

## The role of neutrophil apoptosis during experimentally induced *Streptococcus uberis* mastitis

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**ABSTRACT:** The object of the study was to determine if apoptosis of neutrophils and their subsequent elimination from the mammary gland by macrophages are modulated by an infection of *Streptococcus uberis*. The experiments were carried out in 5 clinically normal Holstein × Bohemian Red Pied crossbred heifers, aged 14 to 18 months. Before the experimental infection mammary glands were stimulated by PBS as a control. The samples of cell populations were obtained by lavages of the mammary glands in 4 intervals (24, 48, 72 and 168 h) after the PBS and after the experimental infection. Flow cytometry was used to determine the Annexin V positive and propidium iodide negative neutrophils (Annexin V<sup>+</sup>/PI<sup>−</sup>). The light microscopy was used to determine apoptotic neutrophils and myeloperoxidase (MPO) positive macrophages. After PBS and *S. uberis* administration the total number of both Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophils and karyopycnotic neutrophils peaked at 24 hours. The highest percentages of Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophils were detected at 72 h after PBS and *S. uberis*, respectively. The highest percentages of karyopycnotic neutrophils were detected at 72 h after PBS and 168 h after *S. uberis*, respectively. The total number of MPO+ macrophages was the highest at 24 h after PBS and 72 h after *S. uberis*. The percentage of MPO+ macrophages was the highest at 72 h after PBS and *S. uberis*. The results of this study demonstrate that during experimental infection of the mammary gland by *S. uberis*, the apoptosis of neutrophils is modulated. Apoptosis of neutrophils and the subsequent phagocytosis of apoptotic neutrophils by macrophages were delayed. This may cause the transition of the acute inflammatory reaction to a chronic state.

**Keywords:** *Streptococcus uberis*; experimental infection; neutrophil apoptosis

*Streptococcus uberis* (*S. uberis*) is an important environmental pathogenic cause of mastitis in dairy cows, particularly during the dry period, the period around calving, and during early lactation, which is not controlled effectively by current mastitis control practices (Oliver et al., 2004).

Bacterial infections of the mammary gland generally induce a non-specific acute-phase inflammatory response, which is initiated by the production and release of proinflammatory cytokines, which mediate the inflammatory response (for detail see in Alluwaimi, 2004). In *S. uberis* infected cows, the first step of the acute inflammatory response is the recruitment of neutrophils and accumulation

of large numbers of these cells in the secretory acini (Thomas et al., 1994). However, the influx of neutrophils into the mammary gland has not been associated with a reduction in the numbers of bacteria present after infection with *S. uberis* (Grant and Finch, 1996; Rambeaud et al., 2003). In contrast to infections with *Escherichia coli* (Hill et al., 1978) and *Staphylococcus aureus* (Schalm et al., 1976), neutrophils were not responsible for the elimination of *S. uberis* from the infected mammary gland (Rambeaud et al., 2003).

Neutrophils accumulated at the site of infection have the potential to damage the surrounding tissue through the continued release of inflammatory me-

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diators by intact cells or through cell death by necrosis with the extrusion of toxic cellular contents (Weiss, 1989). Therefore mammary gland neutrophils undergo apoptosis – programmed cell death – and subsequent phagocytosis by macrophages (Sladek and Rysanek, 2000a,b; 2001). Apoptosis could be critical for the resolution of inflammation during infection, as it would reduce local tissue destruction, diminish further inflammation and recruitment of immune cells, and facilitate the termination of the innate immune response by limiting pro-inflammatory capacity (Kobayashi et al., 2003). Macrophage phagocytosis of apoptotic neutrophils is an essential process in the normal resolution of the inflammatory response (Savill et al., 1989).

The regulation of neutrophil apoptosis within inflammatory foci is not well defined, yet it is critical to the optimal resolution of inflammation (Van Oostveldt et al., 2002). Increased apoptosis is supposed to reduce the proportion of active neutrophils and thus the efficiency of the phagocytic defence of the mammary gland, accelerating resolution of the inflammatory reaction at the same time. Secondly, delayed apoptosis is supposed to favour accumulation of active neutrophils in the mammary gland, leading to increased risk of tissue damage, and allowing the transition of mastitis to an irreversible chronic state (Sladek et al., 2005b). There is growing evidence that several bacterial pathogens of the bovine mammary gland modulate neutrophil apoptosis. Van Oostveldt et al. (2002) show that apoptosis of neutrophils was accelerated during *Escherichia coli*-induced mastitis. In our recent study we detected an effect of *Staphylococcus aureus* (*S. aureus*) experimentally induced mastitis on neutrophil apoptosis (Sladek et al., 2005b).

