Vet. Res. 36 (2005) 629–643
© INRA, EDP Sciences, 2005
DOI: 10.1051/vetres:2005023

Original article

Neutrophil apoptosis during experimentally induced *Staphylococcus aureus* mastitis

Zbysek SLADEK[a]*, Dusan RYSANEK[b], Helena RYZNAROVA[a], Martin FALDYNA[b]

[a] Department of Morphology, Physiology and Veterinary Sciences, Mendel University of Agriculture and Forestry, Zemedelska 1, 613 00 Brno, Czech Republic
[b] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic

(Received 8 October 2004; accepted 14 January 2005)

Abstract – The objective of this study was to determine whether neutrophil apoptosis and their consequent elimination by macrophages from the mammary gland is modulated by an infection caused by *Staphylococcus aureus* (*S. aureus*). The study was performed on twenty mammary glands of 5 virgin heifers. A buffered physiological solution (PBS) was administered as a means of control into the mammary glands of the heifers and after 168 h, the glands were inoculated with *S. aureus*. The samples of cell populations were obtained by lavages of the mammary glands in 4 intervals (24, 48, 72 and 168 h) after the experimental infection. Flow cytometry was used for determination of Annexin-V positivity and propidium iodide (PI) negativity of neutrophils. Light microscopy was used for determination of neutrophil karyopyknosis. Cytochemistry was used for the detection of myeloperoxidase-positive (MPO+) macrophages. Instillation of *S. aureus* resulted in an intramammary infection which persisted during the following experimental period. The total number of both Annexin-V-positive and PI negative neutrophils and karyopyknotic neutrophils peaked at 24 h after both of PBS and *S. aureus* administration. The highest percentages of Annexin-V-positive and PI negative neutrophils and karyopyknotic neutrophils were detected 48 and 168 h after PBS and *S. aureus* administration, respectively. The total number of MPO+ macrophages was the highest 24 h and 48 h after PBS and *S. aureus* administration, respectively; the percentage of MPO+ macrophages was the highest at 72 h in both cases. The dynamics of resolution of mastitis caused by *S. aureus* was very similar to the resolution of inflammatory response of the mammary gland after PBS administration. Mechanisms of cell pathogen elimination as well as inflammation resolution were very intensively involved; nevertheless, the mammary gland infection persisted. An early inclusion of the mechanisms of an acute inflammatory resolution thus paradoxically led to chronic infection.

***Staphylococcus aureus* / mastitis / neutrophil apoptosis**

1. INTRODUCTION

Acute bovine mastitis involves an initial phase, which includes an inflammatory reaction, and a resolution phase. Initiation of the inflammatory reaction is caused by the production and release of chemoattractants by macrophages and epithelial cells for the rapid recruitment of neutrophils in order to eliminate invading bacteria. These interactions result in the accumulation of neutrophils on the site of infection [15]. Neutrophils are

* Corresponding author: sladekz@seznam.cz

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. Neutrophils form the first line of immunological defence of the bovine mammary gland against Gram-positive and Gram-negative pathogens [16].

Neutrophils rapidly accumulate on the site of infection, and there is a concomitant potential to cause severe tissue destruction should they undergo necrotic lysis and release cytotoxic granule contents into mammary gland tissues [2]. Therefore, it follows that timely and vigilant execution of a controlled programmed cell death in neutrophils is important in order to prevent damage to healthy tissues, and is necessary for resolution of infection [9]. Previous elimination of bacterial pathogens, extravasated inflammatory cells (neutrophils) and their contents from tissue are necessary for resolution of acute infection [5]. Therefore, mammary gland neutrophils undergo apoptosis and they are phagocytosed by macrophages [23, 24].

Apoptosis of neutrophils is a non-pathologic mode of cell death characterised by unique morphological and biochemical features that are distinct from oncosis and necrosis. Apoptosis is marked by cellular shrinking, condensation and margination of the chromatin, roughing of the plasma membrane, and translocation of phosphatidylserine on the cell membrane (for a review see Van Cruchten and Van Den Broeck [27]). Apoptosis of neutrophils is accompanied by the loss of a number of fundamental functions: a reduced capability to respond to stimulation, a reduced intensity of phagocytosis (ranging to a total inability to phagocytise), and a lowered degree of degranulation and respiratory burst [31]. Therefore, these fundamental functions are critical for the maintenance of host defences and in reaction to an insult by infectious agents. Under these circumstances, the immune system of the mammary gland may be weakened in its fight against infection and a transition into chronicity may occur.

Some bacterial pathogens moderate neutrophil apoptosis in order to survive and cause disease [3]. One of them is *Staphylococcus aureus (S. aureus),* which is the most important and prevalent contagious mammary pathogen. It causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows [4].

S. aureus possesses a variety of virulence factors: protein A and a capsule or a pseudocapsule (slime), which are antiphagocytic factors, and toxins (alpha and beta) which appear to play a major role in the staphylococcal virulence [26]. These factors interfering with neutrophil function enable a pathogen to develop clinical or chronic infection [4]. A considerable feature of the virulence of bacterial pathogens (e.g. *S. aureus*) is the bacterial modulation of the neutrophil apoptosis differentiation programme [10]. This programme represents the final stage of transcription-regulated neutrophil maturation and regulates multiple processes, including apoptosis [3].

Both proapoptotic and antiapoptotic effects of *S. aureus* on neutrophils has been described in the literature. It was demonstrated that lipoteichoic acid from *S. aureus* inhibits spontaneous apoptosis and, therefore, increases the lifespan of neutrophils [11], and the in vitro cultivation of blood granulocytes with vital *S. aureus* leads to a delay of DNA fragmentation [1]. In contrast, Yamamoto et al. [32] has reported higher proportions of Annexin-V-positive neutrophils following the cultivation of blood neutrophils with heat-killed *S. aureus* bacteria.

