Expression of CD14 and CD44 on bovine polymorphonuclear leukocytes during resolution of mammary inflammatory response induced by muramyldipeptide and lipopolysaccharide

T. Langrova[1,2], Z. Sladek[1,2], D. Rysanek[2]

[1]Mendel University of Agriculture and Forestry, Brno, Czech Republic
[2]Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: The aim of the study was to prove the effect of muramyldipeptide and lipopolysaccharide on the expression of CD14 and CD44 during an induced inflammatory response of the mammary gland and its resolution. The purpose was to clarify whether the CD14 and CD44 expression is controlled by the mechanisms of resolution. The CD44 had previously been judged to be an activation marker along with CD11b on polymorphonuclear leukocytes. The experimental inflammatory response was induced by muramyldipeptide and lipopolysaccharide, while phosphate buffered saline was used as a control. The course of the inflammatory response was monitored at four time points: 24 h and 48 h (initiation of inflammatory response), 72 h and 168 h (resolution of inflammatory response). The total number of cells was determined by a hemocytometer. Flow cytometry was used to determine differential counts of leukocytes, proportions of CD11b+ polymorphonuclear leukocytes, proportions of apoptotic and necrotic polymorphonuclear leukocytes, and proportions of CD14+ and CD44+ polymorphonuclear leukocytes. The proportion of CD11b+ polymorphonuclear leukocytes after induction of inflammation with muramyldipeptide was higher ($P < 0.05$) compared to that after induction by phosphate buffered saline, was highly significantly greater after lipopolysaccharide ($P < 0.01$), and remained at approximately the same level for the whole period of observation (168 h). A higher proportion of CD14+ polymorphonuclear leukocytes was observed 72 h after induction with phosphate buffered saline. A statistically highly significant lower proportion was observed after induction with muramyldipeptide ($P < 0.01$), and a statistically significant lower proportion was observed after induction with lipopolysaccharide ($P < 0.05$). Decrease in the proportion of CD14+ polymorphonuclear leukocytes followed. In the initial phase of the inflammatory response (24 to 72 h) there was a gradual increase in the proportion of CD44+ polymorphonuclear leukocytes, and more so after the phosphate buffered saline. A greatly lower proportion of CD44+ polymorphonuclear leukocytes was observed after administration of muramyldipeptide and lipopolysaccharide: 24 h ($P < 0.01$), 48 h ($P < 0.05$) and 72 h ($P < 0.01$). Compared with muramyldipeptide and lipopolysaccharide, there was a statistically highly significant ($P < 0.01$) lower proportion of CD44+ polymorphonuclear leukocytes observed 168 h after induction with phosphate buffered saline. Hence the proportion of CD44+ polymorphonuclear leukocytes is low in the initial phase of inflammation, and CD44, in contrast with CD11b, does not appear to be a polymorphonuclear marker of activation. The results of the study have shown that expression of CD14 and CD44 is controlled by the factors inducing inflammatory response as well as by the mechanisms of resolution.

Keywords: heifer; mammary gland; inflammation; bacterial toxins; apoptosis

Polymorphonuclear leukocytes (PMN) play an important role in immunity, since they are responsible mainly for nonspecific defense of the body against invading microorganisms, especially bacteria (Smith, 2000). The function of PMN is to eliminate pathogenic microorganisms during

Supported by the Czech Science Foundation (No. 524/03/1531) and the Ministry of Agriculture of the Czech Republic (MZE 0002716201).

the initial phase of acute inflammation, when migration of these cells from blood to the affected tissue occurs. Lipopolysaccharide (LPS), a component of Gram-negative bacteria's cellular wall, and muramyldipeptide (MDP), as a minimal structural unit of peptidoglycan in Gram-positive bacteria, have an ability to induce inflammatory response (Sladek and Rysanek, 2000; Rysanek et al., 2001; Sladek et al., 2002).

The immune system is capable of recognizing these molecules of bacterial toxins by means of two types of "toll like" receptors (TLR). TLR2 recognizes Gram-positive bacterial components (peptidoglycan) while TLR4 recognizes LPS, a component of Gram-negative bacteria (Takeuchi et al., 2000a). Both structural components have an affinity to CD14. Therefore, CD14 is a polyspecific receptor with a manifold recognition potential for Gram-negative and Gram-positive bacteria (Van Miert, 1991; Otterlei et al., 1993). CD14 is expressed as a glycosylphosphatidylinositol-anchored cell surface molecule lacking a transmembrane domain. Engagement of CD14 by ligands like LPS, intact bacteria or apoptotic cells can result in either pro- or anti-inflammatory responses (Schutt, 1999). Although membrane CD14 (mCD14) requires TLR4, soluble CD14 (sCD14) requires both LPS-binding protein (LBP) and TLR4 to induce downstream signaling cascades (Fenton and Golenbock, 1998). MDP binds to CD14 directly (Takeuchi et al., 2000b).

PMN have a short lifespan and inability to remigrate back to the blood stream (Hughes et al., 1997). This creates a potential risk for the surrounding tissue, as the histotoxic content is discharged to the extracellular space during the necrosis of PMN. Therefore, resolution (i.e., termination of inflammation) is necessary to maintain homeostasis of the organ. Resolution is realized by functional and physical elimination of PMN through a process called programmed cell death, or apoptosis. Apoptosis is a physiological manner of cell death, which is genetically controlled, restricts damage to inflamed tissue, and supports resolution of inflammation (Savill, 1997). Resolution is accomplished via phagocytosis of apoptotic cells by macrophages, which prevents release of histotoxic content in granules outside cells. The macrophages do not release proinflammatory mediators to the extracellular space during phagocytosis of apoptotic PMN (Meagher et al., 1992). Although a delay in PMN apoptosis may be advantageous in the initial stage of infection, accelerated PMN apoptosis may be beneficial following elimination of the infection agent (Paape et al., 2003), because activated PMN and their products induce injury to bovine mammary tissue (Capuco et al., 1986).