Persistent accumulation of neutrophils in the udder is a characteristic feature of subclinical and chronic mastitis in dairy cows and may be caused by delayed apoptosis (Boutet et al., 2004). The intramammary infections caused by *S. uberis* are predominantly subclinical and can persist for long periods in a chronic state (King, 1981; Jayarao et al., 1999; Phuektes et al., 2001). In order to have a better understanding of the pathogenesis of *S. uberis* mastitis, it will be important to determine whether *S. uberis* alters the neutrophil apoptosis to facilitate the progression of the disease or contributes to chronic inflammation.

The aim of our study was to determine if apoptosis of neutrophils and their subsequent elimination from the mammary gland by macrophages are

modulated by an infection of *S. uberis*. For this purpose, apoptosis of neutrophils and their phagocytosis by macrophages were studied in models of acute reversible response to the bovine mammary gland caused by PBS and in experimentally induced *S. uberis* mastitis of virgin mammary glands.

## MATERIAL AND METHODS

### Animals

The experiments were carried out on twenty mammary glands of five virgin, clinically healthy Holstein × Bohemian Red Pied crossbred heifers aged 15 to 18 months. 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 certificated. The animals were cared for in compliance with the good care practice protocol.

### Experimental design

Before experimental infection, the mammary glands were treated with phosphate buffered saline (PBS, pH 7.4, 0.01M, NaCl 0.138M; KCl 0.0027M, prepared with apyrogenic water). All four mammary gland sinuses of each heifer were rinsed stepwise with PBS to obtain a cell suspension by the following scheme. The first cell sample was obtained by lavage of the left forequarter 24 h after administration of PBS. The remaining quarters were rinsed stepwise at three 24-hour intervals and one 96-hour interval in the following order: left-rear (48 h) → right-front (72 h) → right-rear (at 168 h). These PBS-treated mammary glands were set as a control to the infection. These same heifers were next week experimentally infected with *S. uberis*. Subsequent lavages of the mammary gland lumens were obtained in the same manner as described.

### Preparation of *S. uberis* bacterial suspension

*S. uberis* strain (Czech Collection of Microorganisms, Masaryk University, Brno, CCM 4617), was used to induce experimental infection of mammary glands. The bacteria were prepared by aero-

bic cultivation of massive inoculum on ram blood agar (BA) medium furnished with a semi-permeable membrane (cellophane) for 18 h at 37°C. The bacteria were then harvested and washed once in apyrogen PBS. The total bacterial cell counts were determined using a haemocytometer; the bacterial suspension was adjusted to  $8 \times 10^6$ /ml in PBS (prepared with apyrogenic water). After appropriate dilutions to  $8 \times 10^2$ /ml, the inocula were aspirated into syringes. Each inoculum was tested by determination of bacterial count (CFU/ml) after 24 h of incubation at 37°C on a BA medium.

### Experimental infection

Briefly, modified urethral catheters (AC5306CH06, Porges S.A., France) were inserted into the teat canal after thoroughly disinfecting the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was injected with 20 ml of PBS and only 2 ml of lavage solution were immediately collected back through the catheter directly into the syringe. The lavages were followed by the administration of 5 ml ( $8 \times 10^2$ /ml) of inoculum through the teat orifice using a syringe.

### Bacteriological examination and *S. uberis* counts

Bacteriological examinations of all the lavages were performed by culture on blood agar plates (5% washed ram erythrocytes) with aerobic incubation at 37°C for 24 hours.

The number of *S. uberis* (CFU/ml) was determined in mammary gland lavages after appropriated decimal dilution in sterile PBS by inoculation of 0.1 ml on Petri dish containing BA medium. Inocula were smeared and incubated aerobically at 37°C. After 24 h the CFU/ml of *S. uberis* was determined.

### Processing of the cells

The total mammary cell counts were determined using the Fossomatic 90 apparatus (Foss Electric, Denmark) and the procedure recommended by the International Dairy Federation (IDF, 1995). The Trypan Blue dye exclusion test demonstrated more than 97.0% cell viability in a fresh cell popula-

tion. The cell suspensions were centrifuged at 4°C and  $200 \times g$  for 10 min. One ml of supernatant was retained for resuspension of the pellet.

### Methods

**Differential cell count.** The differential cell count was enumerated by flow cytometry (FCM; FACS Calibur apparatus, Beckton Dickinson, CA, USA) in accordance with a previously applied method (Sladek et al., 2002).

**Flow cytometry (FCM) assessment of neutrophil apoptosis.** Apoptotic neutrophils were analysed by FCM after being simultaneously stained 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.

**Light microscopy assessment of neutrophil apoptosis.** In oil immersion light microscopy (Olympus BH2, Olympus Optical Co., LTD, Japan), on slides stained panoptically using the Pappenheim method (May-Grünwald-Giemsa stain), apoptosis was assessed by the enumeration of at least 200 neutrophils in accordance with the described morphological features (Sladek and Rysanek, 2000a).