The effect of *S. aureus* on the lifespan of neutrophils has only been studied in in vitro conditions. Therefore, it is not known whether the factors of *S. aureus* virulency are operative in situ during mastitis as modulators of neutrophil apoptosis because no relevant data on this problem exists in the literature.

Our working hypothesis is based on following two main consequences of neutrophil apoptosis modulated by *S. aureus*.

The first, increased apoptosis is supposed to reduce the proportion of active neutrophils and thus the efficiency of the phagocytic defence of the mammary gland, accelerating the resolution of inflammatory reaction at the same time. Second, delayed apoptosis is supposed to favour an accumulation of active neutrophils in the mammary gland leading to increased risks of tissue damage, and allowing a transition of mastitis to an irreversible chronic state.

It is difficult to say how *S. aureus* in vivo moderates neutrophil apoptosis in the mammary gland, because neutrophil apoptosis is an essential step in the resolution of acute inflammation induced not only by bacterial pathogens and their components, but also by non-specific noxa [23, 24].

This data gives rise to a question as to what extent the factors of *S. aureus* virulency, on the one hand, and mechanisms of an acute inflammatory resolution, on the other hand, will participate in the modulation of neutrophil viability during mastitis.

The objective 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. aureus*. For this purpose, apoptosis of neutrophils and their phagocytosis by macrophages were studied in models of acute reversible bovine mammary gland injury caused by PBS and in experimentally induced *S. aureus* mastitis of virgin mammary glands.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were carried out on twenty mammary glands of five virgin, clinically healthy Holstein × Bohemian Red Pied crossbred heifers aged 16 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 certified. The animal care was in agreement with good care practice protocol. All heifers were free of intramammary infections, as demonstrated by a bacteriological examination of mammary lavages.

2.2. Experimental design

Before experimental infection, the mammary glands were treated with phosphate buffered saline (PBS, pH 7.4, 0.01 M, NaCL 0.138 M; KCL 0.0027 M, 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-h intervals and one 96-h 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 then experimentally infected with *S. aureus*. Subsequent lavages of the mammary gland lumens were obtained in the same manner as described. In the lavages after infection with *S. aureus*, the number of colony-forming units (CFU/mL) of *S. aureus* was assessed. The total somatic cell count was assessed by the fluoro-optoelectronic method. The differential leukocyte count in the cell suspension obtained from the lavages was assessed by flow cytometry. Finally, apoptosis of neutrophils was detected in flow cytometry by Annexin-V positivity and in light microscopy by morphological features. Phagocytosis of apoptotic neutrophils by macrophages was assessed by detection of neutrophil associated enzyme myeloperoxidase in macrophages.

2.3. Preparation of the *S. aureus* inoculum

S. aureus, strain Newbould 305 (Czech Collection of Microorganisms, Masaryk University Brno: CCM 6275), was used. The

inoculum was prepared by growing the challenge organism on ram blood agar (BA) medium. Three colonies of this culture were then inoculated into brain heart infusion (BHI) broth and cultivated under continual rotation (30 rot/min) for 18 h at 37 °C. The stock culture was stored at 4 °C until use. On the day of inoculation, 1 mL of the stock culture was inoculated into 5 mL fresh BHI and incubated under continual rotation (30 rot/min) for 4 h in order to allow the bacteria to be in the exponential growth phase. They were then harvested and washed once in PBS (0.01 M, pH 7.4; NaCL 0.138 M; KCL 0.0027 M, prepared with apyrogenic water). Total bacterial cell counts were determined using a haemocytometer; the bacterial suspension was adjusted to 8×10^6/mL in PBS. After appropriate dilutions to 8×10^2/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 BA medium.

2.4. Experimental infection

Briefly, modified urethral catheters (AC5306CH06, Porges S.A., France) were inserted into the teat canal after a thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was injected with 20 mL of PBS (0.01 M, pH 7.4; NaCl 0.138 M; KCl 0.0027 M, prepared with apyrogenic water) and lavages were immediately collected back through the catheter directly to the syringe. The lavages were followed by the administration of 5 mL (800 CFU/mL) of inoculum through the teat orifice using a syringe.

2.5. Bacteriological examination

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 h.

2.6. Processing of the cells

Total mammary cell counts were determined using the Fossomatic 90 apparatus (Foss Electric, Denmark) and the procedure recommended by the International Dairy Federation (IDF) [7]. The Trypan Blue dye exclusion test demonstrated more than 97.0% cell viability in fresh neutrophils by an 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 resuspension of the pellet.

2.7. Differential cell count

The differential cell count was enumerated by flow cytometry (FCM; FACS Calibur apparatus, Beckton Dickinson, CA, USA). Percentages of individual cell types – granulocytes, macrophages and lymphocytes – were read from forward scatter versus side scatter dot plots. Gating was set up based on the expression of a characteristic antigen: CD2 and CD14 (Serotec Ltd, Oxford, United Kingdom).

2.8. Flow cytometry (FCM) assessment of neutrophil apoptosis

Apoptotic 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. [30]. 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 analysed after 15 min of incubation at room temperature by FCM (FACS Calibur apparatus, Becton Dickinson, Mountain View, CA, USA) by differentiation of at least 10 000 cells. The FCM analysis of neutrophils after labelling the

cells with Annexin-V–FITC and PI resulted in the distribution of viable (Annexin V^-/PI^-), apoptotic (Annexin V^+/PI^-) and necrotic cells (Annexin V^+/PI^+) in three different quadrants on dot plots (with FL1 and FL3 axes). Dot plots were evaluated qualitatively and quantitatively using the CellQuest software analysis (Beckton Dickinson, Mountain View, CA, USA).

