Effect of *Staphylococcus aureus* and *Streptococcus uberis* on apoptosis of bovine mammary gland neutrophils *in vitro*

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ABSTRACT: Neutrophils play an important role in the defence of the bovine mammary gland against bacterial infections. In the course of the resolution of mammary gland inflammation, neutrophils undergo programmed cell death – apoptosis. The aim of this study was to confirm whether the co-cultivation of neutrophils of the bovine mammary gland with either *Staphylococcus aureus* or *Streptococcus uberis* leads to signs of apoptosis. In the study, 16 mammary glands of four virgin heifers aged 16 to 18 months were examined. Neutrophils were obtained by lavage after an induced influx. After a three-hour incubation of the neutrophils with bacteria *in vitro*, neutrophil apoptosis was detected by morphological features, by determination of histone-associated DNA fragments (ELISA), and by Annexin-V and propidium iodide positivity (flow cytometry). *S. aureus* and *S. uberis* reduced the incidence of karyopycnotic and zeiotic neutrophils ($P < 0.01$), and insignificantly reduced the concentration of histone-associated DNA fragments ($P > 0.05$). The incubation of neutrophils with bacteria, however, increased the proportion of Annexin-V-positive cells ($P < 0.01$) and Annexin-V and propidium iodide-positive cells ($P < 0.05$). Co-cultivation of neutrophils with either *S. aureus* or *S. uberis* led to the induction of phosphatidylserine translocation characteristic of the early stage of apoptosis. The late signs of apoptosis were delayed by co-cultivation of neutrophils with both pathogens. Therefore it is obvious that although the programmed cell death of apoptosis is initiated by these pathogens, the completion of the program is delayed.

Keywords: *Staphylococcus aureus*; *Streptococcus uberis*; neutrophil; apoptosis

Staphylococcus aureus (hereinafter *S. aureus*) is the most important and prevalent contagious mammary pathogen; it causes clinical and subclinical intramammary infections with serious economic loss and herd management problems in dairy cows (Fox and Gay, 1993). The most prevalent environmental pathogen – *Streptococcus uberis* (hereinafter *S. uberis*) – is also one of the most important pathogens, accounting for a significant proportion of subclinical and clinical intramammary infections in both lactating and non-lactating cows (Pedersen et al., 2003).

Neutrophils form the first line of immunological defence of the bovine mammary gland against bacterial pathogens. Neutrophils are produced in the bone marrow, enter the peripheral blood and migrate through the walls of capillaries into the lumens of mammary glands, where they phagocytose invading bacteria (Paape et al., 2003).

The cytoplasm granules of neutrophils contain a powerful set of bacteriolytic enzymes and bactericidal substances that make the killing of pathogens possible (Bainton, 1975). These are highly histotoxic, however, and, when released to the extracellular space during neutrophil necrosis, may have pro-inflammatory effects and damage the mammary gland tissue (Capuco et al., 1986). Therefore it is important that neutrophils undergo a programmed

Supported by the Grant Agency of the Czech Republic (Grants No. 524/01/1290 and No. 524/03/1531).

cell death or apoptosis. Apoptosis of bovine neutrophils involves an early stage characterized by cell surface exposure of phosphatidylserine and a later stage which includes cell shrinking, nuclear chromatin condensation and DNA fragmentation (Van Oostveldt et al., 1999). Apoptosis is also associated with a loss of neutrophil functions, such as chemotaxis, phagocytosis, degranulation and respiratory burst (Whyte et al., 1993). Apoptotic neutrophils are physically removed from the mammary gland, i.e. phagocytosed, by macrophages. Neutrophil apoptosis is thus an effective protective mechanism that prevents bovine mammary gland tissue damage (Sladek and Rysanek, 2000a,b, 2001).

During bacterial infections of bovine mammary glands, neutrophils are exposed to bacteria and their components, and to bacterial toxins which significantly contribute to the shorter lifespan of neutrophils (Burton and Erskine, 2003). As soon as migrating neutrophils reach the site of inflammation, they are exposed to the effects of various cytokines. Some of the cytokines (GM-CSF, IFN-gamma, IL-1 beta, IL-2) delay neutrophil apoptosis and prolong the neutrophil lifespan by several days (Brach et al., 1992; Colotta et al., 1992; Lee et al., 1993). Other cytokines, e.g. TNF-alpha, delay apoptosis of neutrophils at low concentrations, and accelerate apoptosis at high doses (Takeda et al., 1993; Gon et al., 1996); IL-10 significantly shortens the lifespan of neutrophils by inducing their apoptosis (Cox, 1996).

It has been confirmed that some Gram-positive and Gram-negative pathogens moderate neutrophil apoptosis, including *S. aureus* (for details see Kobayashi et al., 2003b). Apoptosis has been demonstrated in the interaction between *S. aureus* and human neutrophils *in vitro* (Lundqvist-Gustafsson et al., 2001; Yamamoto et al., 2002; Kobayashi et al., 2003b; Nilsdotter-Augustinsson et al., 2004). Finally, the pro-apoptotic effect of enterotoxin by *S. aureus* was described for human blood neutrophils (Moulding et al., 1999).

In the above-mentioned studies, only human blood neutrophils were used. However, similar pro-apoptotic effects of *S. aureus* can also be expected on migrated neutrophils of the bovine mammary gland during infection. Induction of neutrophil apoptosis by bacteria is generally beneficial to the host and should not be looked upon as a virulence factor. Instead, the inhibition of neutrophil apoptosis by some pathogens facilitates pathogenesis (DeLeo, 2004).