**Cytochemistry.** The interaction of macrophages with apoptotic neutrophils was assessed by staining for myeloperoxidase (MPO). Briefly, two smears of mammary lavages of each mammary gland were prepared, dried, fixed for 5 min in 2% glutaraldehyde dissolved in PBS, and stained with dimethoxybenzidine (O-dianisidine HCl, Sigma Chemical Co., St. Luis, USA), hydrogen peroxide (Henson et al., 1978) and subsequently with diluted Giemsa-Romanowski stain using our own modification (Sladek and Rysanek, 2000a).

**Transmission electron microscopy (TEM).** For transmission electron microscopy the cells were prepared according to the procedure as previously described (Sladek and Rysanek, 1999).

### Statistical analysis

The counts and proportions of apoptotic neutrophils and MPO+ macrophages are presented as the statistical means and standard deviations of the twenty mammary glands examined. Significant dif-

ferences in the counts and proportions of apoptotic neutrophils and MPO+ macrophages after PBS treatment and during *S. uberis* infection were determined by paired *t*-tests. The data was processed by STAT Plus software (Matouskova et al., 1992).

## RESULTS

### Experimental infection with *S. uberis* and clinical data

No bacteria were detected in any of the mammary lavages tested in the pre-infection period and 24–168 h after PBS treatment. Following bacterial challenge with *S. uberis*, all the mammary glands of five heifers developed subclinical mastitis. *S. uberis* bacteria were detected bacteriologically throughout the sampling period (Figure 1), with the exception of 1 mammary quarter of one heifer at time point 168 hours. At this time the maximal concentration of bacteria was observed in the remaining mammary glands.

The infected mammary glands showed no swelling during the infection period. The mean maximum rectal temperature was 38.9°C.

### The total and differential leukocyte counts during *S. uberis* infection

Before the challenge, the leukocyte counts averaged  $0.6 \pm 0.2 \times 10^6/\text{ml}$ . Intramammary administration of both PBS and *S. uberis* caused massive neutrophil infiltration from the bloodstream into the challenged mammary glands. Peak leukocyte counts were observed 24 h after the intramammary ad-

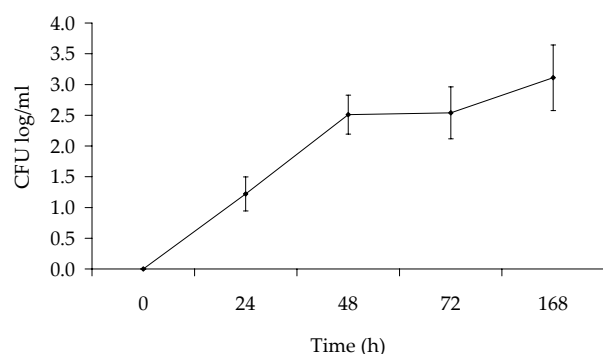


Figure 1. Concentration of bacteria in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation *S. uberis*

ministration of both PBS and *S. uberis* (Figure 2). The number of cells was significantly greater in the *S. uberis*-treated glands in comparison with the PBS-treated mammary glands throughout the sampling period ( $P < 0.05$ ).

In addition to the increase in total cell counts, a large increase was observed in the number and percentage of neutrophils peaking at 24 h after treatment (Figure 2). The proportion of neutrophils was significantly higher ( $P < 0.05$ ) after *S. uberis* in comparison to PBS throughout the sampling period. The peak was followed by a rapid decrease in the total leukocyte count, a corresponding decrease in the percentage of neutrophils (Figure 2), and an increase in the percentage of macrophages (24 h → 168 h:  $2.8 \pm 1.6\% \rightarrow 57.1 \pm 11.7\%$ ) and lymphocytes (24 h → 168 h:  $0.5 \pm 0.3\% \rightarrow 34.1 \pm 7.5\%$ ).

### Dynamics of neutrophil apoptosis during *S. uberis* infection

**Flow cytometry.** Annexin V<sup>+</sup>/PI<sup>−</sup> (apoptotic) neutrophils were detected 24–168 h after PBS and *S. uberis* administration. Total Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophils were the highest at 24 h afterwards with both PBS and *S. uberis*, following the decrease of their count at 48–168 hours. The total Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophil count after *S. uberis* was significantly greater ( $P < 0.01$ : 24–72 h;  $P < 0.05$ : 168 h) when compared to the PBS-treated mammary glands (Figure 3).

