

To the Graduate Council:

I am submitting herewith a thesis written by Magdalena Rambeaud entitled "Dynamics of Leukocytes and Cytokines During Experimentally-Induced *Streptococcus uberis* Mastitis". I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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**DYNAMICS OF LEUKOCYTES AND CYTOKINES DURING
EXPERIMENTALLY-INDUCED *STREPTOCOCCUS*
UBERIS MASTITIS**

A Thesis

Presented for the

Master of Science

Degree

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Magdalena Rambeaud

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DEDICATION

This thesis is dedicated to my parents, Oscar and Cielo Rambeaud, great role models and true examples of hard work and integrity. Thank you for always believing in me and inspiring me to reach higher in order to achieve my goals.

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ABSTRACT

Environmental mastitis is of increasing prevalence in well managed dairy herds throughout the world. Of the environmental pathogens, *Streptococcus uberis* is one of the most prevalent, accounting for a significant proportion of clinical and subclinical intramammary infections in lactating and nonlactating dairy cows and heifers. In spite of this, the pathogenesis of *S. uberis* mastitis is not well understood. The objective of this study was to determine dynamics of leukocytes and cytokines during experimentally-induced *S. uberis* mastitis. Five Jersey and 5 Holstein cows were challenged via intramammary inoculation with *S. uberis* into 2 uninfected mammary glands. One uninfected unchallenged mammary gland from each cow served as a negative control. Rectal temperatures, blood and milk samples were collected frequently for one week after challenge. Clinical status of mammary glands was recorded twice daily during milking. Milk samples were collected to enumerate bacteria and somatic cells, and to determine concentrations of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-8 (IL-8). Neutrophils were isolated from blood to determine expression of CD11b, CD18 and CD62 during the course of experimental infection. Sixteen of 20 challenged mammary glands developed clinical mastitis between 84 and 108 hours after intramammary challenge, and peak clinical signs of mastitis were observed at 144 hours. The number of *S. uberis* in milk from challenged mammary glands increased significantly ($P < 0.05$)

by 48 hours after challenge, in spite of a massive leukocyte infiltration from the bloodstream, evidenced by an increase in the number of somatic cells in milk which began at 18 hours after challenge ($P<0.001$) and remained elevated throughout the study period. Significant amounts of TNF- α , IL-1 β and IL-8 in milk were detected 66 hours after challenge ($P<0.05$). Peak concentrations of TNF- α were observed at 120 hours after challenge, preceding peak clinical signs. Concentrations of IL-1 β in mammary secretions fluctuated considerably from 66 to 144 hours after challenge, but were significantly higher ($P<0.01$) than prechallenge values and values found in milk from control mammary glands. Similar to TNF- α , peak IL-8 concentrations were observed at 120 hours after challenge. The 4 challenged mammary glands that did not become infected exhibited an increase in somatic cells and IL-1 β concentrations in a pattern that appeared to be similar to that of challenged infected mammary glands, but of lesser magnitude. Expression of CD11b was upregulated at 84, 90 and 114 hours after challenge ($P<0.05$), and expression of CD18 was increased 20% at 48 hours after challenge ($P<0.05$). Expression of CD18 decreased sharply 96 hours after challenge and remained depressed until the end of the sampling period ($P<0.01$). Results from this experiment demonstrated that *S. uberis* experimental intramammary infection induced local production of TNF- α , IL-1 β and IL-8, which may play a role in the pathogenesis of *S. uberis* mastitis. Furthermore, an increase in somatic cells in milk occurred earlier than increases in adhesion molecule expression or cytokine production, suggesting that other

mediators may be involved in initial leukocyte recruitment into the mammary gland after infection.

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Chapter 1

INTRODUCTION

Bovine mastitis is the most common and expensive disease affecting dairy cows throughout the world. This disease is typically caused by bacteria and characterized by inflammation of the mammary gland and abnormal appearance of lacteal secretions regardless of the etiologic agent. As a result of mastitis, both milk yield and milk quality are affected greatly. Reduced milk yield and alterations in milk composition due to mastitis are influenced by the severity of the inflammatory response, which in turn is influenced by the mastitis pathogen causing the infection (Oliver and Calvinho, 1995). The National Mastitis Council estimated that mastitis costs dairy producers in the U.S. \$2 billion annually (National Mastitis Council, 1996). Therefore, mastitis continues to be the most significant limiting factor to profitable dairy production worldwide.

Signs of mastitis vary according to severity and duration of infection. Intramammary infection (IMI) may result in clinical or subclinical disease. Mammary glands with subclinical mastitis show no obvious signs of disease. Milk from cows with subclinical mastitis appears normal; however, microorganisms and inflammatory cells are present in high numbers. In many herds, subclinical mastitis is by far the most prevalent and causes the greatest overall loss due to lowered milk production (National Mastitis Council, 1996). Clinical mastitis, on the

other hand, is characterized by abnormal milk, such as clots and clumps, and/or visible alterations of the udder, such as redness and swelling. In severe cases, there may be systemic signs such as elevated temperature, anorexia and septicemia, which may cause death of the animal (Leigh, 1999).

Over 135 infectious agents have been associated with clinical mastitis (Watts, 1988). Mastitis causing organisms have been categorized as contagious or environmental pathogens based on their distinct characteristics of distribution and interaction with the teat and teat duct (Calvinho et al., 1998). Contagious pathogens live and multiply in the cow's mammary gland and are spread from animal to animal primarily during milking. Environmental pathogens are those whose primary reservoir is the environment where cows live and not infected mammary glands (Smith and Hogan, 1993). Contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* species and *Corynebacterium bovis*, while environmental pathogens include a heterogeneous group of bacterial genera, species and strains (Calvinho et al., 1998). The most frequently isolated environmental pathogens are streptococci other than *S. agalactiae* (environmental streptococci) and Gram-negative bacteria (Smith et al., 1985; Oliver, 1988; Smith and Hogan, 1993).

Control of bovine mastitis is very difficult due to the diversity of pathogens capable of causing the disease. Mastitis is present in every herd; however, rates of infection and types of bacteria causing mastitis vary tremendously between herds (Oliver and Mitchell, 1984). Current mastitis control programs developed in

the late 1960's are aimed at reducing the prevalence of mastitis by reducing the incidence, duration and likelihood of transmission of an infection. Therefore, the greatest control has been achieved against those pathogens transmitted from cow to cow. These control programs are based on hygiene including pre- and postmilking teat disinfection, therapeutic and prophylactic antibiotic therapy and culling of chronically infected cows. Acceptance and application of these measures has led to considerable progress in controlling contagious mastitis pathogens. However, these measures have been less effective against environmental pathogens (Smith et al., 1985). Consequently, environmental mastitis has become a major problem, particularly in well-managed dairy herds that have successfully controlled contagious pathogens (Oliver and Mitchell, 1984; Oliver, 1988; Bennet, 1990; Todhunter et al., 1995). It has been hypothesized that the niche vacated by contagious mastitis pathogens becomes occupied by environmental mastitis pathogens, resulting in an increased prevalence of environmental pathogen IMI (Jayarao et al., 1999). Among the environmental streptococci, *Streptococcus uberis* is the most prevalent, accounting for a significant proportion of subclinical and clinical IMI in both lactating and nonlactating cows (Smith et al., 1985; Oliver, 1988; Todhunter et al., 1995; Jayarao et al., 1999; Phuektes et al., 2001).

Therapeutic and prophylactic antibiotic therapy for the control of mastitis presents three major concerns. Firstly, bacteriologic cure rates for therapy during lactation are not as high as in vitro sensitivity data would indicate (Keefe and

Leslie, 1997). Secondly, the presence of antibiotic residues in milk for human consumption is a major concern in today's dairy industry, and mastitis is the number one cause of antibiotic use in dairy cows (Moore and Heider, 1984). Thirdly, there is concern that widespread use of antibiotics for treatment and prevention of mastitis will increase the risk of antibiotic resistance of pathogens that can cause disease in humans. The desire to protect hypersensitive individuals from exposure to specific antibiotics and to reduce the remote possibility of the emergence of antibiotic resistant organisms in milk or in human enteric flora has led to strict and extensive surveillance programs for detection of antibiotic residues in milk (Allison, 1985). Although the risk to human health from antibiotic residues in milk is negligible, sectors of society object to being involuntarily exposed to drug residues. Limiting the use of antibiotics to treat mastitis would greatly benefit dairy farmers, since discarded milk during antibiotic treatment together with decreased milk production account for the largest economic losses associated with mastitis (National Mastitis Council, 1996).

Based on limitations of current methods of mastitis control and concerns regarding use of antibiotics in food producing animals, it seems appropriate to consider alternative non-antibiotic methods for the prevention and control of environmental mastitis. In addition to the required practices of good herd management and hygiene, alternative control measures should include manipulation of host defense mechanisms. This approach requires knowledge of host and pathogen factors involved in the development and establishment of IMI

as well as defense mechanisms within the udder and ways to enhance these mechanisms (Calvinho et al., 1998). However, the pathogenesis of *S. uberis* mastitis is incompletely understood. More insight into mechanisms of IMI by *S. uberis* will ultimately lead to more successful control and prevention of *S. uberis* mastitis in dairy cows.

Chapter 2

LITERATURE REVIEW

CHARACTERISTICS OF *STREPTOCOCCUS UBERIS*

Epidemiology

Streptococcus uberis is ubiquitous in the cow's environment (Smith et al., 1985). It has been isolated from many sites of the cow including tonsils, genital tract, rumen, rectum and skin (Kruze and Bramley, 1982). However, this organism does not contribute significantly to disease outside the mammary gland. It would appear that *S. uberis* has evolved to become a successful and specialized pathogen of the bovine mammary gland (Leigh, 1997). The organism can also be isolated in high numbers from bedding material used for cattle (Bramley, 1982), which is thought to act as an important vector that can contaminate teats. Furthermore, *S. uberis* has been isolated from heavily used pasture in numbers similar to those seen in bedding materials (Harmon et al., 1992).

Intramammary infection with *S. uberis* is common throughout the world. A review by Hogan and Smith (1997) cited four surveys conducted in the previous 10 years in Canada, Ohio, the Netherlands and the United Kingdom, which indicated that *S. uberis* was responsible for 14 to 26% of all clinical mastitis

cases. Interestingly, *S. uberis* is also a significant cause of bovine mastitis in New Zealand, Australia and Argentina, where the dairy industry is primarily pasture based (Williamson et al., 1995; Pankey et al., 1996; Acuña et al., 2001; Phuektes et al., 2001).

Biochemical and serological characteristics

Streptococcus uberis is identified routinely on the basis of colony morphology and esculin hydrolysis; however, subsequent culture confirmation requires additional tests. One of the most widely used systems for the identification of streptococci is the API 20 Strep System (bioMérieux, Vitek Inc.), which consists of 20 miniaturized biochemical tests in a plastic strip. In a study by Jayarao et al. (1991b), this system identified 57 of 60 *S. uberis* isolates correctly. In the same study, the Vitek Gram-positive identification system (GPI, Vitek Systems), using 15 miniaturized biochemical tests, also correctly identified 57 of 60 isolates.

Streptococcus uberis is serologically heterogeneous. Jayarao et al. (1991b) used a streptococcal agglutination system containing Lancefield group A-, B-, C-, D-, F- and G-specific antibodies to determine serogroups of 60 *S. uberis* isolates. Although 16 isolates were classified as belonging to serogroup G, the remaining isolates did not belong to any of the serogroups tested. Consequently, serological tests appear to be of little value for identification of *S. uberis* (Leigh, 1999).

Molecular biology techniques

Using chromosomal DNA hybridization, Garvie and Bramley (1979) demonstrated the presence of two *S. uberis* genotypes (types I and II). Williams and Collins (1990) determined the nucleotide sequences for 16S ribosomal RNA of *S. uberis* genotypes I and II and showed that the two genotypes were phylogenetically distinct and proposed that *S. uberis* genotype II be designated *Streptococcus parauberis*. However, cultural, morphological, biochemical and serological characteristics of the two closely related species are indistinguishable. Techniques developed by Jayarao et al. (1991a; 1992b) based on restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) amplified 16S rRNA genes, indicated that the predominant organism isolated from infected mammary glands was *S. uberis* and that *S. parauberis* occurred infrequently (Oliver et al., 1998).

A number of techniques have been investigated for differentiation and subtyping of individual *S. uberis* strains. Restriction endonuclease fingerprinting of chromosomal DNA (Jayarao et al., 1991c), rRNA gene (rDNA) restriction patterns (Williams and Collins, 1991; Jayarao et al., 1992b) and DNA amplification fingerprinting (Jayarao et al., 1992a) have been shown to be useful procedures for typing *S. uberis*. Each procedure has confirmed the wide variety of strains which are able to infect the mammary gland and substantiates the view that *S. uberis* IMI do not result from contagious transmission of a limited number of virulent strains within a herd (Leigh, 1999).

Virulence factors

For IMI to occur, mechanisms associated with avoidance of phagocytic defenses, adherence to epithelial cells and colonization of mammary gland tissue are probably present (Oliver et al., 1998). By various mechanisms, *S. uberis* is capable of finding beneficial conditions for growth and avoidance of mammary gland defense systems. Potential virulence factors and pathogenic mechanisms for *S. uberis* are presented in Table 1.

1- Capsule

Avoidance of phagocytosis is probably one of the more important mechanisms that bacterial pathogens possess to establish an IMI. Bacterial capsules exert their antiphagocytic functions through mechanisms such as electrostatic repulsion, antigenic mimicry and low immunogenicity, antigen masking, physical barrier between bacteria and phagocyte, binding and depletion of opsonic factors and suppression of phagocyte cell activity (Speert et al., 1983; Almeida et al., 1992). Matthews et al. (1994b) reported that about 50% of *S. uberis* isolates were encapsulated. Almeida and Oliver (1993b) characterized two forms of capsular material from *S. uberis* composed primarily of hyaluronic acid; a higher molecular weight form associated with the bacterial cell and a lower molecular weight form that apparently was released to the surrounding environment.

Table 1. Potential virulence factors of *Streptococcus uberis*. ¹

Virulence factor	Reference
Cell associated factors/activity	
Adherence	(Almeida et al., 1996; Almeida et al., 1999a; Almeida et al., 1999b)
Lactoferrin-binding protein	(Fang and Oliver, 1999)
Collagen-binding protein	(Gilbert et al., 1997)
Fibronectin-binding	(Almeida et al., 1996)
Hyaluronic acid capsule	(Almeida and Oliver, 1993a; Almeida and Oliver, 1993b; Matthews et al., 1994b)
Invasion	(Matthews et al., 1994a)
Laminin-binding	(Almeida et al., 1996)
M-like protein	(Almeida et al., 1996)
PMN toxin	(Leigh, 1994a)
R-like protein	(Groschup and Timoney, 1993)
Plasminogen activator	(Leigh, 1994b)
Extracellular factors	
Hyaluronic acid capsule	(Almeida and Oliver, 1993b)
Hyaluronidase	(Schaufuss et al., 1989)
Uberis factor	(Skalka et al., 1980)

¹adapted from Oliver et al., 1998.

Conflicting reports exist on the role of the capsule in resistance of *S. uberis* to phagocytosis, especially by bovine macrophages. In a study by Grant and Finch (1996), two strains of *S. uberis* that differed in their ability to produce capsule and to resist phagocytosis and killing by neutrophils were both shown to be phagocytosed and killed equally by bovine macrophages. However, Almeida and Oliver (1993a; 1995) reported that phagocytosis and intracellular killing by bovine mammary macrophages was significantly higher for nonencapsulated strains than for encapsulated strains of *S. uberis*. Furthermore, when encapsulated bacteria were opsonized by anticapsular antibodies, the antiphagocytic effect was inhibited (Almeida and Oliver, 1993a). Purified, protease-treated capsular material diminished macrophage phagocytic and killing activity, and this suppressive effect not only protected encapsulated strains but also protected nonencapsulated strains of *S. uberis* from macrophage phagocytosis (Almeida and Oliver, 1993a).