The first step in elimination of apoptotic PMN is their identification by macrophages. For macrophages to recognize apoptotic PMN, there are important biochemical changes on the surface of their cytoplasmic membranes (Messmer and Pfeilschifter, 2000; Akgul et al., 2001; Savill et al., 2003). Macrophages are subsequently equipped with specific receptors, which have an ability to recognize apoptotic PMN (Savill et al., 1993).

During the study of CD14's role in an inflammatory response within the heifer mammary gland, active participation of CD14 during resolution was considered. The role of the membrane receptor CD14 in the heifer mammary gland was studied in mastitis induced by LPS, *S. aureus* and *S. uberis*. It was found that the inflammatory response of the mammary gland to LPS was associated with an expression of CD14 receptors by PMN and macrophages (Paape et al., 1996; Sladek et al., 2002), whereas expression of CD14 receptors greatly depends on the stage of the inflammatory response within the mammary gland. In the initial phase, a decreased expression of CD14 (Paape et al., 1996) was observed, while there was an increasing expression of resolution characteristics in the inflammatory process (Sladek et al., 2002). It has also been found that expression of CD14 on PMN highly correlates with the presence of apoptotic PMN (Sladek and Rysanek, 2006).

Association of LPS with LBP and subsequent creation of CD14-TLR-4 (Takeuchi et al., 1999) could result in an induction of CD44 expression. In recent years, several different observations have implicated CD44 in the regulation of the inflammatory response. Elevated local concentrations of CD44 ligands (such as hyaluronan and fibronectin) that follow tissue injury are likely to be important mediators of macrophage function as the inflammatory response progresses (Vivers et al., 2002). CD44 can mediate some PMN adhesion and emigration, but it does not seem to affect subsequent migration within tissues (Khan et al., 2004). Teder et al. (2002) observed that the cell-surface adhesion molecule and hyaluronan receptor CD44 plays a critical role in resolving lung inflammation. Using hyaluronan-coated beads and erythrocytes coated with anti-CD44 antibodies as the phagocytic prey, Vachon et al. (2006) determined that CD44 medi-

ates efficient phagocytosis in primary murine peritoneal macrophages.

If expression of CD14 on PMN after induction of inflammatory response by LPS is accompanied by expression of CD44, there is a question of whether the toxin of Gram-positive pathogens induces similar expression and whether the CD14 and CD44 expression is controlled by the mechanisms of resolution. The aim of this study was therefore to prove the aforementioned hypotheses. For this purpose an experimental inflammatory response of the mammary gland was used, which was induced by MDP and LPS, which are analogues of Gram-positive and Gram-negative pathogens.

MATERIAL AND METHODS

Experimental animals and design

The study was performed with eight clinically healthy virgin heifers that were crosses of the Holstein and Czech Pied breeds aged 15–18 months. Animals were kept in certified experimental stabling and fed a standard ration of hay and feed supplements. The mammary glands of experimental animals were examined bacteriologically prior to each experiment.

An inflammatory response of the mammary gland was induced in the animals using an inert factor of phosphate buffered saline (PBS), which served as a control, and components of the cellular wall from Gram-positive bacteria (MDP) and Gram-negative bacteria (LPS). The mammary glands were flushed at each time point (see below) and cell suspensions were processed for determining the total leukocyte count and analyzing the viability of PMN in a light microscope. A flow cytometer was used for analyzing CD11b, CD14 and CD44 expression on the surface of PMN and detecting apoptosis and necrosis of PMN.

Induction of inflammatory response

The following were used for inducing inflammatory response: (i) PBS (Sigma, Saint Louis, Missouri, USA) 0.01M, pH 7.4, with a volume of 20 ml/mammary gland, (ii) MDP – synthetic derivative (MurNAc-L-Abu-D-IsoGln, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic) in a concentration of 500 μg in 20 ml PBS, (iii) LPS of *Escherichia coli*, serotype 0128:B12 (Sigma, USA) in a concentration of 5 μg in 20 ml PBS.

Mammary glands were disinfected with 70% ethanol, especially around the external orifice of the teat duct, and subsequently lavaged using adjusted urethral catheters (AC5306CH06, Porges S.A., France). PBS-treated mammary glands were set as a control for the MDP and LPS, as was done in our previous studies (Sladek and Rysanek, 2000; Sladek et al., 2002).

After inducing the inflammatory response, the mammary glands were rinsed according to the following scheme: right front – 24 h, right rear – 48 h, left front – 72 h, left rear – 168 h after using the relevant inductor.

Processing of cell suspension

Collected lavages were immediately evaluated visually and samples were collected for bacteriological examination, for determining total cell counts, and for determining cell viability. Bacteriological examination was performed by cultivating on blood agar with 5% washed ram erythrocytes and aerobic cultivation at 37°C for a period of 24 h. Only results of pathogen-free samples were used for further processing.

The total number of cells was determined by a hemocytometer. Viability of cells in heifers' mammary glands was evaluated by a test of trypan blue exclusion. Cells viability was always higher than 97%. Cell suspension was then centrifuged for a period of 10 min at 200 g and 4°C. One milliliter of supernatant was retained for resuspension of the pellet. The remaining supernatant was recanted.

Detection of PMN apoptosis

Apoptotic PMN were determined by flow cytometer (FCM) using for detection two different biochemical markers for apoptosis:

(i) Staining with Annexin V labeled with FITC (fluorescein isothiocyanate) and PI (propidium iodide) (Vermes et al., 1995). Annexin-V-FLUOS Staining Kit (Boehringer Mannheim, Mannheim, Germany) was used for staining in accordance with the manufacture's instructions.