On the other hand, apoptosis of neutrophils induced by *S. aureus* may be viewed as a virulence factor since such cell death impairs the protective functions of neutrophils (Whyte et al., 1993) because well functioning neutrophils will also finally determine the severity of mastitis following an invasion of bacteria in the mammary gland (Van Oostveldt et al., 2002b). Nevertheless, such a hypothesis has not yet been a subject of experimental interest. Therefore we wanted to know whether the co-cultivation of *S. aureus* with neutrophils of the bovine mammary gland leads to signs of apoptosis of these cells.

Contrary to *S. aureus*, the pro-apoptotic effects of *S. uberis* on neutrophils have not been studied at all. The second question that consequently arises is whether the modulation of neutrophil apoptosis is one of the factors of *S. uberis* virulence. Accordingly, the aim of this study was to confirm whether the co-cultivation of *S. aureus* and *S. uberis* with neutrophils of the bovine mammary gland leads to signs of apoptosis.

MATERIAL AND METHODS

Experimental design and animals

The experiments were conducted with 16 mammary glands (udder quarters) of four clinically healthy, virgin Holstein × Bohemian Red Pied crossbred heifers aged 16 to 18 months. All heifers were free of intramammary infections, as demonstrated by a bacteriological examination of cell suspensions obtained by mammary lavage. The heifers were used as mammary gland cell donors for *in vitro* studies. The model of induced influx was used. Neutrophil populations were incubated with bacteria *in vitro* and then apoptotic neutrophils were detected by light microscopy, transmission electron microscopy (TEM), ELISA of histone-associated DNA fragments (ELISA) and flow cytometry (FCM).

Preparation of neutrophils and processing

Neutrophils from mammary glands were harvested by a procedure previously used by Rysanek et al. (1999) employing a model of an induced influx (Wardley et al., 1976). Briefly, modified urethral catheters (AC5306CH06, Porges S.A., France) were

inserted into the teat canal after thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter (n = 16) was injected with 20 ml of phosphate-buffered saline solution (0.01 M, pH 7.4; PBS) and lavages were immediately collected back through the catheter directly to the syringe. The lavages were followed by the administration of a synthetic analogue of muramyl dipeptide (500 μg/20 ml PBS). The synthetic analogue of muramyl dipeptide (MDP, norMurNAc-L-Abu-D-iGln) was supplied by the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic, Prague. Samples of cell populations were obtained by mammary lavages 18 h after treatment.

Total mammary cell counts were determined using the FOSSOMATIC 90 apparatus (Foss Electric; Denmark) and the procedure recommended by the IDF (1995). The Trypan Blue dye exclusion test demonstrated more than 98.9% cell viability in fresh neutrophils by the enumeration of at least 200 neutrophils. The cell suspensions were centrifuged at 4°C and 200 × g for 10 min. One millilitre of supernatant was retained for the resuspension of the pellet.

Bacteriological examination

Bacteriological examinations of all the lavages, by culture on blood agar plates (5% washed ram erythrocytes) and aerobic incubation at 37°C for 24 h, yielded negative results. No bacteria were detected in any of the mammary lavages tested.

Preparation of *Staphylococcus aureus* and *Streptococcus uberis*

S. aureus (strain Newbould 305 CCM 6275) and *S. uberis* (strain CCM 4617) were used. The inoculum was prepared by growing the organism on ram blood agar (BA) medium. Three colonies of this culture were then inoculated into brain-heart infusion (BHI) broth and cultivated under continuous rotation (30 rpm/min) for 18 h at 37°C. The stock culture was stored at 4°C. On the day of the *in vitro* incubation, 1 ml of the stock culture of *S. aureus* was inoculated into 5 ml fresh BHI and incubated under continuous rotation (30 rpm/min) for 4 h to allow them to be in the exponential growth phase.

A different procedure was used in the case of *S. uberis*, for which 1 ml of the stock culture was inoculated on a cellophane membrane, placed on BA and incubated for 4 h to obtain bacteria in the exponential growth phase.

Both bacterial suspensions were then harvested and washed once with PBS. Total bacterial cell counts were determined using a haemocytometer, and the bacterial suspension was adjusted to a final concentration equalling 1.2×10^7 colony forming units per ml (CFU/ml) in cell culture media. The CFU/ml was determined for each inoculum after 24 h of incubation at 37°C on BA medium.

Cultivation of neutrophils

Fresh mammary gland neutrophils were adjusted (1.2×10^7 cells/ml) in a RPMI 1640 (Sigma, MO, USA) with and without *S. aureus* and *S. uberis* to the ratio of 1 : 1 (the final concentration was 6.10^6 per ml). For light microscopy analysis, the adjusted suspensions were spread on slides (0.5 ml/slide) and placed in a moist chamber. Two specimens of each cell suspension were made. For transmission electron microscopy and flow cytometry, adjusted suspensions were inserted into microplates (6 × 4 Costar Ultraplates, CA, USA). For ELISA, adjusted suspensions were inserted into microplates (12 × 8 Kohinor; Czech Republic). Slides and microplates were incubated at 37°C in a 5% CO_2 atmosphere for one time point – 3 h in accordance with Scaife et al. (2003). After incubation, the cells were stained with May-Grünwald Giemsa stain (Pappenheim method), or processed for TEM, ELISA and FCM.

Methods

Light microscopy. Slides stained by the Pappenheim method (May-Grünwald-Giemsa stain) were examined by light microscopy using oil immersion (Olympus BH2, Olympus Optical Co., LTD, Japan). Apoptosis was assessed by the enumeration of at least 200 neutrophils in accordance with described morphological features (Sladek and Rysanek, 2000a).

