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Cytokine inflammatory response in dairy cows with mastitis caused by *Streptococcus agalactiae*

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Abstract

Introduction: The aim of the study was evaluation of the concentrations of interleukin (IL)-1β, IL-8, IL-12β and tumour necrosis factor alpha (TNF-α) in the serum and milk of cows with mastitis caused by *Streptococcus agalactiae.* **Material and Methods:** A total of 60 milk samples from diseased cows and 30 milk samples from healthy cows were included in the study. Blood and milk samples were taken from Holstein-Friesian cows from three herds (two in tie-stall and one in a free-stall housing system) in Lublin Province in Poland. The concentrations of cytokines in blood serum and quarter milk samples were determined by ELISA. **Results:** The levels of IL-1β, IL-8, IL-12β and TNF-α were significantly higher in the milk of cows suffering from mastitis caused by *S. agalactiae* compared to the milk of healthy cows (263.03 *vs* 55.36 pg/mL, 298.34 *vs* 131.82 pg/mL, 604.10 *vs* 139.17 pg/mL and 460.86 *vs* 78.82 pg/mL, respectively). In the group of sick cows, cytokine levels were significantly higher in milk than in serum (263.03 *vs* 55.25 pg/mL for IL-1β, 298.34 *vs* 164.22 pg/mL for IL-8, 604.10 *vs* 70.34 pg/mL for IL-12β and 460.86 *vs* 104.78 pg/mL for TNF-α). **Conclusion:** The results confirm the involvement of the entire bovine immune system to protect against the bacteria first locally in the udder. The response of the mammary gland to infection caused by *S. agalactiae* is rapid and already very strong at the beginning of the infection.

Keywords: cows, mastitis, *Streptococcus agalactiae*, cytokines.

Introduction

Streptococcus agalactiae (group B *Streptococcus*) is the pathogen most commonly associated with bovine mastitis, a disease which is highly prevalent and economically damaging for the dairy industry because of the need for antibiotic therapy, attendant loss of milk production and other costs (13, 21). It is also a human pathogen that causes serious invasive infections in newborns, pregnant women and the elderly, and may be lethal to immunocompromised adults (21). *Streptococcus agalactiae* belongs to a group of bacteria colonising the udder and spreads from infected to uninfected mammary glands mainly during milking (13, 14). It is capable of infecting many cows in a herd in a short period of time. This bacterium can infect both heifers and older cows and is one of the main causes of economic losses in dairy herds without a control programme and one of the most dangerous microorganisms to the udder (13). It penetrates the epithelium of the milk follicles, thereby causing their swelling. In the next phase of the infection's development, both the milk follicles and the ducts leading milk to the milk sinus close because of the scar tissue resulting from the persistence of infection, which is not fully eradicated because the infection sites are poorly accessible to antibiotics. This process leads to fibrosis and irreversible atrophy of the affected udder, and the infection progresses to a chronic form (14).

Mastitis caused by *S. agalactiae* is often accompanied by signs from the udder (pain and swelling), but also by systemic symptoms (fever and lack of appetite) and significant changes in milk composition (3, 4).

The cow's body responds to a bacterial infection through an inflammatory response initially localised only in the udder but subsequently involving the entire body (24, 26). The primary defence in the mammary gland is leukocytes arriving with blood (10). The duration and severity of the inflammatory process depends primarily on the ability of leukocytes to migrate from the capillaries and the activity of these cells at the site of infection. The predominant leukocyte populations in the milk of cows with mastitis are neutrophils, macrophages and, at a later stage, lymphocytes (12, 24, 26). The migration of these cells from the blood depends on the expression of adhesion receptors on leukocytes and their affinity for ligands on vascular endothelial cells, as well as on the presence of inflammatory mediators in the tissues: cytokines, complement components and leukotrienes (1, 2, 18, 22).

