFA, 90 µL of PMN suspension were preincubated with 10 µ*M* dihydrorhodamine 123 (**DHR**) (phagocytosis per oxidative burst tubes) or DPBS (autofluorescence tubes) for 5 min in a shaking water bath at 37°C. The DHR was purchased from Molecular Probes (Junction City, OR), dissolved in DMSO in the dark at a concentration of 2 m*M*, and stored at –80°C until use. Before use, DHR solution was diluted 1:20 in RPMI 1640 (Life Technologies, Paisley, UK). After loading of leukocytes, 10 µL of PI-labeled bacteria or DPBS and 5 µL of sera pool were added to give a total volume of 115 µL. All experiments were performed over 20-min incubations at 37°C in the dark with a bacteria:PMN of 25:1 (Smits et al., 1997). Phagocytosis was stopped by adding 0.1 m*M* N-ethylmaleimide (Sigma), and samples were stored on ice. Before the analysis, 400 µL of cold DPBS was added to each sample.

A rapid and sensitive 2-color FCT method for simultaneous measurement of phagocytosis rate and oxidative burst activity of isolated bovine PMN was used. In particular, our assay of the oxidative burst activity was able to detect the intracellular fluorescence, which reflected the amount of reactive oxygen species (**ROS**) released into phagosomes. The assay was based on the oxidation of intracellular DHR to green fluorescent rhodamine 123 (**ROD**) by oxidative burst products generated by incubating the PMN with red fluorescent PI-labeled *S. aureus* (Smits et al., 1997; Bassøe et al., 2000). Data were collected from 10,000 cells per sample in the gate set for PMN, and the percentage of fluorescence and the mean fluorescence intensity (**MFI**) were used as quantitative indices of PMN response.

Viability, Apoptosis, and Necrosis Study

In this study, the concentration of PMN suspensions was 10^6 cells per tube. Instead of NEFA, control tubes contained PMN with either DPBS or actinomycin D (Sigma; 16 µ*M*, final concentration). Actinomycin D was dissolved in DPBS to obtain a stock solution of 320 µ*M* and stored at –80°C. Before use, it was thawed and diluted to reach a concentration of 16 µ*M* after addition

Table 3. Effects of NEFA on viability, apoptosis, and necrosis of polymorphonuclear leukocytes[1]

Concentration of NEFA, %	Viability	Apoptosis	Necrosis
0 mmol/L	97.5 ± 5.6[a]	1.96 ± 0.34	0.49 ± 5.73[a]
0.0625 mmol/L	97.5 ± 5.6[a]	1.99 ± 0.34	0.47 ± 5.73[a]
0.125 mmol/L	97.5 ± 5.6[a]	1.98 ± 0.34	0.51 ± 5.73[a]
0.250 mmol/L	97.3 ± 5.6[a]	2.14 ± 0.34	0.54 ± 5.73[a]
0.5 mmol/L	97.2 ± 5.6[a]	2.24 ± 0.34	0.56 ± 5.73[a]
1 mmol/L	96.7 ± 5.6[a]	2.45 ± 0.34	0.81 ± 5.73[a]
2 mmol/L	48.0 ± 5.6[b]	2.45 ± 0.34	49.38 ± 5.73[b]

[a,b]Within columns, means with different letters differ significantly ($P \leq 0.05$) for effect of concentration of NEFA.

[1]Values reported are means ± SEM of 8 cows.

to cell suspensions. Actinomycin D was used, in the light of its ability to induce apoptosis in bovine PMN (Van Oostveldt et al., 1999). The PMN were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C for 3 h.

Apoptosis of bovine PMN was quantified using a dual-color FCT procedure with staining of exposed phosphatidylserine by fluorescein isothiocyanate (**FITC**)-annexin-V (Roche GmbH, Mannheim, Germany) and of cellular DNA using PI.