Transmission electron microscopy. The samples for TEM were prepared by our previously described procedure (Sladek and Rysanek, 1999a). Briefly, the cells were fixed with 3% glutaraldehyde in cacodylate buffer for 2 h and washed three

times for 30 min in 0.1 M cacodylate buffer. They were then postfixed with 0.02 M OsO_4 dissolved in 0.1 M cacodylate buffer, dehydrated in alcohol, and infiltrated with acetone and Durcupan-I overnight. On the subsequent day, the cells were infiltrated with Durcupan-II, embedded and polymerized. Ultrathin sections (90 nm, ultramicrotome LKB; Sweden) were transferred onto grids covered with a formvar membrane. 2% uranyl acetate and Reynold's solution were used for contrast staining. The sections were viewed in the transmission electron microscope (Morgagni 268, Netherlands). AnalySIS (Soft Imaging System, GmbH, Germany) software was used for picture analysis of the cell ultrastructure.

ELISA. Photometric enzyme-immunoassay (quantitative sandwich enzyme immunoassay) was used for the *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) after incubation (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics; Germany). Mouse monoclonal antibodies against DNA and against histone were used. The Sunrise photometer (Tecan, Austria) was used for absorbance measurements at 405 nm wavelength. The manufacturer's laboratory protocol was used, with the exception of the calculation of the "enrichment factor", which was replaced by the "apoptosis and necrosis absorbance rate" introduced by the authors. To calculate this absorbance rate, the sum of apoptotic and necrotic absorbance values was considered as 100%. Apoptosis and necrosis were then expressed as a percentage of that total, and multiplied by the sum of the original apoptotic and necrotic absorbance values.

Flow cytometry. Apoptotic and necrotic neutrophils were analysed by FCM after 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 solutions. The cell suspension was adjusted to 1×10^6 per 1 ml in 100 µl of fresh incubation buffer containing PI and FITC-Annexin-V. The suspension was then analysed after 15 min of incubation at room temperature by FCM (FCM Calibur apparatus, Becton Dickinson, Mountain View, CA; USA) by differentiation of at least 10 000 cells. Dot plots were evaluated qualitatively and quantitatively using the CellQuest software analysis (Beckton Dickinson, Mountain View, CA, USA).

Statistical analysis

The proportions of apoptotic and necrotic neutrophils are presented as statistical means and standard deviations of the sixteen mammary glands examined. Significant differences in the proportion of apoptotic and necrotic neutrophils after cultivation *in vitro* with *S. aureus* and *S. uberis* were determined by the paired *t*-test. The data were processed by STAT Plus software (Matouskova et al., 1992).

RESULTS

Characteristics of the mammary gland neutrophil population

A large cell population ($59.1 \pm 11.7 \times 10^6$/ml) with a relatively high proportion of neutrophils (90.1 ± 11.8%) compared to macrophages (7.3 ± 4.6%) and lymphocytes (2.6 ± 1.1%) was obtained by lavage 18 h after the treatment of mammary glands. The neutrophil population was dominated by structurally and ultrastructurally normal cells. In addition to these normal neutrophils, cells with morphological features of apoptosis (8.4 ± 3.1%) were also found (Figure 1A). The presence of apoptotic cells in the fresh population of neutrophils of the mammary gland was demonstrated by light and transmission electron microscopy, flow cytometry and ELISA assay (see below). Trypan blue-positive neutrophils were detected very sporadically (1.1 ± 0.3%).

Effects of *S. aureus* and *S. uberis* on chromatin condensation and zeiosis in neutrophils

After 3 h incubation, spontaneous apoptosis was observed in 30.8 ± 5.7% of neutrophils cultivated *in vitro* in the absence of bacteria (control). Similarly like apoptotic neutrophils *in vivo* (Figure 1A), the *in vitro* cultivated apoptotic neutrophils were characterized by nuclear chromatin condensation (karyopycnosis) or were in zeiosis.