One of the general mechanisms that has been proposed to explain the role of capsular components in resistance of bacteria to phagocytosis is that the capsule may prevent binding of opsonic factors such as antibody or complement to the bacterial cell (Horowitz and Silverstein, 1980). A capsular and non-capsular phenotype of the same strain of *S. uberis* were shown to bind equal quantities of antibody (Leigh and Field, 1994). In each case, the antibody was intact, bound in the correct orientation and presented the Fc terminus for interaction with receptors on phagocytic cells (Leigh and Field, 1994). Leigh and

Field (1994) suggested that resistance of *S. uberis* to bactericidal action of bovine neutrophils was not related to bound immunoglobulins. In this study, however, bovine mammary macrophages were not evaluated, and there was no indication of whether antiserum reacted against capsular material of *S. uberis*.

2- Plasminogen activator

Streptococcus uberis is highly auxotrophic and depends on acquisition of several amino acids, vitamins, trace elements and a carbohydrate source to grow successfully (Leigh, 1994b). Since milk from lactating mammary glands is deficient in free or peptide associated amino acids, *S. uberis* must possess a mechanism to acquire amino acids for growth. Leigh (1993) demonstrated that *S. uberis* was capable of converting plasminogen to plasmin, which in turn degraded casein and generated by-products needed for optimal bacterial growth. A plasminogen activator, designated PauA, was isolated from *S. uberis* (Leigh, 1994b). However, the relationship between activation of plasminogen and virulence is not clear since a low pathogenic strain which showed caseinolytic activity (Leigh et al., 1990) was susceptible to killing by bovine neutrophils after growth in medium supplemented with casein (Leigh and Field, 1991).

Results from a vaccination study using PauA suggest that this molecule conferred partial protection against experimentally induced *S. uberis* mastitis in dairy cows (Leigh et al., 1999). Three of four animals vaccinated with PauA showed no signs of clinical mastitis, and bacterial shedding in milk was low ($<10^3$

colony forming units/ml of milk) in 5 of the 6 challenged quarters (Leigh et al., 1999). The 5 quarters that resisted clinical infection showed the highest antibody titre to PauA. Furthermore, protection was not associated with influx of large numbers of neutrophils into the mammary gland, evidenced by somatic cell counts for challenged quarters from vaccinated animals that never exceeded 2×10^5 cells/ml of milk (Leigh et al., 1999). These results indicate that although establishment of infection could not be avoided, challenged quarters of vaccinated cows did not develop clinical mastitis, probably by reduction in the rate of colonization of the gland.

3- Lactoferrin-binding proteins

Streptococcus uberis expresses at least two surface proteins, designated lactoferrin-binding proteins, that bind to lactoferrin (Lf) in milk (Fang and Oliver, 1999). Lactoferrin is an iron-binding protein present in bovine milk which increases dramatically in concentration during involution and during infection of the bovine mammary gland (Smith and Oliver, 1981). Additionally, bovine Lf has been found to bind to several types of host cells, including bovine mammary epithelial cells (Rejman et al., 1994). Because *S. uberis* bound to Lf in milk and to purified Lf, it was hypothesized that Lf acted as a bridging molecule between bacteria and mammary epithelial cells or phagocytic cells in the pathogenesis of *S. uberis* mastitis (Fang and Oliver, 1999). More recently, Fang et al. (2000) demonstrated that presence of Lf in the culture medium and in milk enhanced

adherence of *S. uberis* to mammary epithelial cells, providing further evidence to substantiate this hypothesis. Lactoferrin-binding proteins have also been detected in *S. dysgalactiae* subspecies *dysgalactiae* and *S. agalactiae* isolated from cows with mastitis (Park et al., 2002).

4- Adherence of *S. uberis* to mammary epithelium and to extracellular matrix proteins

A common concept in the pathogenesis of infectious diseases is that microorganisms that infect mucosal surfaces should adhere to these surfaces to allow multiplication and colonization of that specific mucosa. Adherence should be strong enough to resist the flushing effect of normal secretions, and sufficiently specific to provide the microorganism with sources of nutrients that support colonization and spreading of the infection (Oliver et al., 1998).

Hasty et al. (1992) proposed a multi-step model for adherence of *Streptococcus* species to epithelial cells which involved two distinct kinetic steps with different affinity interactions between host receptors and bacterial adhesins. In the first step, adherence was weak and of low specificity and involved electrostatic/hydrophobic interactions. Lipoteichoic acid (LTA) was proposed as the model adhesin of this step. The second step involved a strong adhesin-host receptor interaction, was non-hydrophobic, and involved highly specific bacterial cell ligand-host cell receptor interactions. It also provided stability to the attachment between bacteria and host cells. M protein and other ligands such as

fibronectin-binding protein and vitronectin-binding protein were proposed as the main adhesins involved in this step (Hasty et al., 1992).

Almeida et al. (1996) showed that M protein and LTA contributed to adherence of *S. uberis* to mammary epithelial cells and suggested participation of the eukaryotic cell cytoskeleton and interaction between epithelial cell membranes and *S. uberis*. In addition, Almeida et al. (1996) indicated that *S. uberis* bound to extracellular matrix proteins (ECMP). A nonencapsulated strain of *S. uberis* adhered significantly better to mammary epithelial cells, but an encapsulated strain adhered better to laminin, collagen and fibronectin. Of the three ECMP tested in the study by Almeida et al.(1996), both strains showed greater binding to laminin. This agrees with Fillipsen et al. (1990) who showed adherence of *S. uberis* to ECMP and with Thomas et al. (1992) who indicated that adherence of *S. uberis* occurred where the endothelium was coated with fibrin. Gilbert et al. (1997) suggested that laminin, collagen and bovine mammary epithelial cells exert a positive regulation on *S. uberis* which leads to increased synthesis of specific proteins, and that these proteins are probably expressed in vivo during infection. Additionally, incubation of *S. uberis* with ECMP resulted in increased adherence to bovine mammary epithelial cells (Almeida et al., 1999b). Therefore, it is likely that ECMP exert a positive regulation and expression of virulence factors in *S. uberis* that enhances colonization of mammary epithelial cells (Almeida et al., 1999b).

5- *Streptococcus uberis* invasion of mammary epithelial cells

Matthews et al. (1994a) demonstrated that *S. uberis* invaded bovine mammary epithelial cells based on ultrastructural changes in the mammary epithelial cells' cytoplasmic membrane when *S. uberis* was in close proximity or made contact. Endocytic-like invaginations and development of pseudopod-like elongations that extended around and engulfed bacteria were evident where *S. uberis* interacted with epithelial cells. Internalized bacteria were observed enclosed in membrane-bound vacuoles (Matthews et al., 1994a). The invasion process involved mammary cell cytoskeleton elements, specifically F-actin. However, no morphological changes indicative of cell damage were observed following bacterial invasion (Matthews et al., 1994a). Data from experimental infection of bovine mammary glands showed *S. uberis* penetrating secretory epithelium 18 to 24 hours after infection (Thomas et al., 1994), further supporting the findings of *in vitro* studies.

Invasion of mammary epithelial cells by *S. uberis* seems to require *de novo* synthesis of several bacterial proteins (Matthews et al., 1994c). *Streptococcus uberis* synthesized several new bacterial proteins when cocultured with bovine mammary epithelial cells (Matthews et al., 1994c; Fang et al., 1998b). When bacterial protein synthesis was inhibited in coculture with mammary epithelial cells, the ability of *S. uberis* to invade mammary epithelial cells decreased (Matthews et al., 1994c). The ability of *S. uberis* *in vivo* to invade bovine mammary epithelial cells could result in chronic infections, since

internalized bacteria are protected from host defense mechanisms and from the action of most antimicrobials (Oliver et al., 1998).

THE INFLAMMATORY RESPONSE IN THE MAMMARY GLAND

Mammary gland immunity

Defense of the mammary gland against mastitis-causing pathogens is mediated by several anatomical, cellular and soluble protective factors (Sordillo et al., 1997). Once bacteria successfully penetrate the teat orifice, the efficiency of these mechanisms determines the resistance or susceptibility of the mammary gland to the establishment of new IMI.

1- Anatomical defenses

Almost invariably, mastitis occurs when bacteria gain entrance into the mammary gland through the teat canal. The teat canal is typically sealed between milkings by a keratin plug derived from epithelial cells lining the canal. Some components of the keratin plug have known microbicidal activity (Treece et al., 1966), but the major role of this plug is to serve as a physical barrier preventing microbial access to the teat sinus (Capuco et al., 1992).

The teat end contains involuntary sphincter muscles that maintain tight closure between milkings and prevent bacteria from penetrating into the gland

(Hamann and Burvenich, 1994). The importance of the teat canal in prevention of IMI was documented as early as 1953 (Murphy and Stuart, 1953). Under the best of machine milking conditions, the process of milk accumulation and withdrawal from the gland leaves the involuntary teat sphincter muscle in a flaccid state that requires up to 2 hours for muscular tone to return (Schultze and Bright, 1983). Once this musculature contracts to close the teat end, it forms an effective seal of the streak canal and enables reformation of the keratin plug. Increased patency of the teat sphincter muscles is directly related to incidence of mastitis (Murphy and Stuart, 1953). Additionally, teat end lesions can be induced by a variety of traumatic insults, such as improper milking machine performance, chemical damage from teat dips and exposure to cold temperatures and wind (Francis, 1981; Sieber and Farnsworth, 1981; Shearn and Hillerton, 1996). These teat end lesions may represent the primary insult to the teat skin as a prelude to IMI.

2- Soluble defenses

Many soluble factors present in milk act in collaboration with innate and immune defense mechanisms. They include immunoglobulins (Ig), complement fractions, Lf, lysozyme and lactoperoxidase. Immunoglobulins are produced by local plasma cells or selectively transported from serum (Sordillo and Nickerson, 1988b). The concentration of Ig in mammary secretions is dependent upon the stage of lactation, the degree of permeability of secretory tissue and the number

of Ig-producing cells present. In healthy mammary glands, the concentration of Ig is low during lactation but increases during the nonlactating period to reach peak concentration during colostrogenesis (Sordillo and Nickerson, 1988b). Although IgG₁ is the predominant isotype in mammary secretions of healthy cows, neutrophils can transport IgG₂ to the mammary gland as they emigrate towards the site of inflammation (Musoke et al., 1994). Immunoglobulins, together with complement fraction C3b, act as important opsonins that facilitate phagocytosis of bacteria by neutrophils during inflammation (Reiter, 1978; Howard et al., 1980; Targowski, 1983).

Lactoferrin, an iron-binding glycoprotein produced by epithelial cells and neutrophils, is bacteriostatic by its ability to prevent growth of bacteria that have an iron requirement (Smith and Oliver, 1981). However, streptococci are resistant to the antibacterial effects of lactoferrin (Smith and Oliver, 1981; Todhunter et al., 1985) probably because streptococci do not have a high requirement for iron (Weinberg, 1978). Fang and Oliver (1999) showed that different strains of *S. uberis* bound to Lf through specific lactoferrin-binding proteins. *Streptococcus uberis* may utilize Lf as a bridging molecule to adhere and later invade mammary epithelial cells (Fang and Oliver, 1999).

Lysozyme is a bactericidal protein that cleaves peptidoglycans from the cell wall of Gram-positive bacteria as well as the outer membrane of Gram-negative bacteria (Reiter, 1978). However, because bovine milk contains very

low concentrations of lysozyme (Vakil et al., 1969), the real protective role of this enzyme in bovine mastitis is unknown.

Lactoperoxidase, in the presence of thiocyanate and hydrogen peroxide, is bacteriostatic for gram-positive bacteria such as *S. aureus* and streptococci and bactericidal for gram-negative bacteria (Outteridge and Lee, 1988). Lactoperoxidase is present in high concentrations in bovine milk (Reiter, 1978; Reiter and Harnulv, 1984). However, it is generally assumed that milk does not contain hydrogen peroxide (Reiter and Harnulv, 1984), and thiocyanate concentrations may vary greatly depending on external factors such as nutrition (Wood, 1975); therefore, the importance of this system against mastitis causing pathogens is questionable.

3- Cellular defenses

Once bacteria penetrate the teat orifice, they are exposed to mammary gland defense mechanisms that will attempt to eliminate or control bacterial invasion before any serious damage is done to mammary tissue. Resident and newly recruited leukocytes play a key role during the initial stages of pathogenesis.

a- Macrophages

Macrophages are the predominant cell type found in milk from healthy mammary glands (Lee et al., 1980; McDonald and Anderson, 1981; Sordillo and Nickerson, 1988a), and are considered the first line of defense against invading pathogens. Macrophages have the ability of ingesting bacteria, cellular debris and accumulated milk components (Sordillo and Nickerson, 1988a). However, because of indiscriminate ingestion of fat and casein, macrophages suffer degranulation and loss of pseudopodia resulting in dramatically reduced bactericidal ability (Paape and Guidry, 1977; Reinitz et al., 1982). While somewhat helpless against invading bacteria, macrophages have the ability to release a powerful array of cytokines which play a key role in the subsequent inflammatory response to eliminate infection (Craven, 1983).

An effective response requires that macrophages recognize pathogen-associated molecular patterns that distinguish infectious agents from self, and in addition, discriminate among pathogens (Janeway, 1992). Two members of the Toll-like receptor (TLR) family play a key role in this process. TLR4 recognizes LPS, whereas TLR2 recognizes various fungal, gram-positive and mycobacterial components (Hoshino et al., 1999; Lien et al., 1999; Flo et al., 2000; Ozinsky et al., 2000). This family of receptors has the capacity to recognize a wide spectrum of stimuli, providing some “specificity” to the innate immune response. At least eight TLR have been identified (Means et al., 2000). The intracellular domains of TLR are similar to the intracellular domain of the interleukin-1 (IL-1) receptor, and

these regions are referred to as Toll/IL-1R domain. Activation of TLR triggers a multi-step signaling pathway which results in activation of particular target genes, mostly through activation of nuclear factor κ B (NF κ B) (O'Neil and Greene, 1998). This signal transduction pathway is highly similar to the one activated by IL-1 (O'Neil and Greene, 1998). Although likely present, no reports exist on the presence of TLR on bovine mammary gland macrophages.