2.9. Light microscopy assessment of neutrophil apoptosis

Apoptosis was assessed with oil immersion light microscopy (Olympus BH2, Olympus Optical Co., LTD, Japan), on slides stained panoptically by the Pappenheim method (May-Grünwald-Giemsa stain) by the enumeration of at least 200 neutrophils in accordance with the described morphological features [22].

2.10. 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. Louis, USA), hydrogen peroxide [6] and subsequently with diluted Giemsa-Romanowski stain using our own modification [22].

After enumeration of 200 macrophages on each slide, the interaction of macrophages with apoptotic neutrophils was quantified calculating the percentage of MPO-positive (MPO+) macrophages from the total number of macrophages.

2.11. Transmission electron microscopy (TEM)

For transmission electron microscopy the cells were prepared according to the procedure we previously described [21].

2.12. Statistical analysis

The proportions of apoptotic neutrophils and MPO+ macrophages are presented as statistical means and standard deviations of the twenty mammary glands examined. Significant differences in the proportions of apoptotic neutrophils and MPO+ macrophages after PBS treatment and during *S. aureus* infection were determined by a paired *t*-test. The data was processed by STAT Plus software [13].

3. RESULTS

3.1. Clinical data and bacteriological status

Following bacterial challenge with *S. aureus,* all mammary glands developed subclinical mastitis. Infected mammary glands showed no swelling. Rectal temperatures did not increase.

No bacteria were detected in any of the mammary lavages tested in the pre-infection period and 24–168 h after PBS treatment. Bacteria *S. aureus* were detected bacteriologically throughout the sampling period, 24–168 h after instillation.

3.2. The total and differential leukocyte counts during *S. aureus* infection

Before the challenge, leukocyte counts averaged $0.9 \pm 0.5 \times 10^6$/mL. Intramammary administration of both PBS and *S. aureus* caused massive neutrophil infiltration from the bloodstream into the challenged mammary glands. As shown in Figure 1, peak leukocyte counts were observed 24 h after the intramammary administration of both PBS and *S. aureus*. The cellular inflammatory response was significantly greater in the *S. aureus*-treated glands compared to the PBS-treated mammary glands throughout the sampling period ($p < 0.01$). At 48 h, the total cell counts had decreased in both; however the magnitude of the decrease was greater in *S. aureus* infused quarters compared to PBS infused quarters (Fig. 1).

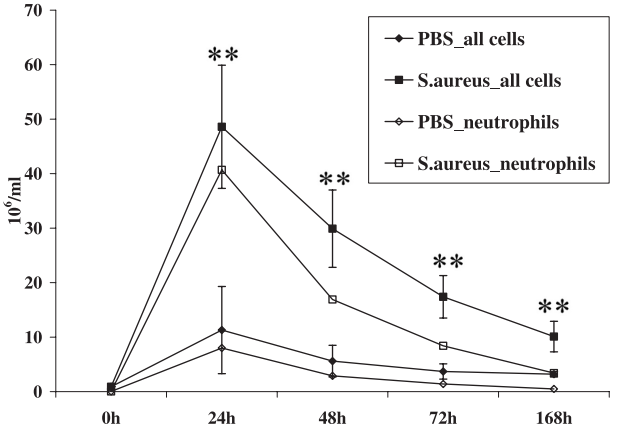

Figure 1. Total number of cells and total number of neutrophils (mean ± S.D.) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. aureus*. Significant between-treatment differences are marked with asterisks for the total number of cells (** $p < 0.01$). Differences in total number of neutrophils are statistically significant for all timepoints ($p < 0.01$).

Table I. Differential cell counts during *S. aureus* mastitis (mean ± SD).

Time points (hours)	Neutrophils (%)	Macrophages (%)	Lymphocytes (%)
	PBS		
Intact	2.5 ± 0.9	68.7 ± 1.4	28.9 ± 3.4
24	70.6 ± 12.0	19.6 ± 6.3	9.8 ± 2.6
48	51.3 ± 9.2	31.4 ± 8.8	17.3 ± 6.3
72	38.0 ± 5.9	43.4 ± 1.8	18.6 ± 7.8
168	14.3 ± 0.6	59.9 ± 1.5	25.8 ± 9.1
	S. aureus mastitis		
24	83.1 ± 59.1	11.6 ± 3.7	6.3 ± 1.8
48	56.6 ± 29.4	25.6 ± 5.3	17.8 ± 2.9
72	48.4 ± 12.2	26.5 ± 11.8	25.1 ± 5.2
168	33.3 ± 3.9	36.6 ± 6.6	30.1 ± 9.3

Macrophages were typically present in larger numbers than the lymphocytes or neutrophils in the mammary glands before the challenge. 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 (Fig. 1 and Tab. I). As shown in Figure 1, the increase was significantly higher in *S. aureus*, when compared to the PBS-treated mammary glands ($p < 0.01$). The number and proportion of neutrophils were significantly higher ($p < 0.01$) after *S. aureus* treatment in comparison to PBS throughout the sampling period (except 48 h). The peak was followed by a rapid decrease in total leukocyte count (Fig. 1), a corresponding decrease in the percentage of neutrophils, and an increase in the percentage of macrophages and lymphocytes (Tab. I).