(ii) Staining with commercial SYTO 13 green fluorescent nucleic acid stain (Molecular Probes,

Eugene, Oregon, USA) was used as described by Dosogne et al. (2003) in a slight modification: 490 µl of cell suspension in RPMI 1640 was stained with 10 µl of diluted (1:40) SYTO 13 solution.

Detection of surface antigens

Mouse anti-ovine CD14 VPM65 (Serotec, Oxford, UK) diluted 1:20 and fluorescein isothiocyanate-labeled goat anti-mouse IgG1-R-PE (SouthernBiotech, Birmingham, Alabama, USA) diluted 1:500 were used as the primary and secondary antibodies, respectively. Negative control samples were stained with the secondary antibody only.

Mouse anti-ovine antibody CD44 BAG40A (VMRD Inc. Pullman, Washington, USA) diluted 1:50 and FITC labeled IgG3 (SouthernBiotech, Birmingham, Alabama, USA) diluted 1:100, and CD11b MM10A (VMRD Inc. Pullman, Washington, USA) diluted 1:20 and FITC labeled IgG2b (SouthernBiotech, Birmingham, Alabama, USA) diluted 1:100 were used as the primary and the secondary antibodies, respectively.

For analysis, the FACS Calibur flow cytometer and CELLQuest™ software (Becton Dickinson, Mountain View, California, USA) were used. Obtained dot plots were subsequently evaluated using the software WinMDI 2.8 (Trotter, 2000). A 20 000 PMN/dot plot was always analyzed.

Statistical analysis

Obtained data were statistically processed with the STAT Plus software program (Matouskova et al., 1992). The differences in percentage of apoptotic, necrotic, CD14+, CD44+ and CD11b+ PMN after induction with PBS, MDP and LPS were statistically evaluated using single factor analysis of variability (ANOVA) and by assessment of significance of differences using Sheffe's method of multiple comparisons. The relationship between CD14+ and CD44+ PMN was performed by correlation analysis.

RESULTS

The inflammatory response of mammary glands to the inductors used

Intramammary administration of PBS, MDP and LPS resulted in an inflammatory response of the mammary gland that was characterized by changes in the total and differential cell counts. The course of inflammatory response was characterized by two phases – initiation and resolution.

The total number of cells in the initial phase of the inflammatory response, 24 h after induction, was significantly increased. The increase was greatest after induction with LPS, less so after MDP, and the least after PBS. The difference compared with PBS was statistically highly significant for LPS and MDP ($P < 0.01$). Gradual decline of the total leukocyte count followed after induction by all inductors. A significant difference relative to PBS ($P < 0.05$) was observed only after induction with LPS (Figure 1).

In the initial phase of inflammatory response (24 h) there were more PMN in the cell population than lymphocytes and macrophages (Figure 2).

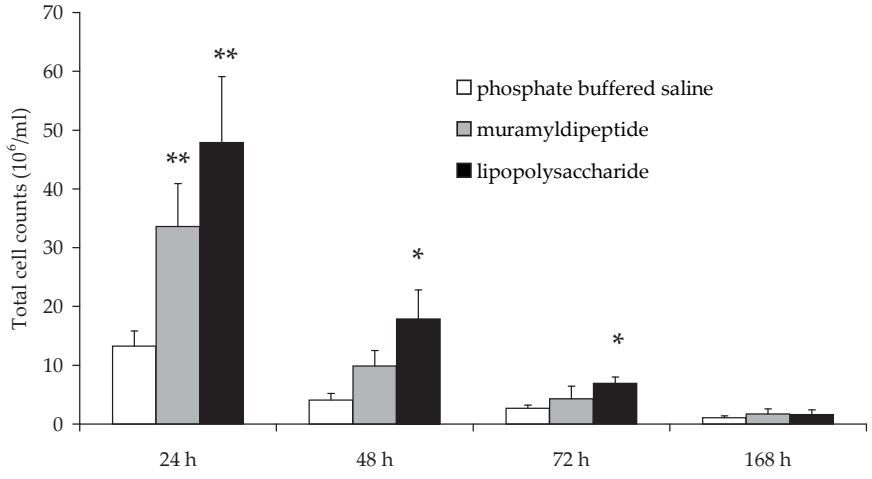

Figure 1. Total cell counts (*$P < 0.05$, **$P < 0.01$)

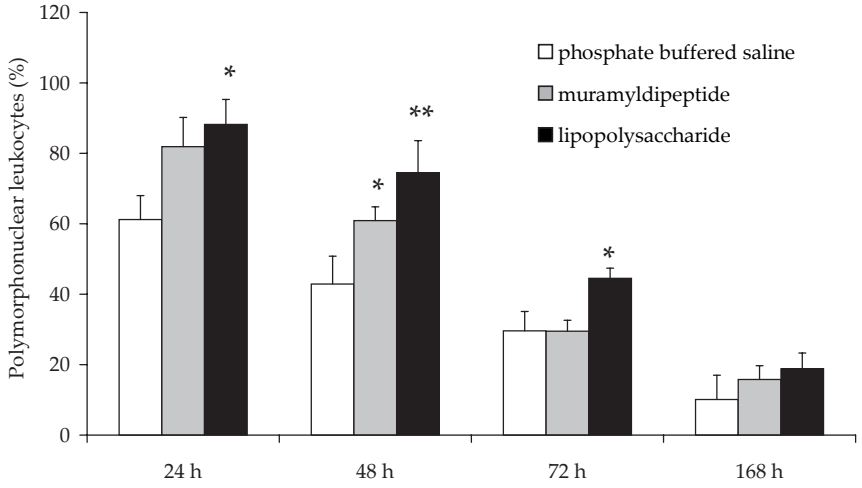

Figure 2. Proportion of polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

A statistically significant difference ($P < 0.05$) was observed between PBS and LPS. The count of PMN gradually decreased through the following time points. There was a statistically highly significant difference observed at 48 h between the inductors PBS and LPS; there was a significant difference at 48 h between PBS and MDP and at 72 h between PBS and LPS. Resolution of the inflammatory response was therefore faster after using the PBS than after using MDP and LPS.

