Veterinary Immunology and Immunopathology 145 (2012) 120–128

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Serum amyloid A isoforms in serum and milk from cows with *Staphylococcus aureus* subclinical mastitis

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ARTICLE INFO

Article history:
Received 4 May 2011
Received in revised form 22 August 2011
Accepted 26 October 2011

Keywords:
Serum amyloid A
Isoforms
Subclinical mastitis
Staphylococcus aureus

ABSTRACT

Serum amyloid A proteins (SAA) are very sensitive acute phase proteins, displaying multiple isoforms in plasma and different body fluids. They are currently under investigation as biomarkers of diseases. The aim of the present study was to compare the concentration and isoform expression of SAA in serum and milk of cows with bacteriologically negative milk (control group) and naturally occurring *Staphylococcus aureus* (*S. aureus*) subclinical mastitis (subclinical mastitis group). Somatic cell count (SCC) and bacteriological analyses were performed to establish the control and subclinical mastitis group. SAA concentration was evaluated using a commercial ELISA kit, while expression of different isoforms (serum A-SAA and milk M-SAA3 isoforms) was visualized by denaturing isoelectrical focusing and immunoblotting. The SAA concentrations in sera and milk of cows in the subclinical mastitis group were three and 100 times higher than in those from the control group of cows, respectively. Cows in the subclinical mastitis group had more acidic SAA isoforms in serum with the most prominent one at p*I* 5.5. This isoform was not detected in sera from the control group. Milk samples in the subclinical mastitis group contained abundant highly alkaline M-SAA3 isoforms and most of the serum isoforms, except for that at p*I* 5.5. In the subclinical mastitis group SAA isoforms with equivalent p*I* as serum isoforms accounted for 20% of the total SAA concentration in milk. There were significant differences in the concentrations and isoform patterns of SAA in serum and milk between the control and subclinical mastitis groups of cows. Also, we demonstrated that serum SAA isoforms were not transferred to milk proportion to their plasma content.

1. Introduction

Serum amyloid A proteins (SAA) comprise a family of apolipoproteins expressed constitutively (C-SAA) and in response to tissue injury (A-SAA). During inflammation, SAA proteins are predominantly associated with high-density lipoproteins (HDL) having a role in increased cholesterol efflux from macrophages at sites of tissue injury (Cabana et al., 1989; Liang and Sipe, 1995; Tam et al., 2008). Moreover, both multiple pro-inflammatory and anti-inflammatory activities of A-SAAs have been detected (rev. Uhlar and Whitehead, 1999). The prompt and intense increase of A-SAA concentration in plasma and/or other body fluids (1000 times) shortly (24 h) after tissue injury, makes this family of proteins potentially useful in veterinary practice as non specific inflammation

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doi:10.1016/j.vetimm.2011.10.015

markers (Eckersall et al., 2001). Among domestic animals, it is of major clinical interest to define markers of subclinical inflammation in ruminants, because their leukocyte acute phase response is often not evident.

It is well established that bovine plasma A-SAA proteins are heterogeneous in their amino acid sequences, molecular weight and isoelectric points—p*I* values (Westermark et al., 1986; Rossevatin et al., 1992; Horadagoda et al., 1993; Alsemgeest et al., 1995). Moreover, it was demonstrated that multiple isoforms of bovine A-SAA occur in diverse plasma concentration ratios during different diseases (Alsemgeest et al., 1995), a finding that could be used to explore more specific SAA isoform patterns of appearance. In all species examined, including ruminants, A-SAA are derived from hepatocytes, but other sites of SAA synthesis have been identified, such as mammary epithelial cells (Kho et al., 2000), gastric epithelial cells (Dilda et al., 2011) and adipocytes (Mukesh et al., 2010). According to recent data, heterogeneity of SAA molecules is also seen in peripheral tissues. Namely, bovine mammary associated SAA3 (M-SAA3) protein identified by Kho et al. (2000) and McDonald et al. (2001) has multiple highly alkaline isoforms (Jacobsen et al., 2005). Prolactin is the main physiological stimulus and infection with *Escherichia coli* (*E. coli*) one of the pathogenic stimuli for expression and secretion of three M-SAA3 isoforms (p*I* > 9.3) (Larson et al., 2005; Jacobsen et al., 2005). Also, mammary epithelial cells *in vitro* produce two more alkaline isoforms with p*I* 8.8 and 7.2, as a response to lipotechoic acid (LTA) from *Staphylococcus aureus* (Weber et al., 2006). Moreover, the complexity of SAA isoforms found in milk during mastitis of different etiology is augmented by the presence of multiple plasma SAA isoforms that are speculated to exude into the inflamed mammary gland (Eckersall et al., 2006). The role of different M-SAA3 isoforms during inflammation still remains elusive.

