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Staphylococcus aureus and *Escherichia coli* Cause Deviating Expression Profiles of Cytokines and Lactoferrin Messenger Ribonucleic Acid in Mammary Epithelial Cells

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ABSTRACT

Pathogens invading the mammary gland cause a complex signaling network that activates the early immune defense and leads to an outcome of inflammation symptoms. To examine the importance of mammary epithelial cells in these regulations and interactions resulting in a pathogen-related course of mastitis, we characterized the mRNA expression profile of key molecules of the innate immune system by quantitative real-time PCR. Mammary gland epithelial cells isolated on d 42 of lactation from 28 first-lactation Holstein dairy cows were cultured separately under standardized conditions and treated for 1, 6, and 24 h with heat-inactivated gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria. Both pathogens increased mRNA expression patterns of proteins involved in pathogen recognition such as Toll-like receptors and nuclear factor-κ B, whereas gram-negatives acted as a stronger stimulus. Furthermore, this could be confirmed by the expression profile of the proinflammatory cytokines tumor necrosis factor alpha, IL-1 beta, IL-6, and chemokines such as IL-8 and RANTES (regulated upon activation, normal T-cell expressed and secreted). Remarkably, at a low level of mRNA expression after 1 h of treatment these cytokines and chemokines were expressed at a significantly higher level in *Staphylococcus aureus* than in *Escherichia coli* affected cells. Lactoferrin showed a deviating expression pattern to pathogen stimulation (i.e., at the 1-h measuring point *Escherichia coli* induced a higher mRNA expression, whereas the highest level was reached after 24 h of stimulation with *Staphylococcus aureus*). Complement factor 3 was the only measured factor that responded equally to both microorganisms. Our data emphasize the role of mammary epithelial cells in the immune defense of the udder and confirm their contribution to pathogen-related different courses of mastitis.
Key words: mammary epithelial cell, cytokine, lactoferrin

INTRODUCTION

Mastitis, defined as an inflammation of the mammary gland, is estimated to be the most prevalent and most costly production disease in dairy herds of developed countries (Seegers et al., 2003). In most cases the inflammation is caused by invading pathogens that influence, in addition to environmental and cow factors, the clinical pattern. An infection with gram-positive bacteria such as *Staphylococcus aureus* can result in a chronic mastitis that persists lifelong (Sutra and Poutrel, 1994). Gram-negative microorganisms, however, especially *Escherichia coli,* can often be isolated from udders of animals suffering from a severe clinical mastitis (Hogan and Smith, 2003).

The last barrier that intramammary pathogens meet after overcoming the teat canal is presented by the epithelial cells covering the inner surface of the mammary gland. On the one hand, epithelial cells are part of the functional unit of the udder because they are responsible for synthesis of many milk components offering a perfect nutritional and immunological supply to the offspring (Korhonen et al., 2000; McManaman and Neville, 2003). On the other hand, these cells provide an important link between the outside environment and the body interior because they are able to recognize conserved molecular patterns of invading microorganisms via pattern recognition receptors, including Toll-like receptors (**TLR**; Rainard and Riollet, 2006). These receptors generate signals affecting activity of the immune system. Thirteen TLR have been identified in mammalian species, and the expression of 10 is known in cattle (Takeda et al., 2003; Menzies and Ingham, 2006).

The TLR2 is activated by peptidoglycan and lipoteichoic acid that are components of the cell membrane of

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Table 1. Sequences, accession numbers of the PCR primers, and length of the PCR products