The percentage of Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophils was the highest 72 h afterwards with both PBS and *S. uberis*, with a subsequent decrease at 168 hours. The proportion of Annexin V<sup>+</sup>/PI<sup>−</sup> neutrophils after *S. uberis* was significantly greater ( $P < 0.05$ : 72–168 h), when compared to the PBS-treated mammary glands (Figure 3). While the proportion of Annexin V<sup>+</sup>/PI<sup>+</sup> neutrophils (secondary necrotic apoptotic neutrophils) was statistically not different after *S. uberis*, when compared to the PBS-treated mammary glands during all experimental period (PBS: 2.8% to 22.1%; *S. uberis*: 2.1% to 19.9%, respectively). However, the proportion was the highest in both treatments at 168 hours.

**Light microscopy.** Neutrophils showing morphological features of apoptosis (Figure 4) appeared in the cell population 24–168 h afterwards with both PBS and *S. uberis*.

The total apoptotic neutrophil count was the highest at 24 h afterwards with both PBS and *S. uberis* and

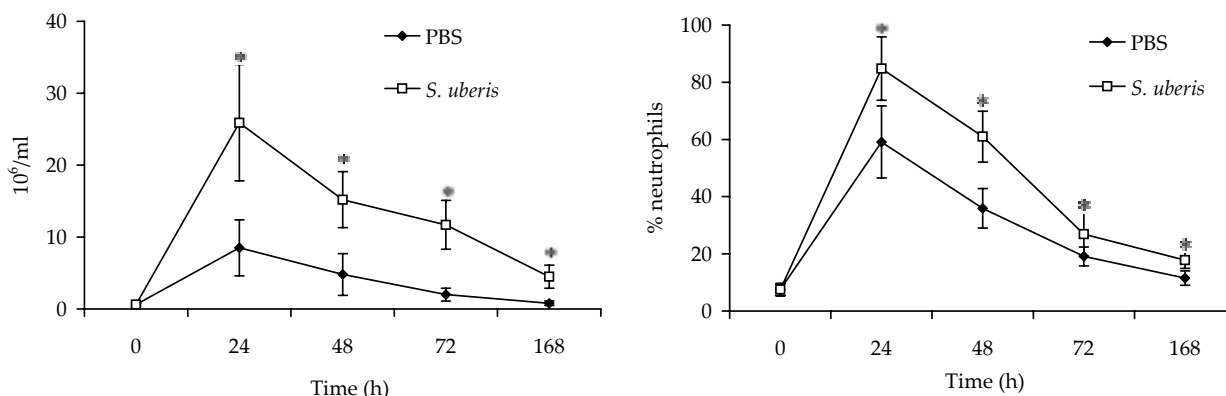


Figure 2. Total number of cells and proportion of neutrophils (mean  $\pm$  S.D.) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. uberis*. Significant between-treatment differences are marked with asterisks (\* $P < 0.05$ ). Differences in the total number of cells and neutrophils are statistically significant at all timepoints ( $P < 0.05$ )

was significantly greater in *S. uberis*-treated glands, compared to the PBS-treated mammary glands ( $P < 0.01$ ). After that (48–168 h), there followed a decrease in the total count of apoptotic neutrophils. The pattern of the total neutrophil apoptosis count corresponds to the total cell count and differential neutrophil count (see Figure 2 and Figure 5). In these cases the dynamics reflect the start of inflammatory response resolution (48–168 h).

After PBS, the percentage of apoptotic neutrophils increased at 48 h and 72 h after treatment, and was significantly ( $P < 0.05$ ; 48h) higher than after *S. uberis*. A decrease in the percentage of apoptotic neutrophils was found 168 h after PBS. In contrast to this a statistically significant ( $P < 0.05$ ) increase in the percentage of apoptotic neutrophils was found 168 h after *S. uberis* (Figure 5).

### Phagocytosis of apoptotic neutrophils during *S. uberis* infection

MPO positive macrophages (MPO+) were detected after both PBS and *S. uberis* throughout the sampling period (24–168 h). The total count of MPO+ macrophages was the highest at 24 h after treatment with PBS, and 72 h after treatment with *S. uberis*. The MPO+ macrophage count in *S. uberis*-treated mammary glands was significantly greater at 48 h, 72 h and 168 h ( $P < 0.01$ ), when compared to the PBS-treated glands (Figure 6).