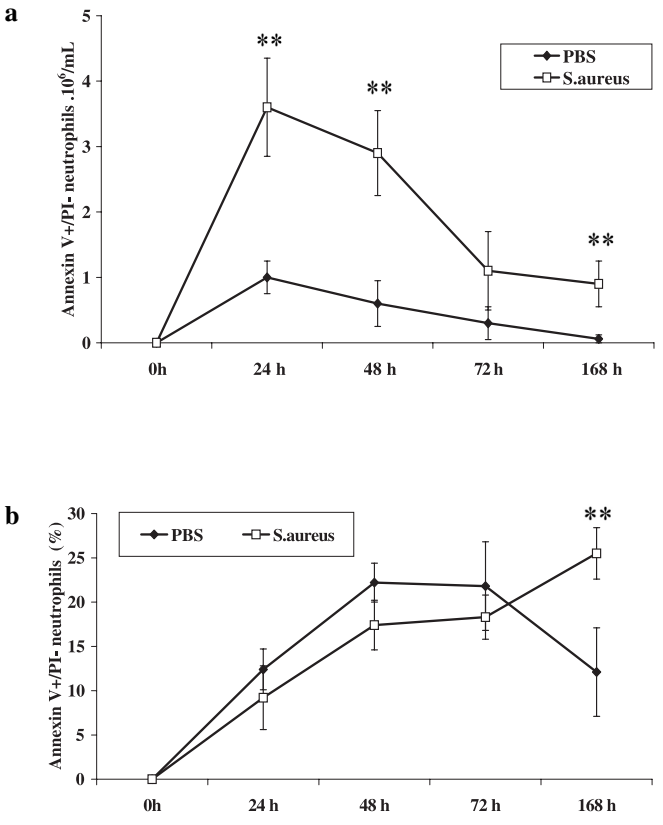

Figure 2. Total number of Annexin V positive and propidium iodide negative neutrophils (a) and relative proportion of Annexin V positive and propidium iodide neutrophils (b) (mean ± SD) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. aureus*. Significant between-treatment differences are marked with asterisks (** $p < 0.01$) (in Figure not marked).

3.3. Dynamics of neutrophil apoptosis during *S. aureus* infection

3.3.1. Flow cytometry

No apoptotic neutrophils (Annexin V^+/PI^-) were found in the mammary glands before challenge. Annexin V^+/PI^- neutrophils were detected 24–168 h after PBS and *S. aureus* administration. Total Annexin V^+/PI^- neutrophils were the highest 24 h after both PBS and *S. aureus* treatment, 48–168 h following the decrease of their count. Total Annexin V^+/PI^- neutrophil count after *S. aureus* was significantly greater ($p < 0.01$), when compared to the PBS-treated mammary glands (Fig. 2).

The percentage of Annexin V^+/PI^- neutrophils was the highest 48 h after PBS with a subsequent decrease at 168 h (Fig. 2). In contrast, after infection by *S. aureus*, a lower percentage, relative to PBS, of Annexin V^+/PI^- neutrophils 24–72 h after instillation was observed, and the highest percentage was observed at 168 h (Fig. 2).

3.3.2. Light microscopy

No apoptotic cells detected in light microscopy were found in the neutrophil

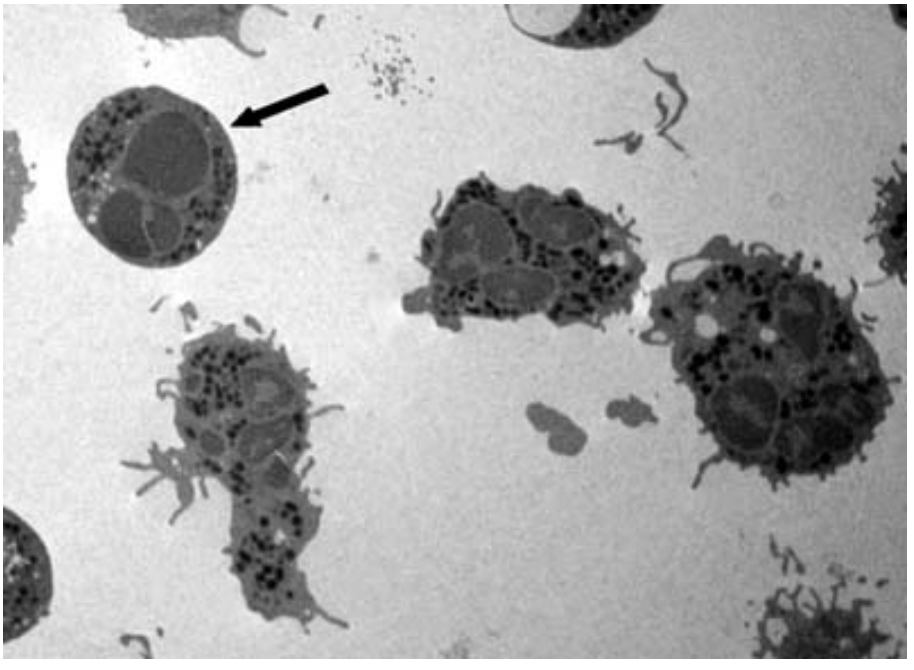

Figure 3. Transmission electron microscopy of apoptotic neutrophil in karyopyknosis (arrow). Magnification 5000 ×.

population in the mammary glands before challenge. Neutrophils showing morphological features of apoptosis (pyknotic nuclei, condensed chromatin, zeiosis – confirmed in TEM, Fig. 3) appeared in the cell population 24–168 h after both PBS and *S. aureus* treatment.

The total apoptotic neutrophil count was the highest 24 h after both PBS and *S. aureus* treatment and was significantly greater in *S. aureus*-treated glands, compared to the PBS-treated mammary glands ($p < 0.01$). After that (48–168 h), a decrease of the total count of apoptotic neutrophils followed. The pattern of the total neutrophil apoptosis count corresponds with the total cell count and differential neutrophil count (see Fig. 1, Tab. I and Fig. 4). In both cases the dynamics reflect the start of inflammatory response resolution (48–168 h).