The proportion of CD11b+ PMN can be seen in Figure 3. The proportion of CD11b+ PMN after induction of inflammation was higher compared with the control (PBS) after induction with MDP ($P < 0.05$); there was a statistically significantly greater difference after LPS ($P < 0.01$) and it was maintained at approximately the same level for the whole period of observation (168 h). The proportion of CD11b+ PMN 72 h after induction with PBS dropped to a level that was maintained even after 168 h. The difference between the control and trial inductors was statistically highly significant after 72 h and 168 h ($P < 0.01$).

Apoptosis of PMN during an inflammatory response of the mammary gland

Apoptosis was detected in the population of PMN during inflammatory responses induced by all factors (Figure 4). The proportion of apoptotic PMN (AnnexinV+/PI– PMN) reached the maximum in the period of observation within 72 h after induction of the inflammatory response. At the same time, the highest increase was observed after induction with PBS. Trial inductors resulted in an increase in the proportion of apoptotic PMN after 48 h. The achieved level was maintained through the whole period of observation (48–168 h). Thus, the statistical significance of the difference between the control

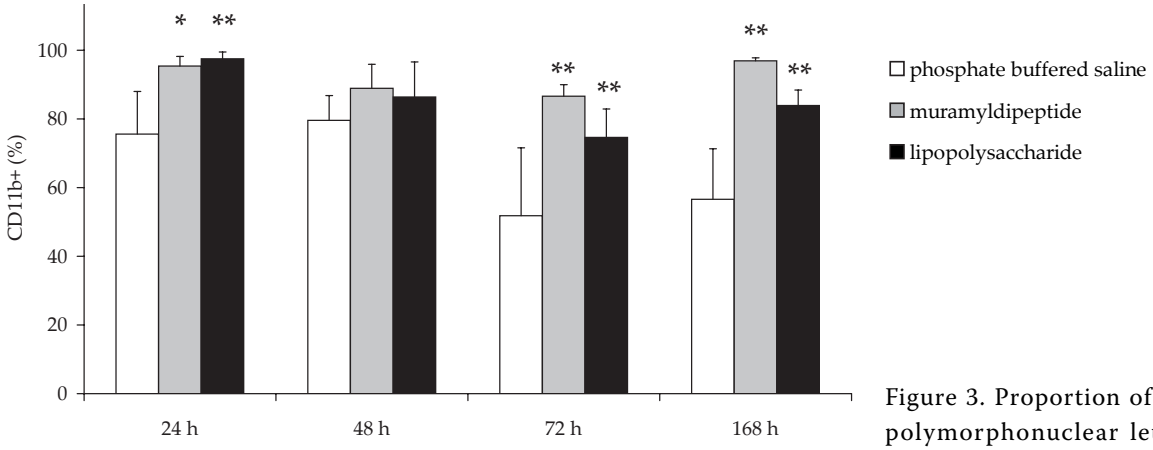

Figure 3. Proportion of CD11b+ polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

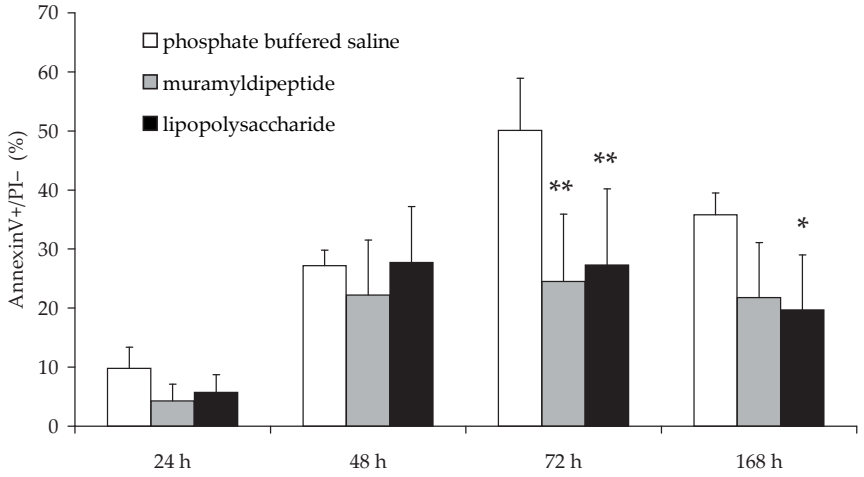

Figure 4. Proportion of AnnexinV+/PI– polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

(PBS) and trial inductors (MDP and LPS) was observed 72 h after induction ($P < 0.01$) (Figure 4).

Similar dynamics of the proportion of apoptotic PMN were also shown by the method using SYTO 13 staining (Figure 5). As in the case of staining with AnnexinV/PI, the highest proportion of apoptotic PMN was seen 72 h after the inductions with PBS. Also, a statistically highly significant difference was observed in the proportion of apoptotic PMN between the PBS and experimental inductors (MDP and LPS) precisely at this time point.

Necrosis of PMN during an inflammatory response of the mammary gland

An increasing proportion of necrotic PMN was detected by AnnexinV/PI staining, with the most pronounced staining following induction with PBS (Figure 6). The difference between PBS and MDP and between PBS and LPS was statistically highly significant ($P < 0.01$) 168 h after the induction.

