Vet. Res. 33 (2002) 271–281

DOI: 10.1051/vetres:2002015

Original article

Evaluation of L-selectin expression and assessment of protein tyrosine phosphorylation in bovine polymorphonuclear neutrophil leukocytes around parturition

Erica MONFARDINI[a], Max J. PAAPE[b]*, Yan WANG[b], Anthony V. CAPUCO[c], Michael HUSHEEM[b], Larry WOOD[c], Christian BURVENICH[a]

[a]Department of Physiology, Biochemistry and Biometrics, Faculty of Veterinary Medicine, University of Ghent, Salysburylaan, 133, Merelbeke 9820, Belgium
[b]Immunology and Disease Resistance Laboratory, USDA-ARS, Beltsville, MD-20705, USA
[c]Gene Evaluation and Mapping Laboratory, USDA-ARS, Beltsville, MD-20705, USA

(Received 10 September 2001; accepted 8 January 2002)

Abstract – Impaired polymorphonuclear neutrophil leukocyte (PMN) function around parturition has been associated with increased clinical mastitis in dairy cows. Rolling and attachment of PMN to the endothelium is the first step in the recruitment process and is accomplished by interaction between L-selectin on PMN and its ligand on endothelial cells. Furthermore, tyrosine phosphorylation is involved in the initiation of many PMN functions. The objective of this work was to determine changes in expression of L-selectin and tyrosine phosphorylation in the perinatal period. Eight clinically healthy Holstein cows were used as PMN donors at d –21, –14, –7, 0 (calving), +1, +2, +7, +14, +28. Evaluation of L-selectin expression was carried out on activated and resting PMN. Anti-bovine L-selectin monoclonal antibody (MAB) and flow cytometric analysis were used to measure the percentage of PMN fluorescing and receptor expression (log mean fluorescent channel, LMFC). Activated and resting PMN showed similar trends in % PMN fluorescence and LMFC. The percentage of PMN fluorescing tended to decrease at parturition, followed by a significant increase at d +14 and +28 ($P < 0.02$). For LMFC a decrease was observed on d +1 followed by an increase through d +28 ($P < 0.01$). Protein tyrosine phosphorylation of lysates prepared from PMN isolated throughout the study was detected by electrophoresis and western blotting using anti-phosphotyrosine MAB. Several protein bands were tyrosine phosphorylated. Two of these bands (42–44 kDa and 90 kDa) varied in intensity over time. The intensity of the 42–44 kDa band gradually increased from d –7, peaked at d +7 ($P < 0.03$), and steadily decreased to d +28 ($P < 0.02$). Antibody to activated mitogen protein kinase reacted with the 42–44 kDa band. Reduced PMN function during the periparturient period

*Correspondence and reprints
Tel.: (1) 301 504 8302; fax: (1) 301 504 9498; e-mail: mpaape@anri.barc.usda.gov

could be related to reduced L-selectin adhesion molecules on the cell surface, and to modulation in the phosphorylation of functionally important molecules.

L-selectin / tyrosine phosphorylation / neutrophil / mastitis / parturition

Résumé – Évaluation de l'expression de la L-sélectine et de la phosphorylation de la tyrosine des protéines des neutrophiles bovins au moment de la parturition. Pendant la parturition, une fonction affaiblie des neutrophiles (PMN) a été associée à une augmentation des mammites cliniques chez les vaches laitières. Roulement et attachement des PMN à l'endothélium est le premier pas dans le processus du recrutement et est accompli par l'interaction entre la L-sélectine sur la membrane des PMN et son ligand sur les cellules de l'endothélium. En outre, dans les PMN, la phosphorylation de la tyrosine est impliquée dans l'initiation de plusieurs fonctions. L'objectif de ce travail était de déterminer les changements d'expression de la L-sélectine et de la phosphorylation de la tyrosine dans la période périnatale. Huit vaches de race Holstein, cliniquement saines, ont été utilisées comme donneuses de PMN aux jours –21, –14, –7, 0 (vêlage), +1, +2, +7, +14, +28. L'évaluation de l'expression de la L-sélectine a été effectuée sur des PMN activés et non activés. Des anticorps monoclonaux anti-L-sélectine bovine (MAB) et la cytométrie en flux ont été utilisés pour mesurer le pourcentage de PMN fluorescents ainsi que l'expression du récepteur (log mean fluorescence channel, LMFC). Les PMN activés et non activés ont montré des changements semblables dans le pourcentage des PMN fluorescents ainsi que dans l'expression du récepteur. Le pourcentage de PMN fluorescents a eu tendance à diminuer au moment de la parturition. Ceci a été suivi par une augmentation significative ($P < 0.02$) aux jours +14 et +28. Pour LMFC une baisse a été observée au jour +1. Ceci a été suivi par une augmentation jusqu'au jour +28 ($P < 0.01$). La phosphorylation de la tyrosine des protéines issue de lysats provenant des PMN étudiés a était détectée par électrophorèse et Western blot en utilisant l'anticorps monoclonal anti-phosphotyrosine. Plusieurs bandes de protéines correspondaient à la tyrosine phosphorylée. Deux de ces bandes (42–44 kDa et 90 kDa) variaient en intensité dans le temps. L'intensité de la bande 42–44 kDa, a augmenté progressivement à partir du jour –7, pour atteindre un maximum au jour +7 ($P < 0.03$), puis a diminué jusqu'au jour +28 ($P < 0.02$). L'anticorps contre la protéine kinase activée par un mitogène a réagi avec la bande de 42–44 kDa. La réduction de la fonction des PMN pendant la période du vêlage peut être mise en rapport avec une expression réduite de la L-sélectine sur la surface cellulaire ainsi qu'avec une modulation de la phosphorylation de molécules fonctionnellement importantes.