The percentage of apoptotic neutrophils was only 13.2 ± 2.9% and 8.5 ± 1.5% 24 h after PBS and *S. aureus,* respectively. After PBS, the percentage of apoptotic neutrophils increased 48 h and 72 h after treatment, and was significantly ($p < 0.01$) higher than after *S. aureus* administration. A dramatic decrease of the percentage of apoptotic neutrophils was found 168 h after PBS. On the contrary, a sharp increase of the percentage of apoptotic neutrophils was found 168 h after *S. aureus* (Fig. 4). The pattern of the relative Annexin V^+/PI^- neutrophil proportions was similar to the relative apoptotic neutrophil proportions detected in light microscopy (see Figs. 2 and 4).

3.4. Phagocytosis of apoptotic neutrophils during *S. aureus* infection

No MPO+ macrophages were found in the mammary glands before the challenge. Macrophages with MPO+ regions in their

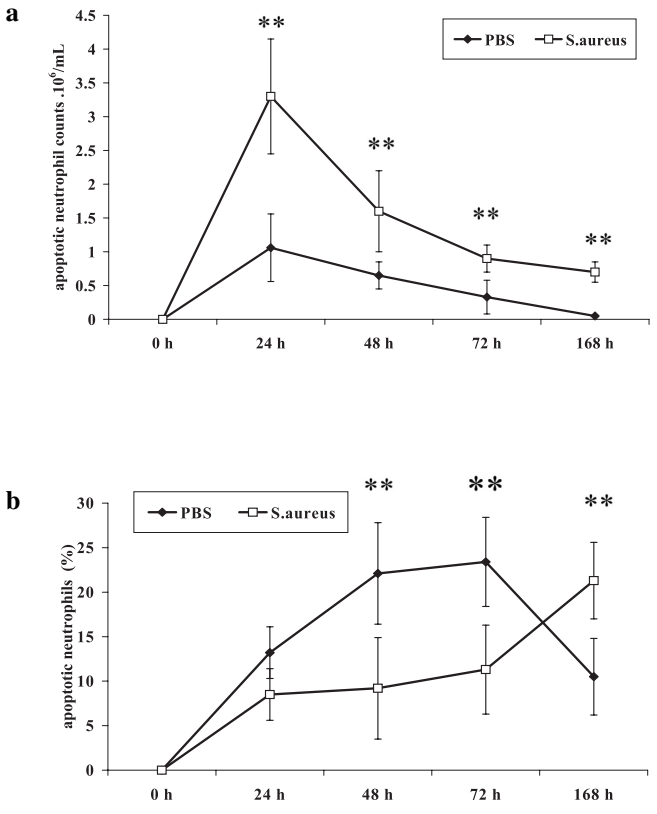

Figure 4. Total number of karyopyknotic neutrophils (a) and relative proportion of karyopyknotic neutrophils (b) (mean ± SD) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. aureus*. Significant between-treatment differences are marked with asterisks (** $p < 0.01$).

cytoplasma were detected after both PBS and *S. aureus* throughout the sampling period (24–168 h). The total count of MPO+ macrophages was the highest 24 h after treatment with PBS, and 48 h after treatment with *S. aureus.* The MPO+ macrophage count in *S. aureus*-treated mammary glands was significantly greater at 48 h ($p < 0.01$), and 72 h and 168 h ($p < 0.05$), when compared to the PBS-treated glands (Fig. 5).

The highest percentage of MPO+ macrophages was found 72 h after both the PBS and *S. aureus* administrations. Thereafter, this percentage decreased slightly with the *S. aureus* administration and more intensively with the PBS administration (Fig. 5). The difference in the percentage of MPO+ macrophages in *S. aureus*-treated mammary glands was significantly greater at 168 h ($p < 0.05$), when compared to the PBS-treated glands (Fig. 5).

3.5. Transmission electron microscopy of phagocytosis of apoptotic PMN

The presence and the degradation of apoptotic neutrophils in the cytoplasm of macrophages were confirmed by TEM. Apoptotic neutrophils present in phagosomes and phagolysosomes of macrophages showed various stages of degradation of

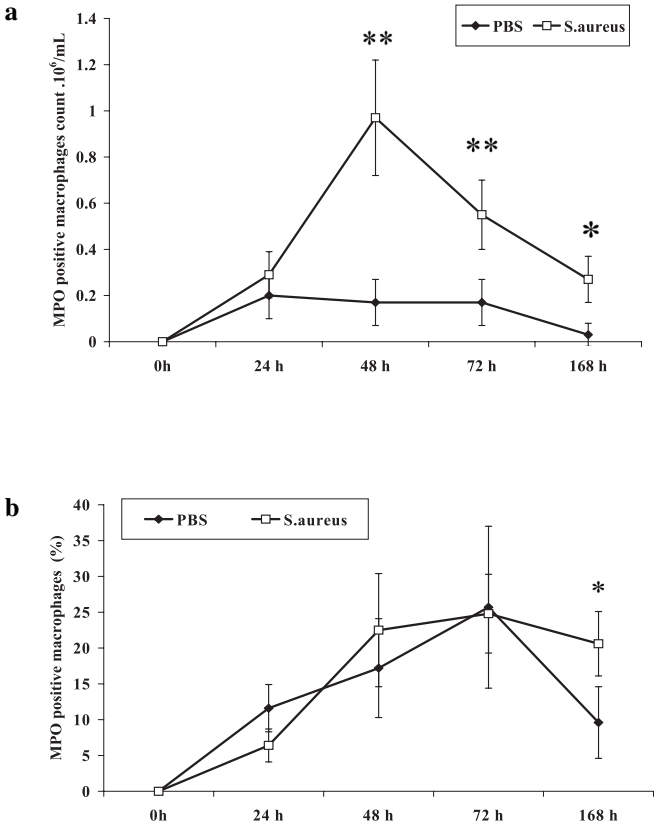

Figure 5. Total number of MPO+ macrophages (a) and relative proportion of MPO+ macrophages (b) (mean ± SD) in mammary lavages collected at 0, 24, 48, 72, and 168 h after intramammary instillation of PBS or *S. aureus*. Significant between-treatment differences are marked with asterisks (* $p < 0.05$; ** $p < 0.01$).

their nuclear and cytoplasmic components. In addition to phagocytosed apoptotic neutrophils, phagocytosed *S. aureus* were also detected in the cytoplasm of macrophages (Fig. 6).