Staining with SYTO 13 revealed an increase in the proportion of necrotic PMN for the whole observed period of inflammatory response after induction with LPS; the proportion of necrotic PMN decreased during resolution of inflammatory response after the induction with PBS. The proportion of necrotic PMN detected after induction with MDP did not significantly change during the course of inflammatory response (Figure 7).

The proportion of CD14+ PMN during inflammatory response

Figure 8 shows that the highest proportion of CD14+ PMN was observed 72 h after the induction with PBS. A statistically highly significant lower proportion was observed after induction with MDP ($P < 0.01$), and a statistically significant lower proportion after induction with LPS ($P < 0.05$). Decrease in the proportion of CD14+ PMN followed.

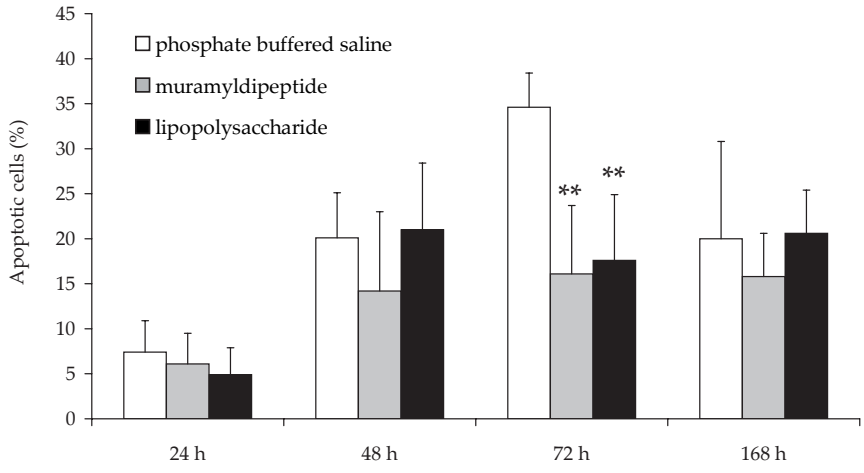

Figure 5. Proportion of apoptotic polymorphonuclear leukocytes – SYTO 13 (**$P < 0.01$)

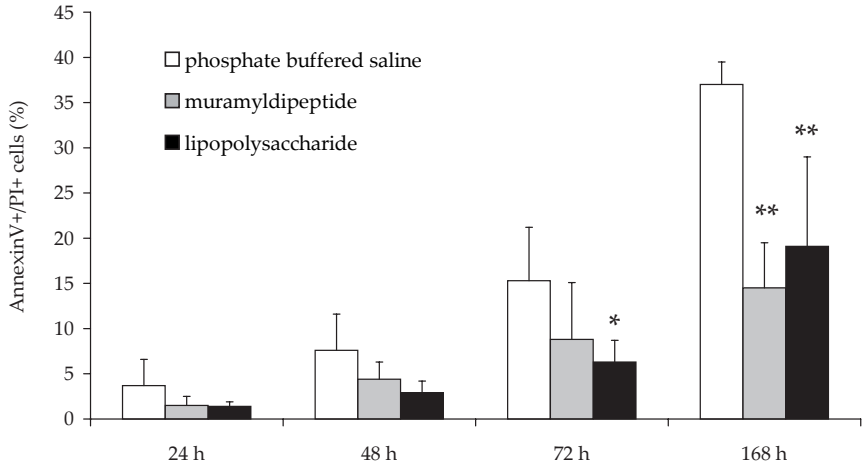

Figure 6. Proportion of AnnexinV+/PI+ polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

The proportion of CD44+ PMN during an inflammatory response

In the initial phase of the inflammatory response (24–72 h) there was a gradual increase in the proportion of CD44+ PMN, and more so following the PBS. Greatly lower proportions of CD44+ PMN were observed after MDP and LPS: 24 h ($P < 0.01$), 48 h ($P < 0.05$) and 72 h ($P < 0.01$). Compared with MDP and LPS, there was a statistically highly significant ($P <$

Table 1. Correlation between proportions of CD14+ and CD44+ polymorphonuclear leukocytes

Inflammatory inductors	Time points (h)	Correlation (r^2)	Significance
MDP	24	0.582	*
	48	0.881	**
	72	0.929	**
	168	0.810	**
LPS	24	0.952	**
	48	0.690	*
	72	0.396	N.S.
	168	0.429	N.S.

MDP = muramyldipeptide, LPS = lipopolysaccharide, N.S. = non significant
*$P < 0.05$, **$P < 0.01$

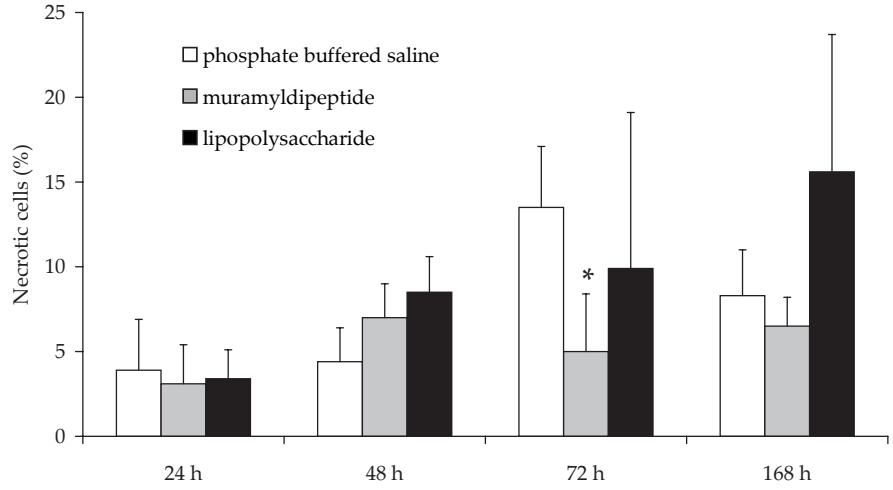

Figure 7. Proportion of necrotic polymorphonuclear leukocytes – SYTO 13 (*$P < 0.05$)