phosphorylation / neutrophile / mammite / L-sélectine / tyrosine

1. INTRODUCTION

Diseases of economic importance in dairy cows such as mastitis and metritis, are related to parturition [5]. Studies performed around parturition have reported an impairment in bovine leukocyte functions [2, 12, 16, 21]. During infection, polymorphonuclear neutrophil leukocytes (PMN) are the first cells recruited, attracted by chemotactic agents such as cytokines released from activated endothelium and macrophages [6]. During this early recruitment process, L-selectin and β_2-integrin adhesion receptors are responsible for rolling and attachment of PMN to the endothelium [36, 40]. Kishimoto and Rothlein [22] described the L-selectin mediated rolling of PMN on the endothelial surface as a passive event. Chemoattractants released from the endothelial surface trigger the shedding of membrane bound L-selectin and results in a firm CD18-mediated adhesion [23, 26]. Besides the well-known adhesion functions mediated by L-selectin and β_2-integrin, the ability of these molecules to transmit signals inside PMN through specific tyrosine residues and phosphorylation of protein kinase domains has recently gained considerable attention [37, 38]. Tyrosine

phosphorylation represents a common activation pathway in PMN in response not only to binding of adhesion receptors, but also in response to chemotactic and inflammatory stimuli [13, 32].

Because a relationship exists between increased susceptibility to intramammary infection and decreased leukocyte function around parturition [5], further investigation into this relationship was warranted. In this research we assessed changes in L-selectin expression and protein tyrosine phosphorylation in bovine PMN around parturition.

2. MATERIALS AND METHODS

2.1. Animals

Eight clinically healthy Holstein cows in their last 2 weeks of gestation were selected for study. Use of animals for this investigation was approved by the Beltsville Agricultural Research Center Animal Care and Use Committees. During the study the normal feeding and housing routine of the herd was maintained. Starting at calving, weekly mammary quarter secretions were aseptically collected for diagnostic bacteriology outlined in the National Mastitis Council's handbook [30] and somatic cell counts (Fossomatic 90, Foss Food Technology, Hillerod, Denmark) [27].

2.2. Blood sample collection

Blood samples (40 mL) were collected at 0700 h from the medial caudal vein into vacutainer tubes containing acid citrate dextrose (ACD) (33.4 g of dextrose, 19.98 g of citric acid, 33 g of sodium citrate in 100 mL of sterile double-distilled water [10 mL blood : 1 mL ACD]). Blood sampling around parturition was scheduled for d –21, –14, –7, 0 (d of calving), +1, +2, +7, +14 and +28.

2.3. Preparation of lipopolysaccharide activated-fetal bovine serum

A suspension of 1 μg/mL of *Escherichia coli* 0128 : B12 lipopolysaccharide (LPS, DIFCO Laboratories, Detroit, MI, USA) in 0.01 M phosphate buffered 0.85% saline solution, pH 7.4 (PBSS) was combined with an equal volume of undiluted fetal bovine serum (FBS) (JRH Sciences, Lenexa, KS, USA), incubated for 30 min at 37 °C and for 30 min at 56 °C, to prepare the crude C5a anaphylatoxin preparation [29]. The LPS-FBS was kept at –80 °C and thawed immediately before use.