4. DISCUSSION

The objective of our experiments was to determine whether apoptosis of neutrophils and their subsequent elimination from mammary glands by macrophages are modulated by an infection caused by *S. aureus*.

Instillation of mammary glands by *S. aureus* was followed by an inflammatory response. The acute-phase response was characterised by massive migration and accumulation of neutrophils in the mammary gland sinuses. After 24 h, the total number of cells increased by more than 50 fold, and the number of neutrophils increased by approximately 900 fold in contrast with a normal mammary gland. Additionally, lower proportions of other cells, i.e. macrophages and lymphocytes, were detected. Such a reaction of the mammary gland is not at all that surprising

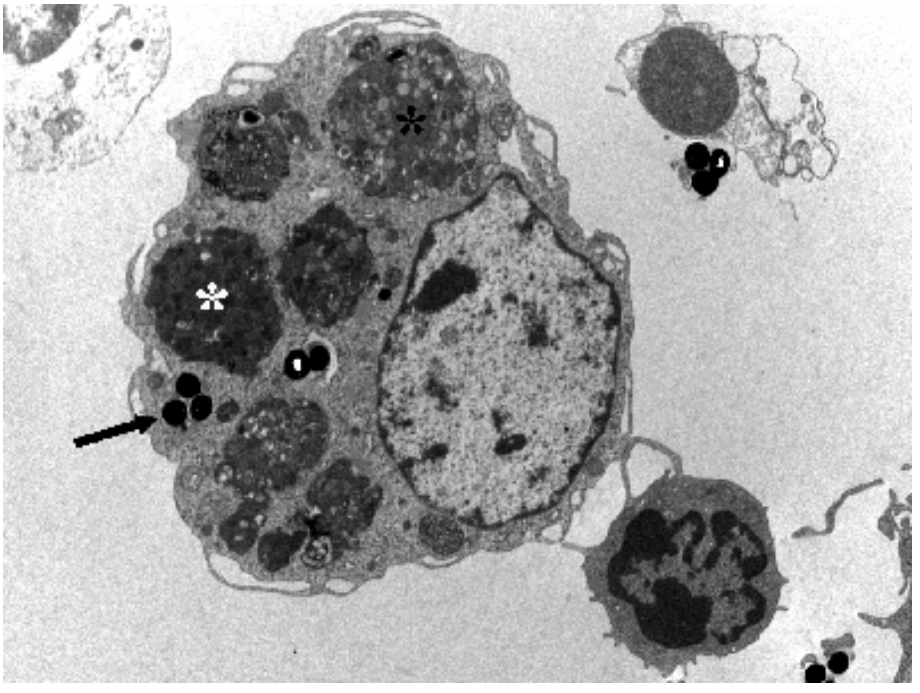

Figure 6. Transmission electron microscopy of macrophage with phagocytosed apoptotic neutrophils (asterisks) and bacteria (arrow). Magnification 5000 ×.

because an increase in the total number of cells and in the proportion of neutrophils occurs during an infection caused by *S. aureus* [18, 19]. No clinical signs were observed, but *S. aureus* bacteria were detected in the lavages during the whole experimental period. Therefore, in this study, instillation of the mammary glands by *S. aureus* induced subclinical mastitis, which became chronic. It is known that *S. aureus* infection often starts with an acute-phase and generally becomes chronic and subclinical [26].

An inflammatory response also occurred 24 h after the intramammary administration of PBS. However, the values of the total cell count and percentages of neutrophils did not reach a value as high as that which was monitored in the *S. aureus* administration, but they were statistically significantly lower.

Neutrophils with biochemical and morphological features characteristic of apoptosis were detected in the mammary gland already during the acute phase of inflammatory response 24 h after *S. aureus* instillation. These neutrophils were found in both the early and late stages of apoptosis, which was verified by the detection of translocated phosphatidylserine in FCM and karyopyknotic nuclei in LM. The presence of apoptotic neutrophils already in the acute-phase response can be explained as follows: (i) neutrophils have a short life-span of 1–2 days and they are predisposed for spontaneous apoptosis [17], (ii) neutrophils are recruited from the bloodstream in various age-phases [8], (iii) apoptosis in neutrophils is induced by migration into the lumen of the mammary gland [28], (iv) another, certainly non-omissible effect is the effect

of the microenvironment of the mammary gland that is represented by a complicated network of biological factors which have not yet been explained [12, 16].

The role of significant inflammatory mediators (for instance TNF-alpha, IL-1-beta, IL-8) in the modulation of neutrophil apoptosis is disputable since these agents were not detected in a soluble form during *S. aureus* infection, in contrast to infection by *E. coli* [18]. This fact is reflected in the symptoms of *S. aureus* mastitis, which were of a subclinical character.

It is not, however, known whether the modulation of apoptosis of neutrophils migrating into the bovine mammary gland occurs in situ during mastitis. No data on the quantitative aspects of neutrophil apoptosis during *S. aureus* mastitis has been found in the available literature. Therefore, the results of this study can only be debated by comparison with the studies in which a modulation of apoptosis of migrated neutrophils of the bovine mammary gland has been detected on models of reversible damage of the bovine mammary gland by bacterial components [23, 24].