Additionally, mammary gland macrophages are important antigen processing and presenting cells (Politis et al., 1992). Bacterial antigens are processed within macrophages and presented on the cell surface together with major histocompatibility complex (MHC) class II molecules. This alignment allows specific lymphocytes to recognize a particular antigen and mount a specific immune response towards it. However, Politis et al. (1992) showed that mammary gland macrophages are less efficient than blood monocytes in cytokine production, antigen presentation and antigen-specific T-cell proliferation. Furthermore, Politis et al. (1992) demonstrated that this inferior ability was not due to suppressive activity of these cells but to an overall decreased functionality. In contrast, Sordillo et al. (1995) reported that mammary gland macrophages have an enhanced ability to produce tumor necrosis factor- α (TNF- α) when compared to blood monocytes during the periparturient period, so it is not that mammary gland macrophages are hyporesponsive altogether. It seems evident that mammary gland macrophages show functional differences compared with blood monocytes. Furthermore, studies by Politis et al. (1992) and

Sordillo et al. (1995) were conducted with two different stimulatory agents for macrophages. It could be possible that macrophages elicit distinct responses to different etiological agents of mastitis.

b- Neutrophils

Polymorphonuclear leukocytes (PMN) or neutrophils generally constitute less than 20% of the cells present in milk from healthy mammary glands (Lee et al., 1980; McDonald and Anderson, 1981). However, in milk from infected mammary glands, neutrophils constitute >90% of total leukocytes (Paape et al., 1981; Sordillo et al., 1987; van Werven et al., 1999). Neutrophils travel from the bloodstream to the site of infection by a tightly regulated migration mechanism. Influx of PMN into the mammary gland occurs at a low level for immune surveillance but increases rapidly in response to bacterial infection, where potent inflammatory mediators guide PMN towards the site of infection (Paape et al., 2000). Neutrophils play a key role in defense of the mammary gland; in fact, it has been shown that the severity and duration of mastitis is related critically to the promptness of leukocyte migration and the bactericidal activity of leukocytes at the site of infection (Hill, 1981; Kehrl et al., 1989). However, similar to macrophages, milk PMN are less phagocytic than blood PMN (Jain and Lasmanis, 1978). This has been attributed to ingestion of milk components such as fat and casein by the PMN (Russel et al., 1976; Paape and Guidry, 1977), and more recently, to a direct effect of diapedesis across mammary epithelium, which

reduced phagocytosis and oxidative burst of bovine neutrophils (Smits et al., 1999). These factors may explain why such high numbers of PMN are required to prevent infection in the bovine mammary gland (Craven and Williams, 1984).

Bovine neutrophils differ somewhat from neutrophils of other species. Like other species, they contain azurophilic and specific granules. The most important antibacterial mechanism derived from azurophilic granules is the myeloperoxidase-hydrogen peroxide-halide system, one of the most potent bactericidal mechanisms of neutrophils (Roth, 1994). A membrane-associated oxidase enzyme complex converts oxygen to superoxide anion, which then undergoes further reactions to form hydrogen peroxide, hydroxyl radical and singlet oxygen (Densell and Mandell, 1990). Myeloperoxidase catalyzes a reaction between hydrogen peroxide and halide ions (iodide, bromide, and chloride), resulting in halogenation and oxidation of bacterial surface components and generation of hypochlorous acid, aldehydes and chloramines (Roth, 1994). Additionally, bovine neutrophils contain a third type of granules, larger and more numerous than the other two classes, which contain a variety of oxygen-independent bactericidal compounds (Gennaro et al., 1983).

Another difference of bovine neutrophils is that they lack Fc receptors for IgG₁ (Howard et al., 1980). Consequently, IgG₁ cannot function as an opsonin for bovine neutrophils. PMNs have membrane receptors for the Fc component of IgM and IgG₂ and for complement fraction C3b, which are necessary for mediating phagocytosis of invading bacteria.

Unlike most mammalian species, bovine neutrophils are not chemotactically attracted to N-formylated bacterial peptides (Craven, 1986), which are fairly unique to prokaryotic organisms. Reasons for this are unknown, but might be related to the important function that microbial flora play in ruminant digestion, and the fact that it would not be beneficial for animals to develop an immune response towards beneficial microbes involved in digestion (Kehrli and Harp, 2001). It is not known how ruminants efficiently recognize the presence of bacterial peptides in undesirable locations; possibly, N-formylated bacterial peptides are recognized by the ruminant immune system in a more modest manner that results in an indirect initiation of an inflammatory response (Kehrli and Harp, 2001).

Inflammation of the mammary gland

Native defenses of the bovine mammary gland are challenged continuously by environmental exposure to bacteria. Once a pathogen has penetrated the teat orifice, innate and adaptive immune systems will attempt to prevent infection before serious damage is caused to secretory tissue. The sequence of events that take place is characterized initially by an acute inflammatory reaction dominated by neutrophil recruitment into mammary secretion. This is evidenced initially by an increase in somatic cell count and appearance of abnormal milk (flakes, clots, clumps).

Initial signals that trigger acute inflammation in the mammary gland are varied and redundant. Many chemical mediators are released within hours of infection and include chemotactic complement fractions, prostaglandins, platelet activating factor, thromboxane A₂ and acute phase cytokines such as IL-1, TNF- α , and interleukin-8 (IL-8) (Giri et al., 1984; Rose et al., 1989; Shuster et al., 1993; Shuster et al., 1997; Riollot et al., 2000). These factors are released by leukocytes in milk or by mammary epithelial cells in response to bacterial products, and play a key role in the battle between the cow's defense mechanisms and the pathogen.

1- Fundamentals of leukocyte recruitment

The first step involved in leukocyte recruitment is the initial contact and loose interaction between leukocytes in the bloodstream and endothelial cells from post-capillary venules. Rolling of leukocytes against the blood vessel wall is mediated by members of the selectin family (Zimmerman, 1992). Molecules of the selectin family are heavily glycosylated, single chain integral membrane proteins that include CD62P, CD62E (both present in endothelial cells) and CD62L (present in leukocytes; Table 2). Rolling of leukocytes is mediated in part by CD62L which binds to mucin-like glycoproteins (GlyCAM-1 and CD34) expressed on postcapillary endothelial cells (Robbins, 1994). CD62L is crucial for

Table 2. Leukocyte-endothelial adhesion molecules. ¹

ENDOTHELIAL MOLECULE	FAMILY	LEUKOCYTE RECEPTOR	FAMILY	DISTRIBUTION
E-selectin (CD62E)	Selectin	Sialyl-Le glycoprotein	Oligosaccharide	Neutrophils T-cells Monocytes
P-selectin (CD62P)	Selectin	Sialyl-Le glycoprotein	Oligosaccharide	Neutrophils Monocytes
ICAM-1	Immunoglobulin	LFA-1 Mac-1	β 2 integrin	All leukocytes
VCAM-1	Immunoglobulin	VLA-4	β 1 integrin	Lymphocytes Monocytes Basophils Eosinophils
GlyCAM-1 CD34	Mucin-like glycoproteins	L-selectin (CD62L)	Selectin	Neutrophils Lymphocytes Monocytes

¹From Robbins, 1994.

recruitment of neutrophils into inflamed tissues; it acts by slowing down leukocytes upon contact with its specific ligands (Butcher, 1991). Additionally, during an inflammatory response, locally produced cytokines stimulate increased expression of CD62P and CD62E on endothelial cells, which interact with undefined ligands on leukocytes and further facilitate margination of leukocytes (von Andrian et al., 1993). Neutrophils activated by cytokines or chemoattractants shed CD62L (Kishimoto et al., 1989), which is a prerequisite step for β_2 - integrin mediated tight adhesion.

Integrins are the major family of cell surface receptors that mediate adhesive cell to cell interactions and attachment to extracellular matrix (Hynes, 1992; Sonnenberg, 1993). Structurally, integrins are heterodimeric glycoproteins made up of alpha and beta chains. The β_2 -integrins are mainly involved in leukocyte-endothelial cell contact (Table 2). The β_2 -integrin family consists of distinct α chains, CD11a, CD11b and CD11c that share a common β chain (CD18). Distribution of β_2 -integrins on leukocyte surfaces varies with cell type and state of activation. The most important β_2 -integrin involved in neutrophil recruitment into inflamed tissue is Mac-1 (CD11b\CD18; Arnaout, 1990). It is predominantly found on granulocytes with some expression on macrophages and natural killer cells (Arnaout, 1990). Mac-1 is predominantly stored in cytoplasmic granules; upon cellular activation these cytoplasmic granules translocate to the cell surface and expression of Mac-1 increases markedly (Miller et al., 1987; Arnaout, 1990). In addition to their critical role in adhesion, it is likely that β_2 -

integrins mediate inside-out signaling and extracellular conformational changes (Miller et al., 1987) with a consequent increase in integrin affinity.

The immunoglobulin family molecules interact with β_2 -integrins expressed on leukocytes. Intracellular adhesion molecule 1 (ICAM-1) is an integral membrane protein that binds to Mac-1 on leukocytes (Diamond et al., 1991). ICAM-1 is expressed in low levels on normal endothelium; however, expression of ICAM-1 increases markedly on cytokine- simulated endothelial cells (Dustin et al., 1986). Increased expression is important for high affinity binding of leukocytes to postcapillary venules and subsequent migration into inflamed tissues (Dustin et al., 1986).

The currently accepted model for neutrophil adhesion and transmigration in acute inflammation postulates the following steps (Robbins, 1994):

- Initial rapid and relatively loose adhesion that accounts for rolling, involving mainly the naturally occurring CD62L and CD62P, and, in cytokine induced endothelium, CD62E.
- Activation of leukocytes by agents made by endothelium or other cells or emanating from the site of injury, to increase avidity of integrins.
- Strong binding of leukocytes and endothelial cells, largely through β_2 -integrin-ICAM pathway, and consequent transmigration.

Mechanisms controlling PMN transepithelial migration in the mammary gland are likely to be unique due to multiple roles of lactating mammary

epithelium. Furthermore, leukocytes must not only traverse the capillary endothelium but also the extracellular matrix and mammary epithelial cells in order to arrive to the alveolar lumen. Smits et al. (2000) investigated the role of Mac-1 in diapedesis across the blood-milk barrier in an in vitro model and reported that neutrophil migration across mammary endothelial cells was almost completely dependent on CD18, the β chain of β_2 -integrins. Neutrophil diapedesis across mammary epithelial cells was dependent to a greater extent on CD11b (Smits et al., 2000). These results provide evidence that different mechanisms exist for neutrophil migration across various cell layers of the blood-milk barrier.

2- Acute phase response mediators

Upon bacterial invasion of the mammary gland, a wide array of inflammatory mediators is released from bacterial cells as well as from host cells that react to the foreign presence and 'give notice' to the immune system in order to attempt to control and eliminate the infection. These inflammatory mediators include a variety of bacterial products; as well as prostaglandins; leukotrienes; histamine; complement fractions; and acute phase cytokines such as IL-1, TNF- α , interleukin-6 (IL-6), interleukin-8 (IL-8) and several colony stimulating factors (Giri et al., 1984; Rose et al., 1989; Shuster et al., 1993; Riollot et al., 2000). These bioactive products have varied effects on local tissues and also direct leukocyte migration into inflamed tissues. Additionally, they exert systemic effects

such as fever, breakdown of the blood-milk barrier and increased bone marrow output of leukocytes to replenish supplies in the bloodstream.

a- Tumor necrosis factor- α

Tumor necrosis factor- α is the principal mediator of the response to gram-negative bacteria (Shuster et al., 1993; Riollot et al., 2000) and may also play a role in innate immune responses to other infectious agents (van den Berg et al., 1999; Malazdrewich et al., 2001). Newly synthesized pro-TNF- α is first displayed on the plasma membrane and then proteolytically cleaved in the extracellular domain to release the mature TNF- α (Kriegler et al., 1988). The 17-kDa soluble TNF- α is the mature form mediating biologic effects. The 26-kDa transmembrane pro-TNF- α , however, is also active and has been shown to mediate the cytotoxic effect of TNF- α through cell-to-cell contact (Kriegler et al., 1988).

Tumor necrosis factor- α synthesis can be stimulated in various cell types by a wide range of stimuli. Although produced predominantly by monocyte-macrophages, TNF- α is also made by other cells such as fibroblasts, epithelial cells, mast cells, neutrophils, natural killer cells and many others (Wang and Tracey, 1999). Exogenous and endogenous factors produced by bacteria, viruses, parasites and tumors are capable of inducing cells to produce TNF- α (Wang and Tracey, 1999). Lipopolysaccharide (LPS) is the classical inducer of TNF- α synthesis in monocytes and macrophages through CD14, a specific LPS

receptor (Wright et al., 1990). Paape et al. (1996) reported the presence of CD14 on bovine mammary PMN and macrophages.

The activity of TNF- α is mediated through at least two types of cell surface receptors, type I (TNFRI) and type II (TNFRII), which are present on virtually all cell types (Hohmann et al., 1989). Most biologic activities of TNF- α are mediated through TNFRI (Espevik et al., 1990), and involve increased rates of transcription of particular target genes, often through activation of NF κ B or AP-1 transcription factors (Malinin et al., 1997).

When small quantities of TNF- α are produced, TNF- α acts locally as a paracrine and autocrine regulator of leukocytes and endothelial cells. Principal biological activities of TNF- α include activation of endothelial cells in the vasculature to express new adhesion molecules that will facilitate neutrophil and subsequently monocyte and lymphocyte migration. Additionally, TNF- α stimulates macrophages and other cell types to secrete chemokines that contribute to leukocyte recruitment, and also activates leukocytes to kill invading microbes (Abbas et al., 1997).

When larger amounts of TNF- α are produced, TNF- α can enter the circulation and have systemic effects. These include induction of fever by increased synthesis of prostaglandins in the hypothalamus, stimulation of macrophages and perhaps vascular endothelial cells to stimulate secretion of IL-1 and IL-6, and stimulation of hepatocyte synthesis of acute phase proteins (Baumann and Gauldie, 1994).

The role of TNF- α as an inflammatory mediator during mastitis has been studied extensively in *E. coli* and endotoxin induced mastitis (Shuster et al., 1993; Shuster et al., 1996; Rainard and Paape, 1997; Blum et al., 2000; Riollet et al., 2000; Paape et al., 2002). As a general rule, after *E. coli* or endotoxin challenge, TNF- α concentrations increase sharply in the inflamed mammary gland, peaking within 24 hours of initial challenge and decreasing sharply afterwards (Shuster et al., 1997; Riollet et al., 2000; Paape et al., 2002). Increased TNF- α concentration in infected mammary glands also correlates with enhanced leukocyte migration from the bloodstream into the mammary gland and with the onset of clinical signs of mastitis (Blum et al., 2000; Paape et al., 2002). TNF- α is clearly associated with acute inflammation, but was not detected in milk of cows with chronic *S. aureus* mastitis (Riollet et al., 2000).

The role of TNF- α during *S. uberis* mastitis has not been investigated. Although different *S. uberis* strains have shown different pathogenicity (Hill, 1988; Oliver, personal communication), *S. uberis* mastitis tends to be less severe than *E. coli* mastitis, although it can cause serious clinical infections. Furthermore, *S. uberis* mastitis can be of long duration as opposed to *E. coli* mastitis, which is usually eliminated by the animal within 5 to 6 days (Hill et al., 1978; Lohuis et al., 1990; Shuster et al., 1996; Shuster et al., 1997).

Streptococcus uberis, as a gram-positive organism, is markedly different from *E. coli*, and its antigenic determinants and virulence factors may likely elicit different effector mechanisms of the inflammatory response. A study by Tissi et

al. (1999) evaluated the role of TNF- α and other cytokines in a mouse model of group B streptococcal arthritis. Interestingly, Tissi et al. (1999) found that TNF- α was not detected in joints of mice injected with group B *Streptococcus* throughout the challenge period, even when articular lesions were most frequent and severe. These findings suggest that TNF- α is not involved in the pathogenesis of streptococcal arthritis. It is possible that other streptococci such as *S. uberis* stimulate similar inflammatory responses.

b- Interleukin-1

Interleukin-1 is the prototype 'multifunctional' cytokine, affecting nearly every cell type, often in concert with TNF- α (Dinarello, 1999). The primary source of IL-1 is the activated macrophage; however, many other cell types such as epithelial cells and endothelial cells can produce IL-1. Specifically for the mammary gland, it has been demonstrated that mammary epithelial cells can produce IL-1 upon LPS stimulation (Boudjellab et al., 2000), and probably upon other stimuli. There are two forms of IL-1, referred to as IL-1 α and IL-1 β , but their biological activity is identical. Most of the IL-1 activity found in the circulation is IL-1 β (Abbas et al., 1997). Two different membrane receptors have been characterized for IL-1, both of which belong to the Ig superfamily, referred to as type I and type II receptors. The type I receptor has slightly higher affinity for IL-1 β , and mediates most of the IL-1 responses (Sims et al., 1993).