The highest percentage of MPO+ macrophages was found at 72 h afterwards with both the PBS and *S. uberis* administrations. Thereafter, this percentage decreased slightly in the *S. uberis* administration and more intensively in the PBS administration

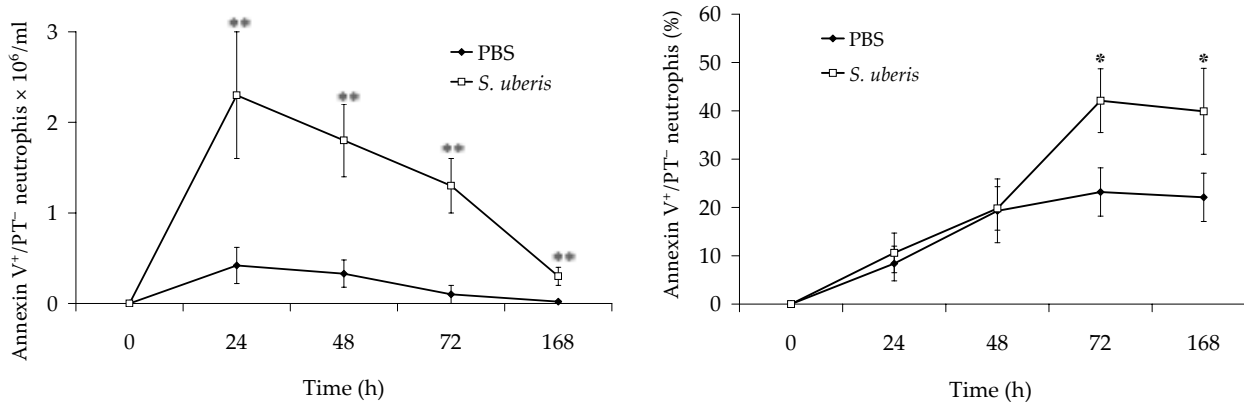


Figure 3. Total number of Annexin V positive and propidium iodide negative neutrophils (a) and relative proportion of Annexin V positive and propidium iodide neutrophils (b) (mean  $\pm$  S.D.) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. uberis*. Significant between-treatment differences are marked with asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ )

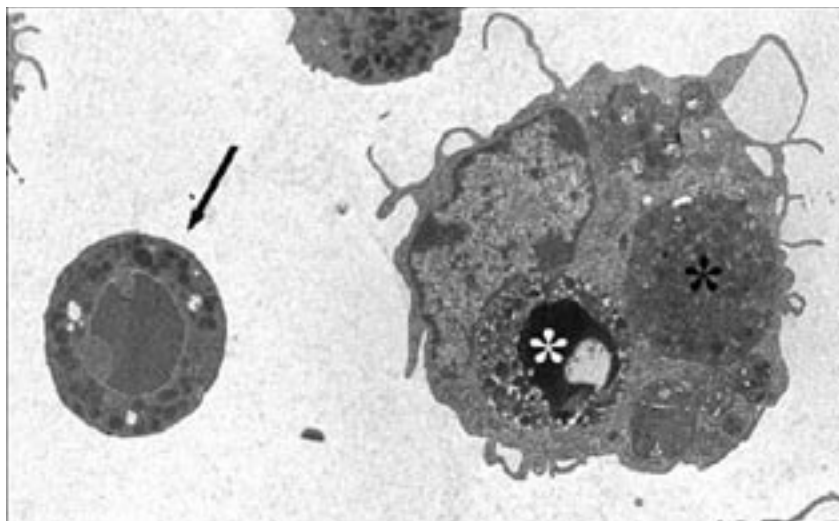


Figure 4. Transmission electron microscopy of apoptotic neutrophil in karyopyknosis (arrow) and macrophage with two phagocytosed apoptotic neutrophils (asterisks) in mammary lavage collected at 24 h after intramammary instillation of *S. uberis*; magnification 5 000×

(Figure 6). The difference in the percentage of MPO+ macrophages in *S. uberis*-treated mammary glands was significantly greater only at 168 h ( $P < 0.05$ ), when compared to the PBS-treated glands (Figure 6).

The presence and degradation of apoptotic neutrophils in the cytoplasm of macrophages were confirmed by TEM (Figure 4).

## DISCUSSION

The objective of our study was to determine if the apoptosis of neutrophils and their subsequent elimination from the mammary gland by macrophages are modulated by an infection of *S. uberis*. To our knowledge, this is the first report about neutrophil apoptosis in the mammary gland during infection by *S. uberis*.

The bacterial strain used in this study to cause experimental *S. uberis* infection induced subclini-

cal mastitis. No clinical signs were observed, but *S. uberis* bacteria were detected in the lavages throughout the entire experimental period. The maximal bacterial concentration was observed as late as 168 h after the challenge just as in the study made by Bannerman et al. (2004a). According to Rambeaud et al. (2003), this fact suggests a lag phase in the ability of *S. uberis* to adapt and grow in the mammary gland.

