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EFFECT OF MURAMYL DIPEPTIDE ON APOPTOSIS OF BOVINE MAMMARY GLAND LYMPHOCYTES *IN VITRO*

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ABSTRACT

The aim of this study was to determine whether apoptosis of lymphocytes is modulated by muramyl dipeptide (MDP) which is the minimal structural unit of peptidoglycans in Gram-positive bacteria. To this aim, apoptosis of lymphocytes was studied in *in vitro* environment (RPMI medium, 37 °C, 5% CO_2) with 10 μg.ml^{-1} MDP. Heifers were used as mammary gland cell donors for *in vitro* studies. Lymphocytes from the intact mammary glands were harvested following influx induction by the phosphate buffered saline intramammary injection. Apoptosis and necrosis of lymphocytes were analysed by flow cytometry following simultaneous staining with Annexin-V and propidium iodide. The results of this study demonstrate that apoptosis of lymphocytes was delayed during *in vitro* incubation with MDP.

Key words: muramyl dipeptide; apoptosis; lymphocytes; mammary gland

INTRODUCTION

Muramyl dipeptide (MDP) is the minimal structural unit of peptidoglycans in Gram-positive bacteria. Infusion of MDP into the bovine mammary gland induces an inflammatory response (Langrova et al., 2008; Sladek and Rysanek, 2008; Sladek and Rysanek, 2009), but there is no data whether MDP modulates lifespan of mammary gland lymphocytes.

Lymphocytes are very important components of the mammary gland immune system. They recognize antigens through membrane receptors specific for invading bacteria (Sordillo et al., 1997). During an acute mastitis caused by Gram-positive bacteria (*Staphylococcus aureus* or *Streptococcus uberis*), an increased number of lymphocytes was detected in the mammary glands (Soltys and Quinn, 1999). In our previous study, we observed delaying of lymphocytes apoptosis during an experimentally induced infection of bovine mammary glands with *S. aureus* or *S. uberis* and during *in vitro* incubation with the same bacteria (Slama et al., 2009).

Bovine strains of *S. aureus* produce staphylococcal enterotoxins such as staphylococcal enterotoxin C (Kenny et al., 1993; Zouharova and Rysanek, 2008). Staphylococcal enterotoxins are among those pyrogenic toxins known as superantigens (Bohach et al., 1990). The interactions of superantigens with the T lymphocytes lead to their apoptosis (Damle et al., 1993; Boshell et al., 1996). *S. aureus* α-toxin are known to induce apoptosis in human peripheral blood mononuclear cells (Haslinger et al., 2003). Park et al. (2006) investigated apoptosis of bovine blood lymphocytes ($CD4^+$ and $CD8^+$) during *in vitro* cultivation with staphylococcal enterotoxin C. Ferens et al. (1998) showed that staphylococcal

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enterotoxin C induced aberrant activation of the $CD8^+$ T cell subset, with a corresponding inversion of the $CD4^+$: $CD8^+$ T cell ratio. Previous information demonstrates that Gram-positive bacterial toxins are studied intensively. In spite of that there is no data about apoptosis of bovine lymphocytes after their MDP stimulation. Apoptosis is an important problem because it decreases the lifespan of cells and impairs their proper functioning (Van Oostveldt et al., 2002).

The aim of our study was to determine whether apoptosis of bovine mammary gland lymphocytes is modulated by MDP during *in vitro* incubation.

MATERIAL AND METHODS

Animals

The experiments were carried out on 12 mammary glands of 3 virgin, clinically healthy, Holstein × Bohemian Red Pied crossbred heifers aged 16 to 18 months. For the *in vitro* studies, heifers were used as cell donors. The heifers were housed in an experimental tie-stall barn and fed a standard ration consisting of hay and concentrates with mineral supplements. The experimental tie-stall used in this study is certified and animal care conformed to good care practice protocols. All heifers were free of intramammary infections.

Experimental design

Intact lymphocytes from the mammary glands were harvested following the phosphate buffered saline (PBS) intramammary injection, using a procedure previously described by Rysanek et al. (1999) that employs a model of an induced influx (Wardley et al., 1976). Fresh mammary gland leukocytes were adjusted (1×10^7 cells.ml^{-1}) in RPMI 1640 medium (Sigma, MO, USA). The samples were incubated with 10 μg.ml^{-1} MDP (MurNAc-L-Abu-D-IsoGln, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic) at 37 °C in a 5% CO_2 atmosphere for 1, 2 and 5 h, as described by Scaife et al. (2003). Incubation in RPMI 1640 medium alone served as control. After incubation, apoptotic and necrotic lymphocytes were detected through flow cytometry (FCM) (Vermes et al., 1995).

FCM assessment of lymphocytes apoptosis and necrosis

Apoptotic and necrotic lymphocytes were analysed by FCM following simultaneous staining with Annexin-V labelled with fluorescein isothiocyanate (FITC) and propidium iodide (PI), as described by Vermes et al. (1995). The commercial Annexin-V-FLUOS staining kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, 500 μL of the incubation buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) was mixed with 10 μL of PI and 10 μL of FITC-Annexin-V solution. After 15 min of incubation at room temperature with fresh buffer containing PI and FITC-Annexin-V, the cell suspension was analysed by FCM with differentiation of at least 20,000 cells. After labelling with Annexin-V–FITC and PI, lymphocyte cells were distributed over three different quadrants of a dot plot analysis (with FL1 and FL3 axes), representing viable (Annexin V^-/ PI^-), apoptotic (Annexin V^+/PI^-), and necrotic cells (Annexin V^+/PI^+). Proportions of apoptotic and necrotic lymphocytes were calculated from the total number of lymphocytes. Dot plots were evaluated qualitatively and quantitatively using WinMDI™ software.