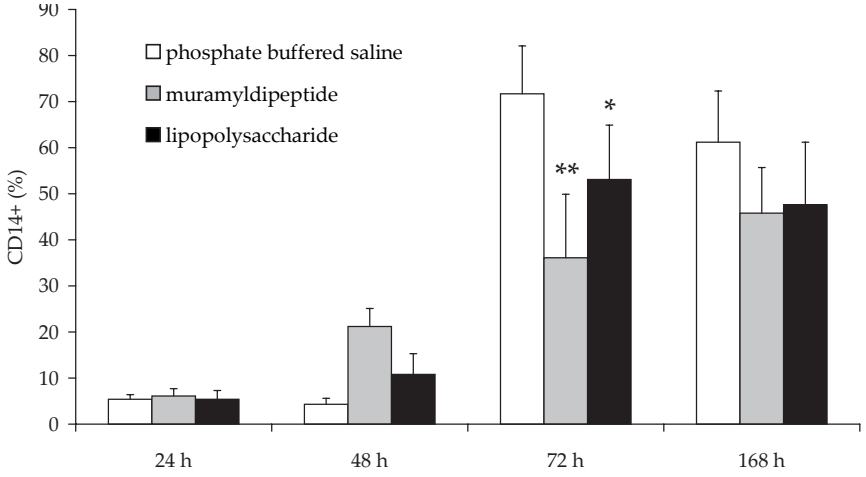

Figure 8. Proportion of CD14+ polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

0.01) lower proportion of CD44+ PMN (Figure 9) observed within 168 h after induction with PBS.

As demonstrated in Table 1, a statistically significant correlation was demonstrated between the proportions of CD14+ and CD44+ PMN during all of the experimental period after the induction with MDP. Induction with LPS showed statistically significant correlation only during the initial phase of inflammatory response.

Hence, the proportion of CD44+ PMN is low in the initial phase of inflammation and, in contrast with CD11b, CD44 does not appear to be a PMN marker for activation.

DISCUSSION

The aim of this study was to determine the dynamics of changes in expressions of CD14 and CD44 PMN in the course of inflammatory response resolution in the heifer mammary gland. The inflammatory response was induced by MDP and LPS.

Intramammary application of PBS, MDP and LPS resulted in an inflammatory response. The inflammatory response had a different character during the initial phase and during resolution. During the initial phase, there was a great influx of PMN into the mammary gland, and 24 h after induction the highest proportion of PMN was observed. Through the further course of the inflammatory response the proportion of PMN was decreasing. These findings were similar to the results of studies already published (Rambeaud et al., 2003; Bannerman et al., 2004; Sladek et al., 2005).

Large differences in the proportions of CD11b+ PMN were observed through the whole period of observation for the inflammatory response. This finding could be explained by the essential role of CD11b in the initial phase of cell influx to the site of inflammation, which is induced by the inductors used (Rysanek et al., 2001). After induction with

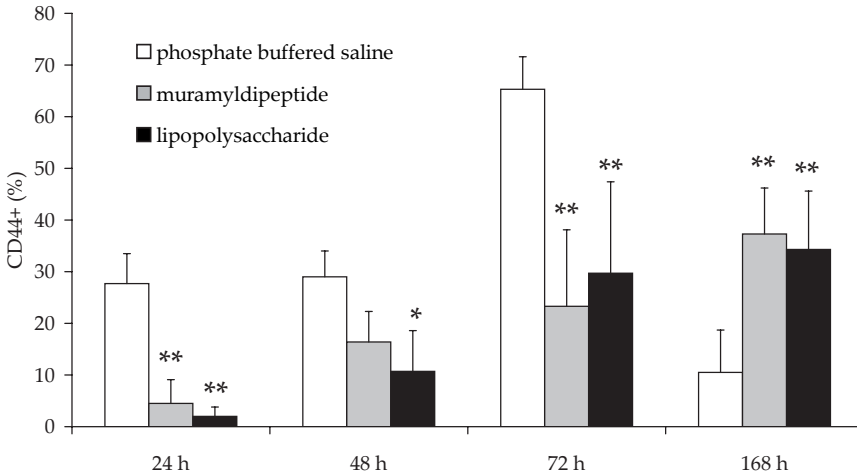

Figure 9. Proportion of CD44+ polymorphonuclear leukocytes (*$P < 0.05$, **$P < 0.01$)

MDP and LPS, there was an influx and expression of β-integrins for the whole observed period of inflammatory response. After induction by PBS, expression of CD11b decreased during the resolution of the inflammatory response. Thus, after an application of MDP and LPS the influx of PMN to the tissues of the mammary gland lasted for a longer period. Influx of PMN to the site of inflammation was compensated by an apoptosis of these cells. CD11b appears more convincingly to be a PMN marker for activation then does CD44.