Experimental infection results in an inflammatory response by the mammary glands, characterised by an increase in the total cell count (more than 40 times greater) with a dominant proportion of neutrophils in the initial stage (24 h). These results are in accordance with other studies using experimental *S. uberis* mastitis (Hill, 1988; Hill et al., 1994; Thomas et al., 1994; Rambeaud et al., 2003; Bannerman et al., 2004a). This higher proportion of migrated neutrophils and increasing concentration of bacteria, on the other hand, suggested

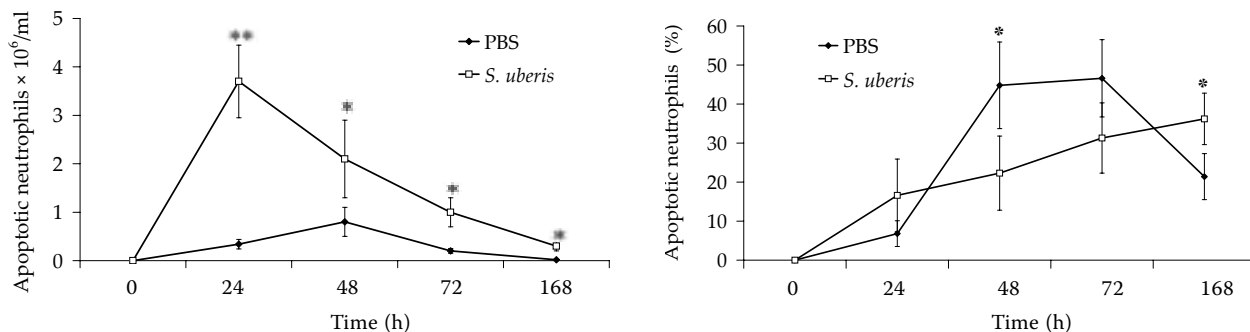


Figure 5. Total number of karyopyknotic neutrophils (a) and relative proportion of karyopyknotic neutrophils (b) (mean  $\pm$  S.D.) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. uberis*. Significant between-treatment differences are marked with asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ )

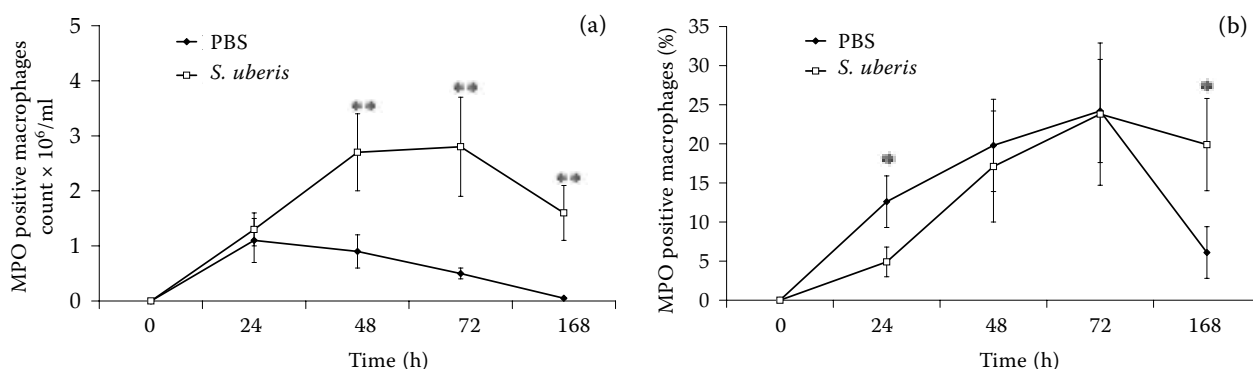


Figure 6. Total number of MPO+ macrophages (a) and relative proportion of MPO+ macrophages (b) (mean  $\pm$  SD) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. uberis*. Significant between-treatment differences are marked with asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ )

the inability to control the growth of *S. uberis* in the mammary gland (Rambeaud et al., 2003). It is known that the influx of neutrophils is not associated with a reduction in the numbers of bacteria *S. uberis* present (Grant and Finch, 1996; Rambeaud et al., 2003). Moreover, macrophages may play a prominent role in the defence of the mammary gland against *S. uberis* mastitis (Hill et al., 1994; Grant and Finch, 1996). Therefore a marked decrease in neutrophil concentration was observed after 48–168 h on one side, elevated counts of bacteria *S. uberis* and increased percentages of macrophages on the other side.

The change in the total numbers of cells and their differential counts indicated the resolution stage of acute inflammatory response of the mammary gland. The decreases in the total counts of neutrophils were caused by apoptosis and their subsequent macrophage phagocytosis. Accordingly, apoptotic cells were detected in the present study in the population of neutrophils, both free in cell suspensions and phagocytized inside macrophages. It is well known that the functional removal of inflammatory cells – neutrophils by apoptosis and their subsequent physical removal by macrophage phagocytosis is the precondition of resolution (Haslett et al., 1994).

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). During the entire experimental period, the apoptotic neutrophils were found in both the early and late stages of apoptosis, which was verified by the

detection of translocated phosphatidylserine in flow cytometry and karyopyknotic nuclei in light microscopy.

