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Effect of *Staphylococcus aureus* and *Streptococcus uberis* on apoptosis of bovine mammary gland lymphocytes

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ABSTRACT

The aim of this study was to determine whether lymphocyte apoptosis is modulated by infections caused by *Staphylococcus aureus* and *Streptococcus uberis*. Samples of cell populations were obtained by lavage of the mammary glands at 4 intervals (24, 48, 72 and 168 h) following infection. The percentage of apoptotic lymphocytes peaked at 168 h after challenge with *S. aureus* or *S. uberis*. Subsequent experiments focused on *in vitro* cultivation of mammary gland lymphocytes with *S. aureus* and *S. uberis*. These experiments showed a lower percentage of apoptotic lymphocytes following 3 h of cultivating cells with bacteria than after cultivation without bacteria. The results demonstrate that during both experimental infection of bovine mammary glands with *S. aureus* or *S. uberis* and during *in vitro* cultivation of lymphocytes with *S. aureus* or *S. uberis*, apoptosis of lymphocytes is delayed.

1. Introduction

Staphylococcus aureus and *Streptococcus uberis* are the most important of the pathogens causing clinical and subclinical bovine mastitis (Fox and Gay, 1993; Pedersen et al., 2003). Resident and incoming leukocytes play an important role in the mammary gland's defence system against such invading pathogens. Macrophages and lymphocytes are the predominant resident cells in the healthy mammary gland, while intramammary infections induce the recruitment of leukocytes (and especially neutrophils) from blood into the mammary gland (Concha et al., 1986; Miller et al., 1991). The role of macrophages and neutrophils in the inflammatory response of bovine mammary glands is well known (Van Oostveldt et al., 2001, 2002; Paape et al., 2002, 2003; Boutet et al., 2004; Sladek et al., 2005, 2006; Sladek and Rysanek, 2006).

Lymphocytes, which recognize antigens through membrane receptors specific for invading bacteria, are very important components of the mammary gland immune system (Sordillo et al., 1997). During bouts of acute mastitis caused by staphylococcal or streptococcal infections, an increased number of lymphocytes are detected in the mammary glands (Soltys and Quinn, 1999). Many authors have described changes in the distribution of the lymphocyte subpopulation in mammary gland secretions, either after induction of an inflammatory response (Soltys and Quinn, 1999; Inchaisri et al., 2000; Leitner et al., 2000b; Ebling et al., 2001; Riollet et al., 2001; Rivas et al., 2001; Faldyna et al., 2006) or during lactation (Park et al., 1992; Van Kampen et al., 1990; Yamaguchi et al., 1999; Asai et al., 1998, 2000; Leitner et al., 2000a). In milk from healthy cows, the lymphocyte population is comprised predominantly of T cells (approximately 60% of lymphocytes) with relatively few B cells (approximately 20% of lymphocytes). The majority of T cells are $CD8^+$ (cytotoxic T cells), with a lower proportion of $CD4^+$ cells (T helper cells) (Taylor et al., 1994; Soltys and Quinn, 1999). CD4 lymphocytes represent a significantly greater percentage of milk-derived lymphocytes in infected mammary glands when compared with normal controls (Taylor et al., 1997). Park et al. (1993); however, showed that the number of activated $CD8^+$ T cells increased in milk obtained from cows experimentally infected with *S. aureus*, and that these cells were responsible for suppressing the proliferative response of milk $CD4^+$ T cells.

Bovine strains of *S. aureus* associated with intramammary infection produce staphylococcal enterotoxins such as staphylococcal enterotoxin C (Kenny et al., 1993; Zouharova and Rysanek, 2008). Staphylococcal enterotoxins are among those pyrogenic toxins known as superantigens (Bohach et al., 1990). The interactions of superantigens with the T cells lead to their activation (Webb and Gascoigne, 1994) and apoptosis (Damle et al., 1993; Boshell et al., 1996). Haslinger et al. (2003) reported that *S. aureus* α-toxin induced apoptosis in human peripheral blood mononuclear cells. Park et al. (2006) investigated apoptosis of two subpopulations of bovine blood lymphocytes ($CD4^+$ and $CD8^+$) during *in vitro* cultivation with staphylococcal enterotoxin C, after previously demonstrating that staphylococcal infections induce immunosuppressive $CD8^+$ T cells *in vivo* (Park et al., 1993; Davis et al., 1996). Similarly Ferens et al.

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(1998), showed that staphylococcal enterotoxin C induced aberrant activation of the CD8⁺ T cell subset, with a corresponding inversion of the CD4⁺:CD8⁺ T cell ratio.

It has also been observed that some viruses may modulate apoptosis of bovine lymphocytes. Bovine herpesvirus 1 can infect CD4⁺ T cells, for example, leading to apoptosis (Winkler et al., 1999). Bruschke et al. (1997) showed that an extra envelope glycoprotein of pestiviruses, which has been designated E^{rns}, can also induce apoptosis of bovine lymphocytes. Bovine leukemia virus; however, has been shown by Dequiedt et al. (1997) to decrease the susceptibility of peripheral blood mononuclear cells to apoptosis.