After the incubation with NEFA mixture, PMN suspensions were centrifuged, and cell pellets (1×10^6 PMN per sample) were resuspended in 100 μL of incubation buffer (10 m*M* HEPES/HCl, pH 7.4, 140 m*M* NaCl, 5 m*M* $CaCl_2$) containing FITC-annexin-V and PI. This solution was freshly prepared adding 20 μL of annexin-V-FITC and 20 μL of a PI solution (50 μg/mL) to 1 mL of incubation buffer. The mixture was incubated for 10 min in the dark at room temperature. Before the analysis, 400 μL of cold DPBS was added to each sample.

To simultaneously identify viable, apoptotic, and necrotic PMN, an FCT technique was also used (Van Oostveldt et al., 2001). This was based on the method of Vermes et al. (1995) developed for apoptosis and necrosis detection in isolated human lymphocytes using FITC-labeled annexin-V and PI. Data were collected from 5,000 cells per sample in the gate set for PMN, and results of PMN viability, apoptosis, and necrosis were reported as the percentage of annexin-V-FITC positive cells (apoptotic cells), PI positive cells (necrotic cells), and negative cells for both kinds of fluorescence (viable cells), respectively.

FCT

For both FCT assays, a FACScan was used (Becton Dickinson Immunocytometry Systems, San Jose, CA). The PMN were selectively gated for analysis by forward scatter and side scatter, which detect cell size and granularity, respectively. The analysis was done using Cell-Quest software (Becton Dickinson Immunocytometry Systems).

Statistical Analyses

Data were analyzed by the mixed model procedure of SAS (SAS, Version 8.1) with cow as random effect and concentration of NEFA as categorical fixed effect. The different NEFA concentrations were compared pairwise using Tukey's multiple comparison method at a global significance level of 5%.

RESULTS

Phagocytosis and Oxidative Burst Study

Phagocytosis was not affected by the tested concentrations of NEFA, neither in terms of percentage nor in terms of MFI (Table 1). In contrast, significant differences were found in the ability of PMN to produce ROS following incubation with NEFA (Table 2). The low and moderate concentrations of NEFA (0.0625, 0.125, 0.25, and 0.5 m*M*) induced a significant decrease ($0.0001 < P < 0.05$) in the phagocytosis-dependent burst activity expressed as a percentage of fluorescence, whereas the highest concentration (2 m*M*) did not cause any significant difference with respect to the control. Instead, the highest concentration of NEFA (2 m*M*) caused an increase ($P < 0.0001$) of MFI after phagocytosis of *S. aureus*.

Figure 1 shows a scatter-dot plot of PMN sample without NEFA or with 2 m*M* NEFA incubated with DHR and PI-labeled *S. aureus* to determine phagocytosis and oxidative burst.

Viability, Apoptosis, and Necrosis Study

The highest concentration of NEFA (2 m*M*) was associated with an increase ($P < 0.0001$) of necrosis, which was concomitant with a decrease ($P < 0.0001$) of viability (Table 3). Conversely, none of the concentrations of NEFA tested in the present study modified the percentage of apoptotic cells.