Primer		Sequence (5′→3′)	Accession no.	Length (bp)
GAPDH	forward	GTC TTC ACT ACC ATG GAG AAG G	NM001034034	201
	reverse	TCA TGG ATG ACC TTG GCC AG		
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	forward	CAG GCT GAG CGA TAT GAT GAC	BC102382	141
	reverse	GAC CCT CCA AGA TGA CCT AC		
Ubiquitin	forward	AGA TCC AGG ATA AGG AAG GCA T	NM174133	198
	reverse	GCT CCA CTT CCA GGG TGA T		
IL-1 beta	forward	AGT GCC TAC GCA CAT GTC TTC	M37211	114
	reverse	TGC GTC ACA CAG AAA CTC GTC		
Tumor necrosis factor alpha	forward	CCA CGT TGT AGC CGA CAT C	NM173966	155
	reverse	CCC TGA AGA GGA CCT GTG AG		
IL-6	forward	GCT GAA TCT TCC AAA AAT GGA GG	NM173923	215
	reverse	GCT TCA GGA TCT GGA TCA GTG		
IL-8	forward	ACA CAT TCC ACA CCT TTC CAC	AF232704	149
	reverse	ACC TTC TGC ACC CAC TTT TC		
RANTES	forward	GCC AAC CCA GAG AAG AAG TG	BC102064	119
	reverse	CTG CTT AGG ACA AGA GCG AGA		
Toll-like receptor 2	forward	CAT TCC CTG GCA AGT GGA TTA TC	AY634629	201
	reverse	GGA ATG GCC TTC TTG TCA ATG G		
Toll-like receptor 4	forward	TAT GAA CCA CTC CAC TCG CTC	DQ839566	207
	reverse	CAT CAT TTG CTC AGC TCC CAC		
Complement factor 3	forward	AAG TTC ATC ACC CAC ATC AAG	NM001040469	191
	reverse	CAC TGT TTC TGG TTC TCC TC		
Lactoferrin	forward	CGA AGT GTG GAT GGC AAG GAA	DQ522305	215
	reverse	TTC AAG GTG GTC AAG TAG CGG		

gram-positive bacteria, whereas gram-negative microorganisms are recognized by TLR4, where LPS acts as a ligand (Pandey and Agrawal, 2006). The activation of several signaling molecules leads to a release of transcription factors, like nuclear factor-κ B (**NFκB**) that translocates into the nucleus and regulates expression of cytokines and other mediators playing a crucial role in the immune defense, cell differentiation, and apoptosis. The NFκB family consists of the following 5 proteins: NFκB1 (p105/p50), NFκB2 (p100/p52), RelA (p65), RelB, and c-Rel. They form homo- and heterodimers in which RelA (p65) has been demonstrated to be of great importance for the antiapoptotic and proinflammatory properties of NFκB (Pahl, 1999; Liang et al., 2004). In numerous in vitro and in vivo studies, proinflammatory signals like tumor necrosis factor alpha (**TNFα**), IL-1β, and IL-6 could be shown being involved in a deviating immune response to infection with *S. aureus* and *E. coli* (Riollet et al., 2000; Bannerman et al., 2004). Furthermore, chemokines such as IL-8 and **RANTES** (regulated upon activation, normal T-cell expressed and secreted, also known as CCL5) participate in the mastitis reaction by attraction of neutrophils to the site of inflammation (Boudjellab et al., 2000; Persson Waller et al., 2003; Pareek et al., 2005). The complement system and lactoferrin also belong to the innate pathogen defense mechanisms in the bovine mammary gland and the complement factor (**C**) 3 and lactoferrin are supposed to be synthesized by bovine mammary gland epithelial cells (**bMEC**) (Hagiwara et al., 2003; Pfaffl et al., 2003; Rainard, 2003; Schmitz et al., 2004; Wellnitz and Kerr, 2004). Beside its antimicrobial properties, lactoferrin was found to have an impact on regulation of proinflammatory cytokines (Berlutti et al., 2006). Expression of lactoferrin seems to be determined by hormonal influences, whereas some studies investigated its relation to bacterial infection directly (Teng, 1999, 2002; Zheng et al., 2005).

In the present investigation we wanted to explore the role of bovine mammary gland epithelial cells from a sufficient number of individuals in the complex network of interactions and regulations of immune defense and we examined its influence on pathogen-related differences in mastitis course. More knowledge about immunological reactions at the epithelial barrier could give evidence on how to interfere pathogen invasions by a therapeutical support of selected factors participating in the signaling pathway and could also be used for genetically assisted selection to improve udder health.

MATERIALS AND METHODS

Primary Cell Culture of Mammary Epithelial Cells

In 28 first lactating German Holstein dairy cows, udder health was controlled continuously by measuring SCC in milk samples and by clinical investigations. Milk samples of each quarter were tested for bacterial infection on slaughtering day. All animals were slaughtered on d 42 of lactation. Primary cell cultures from the mammary gland epithelial cells were obtained as described previously (Wellnitz and Kerr, 2004) with

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