Neutrophil apoptosis during the resolution of bovine mammary gland injury

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SUMMARY

The role of neutrophil apoptosis in the resolution of bovine mammary gland injury induced by intramammary administration of physiological buffered saline (PBS) or lipopolysaccharide (LPS) was investigated. Twenty mammary glands of five non-pregnant heifers were used in the two studies and each animal received both stimuli. Samples of cell populations were collected by mammary gland lavages before and 24, 48, 72 and 96 hours after treatment and examined by light microscopy and staining for myeloperoxidase (MPO). A marked influx of neutrophils into the mammary gland was observed 24 hours after stimulation. At the same time, apoptotic neutrophils and MPO-positive macrophages (MAC) were identified in the samples. The numbers increased to reach maximum values at 48 hours after stimulation with PBS and at 72 hours after stimulation with LPS. The observed differences in the length of the resolution period indicate that neutrophil viability can be modulated by delaying the apoptotic process. Apoptosis of neutrophils and their subsequent phagocytosis by MAC can be regarded as a significant mechanism in the removal of neutrophils from the acutely injured mammary glands and, hence, in the resolution of bovine mastitis. ©2001 Harcourt Publishers Ltd

THE initial phase of inflammation after acute injury of the bovine mammary gland is characterised by a massive influx of neutrophils (Nickerson et al 1986). The resolution of acute inflammation depends on a timely clearance of these inflammatory cells. Most extravasated neutrophils die at the site of inflammation and are cleared away (Weiss 1989). One hypothesis is that the neutrophils undergo necrosis (Nickerson et al 1986, Paape et al 1990). However, necrosis of neutrophils prolongs the inflammation, because of the toxic molecules released by neutrophils in this process (Capuco et al 1986, Weiss 1989). Thus, an alternative mechanism for clearance of inflammatory cells is required during the resolution of acute bovine mammary gland inflammation.

In our early in vivo studies (Sládek and Rysánek 2000a,b) we demonstrated that neutrophils underwent apoptosis (programmed cell death) in an induced influx of bovine juvenile mammary gland. Apoptosis of neutrophils led to the recognition and ingestion of apoptotic neutrophils by macrophages (MAC). It seems that apoptosis of neutrophils is another way of clearing these cells from the mammary gland. The question remains whether apoptosis of neutrophils and the subsequent elimination by MAC from the bovine mammary gland are specific responses to the chemoattractant used in our experiment (Sládek and Rysánek 2000b), or whether this occurs as a general response to other forms of damage to this organ. According to recent reports, neutrophil apoptosis plays an irreplaceable role in the resolution of several inflammatory conditions in man and laboratory animals (for a review see Whyte et al 1999). However, the role of apoptosis in the resolution of inflammation of the bovine mammary gland has not been researched.

The objective of our experiments was to determine if apoptosis of neutrophils and their subsequent elimination from the mammary gland by MAC is a specific response to the stimulus used, or whether these events occur as a general response to damage of this organ. For this purpose, apoptosis of neutrophils, and their phagocytosis by MAC, was studied in a model of acute reversible bovine mammary gland injury caused by two different stimuli.

MATERIALS AND METHODS

Animals

The experiments were carried out in five clinically normal Holstein and Bohemian Red Pied cross-breed heifers, aged 15 to 18 months.

Acute mammary gland injury (MGI)

Two studies were carried out in the same animals, approximately 3 months apart. In both studies, all four glands of each heifer were washed with 20 ml of sterile buffered 0·01 M physiological saline (PBS), pH 7·4, to obtain control samples of cell populations. The washing was followed by intramammary administration of PBS...
(10 ml) in the first study, and of 5 μg of lipopolysaccharide (LPS from *Escherichia coli* serotype 0128:B12, Sigma, St. Louis, MO, USA) in 10 ml of PBS in the second study. Samples of cell populations were obtained by mammary lavages (ML). The first sample was collected from the left forequarter 24 hour after administration of PBS or LPS. Further samples were collected at 24 hours intervals from the remaining quarters in the following order: left hindquarter, right forequarter, right hindquarter. Bacteriological examination of all the lavages, by culture on blood agar plates (5 per cent washed sheep erythrocytes) and aerobic incubation at 37˚C for 24 hours, yielded invariably negative results (no bacteria grew under the culture conditions). Total cell counts were determined using a haemocytometer. The trypan blue dye exclusion test demonstrated that the cells in each ML were ≥ 96 per cent viable. The cell suspensions were centrifuged at 4˚C and 200 g for 10 minutes. One millilitre of supernatant was removed and retained and the remaining supernatant was decanted. The pellet was resuspended in the retained supernatant.