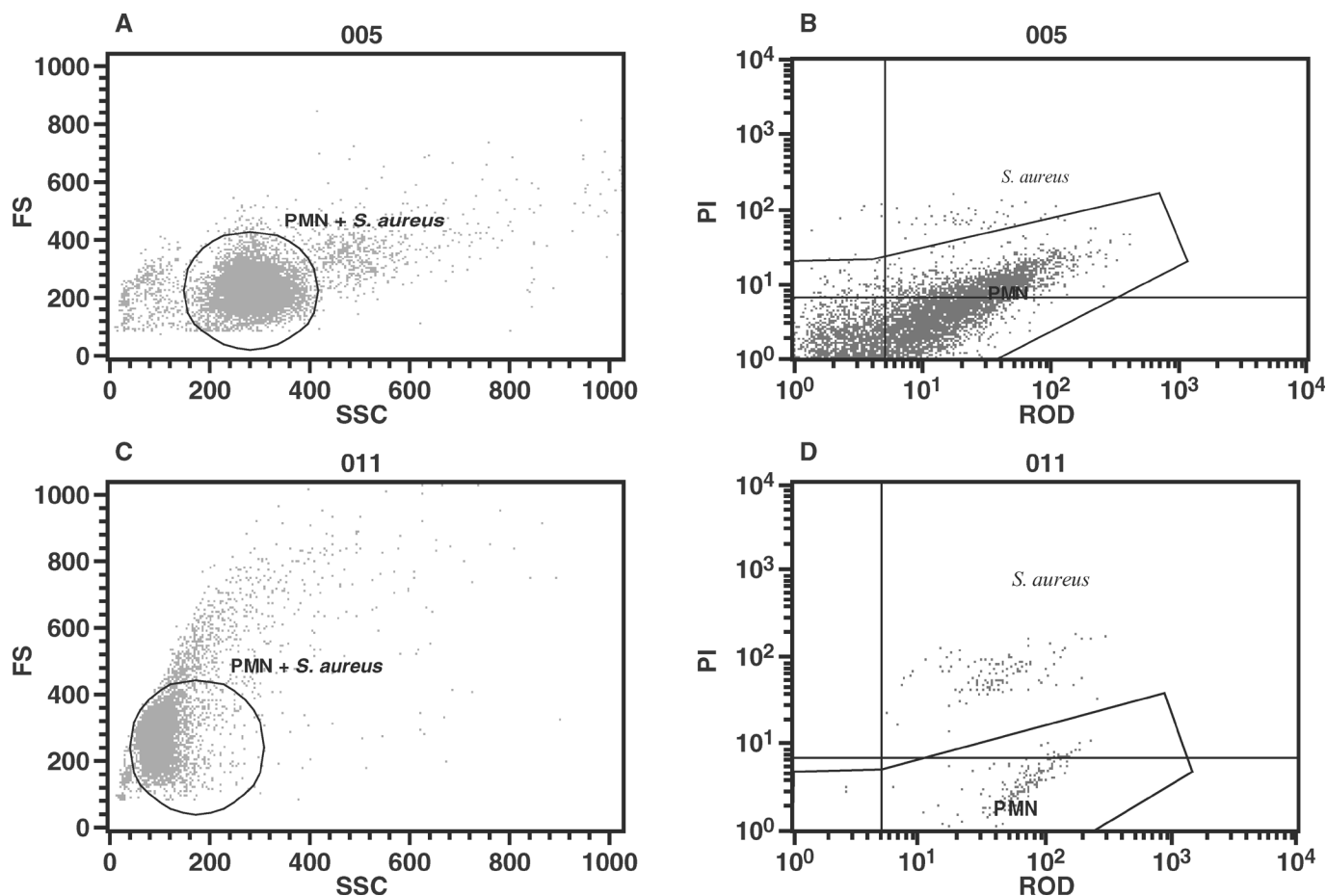Figure 2 shows a scatter-dot plot of PMN sample without NEFA or with NEFA at 2 m*M* incubated with

Figure 1. Representative scatter dot plot (FS = forward scatter; SSC = side scatter) of polymorphonuclear leukocyte (PMN) sample without NEFA (A) or with NEFA at 2 mM (C) incubated with dihydrorhodamine 123 (DHR) and *Staphylococcus aureus* labeled with propidium iodide (PI) to determine phagocytosis and oxidative burst, respectively. The bitmap was set for PMN + *S. aureus*. The concomitant DHR and PI fluorescence patterns were determined (B and D for PMN sample without NEFA or with NEFA at 2 mM, respectively). ROD = rhodamine.

annexin-FITC and PI to determine apoptosis and necrosis, respectively.

DISCUSSION

Hormonal changes (Kehrli et al., 1998) and metabolite fluctuations (Goff and Horst, 1997) are involved in the depression of the immune system observed in periparturient dairy cows.