Apoptosis of PMN and subsequent phagocytosis of apoptotic PMN by macrophages are important for successfully accomplishing the responses of acute inflammation within the mammary gland (Sladek and Rysanek, 2001; Van Oostveldt, 2002). The results of this study reveal that during initiation apoptosis of PMN occurs and shows an increasing trend. This indicates a triggering of the mechanisms of resolution in the mammary gland (Sladek and Rysanek, 2000, 2001). On the other hand, a decline in the proportion of apoptosis of PMN in resolution then indicates its termination. The difference in the proportion of apoptotic PMN was the greatest after PBS, while after MDP and LPS it was manifested with more gradual increase. Analogously, previous studies have shown that MDP and LPS affect apoptosis of bovine PMN *in vitro* (Liu et al., 2005; Rysanek et al., 2005) and *in vivo* (Sladek and Rysanek, 2000, 2001). The results of this study indicate that only MDP and LPS prolong the duration of an acute inflammation. Apart from apoptosis, necrosis was also observed. It showed an increasing tendency, and especially in the resolution phase. This indicates that apoptotic PMN are not completely eliminated through phagocytosis by macrophages and therefore undergo secondary necrosis, as we have reported previously (Sladek et al., 2001a,b; Rysanek and Sladek, 2006; Rysanek et al., 2006).

The initial phase of acute inflammation induced by all three PBS, MDP and LPS was characterized by low proportions of CD14+ and CD44+ PMN. The low proportion of CD14+ PMN may be due to a release of CD14 molecules from the surface of the cell (Paape et al., 1996). The reason for this regulation in the initial phase of inflammatory response after induction with LPS is to prevent production of IL-1 and TNF-α by blocking the LPS-LBP complex (Maliszewski, 1991; Van Miert, 1991). On the contrary, during resolution the proportion of CD14+ PMN increases, and that supports the role of CD14 as a so-called "eat me" signal for macrophages. For the termination of acute inflammation and return of the affected tissue to its original status, it is necessary for PMN to undergo apoptosis and to be removed from tissue. This function is ensured by macrophages, which recognize apoptotic PMN, among others, with the receptor CD14. The role of the CD14 receptor in recognizing apoptotic PMN has been described previously (Gregory and Devitt, 1999; Heidenreich, 1999; Schutt, 1999). CD14 seems to be a candidate receptor for recognition of apoptotic PMN by macrophages in the heifer mammary gland (Sladek et al., 2005; Sladek and Rysanek, 2006).

This study has shown that in the course of induced inflammatory response there is a coincidence in expression of CD14 and CD44 (as shown by the proven positive correlation). This indicates a possible association of CD44 in the "clearing" of apoptotic PMN in resolving inflammation previously proven by Teder et al. (2002). The inflammatory response induced by LPS as well as MDP documented in our study supports these findings. Therefore, it is obvious that MDP plays a similar role as does LPS in expression of PMN CD44+.

This study has proven the coincidence in expression of CD14 and CD44 after induction with MDP and LPS in the course of induced inflammatory response. It is obvious that MDP plays a similar role as does LPS in this process. Expression of CD14 and CD44 after PBS was, however, statistically significantly different. This indicates that expression of CD14 and CD44 is controlled by the factors inducing inflammatory response as well as by the mechanisms of resolution.

Acknowledgement

Authors express thanks to Ing. Lenka Leva and Mrs. Michaela Knakalova for technical assistance.

REFERENCES

Akgul C., Moulding D.A., Edwards S.W. (2001): Molecular control of neutrophil apoptosis. FEBS Letters, 487, 318–322.

Bannerman D.D., Paape M.J., Lee J.W., Zhao X., Hope J.C., Rainard P. (2004): *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune re-

sponses following intramammary infection. Clinical and Diagnostic Laboratory Immunology, 11, 463–472.

Capuco A.V., Paape M.J., Nickerson S.C. (1986): *In vitro* study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows. American Journal of Veterinary Research, 47, 663–668.

Dosogne H., Vangroenweghe F., Mehrzad J., Massart-Leen A.M., Burvenich C. (2003): Differential leukocyte count method for bovine low somatic cell count milk. Journal of Dairy Science, 86, 828–834.

Fenton M.J., Golenbock D.T. (1998): LPS-binding proteins and receptors. Journal of Leukocyte Biology, 64, 25–32.

Gregory C.D., Devitt A. (1999): CD14 and apoptosis. Apoptosis, 4, 11–20.

Heidenreich S. (1999): Monocyte CD14: a multifunctional receptor engaged in apoptosis from both sides. Journal of Leukocyte Biology, 65, 737–743.

Hughes J.R., Johnson A., Mooney C., Hugo K., Gordon J., Savill J. (1997): Neutrophil fate in experimental glomerular capillary injury in the rat. American Journal of Pathology, 150, 223–234.

Khan A.I., Kerfoot S.M., Heit B., Liu L., Andonegui G., Ruffell B., Johnson P., Kubes P. (2004): Role of CD44 and hyaluronan in neutrophil recruitment. The Journal of Immunology, 173, 7594–7601.

Liu J.J., Song C.W., Yue Y., Duan C.G., Yang J., He T., He Y.Z. (2005): Quercetin inhibits LPS-induced delay in spontaneous apoptosis and activation of neutrophils. Inflammation Research, 54, 500–507.

Maliszewski C.R. (1991): CD14 and immune response to lipopolysaccharide. Science, 252, 1321–1322.

Matouskova O., Cigler M., Chalupa K., Hruska K. (1992): STAT Plus – Manual (in Czech). 1st ed. Veterinary Research Institute, Brno. 168 pp.

Meagher L.C., Savill J., Baker A., Fuller R.W., Haslett C.H. (1992): Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxan B2. Journal of Leukocyte Biology, 52, 269–273.

Messmer U.K., Pfeilschifter J. (2000): New insights into the mechanism for clearance of apoptotic cells. Bioassays, 22, 878–881.

Otterlei M., Sundan A., Skjak-Braek G., Ryan L., Smidsrod O., Espevik T. (1993): Similar mechanisms of action of defined polysaccharides and lipopolysaccharides: characterization of binding and tumor necrosis factor-α induction. Infection and Immunity, 61, 1917–1925.