**Assessment of apoptosis**

Two smears of each ML were prepared and stained panoptically by the Papanicolau method (Bessis 1973). Neutrophils showing chromatin condensation, simplification of nuclear structure, vacuolisation and shrinking at light microscopy (oil immersion, objective magnification factor 100×) were classified as apoptotic (Sládek and Rysánek 2000a). Apoptosis was also measured with an Annexin V apoptosis detection kit (Annexin-V-FLUOS staining kit, Boehringer Mannheim, Germany). Flow cytometry (FACS Calibur aparatus, Beckton Dickinson, Mountain View, Canada) was used.

**Histochemistry**

The interaction of MAC with apoptotic neutrophils was quantified using a modified method of staining for myeloperoxidase (MPO) as described by Sládek and Rysánek (2000). Briefly, two smears of each ML were prepared, dried, fixed in 2 per cent glutaraldehyde in PBS for 5 minutes and stained with o-dianisidine in hydrochloric acid with hydrogen peroxide (Sigma, St. Louis, MO, USA). Thereafter, the same smears were stained using a modification of the Giemsa-Romanowski method. The MPO-positive areas in the cytoplasm of MAC acquired a granular structure and a contrasting brownish-grey hue. Moreover, pyknotic and strongly basophilic nuclei of apoptotic neutrophils became visible in the suspension or in the cytoplasm of the MPO-positive MAC. The percentages of MAC containing recently phagocytised apoptotic neutrophils and MAC with indistinguishable phagocytised apoptotic neutrophils and a granular distribution of MPO-positive material were determined in the population of MPO-positive MAC.

**Statistics**

Multifactorial analysis of variance was used to determine significant sources of variability. The significance of differences between means for inflammatory cell numbers, neutrophil numbers, percentage of apoptotic neutrophils and percentage of MPO-positive MAC in response to both stimuli used were determined by Schefee’s method. The correlation between the percentage of apoptotic neutrophils and MPO positive MAC was examined by determination linear regression and correlation coefficients. STAT Plus software (Matoušková et al 1992) was used.

**RESULTS**

**Analysis of sources of variability**

Stimuli and time were found to be significant sources of variability (P < 0.01) whereas mammary glands (udder quarters) were not. The significance of differences between

![Fig 1](#)

Comparison of leukocyte counts in mammary lavages after stimulation with physiological buffered saline (PBS) (open bars) or lipopolysaccharide (LPS) (black bars). Means ± so for five heifers/time point are given. Significant differences are shown (* P<0.05 vs PBS).
responses to stimuli at different time points could therefore be determined (see below).

**Inflammatory cell numbers in inflamed mammary glands**

Acute mammary gland injury was followed by a rapid influx of large numbers of leukocytes into the mammary gland. Peak leukocyte counts were observed 24 hours after the intramammary administration of PBS or LPS. The cellular inflammatory response was significantly greater in the LPS-treated, when compared to the PBS-treated, mammary glands ($P < 0.05$). The cell counts had decreased by 48 hours (Fig 1).

**Differential cell counts in inflamed mammary glands**

Macrophages were typically present in larger numbers than lymphocytes or neutrophils in control mammary glands ($64.6 \pm 10.3$ vs $32.8 \pm 10.9$ vs $2.6 \pm 1.3$ per cent). In addition to the increase in total cell counts, a large increase was observed in the percentage of neutrophils peaking at 24 hours after treatment. As shown in Figure 2, the increase was significantly higher in the LPS-treated, when compared to the PBS-treated, mammary glands ($P < 0.01$). The peak was followed by a rapid decrease in both total leukocyte and neutrophil counts (Figs 1 and 2) and a corresponding increase in the percentage of MAC. At 96 hours after treatment, the neutrophil:MAC ratio approached that observed in control samples (Fig 2). The clearance of neutrophils was more marked and occurred more rapidly in PBS-treated, when compared with LPS-treated, mammary glands.

**Apoptosis of neutrophils**

A highly significant correlation was obtained between neutrophil apoptosis determined morphologically and Annexin V expression when measured over the 96 hours after PBS administration [$r_{(24h)}=0.95$, $r_{(48h)}=0.91$, $r_{(72h)}=0.90$, $r_{(96h)}=0.89$, $n=20$]. As the two methods gave quantitatively similar results, apoptosis was measured by examination of morphological characteristics.