Phagocytes in the inflammatory focus absorb and destroy bacteria and remove cellular aggregates. However, if the microorganisms survive the first reaction of cellular mechanisms, inflammation develops, and pathogens attack the lumen of the mammary gland follicles (24, 26). This leads to damage to the secretory cells and a decrease in milk production, and triggers an acute-phase reaction, during which there is a sharp increase in the production of acute-phase proteins (APPs), which are markers of inflammation (4, 8, 24). Changes in the concentration of APPs in serum are mainly due to changes in the transcription of their genes in hepatocytes. Signals from pathogens are recognised by tool-like receptors (TLRs), and other cell-damaging stimuli activate protein kinases. The main purpose of the acute-phase response is to restore homeostasis in the body by stimulating immune mechanisms. The interaction of the various cellular and humoral mechanisms of the immune response is based on the transmission of stimulatory or inhibitory signals *via* cytokines. Cytokines are small molecule proteins that play a very large role in the body's response to infection and in cell-to-cell communication. In this study, we selected pro-inflammatory cytokines that are very important in the body's early immune reaction: interleukin-1 beta (IL-1β), interleukin-8 (IL-8), interleukin-12 beta (IL-12β) and tumour necrosis factor alpha (TNF-α). Their function is to stimulate all processes involved in cell differentiation, proliferation, degeneration and regeneration (1, 2, 24, 26, 29). After binding to membrane receptors of target cells, the cytokines trigger the synthesis and secretion of APPs (8, 20, 25). Their expression is controlled by regulatory transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (6).

The aim of the study was evaluation of the concentrations of IL-1β, IL-8, IL-12β and TNF-α in the serum and milk of cows with mastitis caused by *Streptococcus agalactiae.*

Material and Methods

Study designs and data collection. Blood and milk samples were taken from Holstein-Friesian cows from three herds (two in a tie-stall and one in a free-stall housing system) in Lublin Province in Poland. The herds numbered 46, 63 and 120 dairy cows, respectively. No five-point mastitis prevention programme was implemented in these herds. Selective dry cow therapy was used in cows with clinical or subclinical mastitis in the current or previous lactation.

All procedures for collecting material for animal testing were recognised by the Local Ethical Committee for Animal Experiments in Lublin as routine veterinary services for dairy cows. Therefore, the study was conducted in accordance with European Union regulations contained in Directive 2010/63/EU on the protection of animals used for scientific purposes.

Samples were taken from the udder quarters of cows affected by mastitis immediately after the beginning of inflammation. No treatment was applied before the samples were taken. Approximately 100 mL of milk was collected from one infected udder quarter of each cow into three sterile tubes. In addition, two blood samples were taken from each cow from the *vena jugularis externa*. The first was collected into a tube with ethylenediaminetetraacetic acid for haematological examination. The second blood sample was collected into a tube with serum coagulation activator (Medlab Products, Raszyn, Poland). The material for laboratory testing (milk and blood samples) was delivered to the laboratory at the University of Life Sciences in Lublin at 4°C in two hours or less. To obtain serum, blood samples were centrifuged at 1,000 × *g* for 20 min. The clear serum was then poured into Eppendorf-type test tubes and stored at −84°C until used in analyses. One fresh milk sample of approximately 30 mL was used to determine the somatic cell count (SCC), which was measured using a SomaCount FC Automatic (Bentley Instruments, Chaska, MN, USA). The second milk sample was centrifuged at 1,000 × *g* for 20 min, then transferred to Eppendorf tubes and frozen at −84°C until cytokine levels were determined in the sample. Milk from the third tube was used for bacteriological testing.

Laboratory analysis. Milk in a volume of 0.01 mL from a sterile tube was inoculated onto agar medium (BTL, Łódź, Poland) with the addition of sterile defibrinated sheep's blood. After a period of incubation of the plates for 24 h at 37°C under aerobic conditions, the morphology of the colonies was evaluated, a catalase test was performed and Gram-stained microscopic slides were prepared. Gram-positive granules arranged linearly and not producing catalase were inoculated onto Aesculin Blood Agar (Oxoid, Basingstoke, UK).

The presence of even one colony of *S. agalactiae* on the medium was considered a positive result. A CAMP (Christie–Atkins–Munch-Petersen) assay was performed, by which bacteria were identified as aesculin-negative, translucent blue, grey or colourless colonies producing β-haemolysin. For unambiguous identification of bacteria, samples were also analysed by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF system; Bruker Daltonics, Bremen, Germany).