A recent in vitro study pointed out a profound reduction of mitogen-induced DNA synthesis, interferon-γ, and IgM secretion in lymphocytes isolated from dairy cows and incubated with various concentrations of NEFA (Lacetera et al., 2004). Additionally, in another study (Lacetera et al., 2005), we reported that overconditioned cows have to be considered at high risk of infection because the intense lipomobilization taking place around calving is associated with alterations of lymphocyte functions.

Reid et al. (1983) demonstrated a direct effect of NEFA on phenotype and functionality of bovine PMN. The present study pointed out no effects of NEFA on PMN phagocytosis. Numerous researchers indicated that PMN have the capacity to metabolize fatty acids through different metabolic pathways, which makes these cells resistant to an overload of fatty acids. Resting PMN can incorporate and store large quantities of exogenous free fatty acids into triacylglycerol or oxidize them to a very large extent (Burns et al., 1976; Phillips et al., 1986). Moreover, other studies indicated that triacylglycerol is stored by PMN to be utilized for phospholipid synthesis (lecithin) during phagocytosis (Elsbach and Farrow, 1969).

Consistent with previous reports (Kakinuma and Minakami, 1978; Listenberger et al., 2001), results reported herein indicated that the highest concentration of NEFA (2 mM) enhanced phagocytosis-induced oxida-
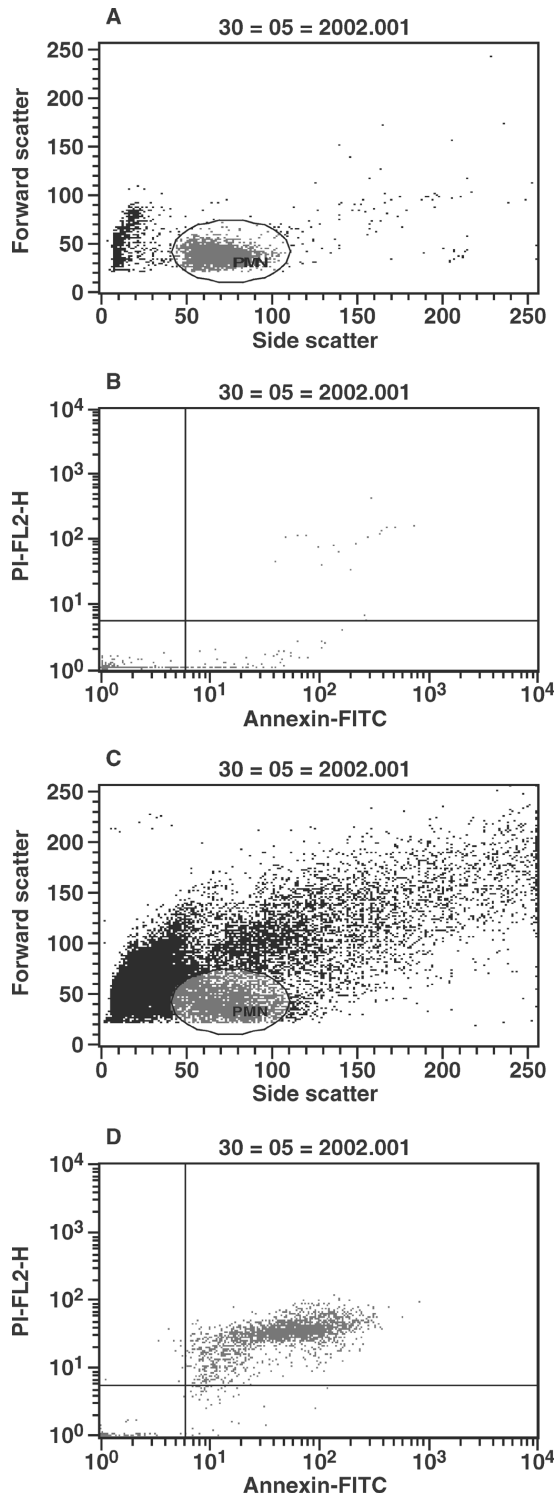
Figure 2. Representative results of scatter dot plot of polymorphonuclear leukocyte (PMN) sample without NEFA (A) or with NEFA at 2 m*M* (C) incubated with annexin-fluorescein isothiocyanate (FITC) and propidium iodide (PI) to determine apoptosis and necrosis, respectively. The ordinate and the abscissa represent cell size and granularity, respectively. The concomitant annexin-FITC and PI fluorescence patterns were determined (B and D for PMN sample without NEFA or with NEFA at 2 m*M*, respectively).