When produced in low amounts, IL-1 acts locally on endothelial cells to promote coagulation and to increase expression of surface molecules that mediate leukocyte adhesion. Interleukin-1 does not directly activate leukocytes, but causes macrophages and endothelial cells to synthesize chemokines that do activate leukocytes, such as IL-6. When secreted into the circulation, IL-1 shares with TNF- α the ability to cause fever, induce hepatic synthesis of acute phase proteins and initiate metabolic wasting (Abbas et al., 1997).

The dynamics of IL-1 production have also been studied in detail during *E. coli* and endotoxin mastitis (Shuster et al., 1993; Shuster et al., 1996; Shuster et al., 1997; Riollot et al., 2000). Interleukin-1 concentrations increased sharply and usually peaked shortly after TNF- α concentrations (Shuster et al., 1997; Riollot et al., 2000). Similarly to TNF- α , no information exists on IL-1 production during *S. uberis* mastitis.

c- Interleukin-8

Interleukin-8 belongs to the chemokine family of cytokines. Chemokines are small molecules, approximately 8 to 10 kDa in size, characterized by their strong chemotactic ability (Oppenheim et al., 1991; Baggiolini et al., 1994). Interleukin-8 has been established as a principal chemotactic factor directing neutrophil recruitment to and activation at the inflammatory focus (Harda et al., 1994). Activated monocytes and macrophages appear to be the predominant cellular sources of IL-8, but several nonimmune cells, such as epithelial cells can

also produce this chemokine (Eckmann et al., 1993). Recently, Boukjjellab et al. (1998) reported IL-8 production by bovine mammary epithelial cells following LPS stimulation. Barber and Yang (1998) indicated presence of IL-8 in mammary secretions from cows with mastitis and reported that the chemotactic ability of those secretions was due to IL-8.

Interleukin-8 is expressed at high levels in milk from cows with coliform mastitis (Riollet et al., 2000). Interleukin-8 production begins more slowly than TNF- α and IL-1 and persists longer (Shuster et al., 1997; Riollet et al., 2000). Barber and Yang (1998) detected IL-8 activity in mammary secretions from a cow clinically infected with *S. aureus*. The role of IL-8 during *S. uberis* mastitis, however, has not been determined.

SUMMARY AND STATEMENT OF THE PROBLEM

Bovine mastitis is the most common and expensive disease affecting dairy cows throughout the world. Studies have shown that as the prevalence of contagious mastitis pathogens was reduced, the proportion of IMI by environmental mastitis pathogens increased markedly. *Streptococcus uberis* is an environmental pathogen that accounts for a significant proportion of clinical and subclinical mammary gland infections worldwide and is not readily controlled by current mastitis control measures. The pathogenesis of *S. uberis* mastitis is incompletely understood. Most pathogenesis studies on bovine mastitis have

been conducted using *E. coli* or endotoxin experimental challenge models; these studies have elucidated the role of blood and milk leukocytes as well as several acute phase inflammatory mediators in the development of *E. coli* mastitis. No information exists on the role of inflammatory mediators during *S. uberis* infection. Therefore, the objective of this study is to evaluate the dynamics of leukocytes and cytokines during experimentally induced *S. uberis* mastitis. A better understanding of the pathogenesis of *S. uberis* mastitis will facilitate development of more efficient control strategies that will minimize economic losses and improve animal health and milk quality.

Chapter 3

DYNAMICS OF LEUKOCYTES AND CYTOKINES DURING EXPERIMENTALLY-INDUCED *STREPTOCOCCUS UBERIS* MASTITIS

INTRODUCTION

Bovine mastitis continues to be the most economically devastating disease affecting the dairy industry (National Mastitis Council, 1996). Of the many bacterial pathogens capable of causing mastitis, environmental pathogens have become increasingly problematic, particularly in herds that have successfully controlled contagious pathogens (Oliver and Mitchell, 1984; Oliver, 1988; Todhunter et al., 1995). Among environmental pathogens, *Streptococcus uberis* is one of the most prevalent, accounting for a significant proportion of subclinical and clinical intramammary infections (IMI) in both lactating and nonlactating cows (Smith et al., 1985; Oliver, 1988; Todhunter et al., 1995; Jayarao et al., 1999; Phuektes et al., 2001).

Acceptance and widespread application of mastitis control practices devised in the 1960's has led to considerable progress in the control of contagious mastitis pathogens. However, these procedures have been less effective against environmental mastitis pathogens. One possible approach to

controlling environmental mastitis involves manipulation of host defense mechanisms. This approach requires knowledge of host and pathogen factors involved in the development and establishment of IMI as well as defense mechanisms within the udder and ways to enhance these mechanisms (Calvinho et al., 1998). However, the pathogenesis of *S. uberis* mastitis is incompletely understood. The role of polymorphonuclear neutrophils (PMN) has been well established in the defense against *Escherichia coli* (Hill et al., 1978) and *Staphylococcus aureus* infection of the bovine mammary gland (Schalm et al., 1976). This has not been established for the control of IMI by *S. uberis*. Furthermore, to our knowledge, a description of inflammatory mediator production during *S. uberis* mastitis does not exist in the literature. The role of cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-8 (IL-8) have been studied extensively during *E. coli* and endotoxin-induced mastitis (Shuster et al., 1993; Shuster et al., 1996; Rainard and Paape, 1997; Blum et al., 2000; Riollot et al., 2000; Paape et al., 2002). No information exists on the role of cytokines during *S. uberis* mastitis. *Streptococcus uberis*, a prevalent gram-positive mastitis pathogen, is markedly different from *E. coli*, and its antigenic determinants and virulence factors likely elicit different effector mechanisms of the inflammatory response. To gain a better understanding of the pathogenesis of *S. uberis* mastitis, the objective of the present study was to evaluate the dynamics of leukocytes and cytokines, if produced, during experimentally induced *S. uberis* mastitis.

MATERIALS AND METHODS

Experimental herds

Lactating cows from two dairy research herds of The University of Tennessee were used in this study. The Dairy Experiment Station (DES) research herd in Lewisburg, TN consists of approximately 170 lactating Jersey cows. The herd is *Streptococcus agalactiae*-negative and has a low prevalence of *S. aureus* but experiences mastitis problems caused by environmental pathogens, in particular *S. uberis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae* and Gram-negative bacteria. Cows in the DES research herd are milked twice daily in a 12-stall trigon milking parlor equipped with automatic milking machine take-offs (Surge, Babson Bros., Oak Brook, IL, USA). Milking machines are backflushed (Surge Backflush II, Babson Bros.) with water after removal from cows. Premilking and postmilking teat disinfection are practiced. Animals are fed rations consistent with stage of lactation and described in the standard operating procedures of the site. Water is provided ad libitum. Lactating cows are housed in free stalls at ambient temperature. Stalls are bedded with dairy waste solids separated from a manure slurry (Alfa-Laval, Inc., Kansas City, MO, USA). Cows are pastured 4 to 6 hours per day when weather and pasture permit. Bovine somatotropin (Posilac, Monanto, St. Louis, MO, USA) is used as a management tool to increase milk production of lactating dairy cows after peak lactation. All cows are dried off approximately 6 to 8 weeks before expected

calving and all mammary glands of cows are infused with an antibiotic preparation approved for use in nonlactating cows following the last milking of lactation.

The Middle Tennessee Experiment Station (MTES) dairy research herd in Spring Hill, TN is composed of approximately 150 lactating Holstein cows. The herd is *S. agalactiae*-negative but experiences mastitis problems caused by *S. aureus* and environmental pathogens, in particular *S. uberis*. Cows in the MTES dairy research herd are milked twice daily in a double-eight parallel parlor equipped with a DeLaval milking machine system (De Laval) with automatic milking machine take-offs. Premilking- and postmilking teat disinfection are practiced. Milking equipment is evaluated routinely and maintained per manufacturer's recommendation. Animals are fed rations consistent with stage of lactation and as described in the standard operating procedures of the site. Water is provided ad libitum. Lactating cows are housed in free stalls at ambient temperature. Stalls are bedded with sawdust. Lactating cows are allowed on pasture for exercise for 4 hours per day when weather permits. Bovine somatotropin (Posilac, Monanto) is used as a management tool to increase milk production of lactating dairy cows after peak lactation. All cows are dried off approximately six to eight weeks before expected calving and all mammary glands of cows are infused with antibiotic preparations approved for use in nonlactating following the last milking of lactation.

Animals

Five clinically healthy Holstein cows from the Middle Tennessee Dairy Experiment Station and 5 clinically healthy Jersey cows from the Dairy Experiment Station were used in this experiment. All cows were approximately 11-38 days in milk, in their 2nd lactation, and had not been exposed previously to *S. uberis*. Each cow was challenged by intramammary inoculation in 2 uninfected mammary glands. One non-challenged mammary gland from each cow served as a within-cow negative control. Selection of uninfected mammary glands for the experiment was based on microbiological examination of mammary secretions 14 and 7 days prior to challenge and SCC of $<2.5 \times 10^5$ cells/ml.

Bacterial suspension and challenge exposure

Streptococcus uberis UT888, isolated originally from a cow with clinical mastitis, was used in the experimental challenge study. Stock bacterial cultures stored at -80°C were thawed, plated on a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Beckton Dickinson Microbiology Systems, Cockeysville MD, USA), and incubated overnight at 37°C in a 5% CO_2 incubator. Approximately two to five colonies were then subcultured at 37°C in Todd Hewitt Broth (Difco, Detroit, MI, USA) for 7 hours to a concentration of approximately 5×10^8 colony forming units (CFU)/ml. Bacterial cultures were then diluted in sterile phosphate-buffered saline (PBS).

Inoculation procedure

Within 0.5 hours after the afternoon milking, teats of cows were cleaned thoroughly with individual disposable paper towels, and teat ends sanitized with swabs containing isopropyl alcohol. The bacterial suspension was infused using sterile disposable syringes fitted with sterile disposable teat cannulas. Five ml of inoculum containing 6,650 CFU (Jersey cows) or 10,500 CFU (Holstein cows) of *S. uberis* in sterile PBS were infused into two uninfected mammary glands of each cow. The infused inoculum was massaged upward into the gland cistern. Teats were immersed in a postmilking teat disinfectant when the above procedure had been completed. The actual number of bacteria infused into mammary glands was determined by viable plate count on blood agar before and after inoculation.

Milk sampling regimen

Milk samples were collected at 0, 12, 18, 24, 36, 42, 48, 60, 66, 72, 84, 90, 96, 108, 114, 120, 144 and 168 hours after challenge (Table 3). When the sampling time point coincided with milking, samples were collected before cows were milked. Samples of foremilk for microbiological examination were collected aseptically as described by Oliver et al. (1994). Before sample collection, teats of cows were cleaned thoroughly with individual disposable paper towels, and teat ends were sanitized with swabs containing isopropyl alcohol. Samples were collected in sterile tubes and stored at –20°C until analysis. Samples for

Table 3. Sampling schedule for cows challenged with *Streptococcus uberis*.

Hours	Micro ¹	SCC ² (challenged mammary glands)	SCC ² (control mammary glands)	Cytokines ³ (challenged mammary glands)	Cytokines ³ (control mammary glands)	Evaluation of animals ⁴	Blood
0	X	X	X	X	X	X	X
12	X	X		X		X	X
18	X	X		X			X
24	X	X	X	X	X	X	X
36	X	X		X		X	X
42	X	X		X			X
48	X	X	X	X	X	X	X
60	X	X		X		X	X
66	X	X		X			X
72	X	X	X	X	X	X	X
84	X	X		X		X	X
90	X	X		X			X
96	X	X	X	X	X	X	X
108	X	X		X		X	X
114	X	X		X			X
120	X	X	X	X	X	X	X
144	X	X	X	X	X	X	X
168	X	X	X	X	X	X	X

¹Milk for microbiological analysis, all mammary glands sampled.

²Milk for determination of the number of somatic cells.

³Milk for cytokine analysis.

⁴Rectal temperature, physical palpation of the udder and strip cup examination of mammary secretions.

determination of numbers of somatic cells (approximately 30 ml) were collected in plastic snap cap vials (Capitol Vial Co.) preloaded with Bronopopl-B as preservative. Samples for determination of numbers of somatic cells were shipped via overnight courier to the Dairy Herd Improvement Association (DHIA) Laboratory, The University of Tennessee, Knoxville, TN, for analysis. Milk samples for cytokine quantification (50 ml) were collected in sterile 50 ml plastic tubes. Samples were refrigerated at 4°C immediately after collection, shipped via overnight courier and whey was obtained as described below upon arrival to the Lactation/Mastitis Laboratory at The University of Tennessee, Knoxville, TN.

Evaluation of animals

Clinical assessment of all animals was performed before experimental challenge and at 0, 12, 18, 24, 36, 42, 48, 60, 66, 72, 84, 90, 96, 108, 114, 120, 144 and 168 hours after challenge. Rectal temperatures were determined at similar time points. Clinical status of all mammary glands and appearance of milk was evaluated using the following scheme:

Milk: 1= normal, 2= a few flakes, 3= small slugs, 4= large slugs/clots, 5= stringy/watery, and 6= bloody.

Udder: 1= normal, 2=slight swelling, 3= moderate swelling, 4= severe swelling, 5= scar tissue, and 6= edema.

Cows that developed clinical mastitis (defined as visible abnormalities in the mammary gland and/or milk) were monitored closely and antibiotic treatment was initiated after day 7 post-challenge or earlier if required. An IMI was defined as a challenged mammary gland where *S. uberis* was isolated at 2 or more occasions during the one week experimental period.

Determination of numbers of somatic cells in milk

Numbers of somatic cells in milk for each challenged and control mammary gland were determined by DHIA personnel at The University of Tennessee, Knoxville, TN, with a Somacount 300 cell counter (Bentley Instruments, Chaska, MN, USA).

Bacteriological examination of mammary gland foremilk samples

Milk samples were examined following procedures recommended by the National Mastitis Council and essentially as described by Oliver et al. (1994). Thawed samples of mammary secretion were mixed thoroughly by vortexing, and 10 µl of each sample were spread onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Beckton Dickinson Microbiology Systems). Plates were incubated at 37°C and bacterial growth was observed and recorded at 24-h intervals for three days. Bacteria were identified tentatively according to colony morphologic features, hemolytic characteristics,

and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase by the tube coagulase method. Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and CAMP reaction. Streptococcal organisms were identified to the species level using the API 20 Strep System (bioMerieux, Vitek Inc., Hazelwood, MO, USA) upon first and last isolation of the organism from experimentally infected mammary glands. Streptococcal organisms isolated between the first and last isolation were identified as *S. uberis* by growth in 6.5% NaCl, hydrolysis of esculin and CAMP reaction. When necessary, milk samples were diluted in PBS and four-10 µl drops of each dilution were plated on trypticase soy agar to determine the number of *S. uberis* per ml of milk.

Preparation of whey samples

Whole milk samples from challenged and control mammary glands of cows in the MTES herd were centrifuged at 48,000 x g for 40 minutes at 4°C. Whey was harvested between the supernatant (fat layer) and the infranatant (casein and cells). Milk samples from cows in the DES herd were centrifuged at 27,000 x g for 30 minutes at 4°C, the fat layer was removed and the sample pH was decreased to 4.5 by addition of glacial acetic acid. Samples were centrifuged at 27,000 x g for 30 minutes at 4°C to precipitate casein and the supernatant was removed by filtration through gauze sponges. The pH was restored to 6.5-7.5 immediately before cytokine analysis by addition of NaOH. All samples were

stored at -80°C until cytokine analysis by enzyme-linked immunosorbent assay (ELISA).