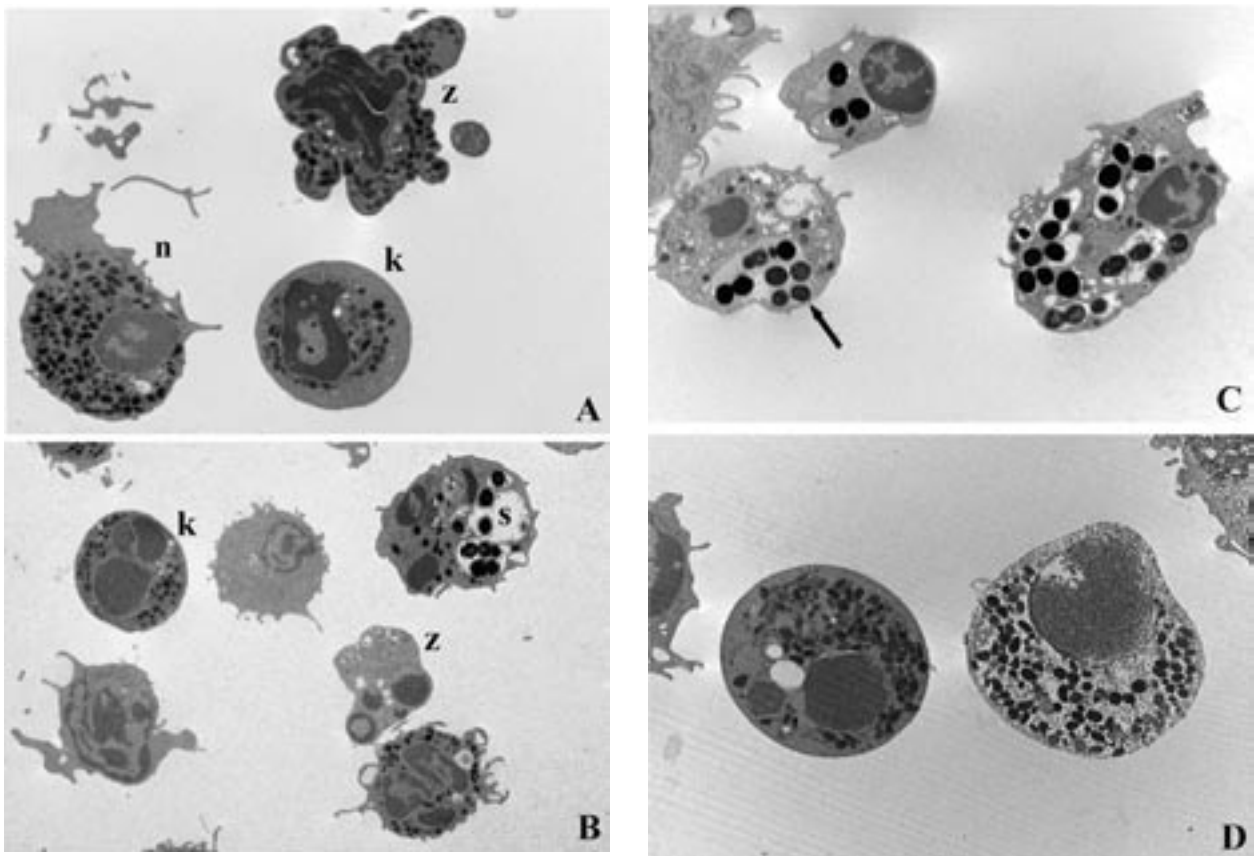


Figure 1. Transmission electron microscopy of bovine mammary gland neutrophils. A – Fresh population of cells obtained by induced influx: normal neutrophil (n), neutrophil in karyopycnosis (k) and neutrophil in zeiosis (z). B – Neutrophils cocultured with *S. aureus* for 3 hours: neutrophil with karyopycnosis (k), zeiosis (z) and with phagocytosed bacteria (s). C – Neutrophils cocultured with *S. uberis* for 3 h: three neutrophils containing phagocytosed bacteria (arrow). D – Neutrophils cocultured with *S. aureus* for 3 hours: karyopycnotic neutrophil (left) and secondary necrotic apoptotic neutrophil in initial stage of lysis (right). Magnification: 5 000× (A), 4 000× (B), 4 500× (C), 6 000× (D)

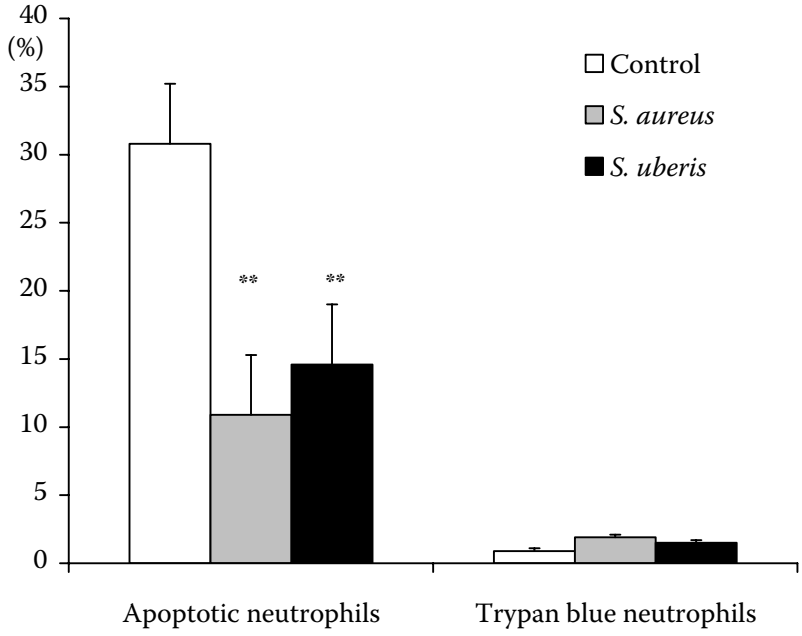


Figure 2. Proportion of neutrophils with karyopycnosis and zeiosis cultured with medium only (control) and medium plus *S. aureus* and *S. uberis* for 3 hours. Apoptotic neutrophils were determined on slides stained by Pappenhaim method. Means ± SD for 16 mammary glands are given. Significant differences are marked with asterisks (**$P < 0.01$)

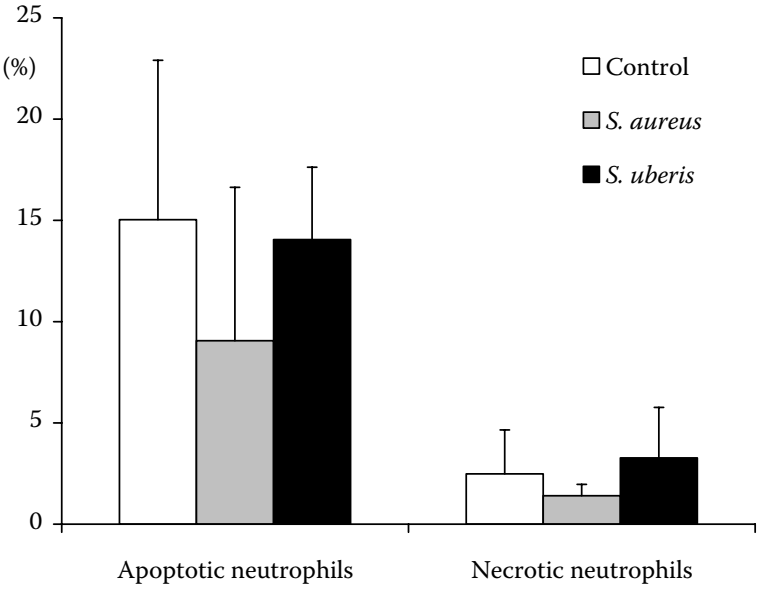


Figure 3. Proportion of apoptotic and necrotic neutrophils after incubation with *S. aureus* and *S. uberis* detected in ELISA. Apoptosis and necrosis were determined by ELISA measuring the concentration of cellular histone-associated DNA fragments. The concentrations were determined in neutrophils cultured with medium only (control) and medium plus *S. aureus* and *S. uberis* for 3 hours. Means ± SD for 16 mammary glands are given