tive burst in terms of MFI. In 2000, de Pablo and de Cienfuegos reported that alteration of the fatty acid composition of cell membranes might cause changes in membrane fluidity, which may modify the activity of some membrane-bound enzymes, such as of NAD(P)H oxidase. Accordingly, Inoguchi et al. (2000) demonstrated that high concentrations of palmitate and oleate stimulate ROS production through protein kinase C-dependent activation of NAD(P)H oxidase in phagocytic cells.

It is not easy to formulate hypotheses to explain why the incubation of PMN with the lowest concentrations of NEFA (0.5, 0.25, 0.125, 0.0625 m*M*) reduced the percentage of positive PMN. However, as reported by others (Kadri-Hassani et al., 1995), fatty acids can exert opposite effects on ROS generation depending on their concentration.

Interestingly, further studies should establish the NEFA effect on the kinetics of PMN ROS, intracellular and extracellular production. The interest of such kind of studies is related to previous findings, which indicated that extracellular deposition of ROS can destroy the surrounding tissues (Passo et al., 1982).

Finally, further studies should also verify whether NEFA might influence other aspects of bovine PMN functions (e.g., chemotaxis or diapedesis) that were not considered in our study. Consistent with this hypothesis, previous studies testing the effects of ketones on sheep PMN demonstrated that BHBA negatively affected chemotaxis, but not phagocytosis (Sartorelli et al., 1999).

The highest concentration of NEFA (2 m*M*), the same inducing the increase of ROS generation, was associated with reduced viability (48.0 vs. 97.5% in the control samples, *P* < 0.0001) and an increase of necrosis (49.38 vs. 0.49% in the control samples, *P* < 0.0001). None of the other concentrations of NEFA exerted significant effects. Several researchers (Kakinuma and Minakami, 1978; Listenberger et al., 2001) documented that high concentrations of fatty acids can increase the generation of ROS that may be responsible for cell damage and death through necrotic processes. To a certain extent in contrast to our findings, Mehrzad et al. (2004) reported a positive correlation between latex-stimulated chemiluminescence and milk PMN viability. Finally, it may also be hypothesized that the higher MFI recorded in PMN incubated with NEFA at 2 m*M* might also be due to nonspecific fluorescence deriving from the breakdown of dead cells. In other words, 2 m*M* of NEFA might have exerted a toxic effect and caused a huge increase in necrotic cells. This might have been associated with loss of cell membranes and increased fluorescence.

Previous studies hypothesized that increased percentages of necrotic PMN may increase the risk for mastitis around calving (Van Oostveldt et al., 2001) and that metabolic events associated with energy insufficiency (increased fat mobilization, etc.) are related to higher risk of metritis (Kaneene et al., 1997). Our study would suggest a possible relationship between the 2 events, in that an intense fat mobilization may be associated with high risk of infections because of the effects of NEFA on PMN viability.