Even though neutrophil apoptosis is a normal phenomenon during neutrophil influx, *S. aureus* infection of the mammary gland, in contrast to PBS administration, caused significant quantitative differences in the numbers and proportion of apoptotic neutrophils occurring in the early and late stages of apoptosis. During persistent infection by *S. aureus*, higher total cell counts, as well as lower percentages, of Annexin V^+/PI^- and karyopyknotic neutrophils, were observed at all time intervals, in contrast to PBS administration.

A lower proportion of Annexin V^+/PI^- and karyopyknotic neutrophils after *S. aureus* infection implies a prolonged influx of neutrophils, only a part of which reaches the early stage of apoptosis. What probably occurs is the so-called dilution effect of an existing cell population by newly migrated neutrophils. This conclusion can be based on the finding of a higher expression of CD11a/CD18 on neutrophils detected during persistent *S. aureus* infection (our unpublished data), which implies a continual influx of newly recruited cells (as described by Riollet et al. [19]), in contrast to PBS administration, in which neutrophil influx is not prolonged [22, 25].

It was verified in the past that *S. aureus* in vitro induces programmed cell death in human blood neutrophils [10]. We have also shown this induced effect of *S. aureus* in migrated neutrophils of the bovine mammary gland in vitro (unpublished data). However, after an incubation of neutrophils with *S. aureus*, we observed an increase in Annexin V^+/PI^- neutrophils and a decrease in the proportion of karyopyknotic neutrophils. Therefore, we suppose that the higher numbers of Annexin V^+/PI^- neutrophils and the lower proportion of karyopyknotic neutrophils 24 h after instillation are related to an interaction of these cells with *S. aureus*. This discrepancy is related to the known fact that in an early stage of apoptosis, neutrophils can phagocytose bacteria. The process of phagocytosis leads to a prolonged early stage of apoptosis and postponed initiation of DNA fragmentation [29, 32]. We verified the occurrence of this process by the finding that the process of bacterial phagocytosis by neutrophils did not occur in PBS-treated mammary glands. Therefore, the initiation of DNA fragmentation was not delayed in the neutrophils of these mammary glands, which led to an increased proportion of karyopyknotic cells in contrast to the same time interval after *S. aureus* infection.

The initiation of resolution of an acute inflammatory reaction occurred 24 h after *S. aureus* instillation and PBS administration. The resolution was characterised by a decrease in the total number of cells and a decrease in the proportion of neutrophils. It is known that induction of apoptosis in the inflammation cells (neutrophils) and their subsequent removal by macrophage phagocytosis is the requirement for resolution of

an inflammatory response [5], which was confirmed by the detection of MPO+ macrophages. The histochemical evidence of MPO positivity in macrophages represents an effective tool for the evaluation of macrophage functional activity regarding the removal of apoptotic cells during the resolution of an acute inflammation of bovine mammary glands [23–25].

MPO+ macrophages were detected in the lavages already at 24 h after experimental *S. aureus* infection and PBS administration. At that time, MPO+ macrophages formed a low proportion and a low count of all macrophages, even though the highest number of apoptotic neutrophils was found in the mammary gland. It is known that an increased proportion of apoptotic neutrophils is parallel with an increased proportion of phagocytosing macrophages [20]. This discrepancy detected by us can be explained by the fact that the gland sinuses were rinsed before PBS administration or by *S. aureus* instillation. By so rinsing, most of the fully immunocompetent macrophages were removed. Because of their immaturity, the newly migrated macrophages do not have the ability to recognise and phagocytose apoptotic neutrophils [14]. The high number of MPO+ macrophages 48 h after *S. aureus* experimental infection is then, in this case, related to the increasing proportion of MPO+ macrophages, which is implied by an increasing proportion of immunocompetent cells.

The observed peak of 72 h in the relative proportions of MPO+ macrophages implies the peak participation of these cells in the process of neutrophil influx resolution after the experimental *S. aureus* infection and PBS administration. The fact that during the persistent *S. aureus* infection the total, as well as relative numbers of MPO+ macrophages, were statistically significantly higher than after PBS administration implies that the process of *S. aureus* mastitis resolution continued further, even after the stated time. Similar results were observed in a previous study during the resolution of an acute inflammatory response after intramammary administration of PBS and LPS [24].

It is important to bring to the attention the fact that at 48–72 h after the experimental *S. aureus* infection a significant decrease of the total number of macrophages and MPO+ macrophages occurred and simultaneously, there was a slight increase of the relative proportion of all macrophages and the relative proportion of MPO+ macrophages. This occurrence can be hypothetically explained as the final stages of clearance of apoptotic neutrophils and migration of inflammatory macrophages into the mammary gland, and the emigration of MPO+ macrophages into regional lymph nodes of the mammary gland [23].

In conclusion, it is important to emphasise that the dynamics of *S. aureus* mastitis resolution is very similar to a resolution of an acute damage of the mammary gland by administration of PBS. Nevertheless, the numbers of apoptotic neutrophils and MPO+ macrophages are significantly higher after experimental *S. aureus* infection. Although the mechanisms of bacterial noxa elimination as well as resolution of infection are very intensively involved, an infection of the mammary gland persists. This verifies that there is an insufficient effectiveness of the means leading to the elimination of bacterial pathogens. In this way the involvement of mechanisms of an acute inflammation resolution paradoxically leads to a chronic infection.

ACKNOWLEDGEMENTS

This study was supported by a Grant Agency of the Czech Republic (GA_R No. 524/03/1531) and by the Ministry of Agriculture (MZE 0002716201). The samples for transmission electron microscopy were prepared in the Department of Histology and Embryology, Masaryk University in Brno. 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. Levá for her assistance with FCM.