IL-1 β ELISA

Bovine IL-1 β was measured by ELISA using commercial antibodies against ovine IL-1 β (Serotec Inc., Raleigh, NC, USA), with the coating monoclonal antibody and the detecting antiserum diluted as suggested by the manufacturer. Flat bottom 96-well plates (Corning Inc., Corning, NY, USA) were coated with 100 μl of monoclonal antibody anti-ovine IL-1 β at a concentration of 5 $\mu\text{g/ml}$ diluted in PBS and incubated at 4°C overnight. Plates were washed three times with PBS-0.01% Tween (PBST), and 200 μl of 5% bovine serum albumin (BSA) in PBST were added to each well to block nonspecific protein binding sites. Plates were incubated for 1 hour at 37°C . After washing plates as before, 100 μl of whey were loaded into each well, together with serial 2-fold dilutions of purified rIL-1 β (10 ng/ml to 0.31 ng/ml) for use as a standard (generously provided by Susan Dunney, Fort Dodge Animal Health, Princeton, NJ, USA). After incubation for 1 hour at 37°C , plates were washed 3 times and 100 μl of rabbit anti-ovine IL-1 β diluted 1:500 in PBST-1%BSA were added to each well. Plates were incubated for 1 hour at 37°C , washed 3 times and 100 μl of goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:20000 in PBST-1%BSA were

added to each well. Plates were incubated for 1 hour at 37°C, washed 3 times and 100 µl of the enzyme substrate, 52 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO, USA) in 0.1M citrate buffer (pH 4.2) with 0.075% hydrogen peroxide were added to each well. Absorbance was read at 405 nm with an ELISA plate reader (Bio-Tek, Winooski, VT, USA) after about 30 minutes at room temperature. Wells that received normal pooled whey from uninfected mammary glands served as blanks. Bovine IL-1 β concentrations were calculated by referring to a standard curve. All samples were assayed in triplicate.

TNF- α ELISA

Concentrations of TNF- α in whey were quantified as described by Paape et al. (2002) with slight modifications. Wells were coated with 100 µl of monoclonal antibody anti-bovine TNF- α (2C4 1D3, generously provided by Dr. Max Paape, ARS, USDA, Beltsville, MD, USA) diluted 1:1000 in carbonate-bicarbonate buffer (pH 9.6). After incubation overnight at 4°C, plates were washed 3 times with PBST and 200 µl of PBS-1%BSA were added to each well to block nonspecific protein binding sites. Plates were incubated for 1 hour at 37°C in a humidified chamber. After washing as before, 100 µl of appropriate dilutions of samples to be tested were added to each well and incubated at 37°C for 2 hours. Plates were then washed 3 times and 100 µl of rabbit serum anti-TNF- α (generously provided by Dr. Ted Elsasser, ARS, USDA, Beltsville, MD,

USA) diluted 1:5000 in PBST-1%BSA were added to each well. Plates were incubated for 2 hours at 37°C in a humidified chamber. After washing the plates as before, 100 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch) diluted 1:20000 in PBST-1%BSA were added to each well and incubated for 2 hours at 37°C in a humidified chamber. Horseradish peroxidase activity was detected by addition of the enzyme substrate, 52 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid; Sigma) in 0.1M citrate buffer (pH 4.2) with 0.075% hydrogen peroxide to each well. Absorbance was read at 405 nm with an ELISA plate reader (Bio-Tek) after about 30 minutes at room temperature. Wells that received normal pooled whey from uninfected mammary glands served as blanks. Two-fold dilutions of cell culture supernatant from LPS stimulated monocytes with a known concentration of TNF- α were used to establish a standard curve. All samples were assayed in triplicate.

IL-8 ELISA

Concentrations of IL-8 in whey were measured by use of a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) essentially as described by the manufacturer. Fifty microliters of standard containing known amounts of recombinant human IL-8 (rhIL-8) or samples were added in duplicate to wells precoated with monoclonal antibody against rhIL-8. A polyclonal antibody anti-rhIL-8 conjugated with horseradish peroxidase was also added to wells. Samples

were incubated for 3 hours at room temperature, washed 6 times and 200 µl of the enzyme substrate tetramethylbenzidine were added to wells. Samples were incubated for 30 minutes at room temperature in the dark, and the enzymatic reaction was stopped by addition of 2N sulfuric acid. The optical density at 450/562 nm was determined with an ELISA plate reader (Bio-Tek). The IL-8 concentration in samples was determined by comparing absorbance obtained with that of the standard curve.

Blood samples

Blood was collected in Holstein cows only. Blood samples for leukocyte total counts and adhesion molecule expression were collected in vacutainer tubes containing 2X Acid Citrate Dextrose (ACD, pH 6.1) in a 20% vol/vol as anticoagulant by puncture of the tail vein. Samples were collected immediately prior to bacterial challenge and at the same time milk samples were taken. Blood was shipped via overnight courier and processed immediately upon arrival at the laboratory.

Leukocyte counts of whole blood

Total leukocyte counts on blood were determined manually with a hemocytometer after lysing erythrocytes with a Unopette microcollection system kit (Beckton Dickinson and Co., Franklin Lanes, NJ, USA).

Neutrophil isolation

Neutrophils were isolated as described previously (Aarestrup et al., 1994) with some modifications. Blood was centrifuged at 860 x g for 30 minutes at 15°C, and the plasma and buffy coat were discarded. Neutrophils were isolated from the remaining erythrocytes by adding an equal volume of double-distilled water for 30 seconds and then by adding a 3X concentration of RPMI 1640 medium (Sigma) to regain isotonicity of the solution. Remaining cells were washed twice with Hank's balanced salt solution (Sigma) and resuspended in FACS solution (0.15M PBS, pH 7.2; 2% heat inactivated fetal bovine serum; 0.03% sodium azide) to a final concentration of 10^7 cells/ml.

Neutrophil adhesion molecule expression

Indirect immunofluorescent analysis of β_2 integrin expression on neutrophils in blood samples was performed by measuring CD18 and CD11b expression using murine monoclonal antibodies and fluorescein-conjugated antibody to mouse IgG. Additionally, expression of selectin CD62 on neutrophils was evaluated. Fifty microliters of neutrophil suspension were added to 96-well round bottom microtitre plates with 50 μ l of FACS solution (negative control), monoclonal antibodies to bovine CD11b (BAQ147A, VMRD Inc., Pullman, WA, USA), bovine CD18 (BAQ30A, VMRD Inc.) or bovine L-selectin (DU1-29, VMRD Inc.). Plates were incubated at 4°C for 30 minutes, washed 3 times with FACS solution, and 100 μ l of goat anti-mouse IgG fluorescein conjugate (Calbiochem,

La Jolla, CA, USA) were added to each well. Samples were incubated at 4°C for 30 minutes, washed as before, and resuspended in 2% formaldehyde in 0.15M PBS. Samples were stored at 4°C in the dark until assayed by flow cytometry.

Flow cytometry analysis

Fluorescence was measured with a FACScan flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). Dot plots were gated for PMN. Leukocytes were assayed for size by forward scattering and for granularity by side scattering. Expression of cell adhesion molecules (i.e., mean fluorescence intensity (MFI)) was calculated after plotting fluorescence of histograms. Fluorescence associated with PMN incubated with FITC-goat anti-mouse IgG secondary antibody in the absence of anti-CD18 or anti-CD11b was considered the control for nonspecific fluorescence. Data were expressed as relative fluorescence intensity units after subtraction of nonspecific (control) fluorescence.

Statistical analysis

Analysis of variance was done with mixed models using SAS software (SAS 8.02, SAS Institute Inc, Cary, NC). A randomized block design with replication was used to determine effect of infection with *S. uberis* over time. When necessary, data were converted to log₁₀ to maintain a normal distribution

for statistical analysis. Data are presented as least square means with associated standard error. Statistical significance was declared at $P < 0.05$, and a trend toward significance was declared at $P < 0.10$.

RESULTS

Experimental challenge and infection with *Streptococcus uberis*

Five Holstein cows and 5 Jersey cows were challenged with *S. uberis* in 2 uninfected mammary glands. Seven cows became infected in both challenged mammary glands, 2 cows became infected in 1 of 2 challenged mammary glands, and 1 cow did not become infected in either challenged mammary gland. There were no significant differences between breeds in response to challenge with *S. uberis* for all parameters evaluated ($P > 0.05$). Therefore, data from all cows were combined for subsequent analysis.

Clinical signs

Following bacterial challenge with *S. uberis*, 16 of 20 challenged mammary glands developed clinical mastitis. Significant changes in mammary and milk scores were first observed at 84 hours after challenge and peaked at 144 hours after challenge ($P < 0.01$; Table 4). Infected mammary glands exhibited swelling and mammary secretions were obviously abnormal, with presence of clots and slugs. Local symptoms were accompanied by a mild systemic

Table 4. Clinical signs in cows challenged with *Streptococcus uberis*.

	Hours																		SE ⁴
	0	12	18	24	36	42	48	60	66	72	84	90	96	108	114	120	144	168	
Temperature (°C)																			
CI ¹ (n=9)	38.7	38.6	38.7	39.3	38.9	38.5	39.1	38.7	38.6	39.2	39.2	39.0	39.7	39.4	38.9	39.3	38.3	38.3	0.3
CU ² (n=1)	38.6	38.9	38.5	39.2	39.1	39.6	39.5	38.9	38.9	38.8	38.1	38.0	38.4	38.8	38.2	38.6	38.8	38.4	
Mammary score																			
CI (n=16)	1 ^a	1 ^a		1 ^a	1 ^a		1.19 ^a	1.19 ^a		1.5 ^a	1.69 ^b		2.44 ^b	2.63 ^b		3.0 ^b	3.13 ^b	2.88 ^b	0.18
CU (n=4)	1	1		1	1		1	1		1	1		1	1		1	1	1	
Control ³ (n=9)	1	1		1	1		1	1		1	1		1	1		1	1	1	
Milk score																			
CI (n=16)	1 ^a	1 ^a		1 ^a	1 ^a		1 ^a	1.13 ^a		1.56 ^a	2.06 ^b		2.18 ^b	2.63 ^b		2.5 ^b	3.63 ^b	3.13 ^b	0.21
CU (n=4)	1	1		1	1		1	1		1	1		1	1		1	1	1	
Control (n=9)	1	1		1	1		1	1		1	1		1	1		1	1	1	

¹Challenged infected mammary glands.

²Challenged uninfected mammary glands.

³Control unchallenged mammary glands.

⁴SE= standard error of the mean.

^{a,b,c}Values within the same row with different superscript differ (P<0.01).

response, evidenced by an increase in rectal temperature in all infected cows. Rectal temperatures did not peak at a similar time point in all cows. Mean maximum rectal temperature was $40.7 \pm 0.3^{\circ}\text{C}$. Unchallenged control mammary glands and 4 challenged mammary glands did not become infected and did not exhibit clinical signs. Based on severity of clinical signs, 3 mammary glands received antimicrobial therapy at 120 hours, 8 mammary glands received antimicrobial therapy at 144 hours and 4 mammary glands received antimicrobial therapy 168 hours after challenge.

Enumeration of bacteria and somatic cells

For the first 42 hours after intramammary inoculation, *S. uberis* was found in very low numbers in challenged infected mammary glands (Figure 1). Since Holstein cows received a slightly higher inoculum dose than Jersey cows, *S. uberis* was isolated at earlier time points in Holstein cows (Table 5). However, bacterial numbers in those isolations were very small (Appendix). Numbers of *S. uberis* in milk increased significantly ($P < 0.05$; Figure 1) by 48 hours after challenge. Between 48 hours and 120 hours after challenge, the time point at which maximal bacterial concentration was reached ($5.03 \log_{10} \pm 0.79$ CFU/ml; $P < 0.01$), numbers of *S. uberis* in mammary secretions increased 1,500-fold. This occurred in spite of massive leukocyte infiltration from the bloodstream into challenged mammary glands (Figure 2). Significant increases in bacterial numbers coincided with appearance of clinical signs (Table 4). After reaching a

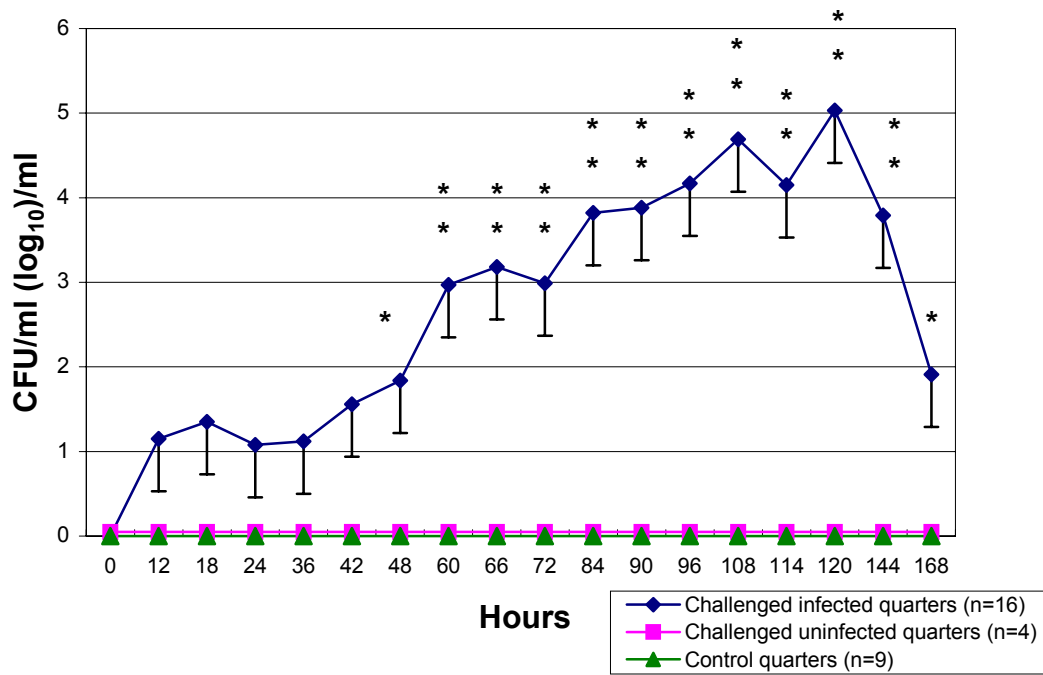


Figure 1. Enumeration of *Streptococcus uberis* in milk (CFU/ml (log₁₀)) from mammary glands of cows challenged with *S. uberis*. Data are means $\pm$ SEM. Asterisks indicate significant differences from prechallenge values (*P<0.05;**P<0.01).

Table 5. Number of infected mammary glands shedding *Streptococcus uberis* at different time points after challenge.

Hours after challenge	Number of challenged infected mammary glands shedding <i>S. uberis</i>	
	Holstein cows ¹ (n=8)	Jersey cows ² (n=8)
0	0	0
12	7	0
18	7	0
24	6	0
36	3	4
42	4	5
48	3	6
60	6	7
66	6	8
72	6	7
84	6	8
90	7	8
96	8	8
108	8	8
114	8	8
120	8	8
144	7	5
168	3	6

¹ Holstein cows received an inoculum dose of 10,500 CFU.

² Jersey cows received an inoculum dose of 6,650 CFU.