Statistical analysis

Arithmetic means and standard deviations were used to describe descriptive statistics of proportions of apoptotic and necrotic lymphocytes. Statistically significant differences in the proportions of these parameters were determined using the paired *t*-test. The data were processed using STATISTICA 8.0 software (StatSoft CR Ltd, Prague, Czech Republic).

RESULTS

The *in vitro* 2 or 5 h of lymphocyte incubation with MDP resulted in the significant decrease ($P < 0.05$ or $P < 0.01$) of apoptotic (Annexin-V^+/PI^-) lymphocytes proportions in comparison with the control (Table 1).

Table 1: Proportion of apoptotic lymphocytes after 1, 2 and 5 h of incubation with muramyl dipeptide (10 μg.ml^{-1}). Significant differences between control (incubation without muramyl dipeptide) and incubation with muramyl dipeptide are marked with plus signs (+ $P < 0.05$; ++ $P < 0.01$)

Incubation time (h)	Control (%) arithmetic mean ± SD	Muramyl dipeptide (%) arithmetic mean ± SD	Statistical significance
1	1.83 ± 1.12	0.99 ± 0.87	-
2	2.37 ± 0.82	0.84 ± 0.65	+
5	3.46 ± 1,03	1.17 ± 1,08	++

Table 2: Proportion of necrotic lymphocytes after 1, 2 and 5 h of incubation with muramyl dipeptide (10 µg.ml^{-1})

Incubation time (h)	Control (%) arithmetic mean ± SD	Muramyl dipeptide (%) arithmetic mean ± SD	Statistical significance
1	3.95 ± 2.27	3.75 ± 3.23	-
2	5,42 ± 5,10	6,81 ± 7,20	-
5	7,52 ± 8,13	8,92 ± 6,93	-

Lymphocytes necrosis ranged from 3.75 to 8.92%, following lymphocytes cultivation with MDP, with no significant differences compared with the control (Table 2).

DISCUSSION

This study focused on answering the question whether apoptosis of bovine mammary gland lymphocytes is modulated by MDP. To this end, apoptosis of lymphocytes was studied in *in vitro* environment with MDP.

The *in vitro* lymphocytes incubation with MDP resulted in the significant decrease of apoptotic lymphocytes proportions. That suggests that apoptosis of bovine lymphocytes is modulated during *in vitro* course with MDP. In our previous study, we found that infection of the mammary gland with *S. aureus* or *S. uberis* led to delaying of lymphocytes apoptosis. This opinion was confirmed by the *in vitro* experiments (Slama et al., 2009). Park et al. (2006) observed a gradual increase in proportions of apoptotic $CD4^+$ and $CD8^+$ T cells cultivated with staphylococcal enterotoxin C over 9 days of *in vitro* cultivation. In the same study, they wrote that some toxins of *S. aureus* induce a prolonged Th-2 biased microenvironment and that, together with the inversion of bovine $CD4^+$:$CD8^+$ T cell ratios, may in part explain the inability of the mammary immune system to establish an effective response to *S. aureus* infections.

The Gram-positive bacteria (*S. aureus* or *S. uberis*) delayed lymphocytes apoptosis both *in vivo* and *in vitro* (Slama et al., 2009). However, Sun et al. (1999) have shown that *Pasteurella haemolytica* leukotoxin, at low concentrations, induces apoptosis of bovine lymphocytes, which may play an important role in the initiation and persistence of *P. haemolytica* infection. Bush and Rosenbusch (2002) have reported that *Mycoplasma bovis* also induces apoptosis of bovine lymphocytes. The production of *M. bovis* protein is necessary for the induction of lymphocytes death.

The Gram-positive bacteria can also induce lymphocytes apoptosis in other species. For example, *Listeria monocytogenes* induces apoptosis of the splenic and lymph-node lymphocytes in mice (Merrick et al., 1997). Human patients infected with the Gram-positive bacterium *Streptococcus pneumoniae* had increased numbers of apoptotic lymphocytes (Kemp et al., 2002). In another study, Popov et al. (2002) described that *Bacillus anthracis* can induce the apoptosis of human peripheral-blood mononuclear cells *in vitro*. α-toxin of *S. aureus* also induces apoptosis of human T-cells *in vitro* (Jonas et al, 1994; Bantel et al., 2001).

There were no significant differences in lymphocytes necrosis following cultivation with MDP. Almost the same result was noticed in cultivation of lymphocytes with *S. aureus* or *S. uberis* (Slama et al., 2009).

CONCLUSION

The results of this study demonstrate that apoptosis of lymphocytes was delayed during an *in vitro* incubation with MDP. Subsequent studies will aim to answer why components of Gram-positive bacteria delay apoptosis of bovine lymphocytes.

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