REFERENCES

[1] Baran J., Guzik K., Hryniewicz W., Ernst M., Flad H.D., Pryjma J., Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria, Infect. Immun. 64 (1996) 4242–4248.

[2] Capuco A.V., Paape M.J., Nickerson S.C., In vitro study of polymorphonuclear leukocyte damage to mammary tissue of lactating cows, Am. J. Vet. Res. 47 (1986) 663–669.

[3] DeLeo F.R., Modulation of phagocyte apoptosis by bacterial pathogens, Apoptosis 9 (2004) 399–413.

[4] Fox L.K., Gay J.M., Contagious mastitis, Vet. Clin. North Am. Food Anim. Pract. 9 (1993) 475–487.

[5] Haslett G., Savill J., Whyte M.K.B., Stern M., Dransfield I., Meagher L.C., Granulocyte apoptosis and the control of inflammation, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 30 (1994) 327–333.

[6] Henson P.M., Zanolari B., Schartzman N.A., Hong S.R., Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitisation and effect of cytochalasin B, J. Immunol. 121 (1978) 851–855.

[7] International Dairy Federation, Milk-Enumeration of somatic cells, International IDF Standard 148A, 1995, 8 p.

[8] Ishii Y., Hashimoto K., Nomura A., Sakamoto T., Uchida Y., Ohtsuka M., Hasegawa S., Sagai M., Elimination of neutrophils by apoptosis during the resolution of acute pulmonary inflammation in rats, Lung 176 (1998) 89–98.

[9] Kobayashi S.D., Voyich J.M., DeLeo F.R., Regulation of the neutrophil-mediated inflammatory response to infection, Microb. Infect. 5 (2003) 1337–1344.

[10] Kobayashi S.D., Braughton K.R., Whitney A.R., Voyich J.M., Schwan T.G., Musser J.M., DeLeo F.R., Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils, Proc. Natl. Acad. Sci. USA 100 (2003) 10948–10953.

[11] Lotz S., Aga E., Wilde I., Van Zandbergen G., Hartung T., Solbach W., Laskay T., Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2, J. Leukoc. Biol. 75 (2004) 467–477.

[12] Lee A., Whyte M.K.B., Haslett C., Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators, J. Leukoc. Biol. 54 (1993) 283–288.

[13] Matouskova O., Chalupa J., Cigler M., Hruska K., STAT Plus, software manual 1.01. Veterinary Research Institute, Brno, 1992, pp. 1–168.

[14] Newman S.L., Henson J.E., Henson P.M., Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages, J. Exp. Med. 156 (1982) 430–442.

[15] Paape M.J., Mehrzad J., Zhao X., Dettileux J., Burvenich C.H., Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes, J. Mammary Gland Biol. Neoplasia 7 (2002) 109–121.

[16] Paape M.J., Bannerman D.D., Zhao X., Lee J.W., The bovine neutrophil: Structure and function in blood and milk, Vet. Res. 34 (2003) 597–627.

[17] Raff M.C., Social controls on cell survival and cell death, Nature 356 (1992) 397–400.

[18] Riollet C., Rainard P., Poutrel B., Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*, Clin. Diagn. Lab. Immunol. 7 (2000) 161–167.

[19] Riollet C., Rainard P., Poutrel B., Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection, J. Dairy Sci. 84 (2001) 1077–1084.

[20] Savill J.S., Willie A.H., Henson J.E., Walport M.J., Henson P.M., Haslett C., Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages, J. Clin. Invest. 83 (1989) 865–867.

[21] Sladek Z., Rysanek D., Ultrastructure of phagocytes from mammary glands of non-pregnant heifers, Anat. Histol. Embryol. 28 (1999) 291–298.

[22] Sladek Z., Rysanek D., Morphology of apoptosis of polymorphonuclear leukocytes isolated from mammary glands of unbred heifers, Vet. Med. Czech 45 (2000) 71–81.

[23] Sladek Z., Rysanek D., Apoptosis of polymorphonuclear leukocytes of the juvenile bovine mammary glands during induced influx, Vet. Res. 31 (2000) 553–563.

[24] Sladek Z., Rysanek D., Neutrophil apoptosis during resolution of bovine mammary gland injury, Res. Vet. Sci. 70 (2001) 41–46.

[25] Sladek Z., Rysanek D., Faldyna M., Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide, Vet. Res. 33 (2002) 191–204.

[26] Sutra L., Poutrel B., Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*, J. Med. Microbiol. 40 (1994) 79–89.

[27] Van Cruchten S., Van Den Broeck W., Morphological and biochemical aspects of apoptosis, oncosis and necrosis, Anat. Histol. Embryol. 31 (2002) 214–223.

[28] Van Oostveldt K., Vangroenweghe F., Dosogne H., Burvenich C., Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early and midlactating healthy cows, Vet. Res. 32 (2001) 617–622.

[29] Van Oostveldt K., Paape M.J., Burvenich C., Apoptosis of bovine neutrophils following diapedesis through a monolayer of endothelial and mammary epithelial cells, J. Dairy Sci. 85 (2002) 139–147.

[30] Vermes I., Haanen C., Stefferns-Naaken H., Reutelingsperger C.P., A novel assay for apoptosis: flow cytometry detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, J. Immunol. 184 (1995) 39–51.

[31] Whyte M.K., Meagher L.C., MacDermot J., Haslett C., Impairment of function in aging neutrophils is associated with apoptosis, J. Immunol. 150 (1993) 5124–5134.

[32] Yamamoto A., Taniuchi S., Tsuji S., Hasui M., Kobayashi Y., Role of reactive oxygen species in neutrophil apoptosis following ingestion of heat-killed *Staphylococcus aureus*, Clin. Exp. Immunol. 129 (2002) 479–484.