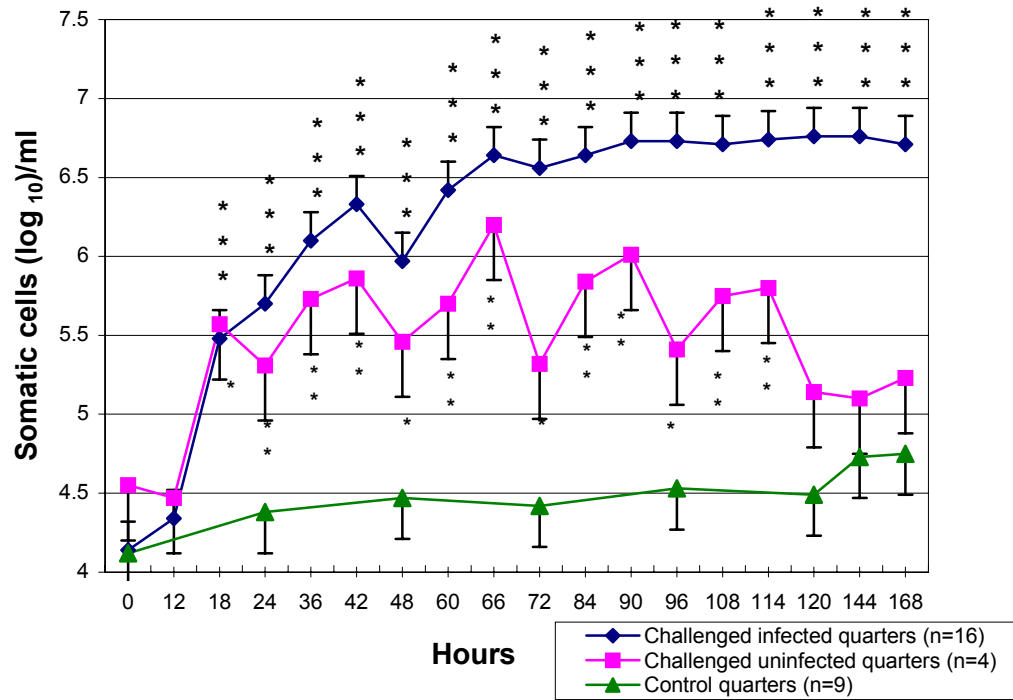


Figure 2. Number of somatic cells (log₁₀/ml) in milk from mammary glands of cows challenged with *Streptococcus uberis*. Data are means $\pm$ SEM. Asterisks indicate significant differences from prechallenge values (*P<0.05;**P<0.01; ***P<0.001).

peak concentration at 120 hours after challenge, bacterial numbers began to decline; this coincided with initiation of antibiotic therapy in 15 of the 16 infected mammary glands between 120 and 168 hours after challenge. *Streptococcus uberis* was not isolated from 4 challenged mammary glands that did not become infected, or from unchallenged control mammary glands.

Leukocyte recruitment to infected mammary glands, as indicated by an increase in the number of somatic cells in milk, began at 18 hours after challenge ($P < 0.001$; Figure 2) and continued throughout the sampling period. Before challenge, the number of somatic cells averaged $4.27 \log_{10} / \text{ml}$; 42 hours after challenge, even when no clinical signs were observed and no bacterial growth had been detected, number of somatic cells in milk increased 100-fold ($6.3 \pm 0.18 \log_{10} / \text{ml}$) and continued to increase to a maximum number of $6.76 \pm 0.18 \log_{10} / \text{ml}$ at 120 hours. Challenged mammary glands which did not become infected ($n=4$) exhibited a significant increase in number of somatic cells ($P < 0.01$; Figure 2). However, the magnitude of the increase was not as great as challenged mammary glands that became infected. No significant changes in the number of somatic cells in milk from unchallenged control mammary glands were detected.

Leukocyte counts in peripheral blood

Blood leukocyte counts varied greatly from cow to cow; however, there was a significant decrease in total blood leukocyte counts at 72 hours after challenge

($P=0.05$; Figure 3). After that, blood leukocyte counts remained suppressed and did not return to baseline levels until 168 hours after challenge.

Tumor necrosis factor- α

During *S. uberis* experimental infection, TNF- α concentrations in whey from infected mammary glands increased significantly by 66 hours following bacterial challenge ($P<0.01$; Figure 4), and remained significantly elevated throughout the remainder of the sampling period. Peak TNF- α concentrations were observed at 120 hours after challenge (461 ± 76 pg/ml). One of four challenged, uninfected mammary glands exhibited a slight increase in TNF- α concentrations; the remainder of the challenged, uninfected mammary glands had TNF- α concentrations below the detection limits of the assay. Only trace amounts of TNF- α were detected in whey from prechallenge samples and in whey from unchallenged control mammary glands.

Interleukin-1 β

Concentrations of IL-1 β in whey from mammary glands experimentally infected with *S. uberis* increased significantly by 66 hours after bacterial challenge ($P<0.01$; Figure 5). Concentrations of IL-1 β fluctuated considerably from 66 to 144 hours after challenge, however IL-1 β concentrations during these times were still significantly ($P<0.01$) higher than prechallenge concentrations. Highest IL-1 β

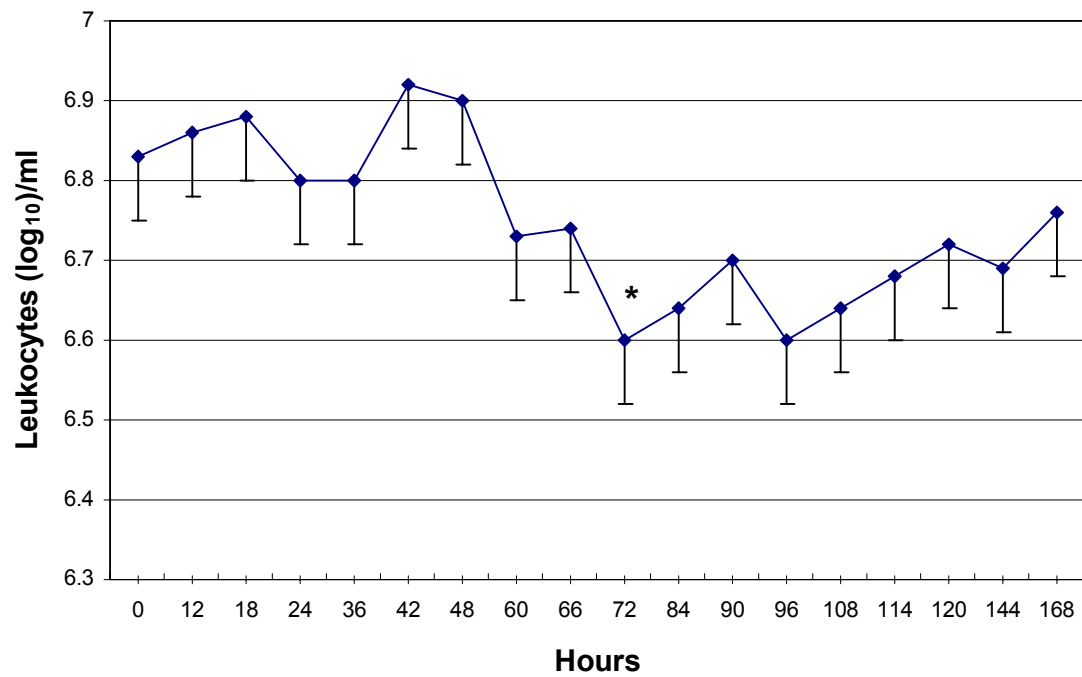


Figure 3. Leukocytes in blood (cells/ml (log₁₀)) from cows experimentally infected with *Streptococcus uberis* (n=9). Data are means ± SEM. Asterisks indicate significant differences from prechallenge values (*P=0.05).

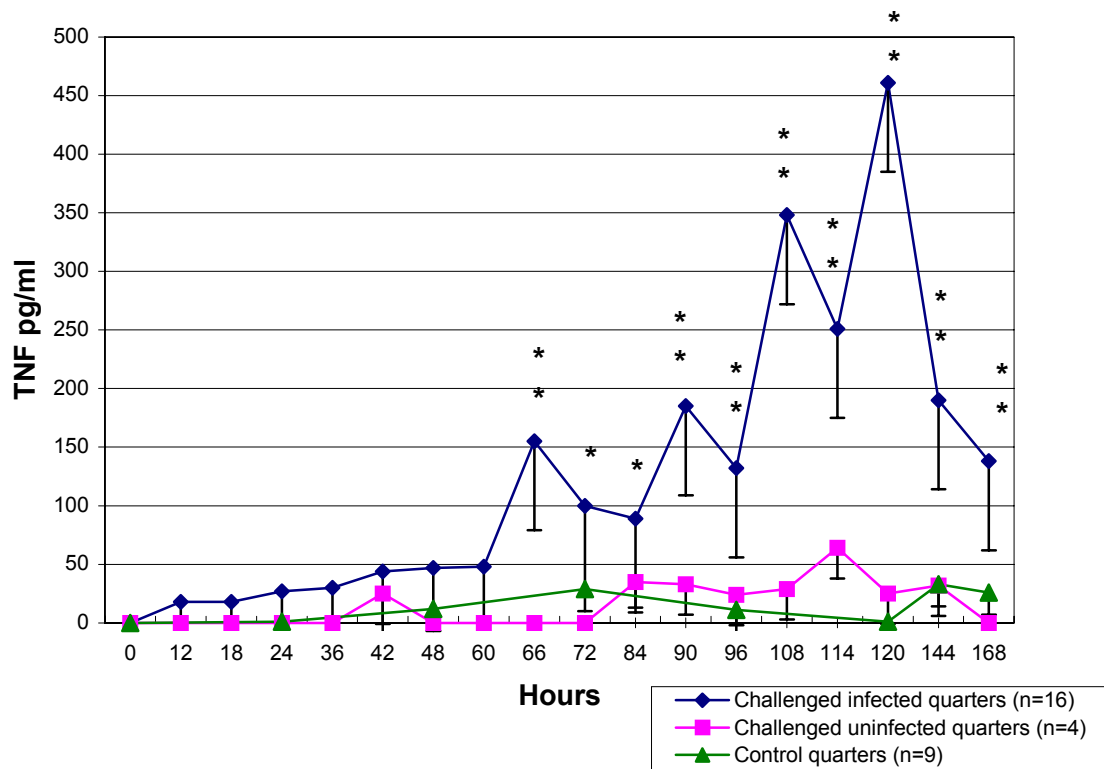


Figure 4. Concentrations of tumor necrosis factor- α (TNF) in whey from mammary glands challenged with *Streptococcus uberis*. Data are means $\pm$ SEM. Asterisks indicate significant differences from prechallenge values (* $P < 0.05$; ** $P < 0.01$).

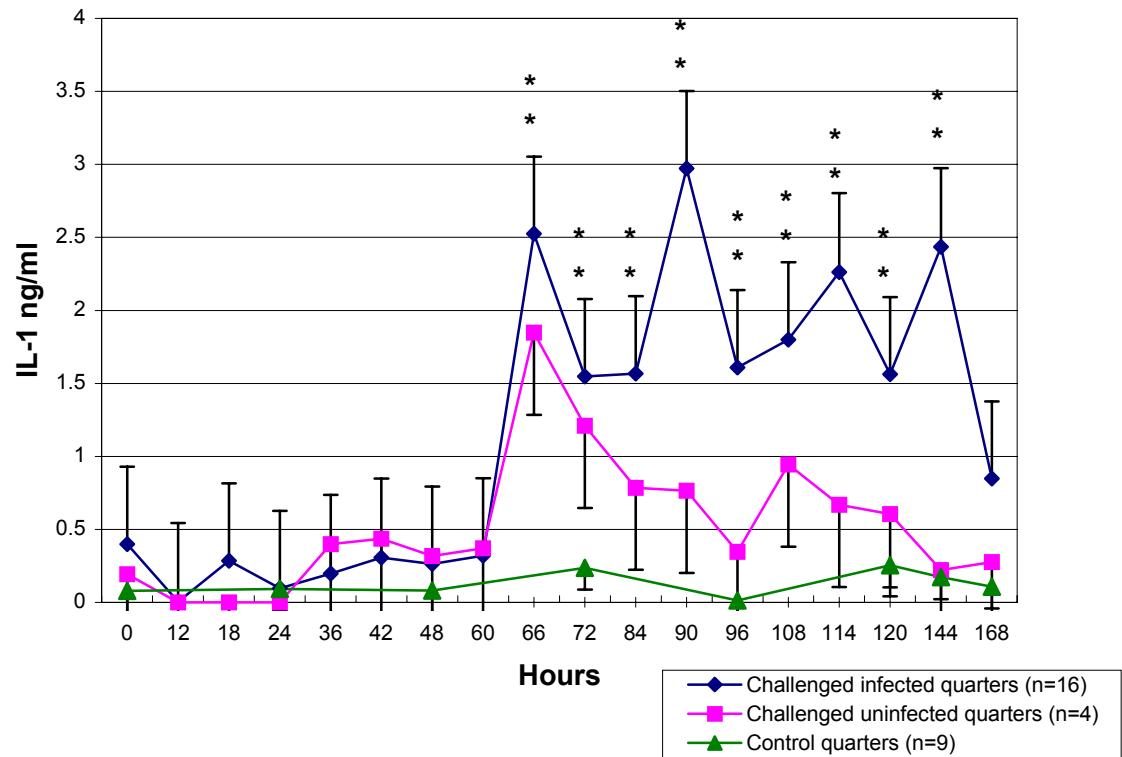


Figure 5. Concentrations of Interleukin-1 β (IL-1) in whey from mammary glands challenged with *Streptococcus uberis*. Data are means $\pm$ SEM. Asterisks indicate significant differences from prechallenge values (*P<0.05; **P<0.01).

concentrations were observed at 90 hours after bacterial challenge (2.97 ± 0.53 ng/ml). The 4 challenged, uninfected mammary glands exhibited an increase in IL-1 β concentrations, and the pattern appeared to be similar to that of challenged infected mammary glands. However, the increase in IL-1 β concentrations in challenged uninfected mammary glands was not significant ($P > 0.05$). Very low concentrations of IL-1 β were detected in control unchallenged mammary glands.

Interleukin-8

The concentration of IL-8 in whey was measured at all time points in Holstein cows and daily in Jersey cows. The concentration of IL-8 was significantly elevated in mammary secretions from challenged infected mammary glands at 66 hours after bacterial challenge ($P < 0.01$; Figure 6). Concentrations of IL-8 fluctuated considerably throughout the remainder of the experimental period. Peak IL-8 concentrations (34.6 ± 6.8 pg/ml; $P < 0.001$) were observed at 120 hours after challenge. No IL-8 was detected in challenged, uninfected mammary glands or in unchallenged control mammary glands.