Selection of animals for the study. The bacteriological examination found milk and serum samples suitable for further analysis. The level of cytokines was examined in the serum and milk of cows from which an *S. agalactiae* strain was isolated. Milk and serum samples from 23 cows with mastitis caused by *S. agalactiae* (and in the milk of which no other mastitis pathogens were detected) were included in the study. Twelve cows had milk abnormalities such as clots and flakes as clinical signs of mastitis, and swelling, redness or soreness of the mammary gland as visible signs. Six of these cows showed general symptoms: lack of appetite, depression, and a slight increase in body temperature. The remaining 11 cows out of the 23 showed subclinical inflammation without signs of disease. The range of SCCs in the milk of these cows was 644,000–3,730,000 cells/mL. The cows were milked twice a day, and their daily milk yields ranged from 20.6 to 58.4 kg. The diseased group included cows that were in different lactations: 4 in their first lactation, 7 in their second, 10 in their third and 2 in their fourth.

Milk and blood samples were also taken from 10 healthy cows showing no signs of disease. This control group consisted exclusively of cows in their first lactation and producing milk with an SCC of less than 100,000 cells/mL in which no microorganisms were found in the bacteriological examination. A haematological examination determining red blood cell and white blood cell counts, haemoglobin (HGB) and hematocrit (HCT) was performed using the Scil ABC+ Vet Animal Hematology Analyzer (Horiba, Kyoto, Japan) and the parameters are shown in Table 1.

Measurements of cytokines in serum and quarter milk samples. The concentrations of cytokines in blood serum and quarter milk samples were determined by ELISA using kits for IL-1β, IL-8, IL-12β and TNF-α (USCN Life Science, Houston, TX, USA). All procedures were performed according to the guidelines provided by the manufacturer. Absorbance readings were taken on an ELx800 automatic microtitre plate reader (Biotek Instruments, Winooski, VT, USA) at 450 nm using 630 nm as the reference. The detection range for cattle of IL-1β, IL-8, IL-12β was 15.6–1,000 pg/mL and that of TNF-α was 7.8–500 pg/mL. The inter- and intra-assay coefficients of variation for all examined cytokines were <12% and <10%, respectively.

Statistical analysis. First, the Shapiro–Wilk test was applied to determine or exclude non-normality of the distribution of trait values in the study groups. Then, the Mann–Whitney test was performed for two independent samples. A P-value < 0.05 was considered significant. The Statistica 12.0 statistical package (StatSoft, Tulsa, OK, USA) was used to perform the calculations.

Results

The following microorganisms were isolated from milk samples taken from infected udder quarters: *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, non-aureus staphylococci, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida* spp.

Evaluation of IL-1β concentration in milk and serum of cows with mastitis caused by *Streptococcus agalactiae* and healthy cows. Levels of IL-1β in the serum of healthy cows ranged from 83.23 to 144.87 pg/mL, with a median of 123.04 pg/mL. In these cows' milk they ranged from 31.73 to 82.03 pg/mL, with a median of 55.36 pg/mL. The content of IL-1β was significantly higher in the milk and significantly lower in the serum of cows with mastitis compared to healthy cows. There was also a significant difference between the concentration of IL-1β in serum and its concentration in milk in both the healthy and streptococcal mastitis cow groups, the milk IL-1β level being significantly lower in healthy cows and significantly higher in diseased cows compared to the serum IL-1β level (Table 2).