In contrast to necrosis, no significant changes in apoptosis were observed in PMN cultured in the presence of NEFA. However, the double labeling method used in our study to detect apoptosis and necrosis was unable to distinguish between primary and secondary necrosis. The latter type of cell death can occur when apoptotic cells cannot be phagocytized by macrophages (Kar et al., 1993; Savill and Haslett, 1995). The hypothesis that in our study PMN death was due to a secondary necrosis consequent to apoptosis would be compatible with the fact that our experiments were carried out on isolated PMN in the absence of macrophages. Finally, we may also hypothesize that the incubation time of PMN with NEFA was too long and permitted the secondary necrosis of apoptotic cells. Therefore, realization of further studies with incubation times shorter than those utilized herein might avoid the occurrence of secondary necrosis and limit phenomena under observation to primary necrosis and apoptosis (Majno and Joris, 1995).

Finally, in contrast to previous findings relative to sheep and bovine lymphocytes (Lacetera et al., 2002a,b, 2004), this study did not show any dose-dependent response of PMN to NEFA and pointed out the most significant effects when PMN were incubated with NEFA at 2 mM. Compared with data relative to lymphocytes, these results confirm (Burns et al., 1976; Phillips et al., 1986) the resistance of PMN to an overload of fatty acids and authorize additional in vitro studies aimed to assess the effects of NEFA on PMN functions at concentrations ranging from 1 and 2 mM (e.g., 1.25, 1.5, and 1.75 mM).

CONCLUSIONS

The present study provided evidence that high concentrations of NEFA can regulate some functions of bovine blood PMN (namely ROS production and viability).

Further in vitro studies should be undertaken to ascertain the effects of NEFA on PMN functions different from those considered in the present study and also whether milk PMN would respond to NEFA in a similar manner. Furthermore, additional studies are needed to elucidate which fatty acids may be responsible for PMN dysfunction and verify their immunopharmacologic potential.

Results reported herein also encourage epidemiology studies to assess whether the intensity of lipomobilization per se is associated with the downregulation of PMN functions and possibly with the higher incidence of periparturient infections in dairy cows. The hypothesis that serum NEFA concentrations have potential as indicators of PMN functionality and risk of infections should also be verified.

REFERENCES

Bassøe, C. F., I. Smith, S. Sørnes, A. Halstensen, and A. K. Lehmann. 2000. Concurrent measurement of antigen- and antibody-dependent oxidative burst and phagocytosis in monocytes and neutrophils. Methods 21:203–220.

Burns, C. P., I. R. Welshman, and A. A. Spector. 1976. Differences in free fatty acid and glucose metabolism of human blood neutrophils and lymphocytes. Blood 47:431–437.

Burvenich, C., M. J. Paape, A. W. Hill, A. J. Guidry, R. H. Miller, R. Heyneman, W. D. Kremer, and A. Brand. 1994. Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced E. coli mastitis in cows immediately after calving. Vet. Q. 16:45–50.

Burvenich, C., V. van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet. Res. 34:521–564.

Busato, A., D. Faissle, U. Kupfer, and J. W. Blum. 2002. Body condition scores in dairy cows: associations with metabolic and endocrine changes in healthy dairy cows. J. Vet. Med. A 49:455–460.

Carlson, G. P., and J. J. Kaneko. 1973. Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med. 142:853–856.

de Pablo, M. A., and A. de Cienfuegos. 2000. Modulatory effects of dietary lipids on immune system functions. Immunol. Cell. Biol. 78:31–39.

Elsbach, P., and S. Farrow. 1969. Cellular triglyceride as a source of fatty acid for lecithin synthesis during phagocytosis. Biochim. Biophys. Acta 176:438–441.

Erb, N. H., and Y. T. Grohn. 1988. Epidemiology of metabolic disorders in the periparturient dairy cow. J. Dairy Sci. 71:2557–2571.

Goff, J. P., and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80:1260–1268.

Hoeben, D., C. Burvenich, A. M. Massart-Leen, M. Lenjou, G. Nijs, D. Van Bockstaele, and J. F. Beckers. 1999. In vitro effect of ketone bodies, glucocorticosteroids and bovine pregnancy-associated glycoprotein on cultures of bone marrow progenitor cells of cows and calves. Vet. Immunol. Immunopathol. 68:229–240.