Adhesion molecule expression on blood neutrophils

Adhesion receptor expression of CD11b, CD18 and CD62 was measured in Holstein cows only and is shown in Table 6. Expression of CD11b, the α -chain from the Mac-1 integrin, tended to show a slight upregulation at 84, 90 and 114

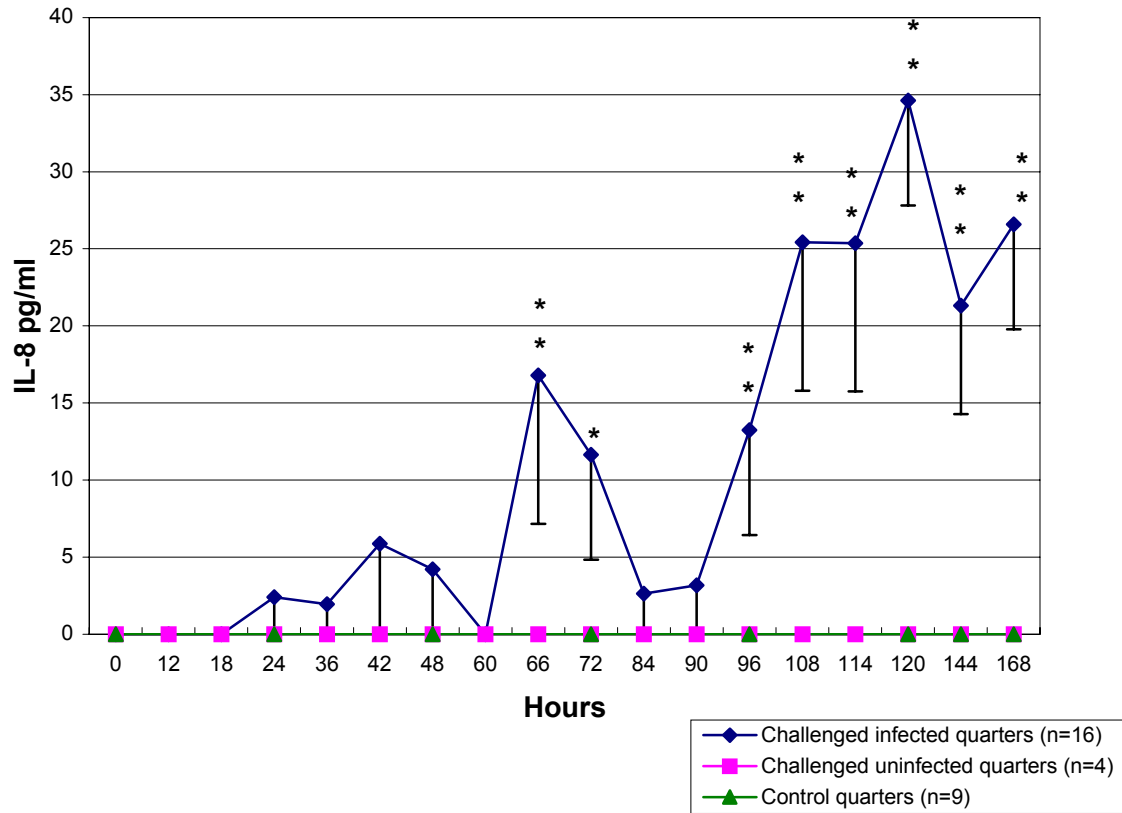


Figure 6. Concentrations of interleukin-8 (IL-8) in whey from mammary glands challenged with *Streptococcus uberis*. Data are means $\pm$ SEM. Asterisks indicate significant differences from prechallenge values (* $P < 0.05$; ** $P < 0.01$).

Table 6. Mean fluorescence intensity (MFI) of CD11b, CD18 and CD62 adhesion molecules on blood polymorphonuclear leukocytes following experimental challenge with *Streptococcus uberis*.

Hours	MFI ¹		
	CD11b ²	CD18	CD62
0	4.6 ^a	70.8 ^a	5.5 ^a
12	6.7 ^a	61.3 ^a	5.8 ^a
18	4.9 ^a	73.4 ^a	4.3 ^a
24	7.1 ^a	66.4 ^a	6.3 ^a
36	6.7 ^a	70.1 ^a	4.7 ^a
42	6.3 ^a	66.8 ^a	3.7 ^a
48	5.7 ^a	84.8 ^b	4.9 ^a
60	4.8 ^a	73.3 ^a	4.9 ^a
66	6.0 ^a	78.0 ^a	8.1 ^a
72	7.1 ^a	66.3 ^a	6.9 ^a
84	7.6 ^b	74.2 ^a	5.3 ^a
90	8.6 ^b	76.3 ^a	6.9 ^a
96	6.4 ^a	48.9 ^c	6.7 ^a
108	5.5 ^a	44.2 ^c	15.2 ^b
114	10.0 ^b	42.9 ^c	8.7 ^a
120	5.4 ^a	42.6 ^c	4.2 ^a
144	4.8 ^a	36.8 ^c	4.5 ^a
168	4.8 ^a	31.4 ^c	5.1 ^a
SE ³	1.3	5	1.7 ^a

^{a,b,c} Values within the same column with different superscript differ (P<0.05).

¹Mean fluorescence intensity (MFI) was measured by flow cytometry.

²MFI for CD11b showed a trend toward significance (P=0.1). ^{a,b,c} Values within this column with different subscript differ (P<0.05) after analysis through specific contrasts.

³SE=standard error of the mean.

hours after challenge ($P=0.1$). When these data were evaluated through specific contrasts, CD11b expression was significantly higher ($P<0.05$) at 84, 90 and 114 hours after challenge. The mean fluorescence intensity for CD18, the β -chain of the Mac-1 integrin, increased 20% by 48 hours after experimental challenge ($P<0.05$). This was followed by a sharp decrease in CD18 MFI 96 hours after challenge which continued until the end of the sampling period at 168 hours after challenge ($P<0.01$). Expression of CD62 fluctuated somewhat after bacterial challenge, and at 108 hours after challenge a significant increase in CD62 expression was observed ($P<0.05$).

Correlation coefficients

A moderate positive correlation was observed between concentrations of TNF- α , IL-1 β and IL-8 in whey, bacterial and somatic cell numbers and the presence of clinical signs of mastitis and from challenged infected mammary glands (Table 7).

DISCUSSION

Streptococcus uberis is an environmental mastitis pathogen that accounts for a significant proportion of IMI in lactating and nonlactating dairy cows. The present study was conducted with cows in early lactation, a time when mammary

Table 7. Correlation coefficients between different clinical and bacteriological parameters of infection during experimental *Streptococcus uberis* mastitis.

	Correlation coefficients						
	IL-1 β ¹	IL-8 ²	Mammary score	Milk score	Temperature	Micro ³	SCC ⁴
TNFα⁵	0.62 ^b	0.63 ^b	0.53 ^b	0.41 ^b	0.35 ^b	0.48 ^b	0.40 ^b
IL-1β		0.56 ^b	0.52 ^b	0.50 ^b	0.23 ^b	0.57 ^b	0.50 ^b
IL-8			0.47 ^b	0.42 ^b	0.24 ^b	0.45 ^b	0.38 ^b
Mammary score				0.82 ^b	0.11 ^a	0.43 ^b	0.48 ^b
Milk score					-0.14 ^a	0.42 ^b	0.46 ^b
Temperature						0.27 ^b	0.18 ^b
Micro							0.53 ^b

¹IL-1 β = interleukin-1 β

²IL-8= interleukin 8

³Micro= enumeration of *Streptococcus uberis* in milk

⁴SCC= number of somatic cells in milk

⁵TNF α = Tumor necrosis factor- α

^a P>0.05

^b P<0.05

glands are highly susceptible to new *S. uberis* IMI (Smith et al., 1985; Todhunter et al., 1995; Jayarao et al., 1999). The bacterial strain used to cause experimental *S. uberis* infection (UT888) induced clinical mastitis in the majority of challenged mammary glands, as reported previously (Fang et al., 1998a; Hockett et al., 2000).

The objective of this study was to study the dynamics of leukocytes and inflammatory cytokines during experimentally-induced *S. uberis* mastitis in order to gain a better understanding of the pathogenesis of this disease in lactating dairy cows. The process of leukocyte recruitment and cytokine production during mastitis has been studied extensively using gram-negative infection models, such as mammary gland bacterial challenge with *E. coli* and *Klebsiella pneumoniae* (Rose et al., 1989; Shuster et al., 1996; Shuster et al., 1997; Blum et al., 2000; Riollet et al., 2000), or endotoxin infusion (Shuster et al., 1993; Rainard and Paape, 1997; Paape et al., 2002). To our knowledge, this is the first report of cytokine production during IMI with *S. uberis*. The present study was conducted to determine the characteristics of the host response to IMI with *S. uberis*, a gram-positive environmental mastitis pathogen, which differs greatly from *E. coli* and other gram-negative pathogens in virulence, growth characteristics and ability to persist in the udder environment.

Data from studies using the *E. coli* mastitis model, to which most comparisons have to be made due to the extensive information published on it, differs considerably from results of the present study. Probably, one of the more

important differences is the clinical course of infection. In the present study, clinical signs of mastitis were not detected until 72 to 108 hours following challenge. With the *E. coli* experimental infection model, clinical signs were evident within the first 8-16 hours after challenge, and signs are usually resolved without any therapeutic intervention within the first week after challenge (Hill et al., 1978; Shuster et al., 1997).

Bacterial challenge of mammary glands with *S. uberis* UT888 provoked clinical mastitis in 16 of 20 challenged mammary glands. Appearance of clinical signs coincided with detection of large numbers of bacteria (>5,000 CFU/ml) in challenged mammary glands. Large numbers of bacteria (>5,000 CFU/ml) in milk were not detected until 84 hours after challenge, suggesting a lag phase in the ability of *S. uberis* to adapt and grow in the mammary gland. One possible explanation for this lag phase is that *S. uberis* may require a period of adaptation to the mammary environment, after which it is able to colonize the mammary gland. *Streptococcus uberis* is highly auxotrophic and depends on acquisition of several amino acids and other nutrients to grow successfully (Leigh, 1994b). Milk from lactating mammary glands is deficient in free or peptide associated amino acids. In order to infect the mammary gland *S. uberis* must possess a mechanism to acquire amino acids for growth. Leigh (1993) demonstrated that *S. uberis* was capable of converting plasminogen to plasmin which in turn degraded casein and generated by-products needed for optimal bacterial growth. Furthermore, a plasminogen activator, designated PauA, was isolated from *S.*

uberis (Leigh, 1994b). Other studies of experimental *S. uberis* IMI with a different strain (*S. uberis* O140J; Hill, 1988; Smits et al., 1998) suggest a similar lag phase although of shorter duration, probably due to a higher pathogenicity of the strain used. Nevertheless, our findings and data from other studies (Hill, 1988; Smits et al., 1998) suggest that upon intramammary inoculation, *S. uberis* requires some time to adjust to the mammary gland environment. Bacteria may then produce plasminogen activator which would activate plasmin and therefore allow better nutrient availability for optimal growth. This “adaptation period” apparently needed by *S. uberis* is in marked contrast with the remarkable ability of *E. coli* to grow in the mammary gland environment even 2 hours after inoculation into the mammary gland (Shuster et al., 1995; Shuster et al., 1996; Riollet et al., 2000).

In contrast to the delay in increases of bacterial numbers, the number of somatic cells in milk increased significantly as early as 18 hours after challenge with *S. uberis*, and were 100-fold greater than prechallenge values at 48 hours after challenge, when *S. uberis* was still detected in very low numbers (<100 CFU/ml). This would suggest inability to control growth of *S. uberis* in the mammary gland in spite of massive leukocyte infiltration from the bloodstream. Interestingly, this contrasts with findings using the *E. coli* experimental infection model, where bacterial numbers increase tremendously during the first hours of infection without apparent recognition by the host and therefore minimal leukocyte recruitment into the mammary gland (Shuster et al., 1995; Shuster et al., 1996; Riollet et al., 2000).

Based on our findings, it seems that host recognition of *S. uberis* during the initial stages of infection is not impaired, as demonstrated by the significant increase in the number of somatic cells in milk from challenged mammary glands within the first 24 hours after challenge. This is in agreement with other studies (Thomas et al., 1994; Smits et al., 1998). Phagocytosis by PMN is a crucial process in the elimination of *E. coli* (Hill et al., 1978) and *S. aureus* (Schalm et al., 1976) from the bovine mammary gland. The inability of PMN to phagocytose and kill certain strains of *S. uberis* has been reported in vitro (Leigh and Field, 1991; Leigh and Field, 1994) and in vivo (Thomas et al., 1994). Resistance of *S. uberis* to the bactericidal action of PMN in vitro was induced by growth of bacteria in media containing casein-derived amino acids and peptides (Leigh and Field, 1991) which may mimic the state within the mammary gland during infection. In a study by Thomas et al. (1994) where pathologic findings of *S. uberis* infection were evaluated in vivo, a large neutrophil response was observed, and considered rather disproportionate to the number of bacteria present in milk. In the same study, very few bacteria were detected inside PMN, raising questions about the role of PMN in elimination of *S. uberis* from the infected mammary gland (Thomas et al., 1994). Results from our experiment also suggest that massive recruitment of PMN was not sufficient to eliminate *S. uberis* from infected mammary glands.

A significant decrease in blood leukocyte counts was observed 72 hours after challenge with *S. uberis*. This decrease was not coincident with an increase

in the number of somatic cells, which occurred earlier, but it resembles findings by Smits et al. (1998) where a significant decrease in blood leukocyte counts was observed 48 and 72 hours after *S. uberis* challenge. However, the study by Smits et al. (1998) was conducted with a more virulent strain of *S. uberis* (strain O140J), which generally causes clinical disease between 24 and 60 hours after challenge (Hill, 1988; Thomas et al., 1994; Smits et al., 1998), earlier than observed in our experiment with *S. uberis* UT888. During experimental *E. coli* or endotoxin induced mastitis, where the inflammatory episode is more marked and sudden, the decrease in circulating blood leukocytes was simultaneous to the increase in the number of somatic cells in the udder (van Werven et al., 1999; Paape et al., 2002).

The importance of cytokines in neutrophil recruitment is well established. However, cytokine production during *S. uberis* mastitis has not been documented. In the present study, cytokine production was assessed in *S. uberis* challenged mammary glands as well as control unchallenged mammary glands. Interestingly, TNF- α , IL-1 β and IL-8 were not detected in significant amounts in milk from infected mammary glands until 66 hours after challenge. All three cytokines measured were significantly elevated by 66 hours, when clinical signs still were not observed. However, at 66 hours after challenge, numbers of somatic cells had almost reached the maximum amount observed in this study (6.64 log₁₀ SCC/ml). It has been demonstrated that, after migration into an inflammatory site, leukocytes begin to synthesize and release their own particular

set of cytokines within target tissue (Lloyd and Oppenheim, 1992). The massive influx of neutrophils into the mammary gland observed in our study may have been responsible for the simultaneous increase in cytokine concentrations at this time, since neutrophils, especially in large quantities, can be an important source of IL-1, TNF- α and IL-8 (Lloyd and Oppenheim, 1992; Xing et al., 1993).

Peak TNF- α production was observed preceding peak clinical signs of mastitis. The TNF- α concentrations tended to increase gradually towards a peak at 120 hours. Great variability was observed between cows, but all challenged infected mammary glands showed detectable amounts of TNF- α . This contrasts with findings reported by Shuster et. al. (1997), where TNF- α bioactivity after challenge with *E. coli* was detected only in mammary glands with severe infection. Similarly, in a previous study by Shuster et al. (1993) where mammary glands were challenged with 10 μ g of endotoxin, TNF- α activity was not detected in any of the challenged mammary glands. In our study, however, even mammary glands with moderate infections showed a significant increase in TNF- α concentration.

Low concentrations of TNF- α were detected as early as 12 hours after challenge in some mammary glands. The relative importance of the very low amounts of TNF- α found in some mammary glands in the early stages of infection is questionable, given the fact that concentrations were close to the lower detection limit of the assay (80 pg/ml) and that low levels of TNF- α were also found in some control unchallenged mammary glands. Nevertheless, TNF- α

biological activity has been observed at 10 pg/ml (Riollet et al., 2000), therefore activity of TNF- α in those samples or even in samples where no TNF- α was detected cannot be excluded. The TNF- α concentrations observed in our study were 30-fold smaller than those reported in *E. coli* or endotoxin experimental infections (Rainard and Paape, 1997; Riollet et al., 2000; Paape et al., 2002). Of note, comparisons between our study and others (Rainard and Paape, 1997; Riollet et al., 2000; Paape et al., 2002) may be of relative value due to differences in sample processing and time of analysis. Nevertheless, the findings that TNF- α concentrations were much lower than reported for *E. coli* experimental infections is not surprising, since other studies using different infection models with other infectious agents have shown little or no detection of TNF- α in spite of the presence of acute inflammatory signs (Baarsch et al., 1995; Tissi et al., 1999).