Table 1. Blood cell counts in cows with mastitis caused by *Streptococcus agalactiae* and in healthy cows

	Cows with mastitis n = 23	Healthy cows n = 10
WBC (× 10^3/mm^3)*	9.1	7.6
Lymphocytes (× 10^3/mm^3)*	2.8	3.6
Neutrophils (× 10^3/mm^3)*	5.4	3.4
Eosinophils (× 10^3/mm^3)*	0.42	0.48
RBC (× 10^6/mm^3)*	5.6	6.6
HGB (g/dL)*	10.2	12.8
HCT (%)*	30	36

* – average counts for all animals from each group; WBC – white blood cell count; RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit

Table 2. The concentration of interleukin-1 beta in milk and in serum from healthy cows and cows with mastitis caused by *Streptococcus agalactiae*

Symbol	Sample	n	Interleukin-1 beta (pg/mL)		
			Median	Min	Max
A	Healthy cows' milk	10	55.36 [B, C]	31.73	82.03
B	*S. agalactiae* mastitic milk	23	263.03 [A, D]	98.58	402.42
C	Healthy cows' serum	10	123.04 [A, D]	83.23	144.87
D	*S. agalactiae* mastitic serum	23	55.25 [B, C]	9.25	122.00

n – number of samples. Superscript letters indicate statistically significant difference (P-value < 0.05) from the equivalent concentration in the group denoted by the letter

Table 3. The concentration of interleukin-8 in milk and in serum from healthy cows and cows with mastitis caused by *Streptococcus agalactiae*

Symbol	Sample	n	Interleukin-8 (pg/mL)		
			Median	Min	Max
A	Healthy cows' milk	10	131.82 [B]	85.36	284.67
B	*S. agalactiae* mastitic milk	23	298.34 [A, D]	127.06	614.20
C	Healthy cows' serum	10	216.33	103.75	288.12
D	*S. agalactiae* mastitic serum	23	164.22 [B]	78.43	404.32

n – number of samples. Superscript letters indicate statistically significant difference (P-value < 0.05) from the equivalent concentration in the group denoted by the letter

Table 4. The concentration of interleukin-12 beta in milk and in serum from healthy cows and cows with mastitis caused by *Streptococcus agalactiae*

Symbol	Sample	n	Interleukin-12 beta (pg/mL)		
			Median	Min	Max
A	Healthy cows' milk	10	139.17 [B]	74.10	288.79
B	*S. agalactiae* mastitic milk	23	604.10 [A, D]	134.05	1,204.60
C	Healthy cows' serum	10	192.51 [D]	117.50	272.93
D	*S. agalactiae* mastitic serum	23	70.34 [B, C]	27.97	140.87

n – number of samples. Superscript letters indicate statistically significant difference (P-value < 0.05) from the equivalent concentration in the group denoted by the letter

Table 5. The concentration of tumour necrosis factor alpha in milk and in serum from healthy cows and cows with mastitis caused by *Streptococcus agalactiae*

	Sample	n	Tumour necrosis factor alpha (pg/mL)		
			Median	Min	Max
A	Healthy cows' milk	10	78.82 [B, C]	15.67	156.50
B	*S. agalactiae* mastitic milk	23	460.86 [A, D]	119.20	1,636.80
C	Healthy cows' serum	10	163.76 [A]	78.09	295.26
D	*S. agalactiae* mastitic serum	23	104.78 [B]	48.20	330.22

n – number of samples. Superscript letters indicate statistically significant difference (P-value < 0.05) from the equivalent concentration in the group denoted by the letter

Evaluation of IL-8 concentration in milk and serum of cows with mastitis caused by *Streptococcus agalactiae* and healthy cows. Serum IL-8 levels in the healthy cows ranged from 103.75 to 288.12 pg/mL, with a median of 216.33 pg/mL. The milk samples from healthy cows contained from 85.36 to 284.67 pg of IL-8 per mL, with a median of 131.82 pg/mL. There was no statistically significant difference between the serum and milk IL-8 levels in the group of healthy cows. However, in the group of diseased cows, the content of IL-8 in milk was significantly higher than the content in serum. In addition, the milk IL-8 level in streptococcal mastitis cows was significantly higher than in the control group of cows. There was no difference in the concentrations of serum IL-8 between the two groups of cows (Table 3).