Low relative numbers and high total counts of apoptotic neutrophils have been observed in the early stage of the inflammatory response (24 h). This is not surprising as it is well known that these cells already exhibit apoptosis during the early stage of the inflammatory response of the mammary gland when the neutrophil influx culminates (Sladek and Rysanek, 2000a,b, 2001; Sladek et al., 2005b). These apoptotic neutrophils represented freshly infiltrated cells as the mammary glands were rinsed before *S. uberis* or PBS were administered. No differences in the relative counts of apoptotic neutrophils were observed between *S. uberis* and PBS 24 h post infection. Similar results were obtained in our previous study when an inert agent (PBS), glucose and bacterial toxins were used (Sladek and Rysanek, 2000b, 2001; Sladek et al., 2005a; Rysanek et al., 2005). This indicates that during the early stage of the inflammatory response the longevity of neutrophils is decreased primarily by migration (Van Oostveldt et al., 2002) and less by the microenvironment of the mammary gland.

The microenvironment of the mammary gland represents a network of various biological effects; their role has not yet been fully recognised. An important role is played by cytokines that exert either a pro- or anti-apoptotic effect (Paape et al., 2003). However, their role is questionable in the early stage of the inflammatory response in mastitis caused by Gram-positive pathogens. The reasons are the minimum detectable levels or absence of the following pro-inflammatory mediators: inter-

leukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-8 (IL-8). In the early stage of the inflammatory response (24 h), elevated levels of pro-inflammatory mediators were not detected in *S. uberis* mastitis (Rambeaud et al., 2003; Bannerman et al., 2004a), in contrast to infection by *E. coli* (Riollet et al., 2000; Bannerman et al., 2004c), by *Serratia marcescens* (Bannerman et al., 2004a) and by *Klebsiella pneumoniae* (Bannerman et al., 2004b). The lack of such a pronounced pro-inflammatory cytokine response during *S. uberis* mastitis is specific to Gram-positive intramammary infections and remains unknown (Bannerman et al., 2004a). Therefore, no significant differences in the proportion of apoptotic neutrophils between *S. uberis* and PBS were observed at the 24 h time point in this study.

In contrast, the effect of the mammary gland challenge with *S. uberis* on apoptosis was observed later (at 48–168 h). Significant differences were primarily observed in the percentages of respective neutrophil apoptosis stages. Higher proportions of neutrophils in the early stages of apoptosis (Annexin V<sup>+</sup>/PI<sup>-</sup>) were detected for *S. uberis* in comparison with PBS 72 h and 168 h post inoculation. Significantly lower numbers of karyopycnotic neutrophils were detected 48 h and 168 h post administration; however, with a further increasing trend. This data indicates delayed apoptosis of neutrophils during *S. uberis* infection. Apoptotic neutrophils were not be phagocytosed by macrophages became secondary necrotic (Savill et al., 1989). We observed these cells during all experimental period, however with no significant difference between PBS and *S. uberis*. The proportion was the highest in both treatments at 168 h and related with decrease of MPO+ macrophages observed in this study, as mentioned below.

In our previous study, analogical results of *in situ* modulation of neutrophil apoptosis were obtained during experimental *S. aureus* infection of the bovine mammary gland (Sladek et al., 2005b). It was demonstrated for *S. aureus* that after phagocytosis, the so called apoptotic differentiation program is induced in neutrophils; this causes a reduction in their longevity (Kobayashi and DeLeo, 2004). Such pro-apoptotic effect was also demonstrated in some strains of streptococci. Some human streptococci in particular modulate an apoptotic differentiation program in neutrophils and alternatively induced apoptosis in other cell types (DeLeo, 2004; Kobayashi and DeLeo, 2004). For

example, the most pathogenic beta-hemolytic *S. pyogenes* from the streptococcus A group, has been shown to invade and survive within epithelial cells, and bacterial internalization is required for the induction of neutrophil apoptosis (Kobayashi et al., 2003). Non-beta-hemolytic *S. pneumoniae* induced neutrophil apoptosis, decreased their survival and consequently increased the susceptibility of patients infected with the influenza A virus to infections with *S. pneumoniae* (Koch et al., 2000; Zysk et al., 2000; Engelich et al., 2001). Lipoteichoic acid of viridans *S. mutans* induced apoptosis of the cells (Wang et al., 2001) and *S. agalactiae*, from the streptococcus B group, induced apoptosis of macrophages (Ulett and Adderson, 2005).