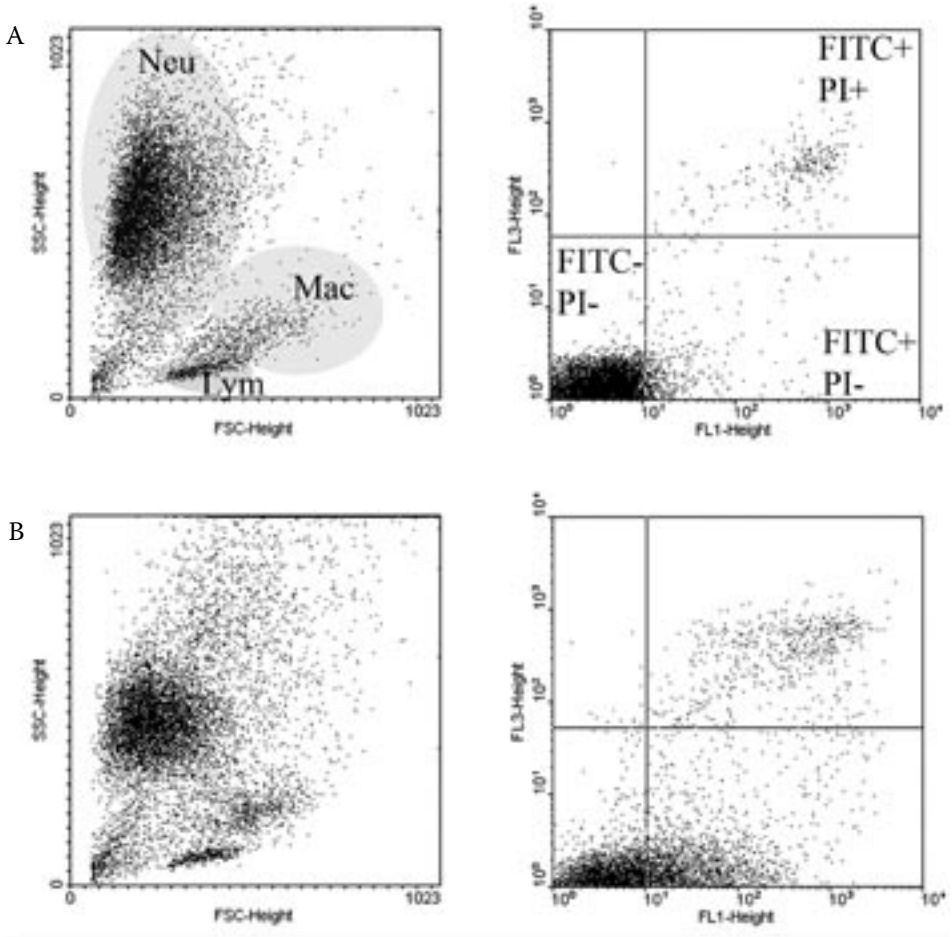


Figure 4. Flow cytometry of apoptotic and necrotic neutrophils after incubation with *S. aureus* and *S. uberis*. Figures in the left column represent dot plots of leukocytes gated for scatter parameters in fresh population (A), and in neutrophils cultured with medium only (B) and medium plus *S. aureus* (C) and medium plus *S. uberis* (D) for 3 hours. Clusters of neutrophils (Neu), lymphocytes (Lym) and macrophages (Mac) are shown. The bit maps (right column) were set for neutrophils only. Flow cytometry analysis of subpopulations of neutrophils shows viable, apoptotic, and necrotic neutrophils gated for Annexin V and propidium iodide. The lower-left quadrant represents

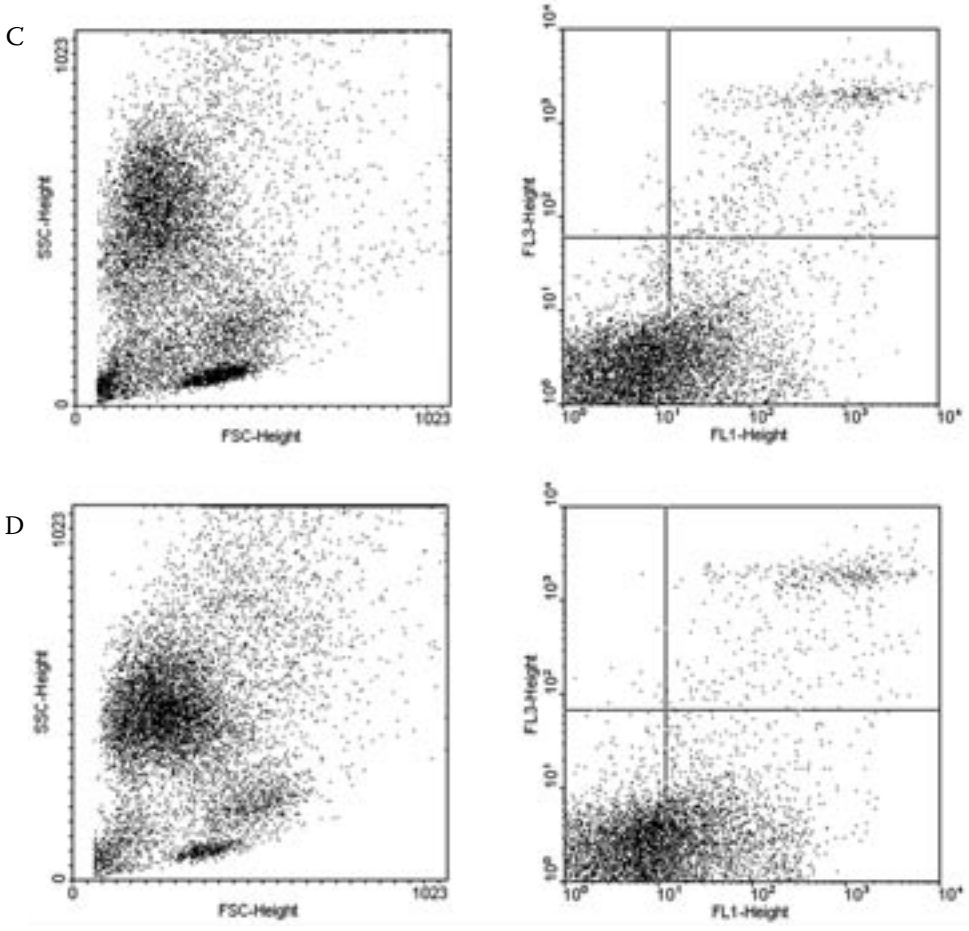
On the other hand, the *in vitro* incubation of neutrophils with *S. aureus* and *S. uberis* resulted in a decreased number of cells with condensed chromatin and of zeiotic cells. Following the incubation with *S. aureus* and *S. uberis*, only 10.9 ± 4.4% and 14.6 ± 4.3% of karyopycnotic and zeiotic neutrophils were found, respectively (Figure 2). The difference in both cases was statistically significant ($P < 0.01$) when compared to the control.