Apoptosis of lymphocytes during intramammary infection has not been investigated; however, resulting in a lack of data on lymphocyte apoptosis during mastitis. Apoptosis, a process that decreases the lifespan of cells and impairs their proper functioning, is an important problem (Whyte et al, 1993; Van Oostveldt et al., 2002) as it can reduce the immunological response of lymphocytes.

The aim of our study was to determine whether apoptosis of lymphocytes is modulated by infection with *S. aureus* and *S. uberis*. To this end, apoptosis of lymphocytes was studied in a model of subclinical mastitis of the bovine mammary gland caused by experimentally induced *S. aureus* and *S. uberis* mastitis.

2. Materials and methods

2.1. Animals

The experiments were carried out on 40 mammary glands of 10 virgin, clinically healthy, Holstein × Bohemian Red Pied crossbred heifers aged 16 to 18 months – 5 heifers for each bacteria (*S. aureus* and *S. uberis*). For the *in vitro* studies, 8 heifers (32 mammary glands) were used as cell donors. The heifers were housed in an experimental tie-stall barn and fed a standard ration consisting of hay and concentrates with mineral supplements. The experimental tie-stall used in this study is certified and animal care conformed to good care practice protocols. All heifers were free of intramammary infections, as demonstrated through a bacteriological examination of mammary lavages.

2.2. Experimental design

Before experimental infection, the mammary glands were treated with phosphate buffered saline (PBS) prepared with apyrogenic water. All 4 mammary gland sinuses of each heifer were rinsed stepwise with PBS to obtain a cell suspension using the following procedure. 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 two 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 for the infections, as undertaken in previous studies (Sladek et al., 2005, 2006). Five heifers were then experimentally infected with *S. aureus* and the remaining 5 heifers with *S. uberis*. Subsequent lavages of the mammary gland lumens were obtained in the same manner as described. The proportion of lymphocytes in the cell suspension obtained from the lavages was assessed through flow cytometry (FCM) (FACS Calibur apparatus, Becton Dickinson, CA, USA).

2.3. Preparation of S. aureus and S. uberis inocula

The two bacterial strains used were *S. aureus* Newbould 305 (CCM 6275; Czech Collection of Microorganisms, Masaryk University, Brno) and *S. uberis* (CCM 4617). The inoculum of *S. aureus* was prepared by growing the organism on ram blood agar (BA) medium. Three colonies of this culture were then inoculated into brain–heart infusion (BHI) broth and cultivated under continuous rotation (30 rpm/min) for 18 h at 37 °C. The stock culture was stored at 4 °C. On the day of experimental infection, 1 ml of the *S. aureus* stock culture was inoculated into 5 ml of fresh BHI and incubated under continuous rotation (30 rpm/min) for 4 h to obtain bacteria in the exponential growth phase.

For *S. uberis*, 1 ml of the stock culture was inoculated onto a cellophane membrane, which was placed on BA and incubated for 4 h to obtain bacteria in the exponential growth phase. Both bacterial suspensions were then harvested and washed once with PBS. Total bacterial cell counts were determined using a haemocytometer, and the bacterial suspension was adjusted to a final concentration equalling 8.0×10^6 CFU/ml in PBS. The CFU/ml was verified for each inoculum after 24 h of incubation at 37 °C on BA medium. After dilution of the bacterial suspensions to 800 CFU/ml, the inocula were adjusted in the syringes.

2.4. Experimental infection

Modified urethral catheters (AC5306CH06, Porges SA, France) were inserted into the teat canal following thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was injected with 20 ml of PBS and 2 ml of lavage solution was immediately collected back through the catheter directly to the syringe and subsequently used for bacteriological examination. Lavages were followed by the administration of 5 ml of inoculum (800 CFU/ml) through the teat orifice using a syringe.

2.5. Bacteriological examination

Bacteriological examination of all lavages was performed through culture on blood agar plates (5% washed ram erythrocytes) with aerobic incubation at 37 °C for 24 h.

2.6. Processing of cells

The trypan blue dye exclusion test demonstrated more than 98% cell viability in fresh lymphocytes by an enumeration of at least 200 cells. The cell suspensions were centrifuged at 4 °C and 200g for 10 min. One millilitre of supernatant was retained for resuspension of the pellet.

2.7. Proportion of lymphocytes

The percentage of lymphocytes was enumerated by FCM by reading from forward scatter versus side scatter dot plots (Sladek et al., 2002). Gating was set up based on the expression of CD14 for differentiating lymphocytes from macrophages (Riollet et al., 2001).

2.8. FCM assessment of lymphocyte apoptosis and necrosis

Apoptotic and necrotic lymphocytes were analysed by FCM following simultaneous staining with Annexin-V labelled with fluorescein isothiocyanate (FITC) and propidium iodide (PI), as described by Vermes et al. (1995). The commercial Annexin-V-FLUOS staining kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, 500 μL of the incubation buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) was mixed with 10 μL of PI and 10 μL of FITC-Annexin-V solution. After 15 min of incubation at room temperature with fresh buffer containing PI and FITC-Annexin-V, the cell suspension was analysed by FCM with differentiation