2.4. L-selectin binding and expression

Whole blood (100 μL) was incubated with 10 μL of PBSS or 10 μL of LPS-FBS for 10 min at 39 °C. Blood was then incubated for 30 min at 4 °C with either 10 μL of mouse IgG_1 isotype control (Sigma Chemical Co., St Louis, MO, USA, 0.2 mg/mL) or 10 μL of anti-bovine L–selectin MAB 11G10 (ammonium sulfate purified, total protein 0.1 mg/mL) [39]. Each treatment was performed in duplicate. After incubation, cells were washed twice with 3 mL of ice cold PBSS and centrifuged (4 °C, 600 × *g*, 5 min). After aspiration of the supernatant, the cell pellet was resuspended in 500 μL of PBSS. The red blood cells (RBC) were lysed by adding 1 mL of sterile water for 45 s and restoring isotonicity with 500 μL of 2.7% saline. After centrifugation (4 °C, 1 300 × *g*, 5 min), fluorescein-labeled goat anti-mouse IgG plus IgM (heavy light chain) (Kirkegaard & Perry Inc, Gathersburg, MD, USA, 0.025 mg/mL) was added (100 μL) to the cell pellet and incubated for 45 min at 4 °C. The cells were washed twice with 2 mL of ice cold PBSS with centrifugation (4 °C, 660 × *g*, 5 min). One milliliter of 1% paraformaldehyde in PBSS was added to the cell pellet. Cells were kept in the dark at 4 °C until analyzed by flow cytometry (Coulter Profile, Coulter Electronics Inc., Hialeah, FL, USA).

2.5. Flow cytometry

To measure MAB binding, a flow cytometer (Coulter Profile, Coulter Electronics Inc., Hialeah, FL, USA) equipped with an air-cooled argon ion laser was used. The laser was set at 488-nm wavelength, 7.0 to 7.5 A current and 15mW power. The cytometer was aligned daily using 9.7 μm fluorospheres (Coulter Electronics Inc., Hialeah, FL, USA). The PMN were gated on the basis of light scattering properties. Green fluorescence was measured using a 525 nm band pass filter. The percentage of PMN fluorescence and log mean fluorescent channel (LMFC), which measures receptor density, were determined by measuring the green fluorescence associated with gated PMN and correcting for background (mouse IgG_1 isotype control).

2.6. Preparation of PMN cell lysate

Bovine PMN were isolated from each of the eight cows by differential centrifugation and hypotonic lysis of RBC using established procedures [8]. Total leukocyte counts were determined electronically (Multisizer, Coulter Electronics Inc., Hialeah, FL, USA). Differential cell counting was performed by microscopic evaluation of Wright-stained smears. Two hundred cells were counted on each of two slides. Viability was assessed by trypan blue dye exclusion test. On average, 95% were PMN with a viability of 98%. Cell suspensions were adjusted to 5×10^6 viable PMN/mL of PBSS. Blood and cells were maintained at 4 °C throughout the isolation.

For preparation of cell lysates, 500 μL of PMN suspension were aliquoted into Ependorff tubes and centrifuged at 31 000 × *g* for 1 min. The supernatant was discarded and the pellet was resuspended in 50 μL of PBSS. One hundred and fifty microliters of 2 × Laemmli buffer (2% sodium dodecyl sulfate [SDS], 5 mM sodium phosphate, 10% glycerol, 0.1 M dithiothreitol, 5% β-mercaptoethanol, 0.01% bromophenol blue) were added and the solution was passed twice through a syringe (once through a 20 gauge needle and once through a 27 gauge needle). The preparation was heated at 100 °C for 5 min and stored at –80 °C until analysis.

2.7. Tyrosine phosphorylation and activation of mitogen activated protein kinase

For each of the eight cows, a gel was run with PMN lysates from all the sampling times for a single cow. The order of the samples added to the gel (10 μL) corresponded to the blood sampling during the study. Cell lysates were separated on a 10% or 12% Tris-glycine gel (Invitrogen Corp, Carlsbad, CA, USA), and then transferred onto a nitrocellulose membrane. After blocking the membrane with 1% BSA in PBSS containing 0.01% Tween 20 (PBSS-T) for 1 h at room temperature with gentle rocking, the membrane was probed with either anti-phosphorylated tyrosine MAB 4G10 (1 μg/mL; Upstate Biotech, Lake Placid, NY, USA), anti-mitogen activated protein kinase (MAPK) polyclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) or IgG_1 isotype control MAB (Sigma Chemical Co., St. Louis, MO, USA). The membrane was washed three times with PBSS-T, and then incubated with biotin-sp (long space)- conjugated goat-anti-mouse antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). After washing, the membrane was incubated with HRP-conjugated streptavidin (1:1500, Amersham Life Sciences, Piscataway, NJ, USA). The membrane was developed with ECL Western blotting reagent (Amersham Life Sciences, Piscataway, NJ, USA).