Hoeben, D., R. Heyneman, and C. Burvenich. 1997. Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet. Immunol. Immunopathol. 58:165–170.

Hoeben, D., E. Monfardini, G. Opsomer, C. Burvenich, H. Dosogne, A. De Kruif, and J. F. Beckers. 2000. Chemiluminescence of bovine polymorphonuclear leukocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. J. Dairy Sci. 67:249–259.

Inoguchi, T., P. Li, F. Umeda, H. Y. Yu, M. Kakimoto, M. Imamura, T. Aoki, T. Etoh, T. Hashimoto, M. Naruse, H. Sano, H. Utsumi,

and H. Nawata. 2000. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes 49:1939–1945.

Kadri-Hassani, N., C. L. Leger, I. Vachier, and B. Descomps. 1995. Bimodal action of fatty acids on PMA-stimulated O2-production in human adherent monocytes. J. Lipid Mediat. Cell Signal 11:159–173.

Kakinuma, K., and S. Minakami. 1978. Effects of fatty acids on superoxide radical generation in leukocytes. Biochim. Biophys. Acta 538:50–59.

Kaneene, J. B., R. A. Miller, T. H. Herdt, and J. C. Gardiner. 1997. The association of serum nonesterified fatty acids and cholesterol, management and feeding practices with peripartum disease in dairy cows. Prev. Vet. Med. 31:59–72.

Kar, S., Y. Ren, J. S. Savill, and C. Haslett. 1993. Inhibition of macrophage phagocytosis in vitro of aged neutrophils increases release of neutrophil contents. Clin. Sci. 85:27–33.

Kehrli, M. E., K. Kimura, Jr., J. P. Goff, J. R. Stabel, and B. J. Nonnecke. 1998. Periparturient immunosuppression in dairy cows: Nutrition and lactation effects. Pages 41–55 in Proc. 10th Int. Conf. Production Diseases in Farm Animals, Utrecht, The Netherlands.

Lacetera, N., O. Franci, D. Scalia, U. Bernabucci, B. Ronchi, and A. Nardone. 2002a. Effects of nonesterified fatty acids and β-hydroxybutyrate on functions of mononuclear cells obtained from ewes. Am. J. Vet. Res. 63:414–418.

Lacetera, N., O. Franci, D. Scalia, U. Bernabucci, B. Ronchi, and A. Nardone. 2002b. Effects on functions of ovine blood mononuclear cells for each of several fatty acids at concentrations found in plasma of healthy and ketotic ewes. Am. J. Vet. Res. 63:958–962.

Lacetera, N., D. Scalia, O. Franci, U. Bernabucci, B. Ronchi, and A. Nardone. 2004. Effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J. Dairy Sci. 87:1012–1014.

Lacetera, N., D. Scalia, U. Bernabucci, B. Ronchi, D. Pirazzi, and A. Nardone. 2005. Lymphocyte functions in overconditioned cows around parturition. J. Dairy Sci. 88:2010–2016.

Listenberger, L. L., D. S. Ory, and J. E. Schaffer. 2001. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J. Biol. Chem. 276:14890–14895.

Majno, G., and I. Joris. 1995. Apoptosis, oncosis and necrosis: an overview of cell death. Am. J. Pathol. 146:3–15.

Mehrzad, J., L. Duchateau, and C. Burvenich. 2004. Viability of milk neutrophils and severity of bovine coliform mastitis. J. Dairy Sci. 87:4150–4162.

Mehrzad J., L. Duchateau, and C. Burvenich. 2005. High milk neutrophil chemiluminescence limits the severity of bovine coliform mastitis. Vet. Res. 36:101–116.

Noble, R. C., W. Steele, and J. Moore. 1971. The plasma lipids of the ewe during pregnancy and lactation. Res. Vet. Sci. 12:17–53.