In addition to apoptotic neutrophils (Figure 1B,C), neutrophils in various stages of lysis of their nuclei and cellular components were found. The lysed neutrophils exhibited a number of ultrastructural attributes of apoptotic neutrophils, e.g. defragmented nuclei, spherical shape and intact granules (Figure 1D), which shows that they were secondary necrotic apoptotic neutrophils.

No significant differences in the proportion of trypan blue positive neutrophils were observed after 3 h incubation in the control samples and in the experimental samples with *S. aureus* and *S. uberis.*

Effects of *S. aureus* and *S. uberis* on DNA fragmentation in neutrophils

Apoptosis of neutrophils was determined by ELISA measuring the concentration of histone-associated DNA fragments (HA-DNA) in the cellular fraction. We detected a statistically insignificant ($P > 0.05$) decrease of HA-DNA after 3 h incubation of neutrophils with *S. aureus* and a statistically insignificant ($P > 0.05$) decrease in HA-DNA with *S. uberis* in comparison with the control (Figure 3).



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apoptotic, and necrotic neutrophils gated for Annexin V and propidium iodide. The lower-left quadrant represents intact-viable neutrophils which exclude PI and do not bind Annexin-V (FITC–/PI–), the lower-right quadrant represents apoptotic neutrophils binding Annexin-V, but with preserved cell membrane integrity (FITC+/PI–), and the upper-right quadrant represents necrotic neutrophils binding Annexin-V and showing PI uptake (FITC+/PI+). One representative out of the sixteen experiments done is shown

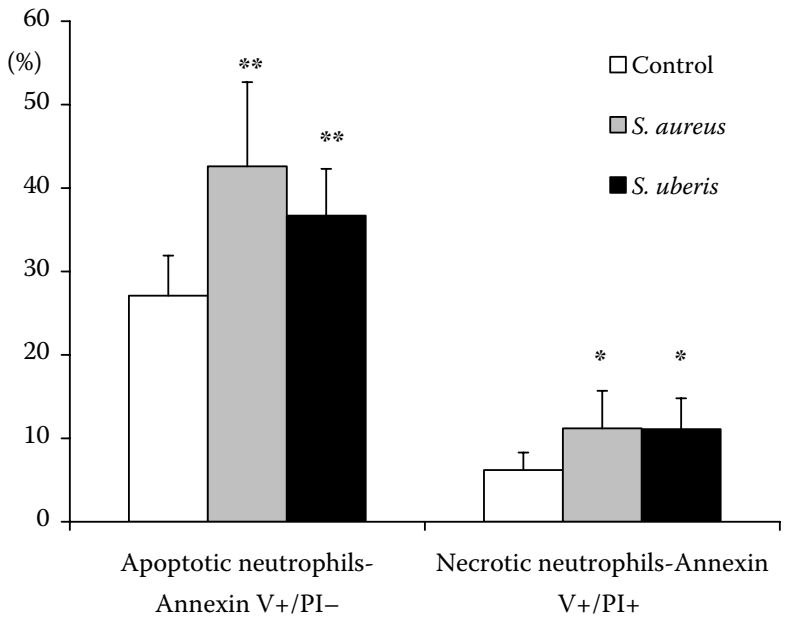

Figure 5. Proportion of apoptotic and necrotic neutrophils after incubation with *S. aureus* and *S. uberis* detected in the flow cytometer. Apoptosis and necrosis of neutrophils were measured by the proportion of Annexin V positive (apoptotic) and Annexin V and PI positive (necrotic) cells in neutrophils cultured with medium only (control) and medium plus *S. aureus* and medium plus *S. uberis* for 3 hours. Means ± SD for 16 mammary glands are given. Significant differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$)

Necrosis of neutrophils following the cultivation with *S. aureus* was less extensive than in the case of *S. uberis*, but the difference was not statistically significant (Figure 3).

Effects of *S. aureus* and *S. uberis* on Annexin-V and propidium iodide positivity in neutrophils

The FCM analysis of neutrophils after labelling the cells with Annexin-V – FITC and PI resulted in the distribution of viable, apoptotic and necrotic cells in three different quadrants on dot plots (with FL1 and FL3 axes) (Figure 4).