2.8. Scanning of blots

The intensity of the bands present in the blots was determined by scanning with a Shimadzu CS-9000 dual wavelength flying-spot scanner (Shimadzu

Scientific Instruments, Inc. Columbia, MD, USA) equipped with a tungsten lamp at a wavelength of 370 nm. The scanner measures absorbance by reflectance spectrophotometry over the distance the beam of light moves along the lane measured, in mm. The measurement reported is the area under the curve of the peak that is proportional to the density of the band measured. The area under the curve was expressed as "$\log_{10}$". Because of distortion of the left lane on the blots, a reliable scan of the 21d band could not be obtained.

2.9. Statistical analysis

Residuals were examined for homogeneity using PROC MIXED in SAS (SAS Inst. Inc., Cary, NC, USA). Homogeneity of variance was examined by comparing a covariance structure with constant variance on the diagonal to a structure with heterogeneous variance. Lack of independence of residuals (i.e. correlated residuals) was examined by comparing a covariance structure with constant correlation to a structure with variable correlations. Least-squares analysis of variance was conducted to determine significance, by time of sample collection for LMFC, % cells fluorescing and band intensity in the blots. The Duncan multiple-range test was used to assess individual differences among treatment means.

3. RESULTS

3.1. Binding of anti-L-selectin MAB to PMN and receptor expression

For unstimulated PMN in group 1 (Fig. 1) 93.50 to 98.73% of the PMN isolated 2 weeks before to 4 weeks after calving bound anti-bovine L-selectin MAB. The percentage of PMN binding anti– L-selectin MAB tended to decrease ($P < 0.10$) from 96.17% on d –14 and 97.06% on d –7 to 93.81% at calving. After parturition a gradual increase in percentage PMN binding MAB was observed that reached maximum ($P < 0.02$) at d +14 (98.73%) and d +28 (98.63%) when compared to d 0 (93.81%).

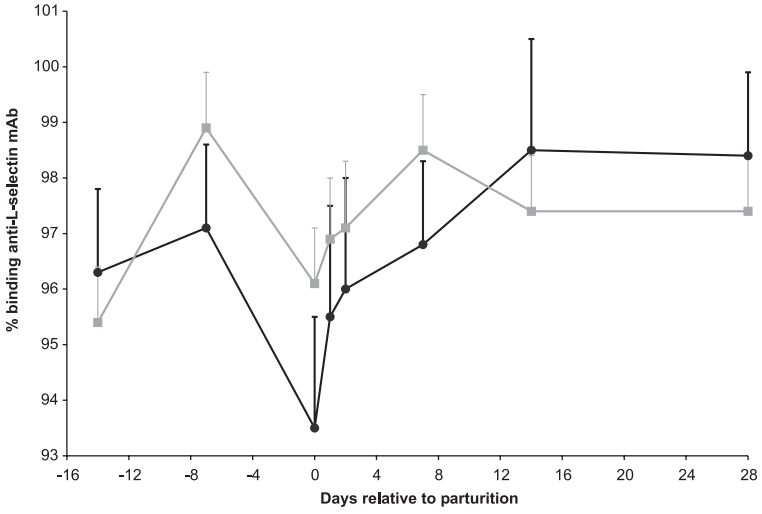

Figure 1. Percentage of resting (●) and lipopolysaccharide-activated ( ) PMN binding anti-bovine L-selectin MAB around parturition. The data from eight cows are expressed as means and standard errors.

Following parturition, receptor expression per cell decreased 34% ($P < 0.05$) from d –7 to d +1 then gradually increased 30% ($P < 0.01$) from d +1 to d +28 (Fig. 2). Trends in% PMN binding and receptor expression of LPS-FBS stimulated PMN were not significantly different from unstimulated PMN (Figs. 1 and 2).

3.2. Tyrosine phosphorylation

Electrophoretic separation of unstimulated PMN lysates followed by Western blotting using anti-phosphotyrosine MAB 4G10 resulted in the appearance of 11 distinct protein bands with extrapolated kDa of 12, 17, 21, 26, 33, 42–44, 50, 60, 70, 83, and 90 (Fig. 3). Samples collected at each time period showed variation in the intensity of the 42–44 and the 90 kDa bands. Intensity of the 42–44 kDa band increased from d –21 to d –14, decreased on d –7 through d +2, increased on d +7 then decreased to lowest on d +28. Although intensity of this band tended to decrease between d +7 and +28 ($P < 0.06$), a reduction was observed between samples from d +7 and +28 ($P < 0.02$). Intensity of the 90 kDa band was more variable and no consistent trend relative to parturition could be established.