Evaluation of IL-12β concentration in milk and serum of cows with mastitis caused by *Streptococcus agalactiae* and healthy cows. Serum IL-12β levels in the healthy cows ranged from 117.5 to 272.93 pg/mL with a median of 192.51 pg/mL. The range of IL-12β levels in these cows' milk was 74.1–288.79 pg/mL, with a median of 139.17 pg/mL. There was no significant difference in the concentrations of IL-12β between the serum and the milk of healthy cows. The milk IL-12β level in cows with mastitis was significantly higher than in control cows, while the content of this cytokine in the serum of diseased cows was significantly lower than the content in the serum of healthy cows. In the group of streptococcal mastitis cows, the concentration of IL-8 was significantly lower in serum than in milk (Table 4).

Evaluation of TNF-α concentration in milk and serum of cows with mastitis caused by *Streptococcus agalactiae* and healthy cows. Tumour necrosis factor alpha levels in the serum of healthy cows ranged from 78.09 to 295.26 pg/mL, with a median of 163.76 pg/mL, and differed significantly from those in milk (15.67–156.50 pg/mL, with a median of 78.82 pg/mL). However, in the group of cows with mastitis, the level of TNF-α in milk was significantly higher than the level in serum. The content of TNF-α in the milk of cows with mastitis was also significantly higher than that in the milk of control cows, while there was no statistically significant difference between serum TNF-α levels in the two groups of animals (Table 5).

Discussion

In the present study, the concentrations of IL-1β, IL-8, IL-12β and TNF-α were investigated in serum and in milk obtained from cows suffering from mastitis caused by *S. agalactiae.* For many years, *S. agalactiae* was described as the main cause of mastitis in cows (13, 14, 21). However, current studies demonstrate that the incidence of this pathogen in udder infections is much lower than it previously was, most likely because of the raising of hygiene standards in milk production and the treatment of dry cows (7, 15, 28). Unfortunately, there are still herds in which the five-point mastitis prevention plan has not been implemented, and for this reason a large percentage of mastitis cases in these herds is caused by infectious pathogens. *Streptococcus agalactiae* has not lost its pathogenic characteristics and can cause mastitis with a severe clinical course, as proved by the results of our previous studies (3, 4).

In the course of infection caused by *S. agalactiae*, the immune system is activated in the mammary gland, as evidenced by a significant increase in the level of APPs in milk (4, 8, 26). Once the bacteria are recognised, the production is induced of immune mediators, especially cytokines, from local cell populations in the mammary gland (2). The immune system recognises and responds to a variety of pathogens, even if they attack a cow's udder for the first time. Specific cell membrane receptors recognise various bacterial cell wall structures – lipopolysaccharide (LPS), peptidoglycan and lipoteichoic acid – which form pathogen-associated molecular patterns (PAMPs) (2, 17, 22). Microorganisms in the udder interact with both epithelial cells and macrophages, and the latter are induced to produce and secrete TNF-α by the binding of PAMPs to TLRs. The most important functions of TNF-α are to stimulate the synthesis of other cytokines, induce inflammatory reactions, control cellular processes and maintain tissue homeostasis (17, 23, 29). In the process of udder inflammation, recruitment of neutrophils, stimulation of their pathogen-fighting activity and promotion of the secretion of IL-1, IL-6 and arachidonic acid metabolites by TNF-α are very important (17). Interleukin-1 production is induced upon recognition of the presence of bacteria by TLRs on macrophages and epithelial cells, *i.e.* soon after the increase in TNF-α (17, 20, 25, 32). In experimental acute mastitis in cattle, IL-1α has been found to exhibit local modulation of such first-line defence mediators as prostaglandin (PG-F2α) and leukotriene (LT-B4), while IL-1β is released into the blood to produce systemic effects (19).

In our study, we found a significant increase in the levels of both TNF-α and IL-1β in the milk of cows suffering from mastitis caused by *S. agalactiae* compared to the levels in the milk of the control group (5.8-fold for TNF-α and 4.8-fold for IL-1β). Elevated TNF-α and/or IL-1 levels were also recorded by other authors in the early stages of udder infections caused by different pathogens (1, 2, 12, 20, 24, 31). When LPS was experimentally introduced into the udder, it resulted in an increase in IL-1 levels as early as 2.5 to 4 h after application (25). The high level of cytokines in the milk of diseased cows confirms that the body attempts to fight the infection first locally in the udder before a systemic response is activated. This is also confirmed by the higher concentrations of cytokines in milk than in serum in mastitic cows which were shown in our study (4.4-fold for TNF-α and 4.8-fold for IL-1β). It should be noted that in the group of healthy cows, the levels of TNF-α in serum and milk were the same, but those of IL-1β were more than twice as high in serum as in milk.