Data concerning the presence of plasma SAA isoforms and M-SAA3 isoforms in mastitic milk were obtained in experimentally induced *E. coli* mastitis (Jacobsen et al., 2005). The M-SAA3 isoform pattern upon LTA stimulation was evaluated on a mammary epithelial cell line (Larson et al., 2005; Weber et al., 2006). However, there are no data on serum A-SAA isoforms and M-SAA3 isoforms in milk during naturally occurring *S. aureus* subclinical mastitis, a frequent pathological condition on dairy farms. The aim of this study was to compare the concentration and isoform expression of SAA in serum and milk from cows with and without *S. aureus* subclinical mastitis.

2. Materials and methods

2.1. Animals and sample collection

The investigation was performed on a dairy farm representative for the dairy industry in Serbia. The blood and milk samples were obtained in the winter at a daily ambient temperature around 4 °C from 34 clinically healthy midlactation, multiparous (between parities two and five), Holstein Cows (150 ± 10 days of lactation) with no signs of inflammation of the mammary glands. The average milk production per cow on the farm was 4000 L per year. Milking frequency was twice a day. Samples were collected during a routine mammary gland health screening procedure with the California mastitis test. Teats were disinfected with 70% ethanol. The first milk jets were discarded and then approximately 15 mL of milk was aseptically collected from each udder quarter by hand milking before the normal afternoon milking. All 136 milk samples were divided into three parts: one was used for the somatic cell count (SCC), the second part for bacteriological identification and the third part for determination of the concentration and presence of isoforms of SAA. This third subsample was stored at −20 °C until analysis. Blood serum was separated 2 h after venepuncture (*v. jugularis*), centrifuged at 2500 × *g* for 15 min and stored at −20 °C until analysis.

2.2. Somatic cell count, bacteriology and allotment into groups

Currently, SCC is accepted as the standard indicator for the dairy industry in the diagnosis of subclinical mastitis. We used it as a parameter to estimate mammary gland health. SCC in the milk samples was measured on a Fossomatic 360 instrument (Foss Electric, Hillerad, Denmark).

Samples for bacteriology were processed according to Tolle et al. (1977). Briefly, aseptically collected milk samples were plated onto blood agar plates and incubated for 24 h at 37 °C. White colonies with a hemolysis zone characteristic for Gram-positive cocci were tested for catalase and coagulase activity. Catalase positive and coagulase positive cocci were marked as *S. aureus*. Milk samples with no bacterial growth were designated as bacteriologically negative.

Cows for the control and subclinical mastitis groups were chosen as follows:

S. aureus positive group (subclinical mastitis group): Among 34 cows ten had two quarters (2 quarters/cow for 10 cows) with milk samples positive for *S. aureus*. Samples with more than 500,000 SCC and thus defined as subclinical mastitis samples (IDF, 1971), were selected for determination of SAA concentration. The remaining 20 quarters that were not infected were not included in this study.

Bacteriologically negative group (control group): It was previously shown that if the quarters were examined only once, the bacteriological results could be falsely negative, due to variation in the number of bacteria present at different time points during mastitis (Sears et al., 1990). As we did not perform multiple consecutive bacteriological analyses, we used SCC as a supplemental parameter to estimate mammary gland health. In a meta-analysis on quarter milk SCC in infected cows Djabri et al. (2002), as well as other authors reviewed by Pyörälä (2003), reported that healthy quarters had less than 100,000 SCC. Using this criterion we identified ten animals with bacteriologically negative milk samples from all quarters, and a very low somatic cell count (under 30,000 SCC). Furthermore, two milk samples per animal (2 quarters/cow for 10 cows) were randomly chosen for determination of SAA concentration.