3.3. Detection of a 42–44 kDa band using anti-MAPK antibodies

By probing identical blots as above using polyclonal antibody to human MAPK the 42–44 kDa band was tentatively identified as MAPK (Fig. 4). The doublet bands, in this representative blot for one cow, appeared to correspond to ERK-1 and ERK-2 as suggested by probing with polyclonal antibodies against human MAPK. Control blots, which were run in the absence of primary antibody, indicated the specificity of the antibody for MAPK (data not shown). The average intensity of the bands present in the blots for the eight cows is shown (Fig. 5). The intensity of the 42–44 kDa band gradually increased from d –7, peaked at d +7 ($P < 0.03$), and steadily decreased to d +28 ($P < 0.02$).

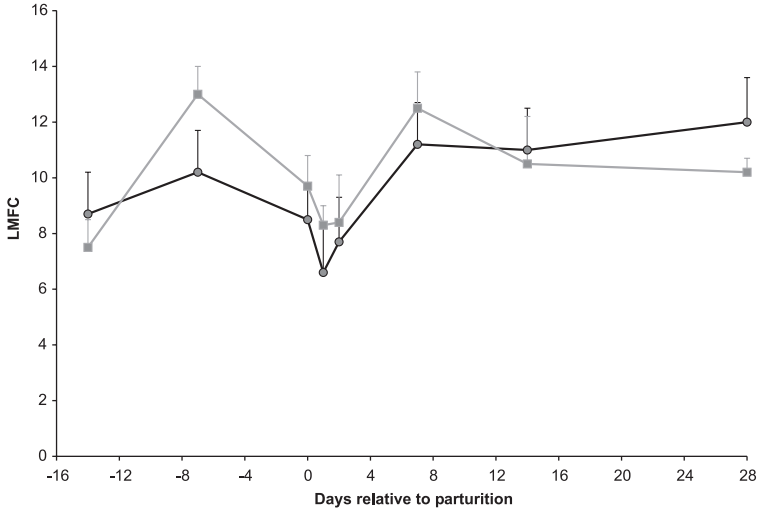

Figure 2. Log mean fluorescent channel (LMFC) of L-selectin expression on resting (●) and lipopolysaccharide-activated ( ) PMN around parturition. The data from eight cows are expressed as means and standard errors.

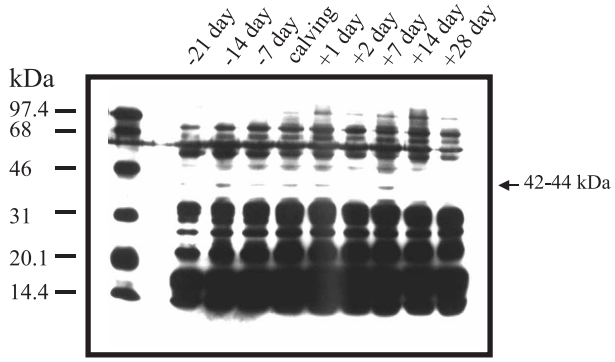

Figure 3. Western blot of unstimulated PMN lysates after electrophoretic separation and staining with anti-phosphotyrosine MAB 4G10. This blot was selected at random from blots obtained from eight cows.

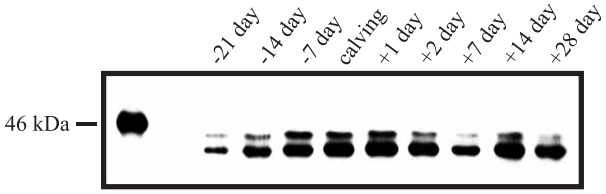

Figure 4. Staining of the 42-44 kDa band with polyclonal antibody to human mitogen activated protein kinase. This blot was selected at random from blots obtained from eight cows.

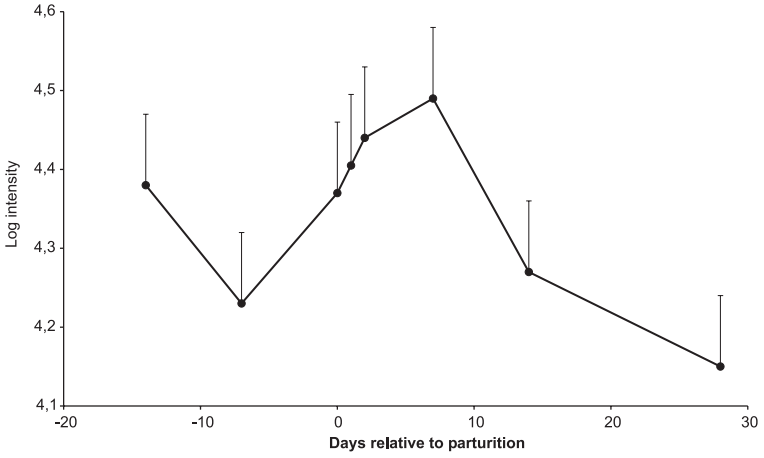

Figure 5. Intensity of the 42-44 kDa tyrosine phosphorylated band from bovine neutrophils around parturition. Because of distortion of the left lane on the blot, a reliable scan of the –21 d band could not be obtained. The data from eight cows are expressed as means and standard errors.