In the inflammatory process, the pro-inflammatory cytokines TNF-α and IL-1α and β induce leukocyte infiltration by regulating the expression of leukocyte adhesion molecules on vascular endothelial cells. Recruitment and emigration of circulating leukocytes has been shown to depend on a multi-step cascade of events involving their binding, rolling, and strong adhesion before emigration, and these steps are mediated by various adhesion molecules and activation pathways (32). The synergistic effect of IL-1β and TNF-α stimulates IL-8 release, inducing neutrophil migration and stimulating these cells' fighting activity (2, 6, 9, 27, 31).

The results of our study showed nearly twice (1.8-fold) as high levels of IL-8 in the milk as those in the serum of cows suffering from mastitis caused by *S. agalactiae*, and more than twice (2.2-fold) as high levels as those in the milk of healthy cows. The findings provide evidence of mobilisation of the overall immune system for local defence of the udder because IL-8 plays an extremely important role in the fight against pathogens. It enables the recruitment of leukocytes, especially neutrophils, stimulates their migration to the site of infection because of adhesion to endothelial cells, and regulates host-cell survival, usually by preventing apoptosis and prolonging neutrophil viability, thereby maintaining for longer the body's ability to eradicate pathogens (5, 11).

The results of our study correlate with the studies of other authors. High levels of IL-8 in milk were recorded in udder infections caused by various microorganisms (*E. coli*, *S. uberis*, and *Staphylococcus aureus*), but also in the experimental introduction of LPS into the udder and even using cultures of primary mammary gland epithelial cells (1, 2, 5, 9, 20, 25, 30, 31). A predictor of high IL-8 levels is an increase in IL-8 gene transcription, and as experimental proof of this, a 1,054-fold increase in IL-8 gene transcription has been reported in mastitis caused by *S. uberis* (16).

Neutrophils recruited to the site of infection phagocytose the bacteria and produce reactive oxygen species, low-molecular-weight antimicrobial peptides and defensins, which eliminate a wide range of pathogens that cause mastitis (18, 26). If the infecting bacteria survive, the neutrophil infiltrate is replaced after a short time by T and B lymphocytes and monocytes. The mediator between innate and acquired immunity is

IL-12, which regulates T-lymphocyte proliferation, activation and differentiation, stimulates interferon gamma production and induces natural killer cells to produce cytokines (17, 18). Our results showed high concentrations of IL-12β in the milk of diseased cows (4.3 times higher than in the serum of these cows and 8.6 times higher than in the milk of healthy cows).

In this study, we found that the levels of IL-1β and IL-12β were significantly lower in the serum of cows with mastitis than in the serum of healthy cows. It is likely that in the early stages of infection, serum levels of these cytokines decrease significantly because of the increased migration of macrophages from peripheral blood to the inflammatory focus in the udder. However, there was no statistically significant difference in the serum concentrations of IL-8 and TNF-α between diseased and healthy animals. There were also no values of individual parameters of the red blood cell system and the peripheral blood smear yielded in the haematological examination of mastitic cows' samples which fell outside the reference ranges.

Conclusion

Our results showed that in healthy cows, the concentration of cytokines in serum was the same as (for IL-8 and IL-12β) or significantly higher than (2.2-fold for IL-1β and 2.0-fold for TNF-α) in milk. On the other hand, in cows suffering from mastitis caused by *S. agalactiae*, we found significantly higher levels of the tested cytokines in milk than in serum, which confirms that the cow's body mounts its first defence against the bacteria locally in the udder. In addition, we showed that the response of the mammary gland is already powerful at the beginning of mastitis caused by *S. agalactiae* infection, as evidenced by the high milk IL-1β, IL-8, IL-12β and TNF-α levels compared to the levels in healthy cows' milk.

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