Paape M.J., Lilius E.M., Wiitanen P.A., Kontio M.P., Miller R.H. (1996): Intramammary defense against infections induced by *Escherichia coli* in cows. American Journal of Veterinary Research, 57, 477–482.

Paape M.J., Bannerman D.D., Zhao X., Lee J. (2003): The bovine neutrophil: Structure and function in blood and milk. Veterinary Research, 34, 597–627.

Rambeaud M., Almeida R.A., Pighetti G.M., Oliver S.P. (2003): Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. Veterinary Immunology and Immunopathology, 96, 193–205.

Rysanek D., Sladek Z., (2006): The image of exocytosis during neutrophils and macrophages phagocytic activities in inflammation of mammary gland triggered by experimental *Staphylococcus aureus* infection. Anatomia Histologia Embryologia, 35, 171–177.

Rysanek D., Babak V., Sladek Z., Toman M. (2001): Variations among unbred heifers in the activities of polymorphonuclear leukocytes from the mammary gland and blood. Journal of Veterinary Medicine, Series B, 48, 31–41.

Rysanek D., Sladek Z., Vasickova D., Faldyna M.: (2005): Effects of certain inducers of leukocytes migration into the bovine mammary gland on neutrophil apoptosis manifestation in a subsequent *in vitro* cultivation. Physiological Research, 54, 305–312.

Rysanek D., Sladek Z., Babak V., Vasickova D., Hubackova M. (2006): Spontaneous and induced cytolysis of leukocytes from bovine mammary gland in the course of cultivation *in vitro* – the correlation with neutrophil granulocytes apoptosis. Veterinarni Medicina, 51, 265–277.

Savill J.S. (1997): Recognition and phagocytosis of cell undergoing apoptosis. British Medical Bulletin, 53, 491–508.

Savill J.S., Fadok V., Henson P., Haslett C. (1993): Phagocyte recognition of cells undergoing apoptosis. Immunology Today, 14, 131–136.

Savill J.S., Gregory C., Haslett C. (2003): Cell biology. Eat me or die. Science, 302, 1516–1517.

Schutt Ch. (1999): Molecules in focus, CD14. The International Journal of Biochemistry and Cell Biology, 31, 545–549.

Sladek Z., Rysanek D. (2000): Apoptosis of polymorphonuclear leukocytes of the juvenile bovine mammary gland during induced influx. Veterinary Research, 31, 553–563.

Sladek Z., Rysanek D. (2001): Neutrophil apoptosis during the resolution of bovine mammary gland injury. Research in Veterinary Science, 70, 41–46.

Sladek Z., Rysanek D. (2006): The role of CD14 during resolution of experimentally induced *Staphylococcus aureus* and *Streptococcus uberis* mastitis. Comparative Immunology, Microbiology and Infectious Diseases, 29, 243–262.

Sladek Z., Rysanek D., Faldyna M. (2001a): Light microscopic and flow cytometric detection of apoptosis and necrosis of neutrophils in the mammary gland of the virgin heifer. Acta Veterinaria Brno, 70, 149–155.

Sladek Z., Rysanek D., Faldyna M. (2001b): Leukocytes in bovine virgin mammary gland: flow cytometry imaging during development and resolution of induced influx. Veterinarni Medicina, 46, 190–198.

Sladek Z., Rysanek D., Faldyna M. (2002): Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide in heifers. Veterinary Research, 33, 191–204.

Sladek Z., Rysanek D., Ryznarova H., Faldyna M. (2005): Neutrophil apoptosis during experimentally induced *Staphylococcus aureus* mastitis. Veterinary Research, 36, 629–643.

Smith G.S. (2000): Neutrophils. In: Feldman B.F., Zinkl J.G, Jain N.C. (eds.): Schalm´s Veterinary Hematology. Lippincott Williams and Wilkins, Philadelphia. 281–296.

Takeuchi O., Hoshino K., Kawai T., Sanjo H., Takada H., Ogawa T., Takada K., Akira S. (1999): Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity, 11, 443–451.

Takeuchi O., Hoshino K., Akira S. (2000a): Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. Journal of Immunology, 165, 5392–5396.

Takeuchi O., Takeda K., Hoshino K., Adachi O., Ogawa T., Akira S. (2000b): Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. International Immunology**,** 12, 113–117.

Teder P., Vandivier R.W., Jiang D., Liang J., Cohn L., Pure E., Henson P.M., Noble P.W. (2002): Resolution of lung inflammation by CD44. Science, 296(5565), 155–158.

Trotter J. (2000): WinMDI 2.8 software. Windows Multiple Document Interface Flow Cytometry Application.

Vachon E., Martin R., Plumb J., Kwok V., Vandivier R. W., Glogauer M., Kapus A., Wang X., Chow C.W., Grinstein S., Downey G.P. (2006): CD44 is a phagocytic receptor. Blood, 107, 4149–4158.

Van Miert A.S. (1991): Acute phase response and non cellular defense mechanisms. Flemish Veterinary Journal, 62, 69–91.

Van Oostveldt K., Paape M.J., Dosogne H., Burvenich C. (2002): Effect of apoptosis on phagocytosis, respiratory burst and CD18 adhesion receptor expression of bovine neutrophils. Domestic Animal Endocrinology**,** 22, 37–50.

Vermes I., Haanen C., Steffens-Nakken H., Reutellingsperger C. (1995): A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of Immunological Methods**,** 184, 39–51.

Vivers S., Dransfield I., Hart S.P. (2002): Role of macrophage CD44 in the disposal of inflammatory cell corpses. Clinical Science**,** 103, 441–449.

Received: 2007–04–16
Accepted after corrections: 2008–01–02

Corresponding Author:

MVDr. Dusan Rysanek, CSc., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 501, e-mail: rysanek@vri.cz