4. DISCUSSION

Neutrophils are considered as the first line of defense against infection, and their ability to react to inflammatory stimuli is dependent on functionality and integrity of the cell. PMN migrate within minutes to the site of inflammation, and the processes of rolling, adhesion and migration are the first PMN functions involved in immune defense [33, 36]. In this study, effects of parturition on the expression of membrane bound L-selectin by resting and activated PMN, and assessment of changes in intracellular protein tyrosine phosphorylation were investigated. Results indicated a reduced expression of L-selectin around parturition when compared with weeks 2 and 4 after calving. We think that this reduction in L-selectin receptors can have a negative impact on the ability of PMN to be recruited into the mammary gland. Random migration and chemotaxis are impaired during the first week after calving [2, 28], as are phagocytosis [16, 21, 34] and respiratory burst activity [12, 19, 21]. A recent study reported a significant drop in L-selectin expression between 9 and 24 h after calving [25]. Our results confirm and extend those findings. Other studies [7, 24] have shown that a pre-calving defect in PMN superoxide production and chemotaxis were predisposing factors to the outcome and severity of mastitis postpartum. Moreover, other authors [35] concluded that the outcome of experimentally induced *E. coli* mastitis during the periparturient period involved not only impaired chemotaxis, but also defects in up-regulation of CD18 adhesion receptors. The totality of these findings could contribute to the high susceptibility of intramammary infection in dairy cows during the periparturient period.

Increased plasma cortisol and estrogen around parturition [16] may also contribute to L-selectin shedding. Treatment with dexamethasone and cortisol resulted in down-regulation of membrane bound L-selectin [4] on resting and activated PMN. Burton and Kehrli [3] also observed that glucocorticoid-mediated down regulation of membrane bound L-selectin and CD18 on PMN increased mammary gland susceptibility to *Staphylococcus aureus* following experimental challenge. Studies performed on human endothelial cells demonstrated that 17β-estradiol inhibited IL-1-mediated endothelial cell adhesion molecule transcription and resulted in diminished membrane protein expression and reduced IL-1-induced leukocyte adhesion [9]. Another factor contributing to shedding of L-selectin at parturition is the high plasma levels of cytokines (IL-1, IL-6, IFN-γ, TNF) in dairy cows during the periparturient period [14]. Kishimoto and Rothlein [22], Lynam et al. [26], Griffin et al. [15], demonstrated a clear shedding of membrane bound L-selectin after PMN activation with different agents such as PMA, C5a, LTB_4, IL-1, LPS and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Cleavage of L-selectin would be intended either as a signal to switch to a β_2-integrin dependent adhesion, or to serve as a regulatory balance in PMN adherence by this receptor to the endothelium [20]. During our study, a group of PMN were activated with LPS-FBS, which generates the chemoattractant C5a. Treatment of PMN with LPS-FBS did not affect expression of L-selectin when compared to resting PMN. In the study by Lee and Kehrli [25], they also found no effect on L-selectin expression in PAF (platelet activating factor) stimulated bovine PMN compared to resting PMN obtained during the periparturient period. A possible explanation for the lack of additional shedding of L-selectin from PMN after incubation with LPS-FBS could be that the PMN were already activated by the high concentrations of cytokines in plasma during late pregnancy and parturition [14]. This activation would result in a rapid decrease in L-selectin expression as a result of proteolysis at the cell membrane

level [20], rendering the PMN resistant to further shedding of L-selectin by the C5a generated by LPS-FBS.

The assessment of tyrosine phosphorylation in resting bovine PMN during the periparturient period in this study revealed many bands that reacted with anti-phosphotyrosine MAB. The bands that we considered of interest were the ones corresponding to 42–44 kDa and 90 kDa. The 42–44 kDa band increased in intensity starting from d –7 to d +7 of parturition. Having found a clear cross-reactivity of this band with anti MAPK polyclonal antibody leaves us to conclude that this band corresponds to MAPK. The MAPK are a group of protein serine/threonine kinases that are involved in cell proliferation and differentiation. The MAPK family includes three subgroups; 42–44 kDa ERK1/ERK2, JNK/SAPK, and p38 kinase. Activation of MAPK requires phosphorylation of both tyrosine and threonine residues [1]. Shedding of L-selectin from the cell surface of PMN indicates increased adhesiveness and aggregation of PMN. Activation of MAPK occurs following shedding of L-selectin [10, 37] similar to what was observed in the present study. The involvement of tyrosine phosphorylation on adherence of human PMN was demonstrated by others [11, 31]. Hidari et al. [18] also reported that the ligation of P-selectin glycoprotein ligand-1 (PSGL-1), activated ERK1/ERK2 in human PMN. Interaction between PSGL-1 and L-selectin contributes to shear-dependent PMN aggregation [17]. The increased phosphorylation indicated the activation of PMN which was consistent with our finding in this study that PMN did not shed L-selectin after treatment with LPS-FBS. The 90 kDa band that was expressed in PMN at calving still has to be verified. Nevertheless, this band is similar in MW to membrane bound epitopes that bind L-selectin in bovine PMN lysates run under reducing conditions [39].