The fresh population of neutrophils was characterized by 12.9 ± 4.1% apoptotic cells (FITC+/PI–) and 4.3 ± 2.6% necrotic cells (FITC+/PI+). The 3 h incubation of neutrophils without bacteria (control) increased the number of FITC+/PI– neutrophils and FITC+/PI+ neutrophils to 27.1 ± 4.8% and 6.2 ± 2.1%, respectively (Figure 4).

The dot plots show that the presence of the two pathogens during cultivation resulted in a statistically significant increase in the number of both FITC+/PI– cells ($P < 0.01$) and FITC+/PI+ cells ($P < 0.05$). *S. aureus* was responsible for an increase in FITC+/PI– neutrophils to 42.6 ± 10.1% and an increase in FITC+/PI+ neutrophils to 11.2 ± 4.5%. *S. uberis* was responsible for an increase in FITC+/PI– neutrophils to 36.7 ± 5.6% and an increase in FITC+/PI+ neutrophils to 11.1 ± 3.7% (Figure 4).

The numbers of FITC+/PI– and FITC+/PI+ neutrophils during incubation are summarized in Figure 5.

DISCUSSION

The aim of this study was to confirm whether the co-cultivation of neutrophils of the bovine mammary gland with either *S. aureus* or *S. uberis* leads to the early and/or late signs of apoptosis.

As a part of more general research into the role of neutrophil apoptosis during the resolution of an acute inflammation of the bovine mammary gland, important bacterial pathogens, i.e. *S. aureus* and *S. uberis*, were used in the study to examine their effects on modulation of neutrophil apoptosis. Compared with other studies on the modulation of the apoptosis of blood neutrophils with bacteria, this study focused on the neutrophils of the bovine mammary gland.

The synthetic derivative muramyl dipeptide (MDP) was intentionally used in the experiment because it is a cell wall toxin analogue of Gram-positive bacteria (Bahr and Chedid, 1986). Leukocytes with a high proportion of neutrophils migrated from the blood to the mammary gland in response to MDP. In contrast to blood neutrophils, mammary neutrophils represent a population of relatively older cells. Because neutrophils are short-lived postmitotic cells unable to migrate back to the blood stream, they undergo apoptosis (Raff, 1992). Apoptosis of

neutrophils is characterized by specific morphological and biochemical changes (for review see Simon, 2003). The most important change in the early stage of apoptosis includes phosphatidylserine translocation. In the late stage, nuclear chromatin condensation, membrane blebbing and DNA fragmentation are the most important changes. For these reasons, neutrophil apoptosis detection in this study relied on flow cytometry for phosphatidylserine translocation imaging, on light and transmission electron microscopy, and on ELISA determination of histone-associated DNA fragments.

Using the above-mentioned methods, in the fresh population of neutrophils obtained by an induced influx, apoptotic and also some necrotic cells were detected. This finding is not surprising because apoptosis is well known as an effective mechanism providing protection to the bovine mammary gland against damage. This explains why, in the initial stage of the inflammatory response when the influx of neutrophils is peaking, some of the cells are undergoing apoptosis (Sladek and Rysanek, 2000b, 2001).

Bovine mammary gland neutrophil viability, apoptosis and necrosis are influenced by many factors.

Neutrophil apoptosis is induced already during the influx of these cells due to energy depletion during their migration (Watson et al., 1997; Van Oostveldt et al., 2002a). Therefore apoptotic cells are found already in the fresh population of neutrophils 18 h after the influx induction by MDP (Sladek and Rysanek, 2000a,b, 2001). It is not known whether MDP has a pro- or anti-apoptotic effect on mammary gland neutrophils *in vitro*. In our earlier studies, we observed no statistically significant differences in the proportion of apoptotic neutrophils obtained after intramammary instillation of PBS and MDP (Sladek and Rysanek, 2000b) and after subsequent cultivation *in vitro* (Rysanek et al., in press). However, LPS from Gram-negatives increases viability of bovine neutrophils. LPS delays apoptosis of bovine blood neutrophils *in vitro* (Van Oostveldt et al, 2002b) and mammary gland neutrophils *in vivo* (Sladek and Rysanek, 2001). Finally, parity and stage of lactation of cows can influence neutrophils viability as reported by Mehrzad et al. (2001, 2002).

We considered a 3h incubation time to be quite sufficient for the expression of apoptosis modulation as in our previous study aimed at the aging of neutrophils we demonstrated spontaneous apoptosis in one third of neutrophils of bovine mammary gland after 3 h of incubation (Sladek et al., 2002). A similar incubation time during investigation of apoptosis of activated neutrophils was used by Yang et al. (1998).

When neutrophils alone were cultivated, morphological methods indicated that about one third of the cells were karyopycnotic or zeiotic. These findings were not very surprising either, however, because spontaneous apoptosis resulting from the natural aging of these cells was demonstrated earlier by *in vitro* cultivations of human blood neutrophils (Savill et al., 1989; Payne et al., 1994) and in bovine blood neutrophils (Van Oostveldt et al., 1999) and bovine mammary gland neutrophils (Sladek et al., 2002).