Concentrations of IL-1 β were also significantly elevated 66 hours after challenge with *S. uberis*. The pattern of increase of IL-1 β was not gradual; this may be explained by differences in peak concentrations for individual mammary glands, which did not occur at the same time. Nevertheless, IL-1 β remained significantly elevated until 120 hours. Interestingly, the 4 challenged mammary glands that did not become infected exhibited an increase in IL-1 β concentrations in a pattern that was similar to that of infected mammary glands. This observation was surprising, considering that IL-1 β is one of the acute phase cytokines responsible for clinical signs of inflammation (Baumann and Gauldie, 1994) , and

the 4 uninfected mammary glands did not show any clinical signs of inflammation. Interleukin-1 β is one of the main cytokines found during experimental *E. coli* mastitis, with peak concentrations coinciding with the establishment of clinical signs (Shuster et al., 1997; Riollot et al., 2000). Since IL-1 β concentrations in control unchallenged mammary glands remained significantly lower than challenged mammary glands, findings of increased IL-1 β concentrations and numbers of somatic cells in the 4 challenged uninfected mammary glands indicate that a mild inflammatory response did develop, but this response might have been enough to control the infection without any detectable clinical signs.

Similar to TNF- α , small amounts of IL-1 β were detected in some prechallenge and control unchallenged samples. Other investigators have reported low concentrations of TNF- α and IL-1 β in secretions of noninflamed mammary glands or unstimulated mammary epithelial cultures (Sordillo et al., 1990; Basolo et al., 1993; Shuster et al., 1997). The physiological significance of basal levels of these mediators is unknown. Shuster et. al. (1997) suggested that these mediators may contribute to the small influx of leukocytes that occurs even in uninfected mammary glands.

Interleukin-8 was detected only in challenged mammary glands that became infected. The IL-8 increase was observed simultaneously to increases in TNF- α and IL-1 β concentrations. Others have shown that TNF- α and IL-1 β stimulate IL-8 secretion (Larsen et al., 1989; Boudjellab et al., 2000). Riollot et.

al. (2000) reported that high levels of IL-8 were found not only in mammary secretions but also in mammary cells from an *E. coli* infected cow, suggesting that the real quantities of IL-8 present in mammary secretions from infected glands may be underestimated. Since IL-8 has the ability to link itself to cell membrane receptors or to be rapidly internalized by cells (Marie et al., 1997), it is possible that larger amounts of IL-8 may have been produced, but not detected in the mammary secretion analysis.

Cytokine involvement in leukocyte migration to an inflammatory site is well documented (Cybulsky et al., 1988; Baggiolini et al., 1989; Persson et al., 1993; Shuster et al., 1997). Cytokines mediate leukocyte recruitment to inflammatory sites by their chemotactic ability and by activation of adhesion molecules on circulating leukocytes and endothelial cells in the adjacent vasculature (Shuster et al., 1993; Shuster et al., 1997). Shuster et. al. (1997) reported that production of inflammatory mediators during experimental *E. coli* mastitis was coincident with a dramatic increase in the number of somatic cells, suggesting that upon bacterial recognition by the host, inflammatory mediators were produced and leukocytes recruited into the infected mammary gland. In our study, however, leukocyte recruitment into challenged mammary glands occurred earlier than significant local production of TNF- α , IL-1 β or IL-8. These three cytokines, on the other hand, are not the only soluble factors involved in the inflammatory response. Many other mediators such as histamine, serotonin, complement fragments, prostaglandins and leukotrienes may be responsible for increased

vascular permeability and leukocyte migration during the early phase of mastitis. Giri et. al. (1984) reported that prostaglandin concentrations in endotoxin-infused mammary glands preceded increased leukocyte counts. Additionally, Rose et. al. (1989) suggested that leukotriene B₄ (LTB₄) may be the principal neutrophil chemotactic factor in mastitis, although increased numbers of somatic cells preceded increases in milk LTB₄. Furthermore, Shuster et. al. (1993) reported that swelling and increases in mammary gland permeability after endotoxin infusion preceded detection of TNF- α , IL-1 β or IL-6, suggesting that these clinical changes were mediated by other inflammatory mediators. No information exists about the role of other inflammatory mediators in *S. uberis* mastitis.

Rose et al. (1989) suggested that higher concentrations of inflammatory mediators in the tissue of a mastitic mammary gland may precede any increases observed in milk. The exact site of inflammatory mediator production in the infected mammary gland is unknown. Macrophages produce many inflammatory mediators upon bacterial stimulation, but epithelial cells can also generate a variety of inflammatory mediators upon interaction with bacteria (Agace et al., 1993; Eckmann et al., 1993). A study showed that release of inflammatory cytokines by epithelial cells was directed toward the basolateral surface, the direction from which host defenses would be recruited (Eckmann et al., 1993). Specifically, mammary epithelial cells have been shown to produce IL-1 β and IL-8 in vitro (Boudjellab et al., 2000). *Streptococcus uberis* has been shown to adhere to and invade mammary epithelial cells in vitro (Matthews et al., 1994a;

Almeida et al., 1996). Therefore, as suggested by Shuster et. al. (1997), it is possible that cytokines were produced initially by epithelial cells upon contact with bacteria and that their concentrations may have been greater on the basolateral surface. As a consequence of this, their biological effects may have been induced in the interstitium. In this case, cytokine concentration in milk may have not been an accurate reflection of cytokine production and potential biological activity, and might explain the apparent delay observed in this study between cytokine detection in mammary secretions and leukocyte migration into challenged mammary glands.

The role of adhesion molecules CD11b/CD18 and CD62 was also evaluated during *S. uberis* experimental infection. For CD11b, expression was slightly upregulated at 84, 90 and 114 hours after challenge. The increase in MFI was rather small, but differences of similar proportion have been reported during experimental *S. uberis* mastitis with strain O140J (Smits et al., 1998). The MFI for CD18 was upregulated 20% at 48 hours after challenge, similar to that reported by Smits et. al. (1998) during experimental *S. uberis* mastitis. In our study, however, increased expression of CD11b and CD18 was not coincident with an increase in number of somatic cells, which occurred earlier. Interestingly, Smits et. al. (1998) also reported that diapedesis of PMN from cows challenged with *S. uberis* was dramatically diminished, in spite of upregulation of CD11b and CD18. It is possible that other adhesion receptors may be involved in PMN migration into the mammary gland. The MFI for CD18 decreased dramatically 96

hours after challenge, and remained below prechallenge values. Since this is the period of time when cows were showing clinical signs, it is possible that endogenous release of cortisol upon onset of clinical signs caused downregulation of CD18 expression, as suggested by others (Burton and Kehrli, 1995; Smits et al., 1998). Using the same mastitis model described in the present study, Hockett et al. (2000; 2002a; 2002b) reported a significant increase in serum cortisol levels 96 hours after challenge with *S. uberis* UT888 and serum cortisol remained significantly elevated at 168 hours after challenge (Hockett et al., 2000; Hockett et al., 2002a; Hockett et al., 2002b). This time period between 96 and 168 hours after bacterial challenge is coincident with the marked downregulation of CD18 observed in our experiment. Therefore, it is possible that increased serum cortisol may have caused downregulation of CD18. No significant variation was detected in CD62 expression, except for an unexplainable increase in MFI at 108 hours after challenge.

In conclusion, this study provides insight into the inflammatory response during experimental *S. uberis* mastitis. Leukocyte recruitment into mammary glands was prompt and significant, though appeared to be ineffective in controlling the onset of infection. Intramammary infection with *S. uberis* elicited local production of cytokines, and peak concentrations of TNF- α , IL-1 β and IL-8 were reached once clinical signs had been established. Expression of adhesion molecule CD11b/CD18 was upregulated, but a significant increase in the number of somatic cells was observed before upregulation of CD11b/CD18. These

findings suggest that other factors may be involved in initial leukocyte recruitment into mammary glands after *S. uberis* infection, and that TNF- α , IL-1 β and IL-8 may be responsible, at least in part, for the observed clinical signs. Future studies focused on the early events that take place after bacterial challenge may help to elucidate the pathogenesis of *S. uberis* mastitis and the development of more effective control measures for *S. uberis* mastitis in dairy cows.

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APPENDIXES

Appendix 1. Enumeration of *Streptococcus uberis* (colony forming units/ml) in milk from mammary glands experimentally infected.

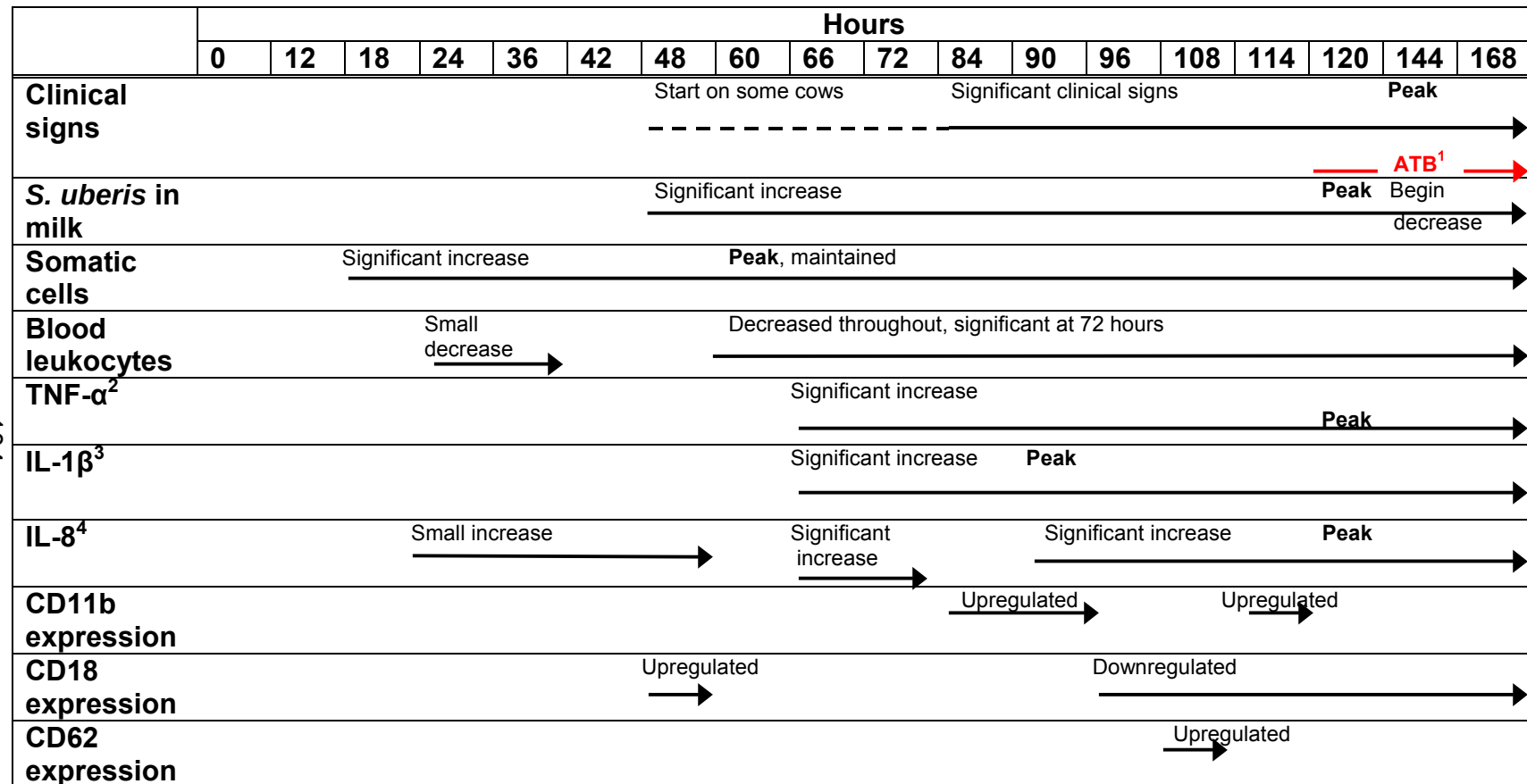
Hours after challenge	Holstein challenged infected mammary glands ¹								Jersey challenged infected mammary glands ²							
	61 LR ³	61 LF ⁴	87 RF ⁵	87 RR ⁶	91 RF	91 RR	116 LR	116 LF	562LF	769RF	800RF	800LR	826RR	826LR	840RF	840LF
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1100	300	1300	500	200	200	300	0	0	0	0	0	0	0	0	0
18	2800	2500	0	1300	500	2700	3000	100	0	0	0	0	0	0	0	0
24	2600	500	0	800	0	100	700	2300	0	0	0	0	0	0	0	0
36	400	200	0	100	0	0	0	0	0	0	0	400	1900	300	500	0
42	400	100	0	200	0	0	4800	0	15	51000	0	100	0	0	1600	1600
48	700	0	2400	0	0	0	100	0	4250	27250	0	4750	700	0	2300	1750
60	3900	5300	10000	2000	0	0	4100	2300	110000	55750	100	1200	0	1200	2750	30000
66	1300	1300	6500	50000	0	0	17300	4400	270000	407500	100	2300	800	100	900	10000
72	1000	1000	8000	7750	0	0	12800	6500	4750	975000	200	2600	900	0	5000	10250
84	67500	37500	10000	10000	0	0	13000	46500	2275000	82500	200	10750	8900	1500	700000	25000
90	40000	14500	19300	5000	7500	0	6750	4000	262500	1200000	600	9250	82500	3500	3250	6500
96	14800	13800	7500	5000	2100	200	368000	24500	270000	2975000	3600	12500	12500	5500	24500	3250
108	82500	3750	220000	16000	1700	2000	900000	1000000	690000	390000	500	625000	26500	500	975000	317500
114	17300	21000	14000	208000	2900	2900	45300	5500	1300000	15500	2500	44250	5250	2500	21300	1800
120	10500	6500	1400000	4000	5000	200	26800	363000	3025000	180000	40500	187500	1925000	1650000	290000	21750000
144	210000	16800	0	165000	230000	800	87500	2850000	54500	0	20250	1825000	260000	300000	0	0
168	0	0		0	8000	0	62500	5000	0	100	10000	2425000	100	100	0	0

¹ Holstein cows were challenged with 10,500 CFU/ml of *S. uberis* UT888.

² Jersey cows were challenged with 6,650 CFU/ml of *S. uberis* UT888.

³ LR= Left rear mammary gland; ⁴ LF= Left front mammary gland; ⁵ RF= Right front mammary gland; ⁶ RR= Right rear mammary gland.

Appendix 2. Timeline of events during experimentally-induced *Streptococcus uberis* mastitis.



¹ ATB=antibiotic therapy. Three mammary glands received antibiotic therapy at 120 hours, 8 mammary glands received antibiotic therapy at 144 hours and 4 mammary glands received antibiotic therapy at 168 hours after intramammary inoculation with *S. uberis*.

² TNF α = tumor necrosis factor-alpha

³ IL-1 β = interleukin-1 beta

⁴ IL-8= interleukin-8

VITA

Magdalena Rambeaud was born March 14, 1975 in Bariloche, Argentina. She attended primary and secondary school in City Bell, Argentina. Magdalena graduated from Jose Manuel Estrada High School in 1992. From there, she went to The National University of La Plata where she received a Doctor in Veterinary Medicine degree in 1998. In the fall of 2002, she graduated with a Master of Science degree in Animal Science from The University of Tennessee.