Oltenacu, P. A., and I. Ekesbo. 1994. Epidemiological study of clinical mastitis in dairy cattle. Vet. Res. 25:208–212.

Passo, S. A., S. A. Syed, and J. Silva. 1982. Neutrophil chemiluminescence in response to Fusobacterium nucleatum. J. Periodont. Res. 17:604–613.

Phillips, W. A., H. Mossmann, and E. Ferber. 1986. Changes in the incorporation of free fatty acids upon the stimulation of human polymorphonuclear leukocytes. J. Leuk. Biol. 39:267–284.

Reid, I. M., R. A. Collins, A. M. Dew, and A. W. Hill. 1983. Peripheral leukocyte numbers and function in cows with fatty liver. J. Pathol. 141:515–516.

Roberts, C. J., I. M. Reid, G. J. Rowlands, and A. Patterson. 1981. A fat mobilization syndrome in dairy cows in early lactation. Vet. Rec. 108:7–9.

Roets, E., C. Burvenich, A. Diez-Fraile, and E. N. Noordhuizen-Stassen. 1999. Evaluation of the role of endotoxin and cortisol on modulation of CD18 adhesion receptors in cows with mastitis caused by *Escherichia coli*. Am. J. Vet. Res. 60:534–540.

Sartorelli, P., S. Paltrinieri, and F. Agnes, 1999. Non-specific immunity and ketone bodies. I: In vitro studies on chemotaxis and phagocytosis in ovine neutrophils. J. Vet. Med. A 46:613–619.

Sartorelli, P., S. Paltrinieri, and S. Comazzi. 2000. Non-specific immunity and ketone bodies. II: In vitro studies on adherence and superoxide anion production in ovine neutrophils. J. Vet. Med. A 47:1–8.

Savill, J., and C. Haslett. 1995. Granulocyte clearance by apoptosis in the resolution of inflammation. Semin. Cell Biol. 6:385–393.

Smits, E., C. Burvenich, and R. Heyneman. 1997. Simultaneous flow cytometric measurement of phagocytotic and oxidative burst activity of polymorphonuclear leukocytes in whole bovine blood. Vet. Immunol. Immunopathol. 56:259–269.

Strang, B. D., S. J. Bertics, R., R. R. Grummer, and L. E. Armentano. 1998. Effect of long-chain fatty acids on triglyceride accumulation, gluconeogenesis, and ureagenesis in bovine hepatocytes. J. Dairy Sci. 81:728–739.

Suriyasathaporn, W., C. Heuer, E. N. Noordhuizen-Stassen, and Y. H. Schukken, 2000. Hyperketonemia and the impairment of udder defense: A review. Vet. Res. 31:397–412.

Van Oostveldt, K., H. Dosogne, C. Burvenich, M. J. Paape, V. Brochez, E. Van den Eeckhout. 1999. Flow cytometric procedure to detect apoptosis of bovine polymorphonuclear leukocytes in blood. Vet. Immunol. Immunopathol. 70:125–133.

Van Oostveldt, K., F. Vangroenweghe, H. Dosogne, and C. Burvenich. 2001. Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early and midlactating healthy cows. Vet. Res. 32:617–622.

Veenhuizen, J. J., J. K. Drackley, M. J. Richard, T. P. Sanderson, L. D. Miller, and J. W. Young. 1991. Metabolic changes in blood and liver during development and early treatment of experimental fatty liver and ketosis in cows. J. Dairy Sci. 74:4238–4253.

Vermes, I., C. Haanen, H. Stefferns-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin-V. J. Immunol. 184:39–51.

Zerbe, H., N. Schneider, W. Leibold, T. Wensing, T. A. Kruip, and H. J. Schuberth. 2000. Altered functional and immunophenotypical properties of neutrophilic granulocytes in postpartum cows associated with fatty liver. Theriogenology 54:771–86.