The results from this study indicate that around the time of parturition bovine PMN express reduced amounts of the adhesion molecule L-selectin. Reduced surface expression of L-selectin could render PMN less capable of transendothelial migration, rendering the gland more susceptible to infection. Electrophoretic separation of PMN lysates followed by Western blotting using anti-phosphotyrosine MAB resulted in the appearance of 11 distinct protein bands. Intensity of the 42–44 kDa band, tentatively identified as MAPK, decreased in intensity starting 7 days following parturition. Because MAPK are a group of protein kinases involved in cell proliferation and differentiation, their reduction could have a negative impact on neutrophil function.

REFERENCES

[1] Anderson N.G., Maller J.L., Tonks N.K., Sturgill T.W., Activation of MAP kinase requires integration of signals from two distinct phosphorylation pathways, Nature 343 (1990) 651-653.

[2] Berning L.M., Paape M.J., Peters R.R., Functional variation in endogenous and exogenous immunoglobulin binding to bovine neutrophils relative to parturition, Am. J. Vet. Res. 54 (1993) 1145-1153.

[3] Burton J., Kehrli M.E., Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol– and dexamethasone-treated cows, Am. J. Vet. Res. 56 (1995) 997-1006.

[4] Burton J., Kehrli M.E., Kapil S., Horst R.L., Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone, J. Leukoc. Biol. 57 (1995) 317-325.

[5] Burvenich C., Detilleux J., Paape M.J., Massart-Leën A.M., Physiological and genetic factors that influence the cows resistance to mastitis, especially during early lactation, Proc. Symposium on Immunology of Ruminant Mammary Gland, Stresa, Italy, June 11–14, 2000, pp. 9-20.

[6] Butcher E.C., Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity, Cell 67 (1991) 1033-1036.

[7] Cai T.Q., Weston P.G., Lund L.A., Brodie B., McKenna D.J., Wagner W.C., Association between neutrophil functions and periparturient

disorders in cows, Am. J. Vet. Res. 55 (1994) 934-943.

[8] Carlson G.P., Kaneko J.J., Isolation of leukocytes from bovine peripheral blood, Proc. Soc. Exp. Biol. Med. 142 (1973) 853-856.

[9] Caulin-Glaser T., Watson C.A., Pardi R., Bender J.R., Effects of 17β-estradiol on cytokine-induced endothelial cell adhesion molecule expression, J. Clin. Invest. 98 (1996) 36-42.

[10] Crockett-Torabi E., Selectins and mechanisms of signal transduction, J. Leukoc. Biol. 63 (1998) 1-14.

[11] Fourtes M., Jin W.W., Nathan C., Adhesion-dependent protein tyrosine phosphorylation in neutrophils treated with tumor necrosis factor, J. Cell Biol. 120 (1993) 777-784.

[12] Gilbert R.O., Grohn Y.T., Miller P.M., Hoffman D.J., Effect of parity on periparturient neutrophil functions in dairy cows, Vet. Immunol. Immunopathol. 36 (1993) 75-82.

[13] Girard D., Paquin R., Naccache P.H., Beaulieu A.D., Effects of interleukin-13 on human neutrophil functions, J. Leukoc. Biol. 59 (1996) 412-419.

[14] Goto M., Maruyama M., Kitadate K., Kirisawa R., Obata Y., Koiwa M., Iwai H., Detection of interleukin-1β in sera and colostrum of dairy cattle and in sera of neonates, J. Vet. Sci. 59 (1997) 437-441.

[15] Griffin J.D., Spertini O., Belvin M.P., Levine H.B., Kanakura Y., Tedder T.F., Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursor, J. Immunol. 145 (1990) 576-584.

[16] Guidry A.J., Paape M.J., Pearson R.E., Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow, Am. J. Vet. Res. 37 (1976) 1195-1200.

[17] Guyer D.A., Moore K.L., Lynam E.B., Schammel C.M., Rogelj S., McEver R.P., Sklar L.A., P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation, Blood 88 (1996) 2415-2421.

[18] Hidari K.I.-P.J., Weyrich A.S., Zimmerman G.A., McEver R.P., Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils, J. Biol. Chem. 272 (1997) 28750-28756.