Unfortunately, the literature contains no data about the pro-apoptotic effect of *S. uberis*. Nevertheless, we may hypothesise that the modulation of neutrophil apoptosis in this study is probably related to an interaction of these cells with *S. uberis* as *in vitro* cocultivation of bovine mammary gland neutrophils with *S. uberis* lead to an increase in Annexin V<sup>+</sup>/PI<sup>-</sup> neutrophils and a decrease in karyopycnotic neutrophils (Sladek et al., 2005a). We observed the same effect of *S. uberis* in this study. Accordingly, a higher proportion of Annexin V<sup>+</sup>/PI<sup>-</sup> neutrophils at 72 h and at 168 h and a lower proportion of karyopycnotic neutrophils at 48 h and at 72 h after *S. uberis* challenge was detected. The effect of *S. uberis* on the proportion of Annexin V<sup>+</sup>/PI<sup>-</sup> neutrophils at 72 h and 168 h related to the maximal concentration of bacteria and related to the higher ratio between the neutrophils and *S. uberis* bacteria.

Furthermore, we hypothesised that a lower proportion of karyopycnotic neutrophils after the *S. uberis* challenge was caused by the prolonged influx of newly migrated neutrophils. Thomas et al. (1994) indicate that 6 days after infection by *S. uberis*, the neutrophil response was still evident. What probably occurs is the so-called dilution effect of an existing neutrophil population by newly migrated neutrophils (Sladek et al., 2005b). Secondly, phagocytosis of bacteria by neutrophils can leads to a prolonged early stage of apoptosis and the postponed initiation of DNA fragmentation (Van Oostveldt et al., 2002; Yamamoto et al., 2002). Finally, Rambeaud et al. (2003) and Bannerman et al. (2004a) saw an increase in the level of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in *S. uberis* mastitis 60h–120 h after the challenge. These pro-inflammatory mediators can delay the neutrophil apoptosis and subsequently prolong the resolution of inflammatory reaction



(Tatsuta et al., 1996; Murray et al., 1997; Kettritz et al., 1998).

The percentage of apoptotic neutrophils present during the resolution of the inflammatory reaction of the mammary gland does not directly reflect the actual dynamics of apoptosis as apoptotic neutrophils are immediately phagocytosed by macrophages (Sladek and Rysanek, 2001). The intensity of phagocytosis by macrophages depends on the amount of apoptotic neutrophils available (Savill, 1992). Therefore, the most intensive clearing of apoptotic neutrophils by macrophages coincided with the maximum expression of apoptosis as observed in this study. For this purpose we used staining for MPO as this method gives excellent concordance with the total uptake of apoptotic neutrophils (Savill et al., 1989). However, a different proportion of MPO+ macrophages was discovered during the initiation and resolution of the inflammatory response after *S. uberis* and PBS.

During the early stage of the inflammatory response, a higher proportion of MPO+ macrophages ( $12.6 \pm 3.3\%$ ) was observed for PBS in comparison with *S. uberis* ( $4.9 \pm 1.9\%$ ). However, the total counts of MPO+ macrophages were not significantly different from a statistical point of view. This resulted in a different ratio of the total counts of apoptotic neutrophils to MPO+ macrophages; this was higher for *S. uberis* (cca 3:1) compared with PBS (cca 0.5:1). This fact can be explained primarily by the different proportions of macrophages in differential cell counts (PBS  $12.6 \pm 8.1\%$  versus *S. uberis*  $2.8 \pm 2.6\%$  at 24 h). Secondly, it is known, that macrophages are the primary phagocytic cell during *S. uberis* mastitis (Thomas et al., 1994). The participation of macrophages in the phagocytosis of bacteria during infection results in the macrophages being activated by the so called conservative route. That activation of macrophages exerts a pro-inflammatory effect and does not lead to the phagocytosis of apoptotic neutrophils (Duffield, 2003; Mantovani et al., 2004).

During resolution of the inflammatory response after *S. uberis* the peak of MPO+ macrophages coincides only with the peak of Annexin V<sup>+</sup>/PI<sup>-</sup> neutrophils and was observed at 72 h, while in PBS the peak of MPO+ macrophages coincided with the peak of Annexin V<sup>+</sup>/PI<sup>-</sup> neutrophils and the peak of karyopyknotic neutrophils at 72 h together. After that, the proportion of MPO+ macrophages decreased dramatically, suggesting that the resolution of the inflammatory reaction to PBS is gradu-

ally brought to an end (Sladek and Rysanek, 2000b; Sladek and Rysanek, 2001). Whereas in *S. uberis*, the proportion of MPO+ macrophages decreases very slightly and this suggests the resolution of the inflammatory reaction to *S. uberis* is prolonged and can lead to chronicity.

The results of this study demonstrate that during experimental infection of the mammary gland by *S. uberis*, the apoptosis of neutrophils is modulated. Apoptosis of neutrophils and the subsequent phagocytosis of apoptotic neutrophils by macrophages were delayed. This may cause the transition of the acute inflammatory reaction to a chronic state.

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