The cultivation of neutrophils with *S. aureus* and *S. uberis* resulted in a statistically significant ($P < 0.01$) decrease in the proportion of karyopycnotic and zeiotic neutrophils. It is still unclear whether ultrastructural changes characteristic of apoptosis may develop in migrated neutrophils as a result of their interaction with Gram-positive bacteria. Data on the induction of morphological signs of migrated neutrophil apoptosis by Gram-positive bacteria is not currently found in the literature. Therefore it is impossible to compare the results of our study with those of other authors. For that reason we can only note that our results are in contrast to the described effect of Gram-negative bacteria on blood neutrophils (Stevens and Czuprynski, 1996; Watson et al., 1996; Yang et al., 1998). With regard to the DNA fragmentation, a lower concentration of histone-associated DNA fragments in the neutrophil lysate was observed in the interaction between neutrophils and *S. aureus* compared with the control in this study. This corroborates the findings of Baran et al. (1996), who demonstrated that neutrophils did not become apoptotic after the phagocytosis of *S. aureus*, and that spontaneously occurring degradation of their DNA was delayed in populations which took up bacteria.

In contrast to findings on *S. aureus*, no study concerning the effects of *S. uberis* on the signs of neutrophil apoptosis has been published.

In neutrophils cultivated in the absence of bacteria (the control) and stained with Annexin-V and PI, FCM showed that approximately one quarter of the cells were apoptotic. Compared with the control, however, the cultivation of neutrophils with either *S. aureus* or *S. uberis* resulted in a statistically significant ($P < 0.01$) increase in the proportion of Annexin-V-positive (i.e. apoptotic) cells. The discrepancy between the proportion of apoptotic neutrophils ascertained by microscopy and FCM is not

very surprising, however. Differences in the quantities of apoptotic neutrophils detected by morphological methods and fragmented DNA assays compared with proportions of Annexin-V-positive cells are well known. Annexin-V has a very high affinity for phosphatidylserine, which is located on the inner leaflet of the cytoplasmic membrane. The translocation of phosphatidylserine is a mark of the early changes of apoptosis (Zhang et al., 1997; Stuart et al., 1998). Therefore Annexin-V is a powerful selective tool for the detection of apoptotic cells, and is particularly suited for the detection of early apoptotic changes (Balasubramanian and Schroit, 2003). Further, this discrepancy in the results of our study can be explained by the well-known fact that in the early stage of apoptosis neutrophils are still capable of bacteria phagocytosis. The process of phagocytosis could prolong the early stage of apoptosis and delay the initiation of DNA fragmentation as mentioned by DeLeo (2004). With regard to the fact that the process of phagocytosis did not occur in the control in our study (*in vitro* cultivation of neutrophils in the absence of bacteria), the initiation of DNA fragmentation was not delayed. That is why, after 3 h of incubation, higher proportions of karyopycnotic neutrophils in the light microscope and higher concentrations of DNA-histone in ELISA were detected.

Yamamoto et al. (2002) reported higher proportions of Annexin-V-positive neutrophils following the cultivation of blood neutrophils with *S. aureus*. This implies that phosphatidylserine binding is expressed earlier on apoptotic neutrophils following the ingestion of bacteria, thus facilitating phagocytosis of the apoptotic cell by the phosphatidylserine receptor bearing macrophages (Fadok et al., 2001; Kagan et al., 2002). Watson et al. (1996) also believed that, compared with a spontaneous apoptosis, the *in vitro* apoptosis of neutrophils was accelerated by phagocytosis and subsequent ingestion of *E. coli*. Recently, it has been verified that an acceleration of neutrophil apoptosis is accomplished by expression of genes that comprise a common apoptosis differentiation program after phagocytosis of *S. aureus* (Kobayashi et al., 2003b). It should be noted that the mechanism of induction of apoptosis in bovine mammary gland migrated neutrophils is still largely unknown.

In addition to apoptosis, different amounts of necrotic neutrophils were found in the control (neutrophils cultivated in the absence of bacteria) and in cultivations with *S. aureus* and *S. uberis*. A statistically significant ($P < 0.05$) increase in the number of necrotic neutrophils was ascertained by FCM. We hypothesize that in conditions *in situ* and *in vivo* apoptotic neutrophils are phagocytosed by macrophages as we observed in our earlier studies (Sladek and Rysanek, 2000a,b, 2001). However, when neutrophils were cultivated *in vitro* in the absence of macrophages apoptotic neutrophils underwent secondary necrosis (Payne et al., 1994). After apoptotic cells have exhausted their energy supplies, they lose the ability to maintain the integrity of their membranes and undergo necrosis. Apoptotic neutrophils undergoing secondary necrosis are morphologically characterised by a total destruction of their nuclear and cytoplasmic components and exhibit all the manifestations and signs of necrosis (Payne et al., 1994). The hypothesis of a secondary necrosis is also corroborated by the fact that no typical morphologic manifestations of a primary necrosis of bovine neutrophils described earlier (for example: Nickerson et al., 1985; Lintner and Eberhart, 1990; Paape et al., 1990; Sladek and Rysanek, 1999b) were observed in this study. Larger numbers of secondary necrotic apoptotic neutrophils are probably a consequence of larger numbers of apoptotic neutrophils after their incubation with *S. aureus* and *S. uberis* compared with the control.

In conclusion it can be noted that the co-cultivation of *S. aureus* and *S. uberis* with neutrophils of the bovine mammary gland leads to the induction of phosphatidylserine translocation characteristic of the early stage of apoptosis. The late signs of apoptosis, karyopycnosis and zeiosis, were delayed by the co-cultivation of neutrophils with both pathogens. Therefore it is obvious that although the programmed cell death of apoptosis is initiated by these pathogens, the completion of the program is delayed. Neutrophils could stay functioning and capacity of pathogens to induce such stage of apoptosis does not contribute to their virulence.

Acknowledgements

The samples for transmission electron microscopy were prepared in Department of Histology and Embryology, Masaryk University, Brno, Czech Republic. We are very grateful to Ms. D. Hradilova and Mr. I. Ilkovics for their excellent technical assistance in the application of TEM and to L. Leva for her assistance with FCM.

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Received: 04–10–25
Accepted after corrections: 04–12–13

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