No apoptotic cells were seen in the population of neu-
trophils collected from control mammary glands. Morphological changes typical of apoptosis i.e. condensation of chromatin (karyopyknosis), cell shrinking, loss of pseudopodia and fragmentation into apoptotic bodies were observed at 24 hours after treatment with PBS or LPS (Fig 3). At the same time, MAC containing phagocytosed apoptotic neutrophils in their cytoplasm appeared in the cell suspensions (see below). The percentage of apoptotic neutrophils was highest at 48 and 72 hours after the treatment with PBS and LPS, respectively (Fig 4).

Engulfment of apoptotic neutrophils by MAC

All MAC in cell suspensions collected prior to administration of PBS or LPS were MPO-negative. Myeloperoxidase-positive MAC were found in cell populations collected at 24 hours after the treatment with either PBS or LPS (Fig 5). Less than one half of the MPO-positive MAC contained pyknotic cell nuclei in their cytoplasm which is indicative of recent phagocytosis of apoptotic cells (Fig 3).

The highest percentages of MPO-positive MAC were observed at 48 and 72 hours after the treatment with PBS and LPS, respectively. There was a highly significant correlation between the percentages of MPO-positive MAC and apoptotic neutrophils throughout the 96 hours observation period (n=20, r=0.68, P<0.01 for the treatment with LPS).

DISCUSSION

In the present study we used a method of induction of MGI using PBS or LPS, which is characterised by a massive and time dependent influx of neutrophils into the mammary gland (Wardley et al 1976).

Several studies have investigated the mechanism of mammary gland clearance of neutrophils by necrosis (Nickerson et al 1986, Paape et al 1990). Apoptosis was not studied in
Neutrophil apoptosis in resolution of injury

The resolution of acute MGI does not directly reflect the actual dynamics of apoptosis because neutrophic neutrophils are immediately phagocytosed by MAC. The peak percentages of both apoptotic and MPO-positive MAC were observed at 24 hours after administration of PBS or LPS. It seems that this time corresponds to the life span of neutrophils which has been estimated at 1–2 days (Squier et al. 1991). The early occurrence of apoptotic neutrophils at 24 hours after administration of PBS or LPS indicates that some neutrophils were recruited from the blood stream at an advanced age, as noted by Ishii et al. (1998).

The percentage of apoptotic neutrophils was highest at 48 and 72 hours after the treatment with PBS and LPS, respectively. The delay in reaching peak values observed after treatment with LPS could be explained by the fact that this bacterial product is known to extend the life span, and delay apoptosis, of neutrophils (Haslett et al. 1991, Lee et al. 1993, Yamamoto et al. 1993).

The percentage of apoptotic neutrophils present during the resolution of acute MGI does not directly reflect the actual dynamics of apoptosis because neutrophic neutrophils are immediately phagocytosed by MAC. Compared with the rather slow development of detectable morphological changes indicative of apoptosis, the recognition by MAC, phagocytosis and degradation of apoptotic neutrophils are extremely rapid events (Newman et al. 1982). Further studies have demonstrated that MAC begin to phagocytose neutrophils at an early stage of apoptosis when the cytoplasmatic membrane is intact (Savill 1994). Non-degraded, recently phagocytosed neutrophils with pyknotic nuclei in the cytoplasm of MAC were observed also in our recent studies in juvenile bovine mammary glands (Sládek and Ryšánek 2000). This could explain the relatively low percentage of neutrophils showing typical morphological signs of apoptosis in ML and suggest that the majority of apoptotic cells may have already been engulfed and degraded by MAC (see Savill et al. 1989). This agrees with the distribution of MPO granular pattern observed in most MPO-positive MAC.

The high percentage of apoptotic neutrophils and MPO-positive MAC, as well as the strong positive correlation between them, suggest a significant role of MAC in the resolution of acute MGI. The peak percentages of both apoptotic neutrophils and MPO-positive MAC were observed at 48 and 72 hours after the treatment with PBS and LPS, respectively. So, the most intensive clearing of apoptotic neutrophils coincided with the maximum expression of apoptosis. As stated by Savill (1992b), the intensity of phagocytosis by MAC depends on the amount of available apoptotic neutrophils.

Necrotic neutrophils were also observed sporadically in the cell suspensions obtained from the injured mammary glands, but the percentage remained almost unchanged and did not correspond to the percentage of MPO-positive MAC (data not shown). Nevertheless, the fact that these were necrotic neutrophils suggests that tissue damaging factors are present in the mammary gland.

It can be concluded from the results presented here that apoptosis of neutrophils and their subsequent phagocytosis by MAC is a general response to the resolution of damage to this organ. However, this effect appears to be delayed by LPS.

ACKNOWLEDGEMENTS

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REFERENCES


Author Query

1. Please check references Savill et al 1992 a and b are correct in the text
2. Update Sládek & Rysánek 2000b if possible