[19] Hoedemaker M., Lund L.A., Wagner W.C., Function of neutrophils and chemoattractant properties of fetal placental tissue during the last month of pregnancy in cows, Am. J. Vet. Res. 53 (1992) 1524-1529.

[20] Jutila M.A., Rott L., Berg E.L., Butcher E.C., Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1, J. Immunol. 143 (1989) 3318– 3324.

[21] Kehrli M.E., Nonnecke B.J., Roth J.A., Alterations in bovine neutrophil function during the periparturient period, Am. J. Vet. Res. 50 (1989) 207-214.

[22] Kishimoto T.K., Rothlein R., Integrins, ICAMs, and selectins: role and regulation of adhesion molecules in neutrophil recruitment to inflammatory sites, Adv. Pharmacol. 25 (1994) 117-169.

[23] Kishimoto T.K., Jutila M.A., Berg E.L., Butcher E.C., Neutrophil MAC-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors, Science 245 (1989) 1238-1241.

[24] Kremer W.D.J., Noordhuizen-Stassen E.N., Grommers F.J., Daemen A.J.J.M., Henricks P.A.J., Brand A., Burvenich C., Preinfection chemotactic response of blood polymorphonuclear leukocytes to predict severity of *Escherichia coli* mastitis, J. Dairy Sci. 76 (1993) 1568-1574.

[25] Lee E.K., Kehrli M., Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves, Am. J. Vet. Res. 59 (1998) 37-43.

[26] Lynam E.B., Simon S.I., Rochon Y.P., Sklar L.A., Lipopolysaccharide enhances CD11b/CD18 function but inhibits neutrophil aggregation, Blood 83 (1994) 3303-3311.

[27] Miller R.H., Paape M.J., Acton J.C., Comparison of milk somatic cell counts by Coulter and Fossomatic counters, J. Dairy Sci. 69 (1986) 1942-1946.

[28] Nagahata H., Makino S., Takeda S., Takahashi H., Noda H., Assessment of neutrophil function in the dairy cow during the perinatal period, J. Vet. Med. B 35 (1988) 747-751.

[29] Naidu T.G., Newbould F.H.S., A simple method for quantitation of chemotaxis of polymorphonuclear neutrophils in vitro, Immunol. Commun. 3 (1974) 457-468.

[30] National Mastitis Council, Inc., Microbiological procedures for the diagnosis of bovine udder infection, 3rd edition, Natl. Mastitis Counc. Inc., Arlington, VA, USA, 1990.

[31] Rafiee P., Lee J.K., Leung C.C., Raffin T.A., TNF-γ induce tyrosine phosphorylation of mitogen-activated protein kinase in adherent human neutrophils, J. Immunol. 154 (1995) 4785-4792.

[32] Rollet E., Caon A.C., Roberge C.J., Liao N.W., Malawista S.E., McColl S.R., Naccache P.H., Tyrosine phosphorylation in human neutrophils. Comparison of the effects of different classes of agonists and identification of the signaling pathways involved, J. Immunol. 153 (1994) 353-363.

[33] Rosales C., Juliano R., Signal transduction by cell adhesion receptors in leukocytes, J. Leukoc. Biol. 57 (1995) 189-198.

[34] Saad A.M., Concha C., Astrom G., Alterations in neutrophil phagocytosis and lymphocyte blastogenesis in dairy cows around parturition, J. Vet. Med. B 36 (1989) 337-345.

[35] Shuster D.E., Lee E.K., Kerhli M., Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation, Am. J. Vet. Res. 57 (1996) 1569-1575.

[36] Tedder T.F., Steeber D.A., Chen A., Engel P., The selectins: vascular adhesion molecules, FASEB J. 9 (1995) 866-873.

[37] Waddel T.K., Fialkow L., Chan C.K., Kishimoto T.K., Downey G.P., Signaling functions of L-selectin. Enhancement of tyrosine phosphorylation and activation of MAP kinase, J. Biol. Chem. 270 (1995) 15403-15411.

[38] Walzog B., Offermans S., Zakrzewicz A., Gaehtgens P., Ley K., β_2 integrins mediate protein tyrosine phosphorylation in human neutrophils, J. Leukoc. Biol. 59 (1996) 747-753.

[39] Wang Y., Paape M.J., Leino L., Capuco A.V., Narva H., Functional and phenotypic characterization of monoclonal antibodies to bovine L-selectin, Am. J. Vet. Res. 58 (1997) 1392-1401.

[40] Wardlaw A.J., Walsh G.M., Neutrophil adhesion receptors, Immunopharmacology of Neutrophils 7 (1994) 133-157.