5-1997

Interaction of Streptococcus dysgalactiae with bovine mammary epithelial cells

Luis Fernando Calvinho

To the Graduate Council:

I am submitting herewith a dissertation written by Luis Fernando Calvinho entitled "Interaction of Streptococcus dysgalactiae with bovine mammary epithelial cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Stephen P. Oliver, Major Professor

We have read this dissertation and recommend its acceptance:

Philip Bochsler, Judith Grizzle, Fred Hopkins, Alan Mathew, Kelly Robbins

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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INTERACTION OF *STREPTOCOCCUS DYSGALACTIAE* WITH BOVINE MAMMARY EPITHELIAL CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Luis Fernando Calvinho

May, 1997

DEDICATION

This dissertation is dedicated to my wife Patricia, my children Francisco and Sofía and my parents, Luis and Elda.

ACKNOWLEDGMENTS

I wish to express my gratitude and appreciation to Dr. Stephen P. Oliver for his continuing support, good humor and sound direction over the past years.

Thanks also to Drs. Philip Bochsler, Judith Grizzle, Fred Hopkins, Alan Mathew and Kelly Robbins for serving as doctoral committee members and for their evaluation of this research.

I would like to thank Barbara Gillespie, Douglas Luther and Bhushan Jayarao for technical assistance during the completion of this project and to Mark Lewis for the many times he helped me solve big or small problems to continue with my laboratory work.

To my colleague Raúl Almeida for his understanding, support and friendship during the good and hard times we shared over the past years.

Lastly, I wish to express my love and gratitude to my wife Patricia for her support and encouragement, and to my children Francisco and Sofía for their patience during the completion of this degree.

ABSTRACT

Streptococcus dysgalactiae accounts for a significant number of intramammary infections during lactation and the nonlactating period. In spite of its high prevalence, little information is available about factors that contribute to the virulence of *S. dysgalactiae*. This organism adheres to bovine mammary epithelial cells and to extracellular matrix proteins *in vitro*, and invades bovine mammary epithelial cells in culture; all of which can be potentially important pathogenic mechanisms. Experiments were conducted to characterize factors and mechanisms involved in adherence and invasion of *S. dysgalactiae* to bovine mammary epithelial cells *in vitro*.

Adherence of *S. dysgalactiae* was affected by both the mammary epithelial cell type and bacterial strain used. Bacterial adherence was mediated by saturable cell receptors, and reduction of bacterial adherence following cell fixation indicated that cell surface proteins played a major role in this process. Delineation of bacterial and epithelial cell factors influencing adherence of *S. dysgalactiae* to mammary epithelial cell monolayers resulted in development of an *in vitro* method for quantifying bacterial adherence. Presence of two bacterial factors, lipoteichoic acid (LTA) and M-like protein, potentially involved in adherence of *S. dysgalactiae* to mammary epithelial cells, was detected in strains of *S. dysgalactiae* isolated from bovine intramammary infections. Bacterial surface proteins played a major role in adherence of *S. dysgalactiae* to mammary epithelial cells. However, use of antibodies directed against streptococcal M24 protein did not affect adherence of *S. dysgalactiae* to mammary epithelial cells. LTA appeared to play a minor role in adherence of one strain of *S. dysgalactiae* to mammary epithelial cells but had no effect on another strain of *S. dysgalactiae*.

Streptococcus dysgalactiae invasion into mammary epithelial cells increased with inoculum size; however, number of intracellular bacteria was not proportional to

inoculum size indicating that a finite number of organisms are capable of invading epithelial cells. No net increase of intracellular organisms was detected at several bacterial densities evaluated; however, *S. dysgalactiae* remained viable throughout the period of evaluation. In addition, *S. dysgalactiae* did not appear to cause cell injury at any bacterial density and time point evaluated. These data suggest that *S. dysgalactiae* can survive within mammary epithelial cells for extended periods of time without losing viability or damaging the eukaryotic cell. This feature may be associated with development of persistent infection, protection of organisms from antimicrobial drugs and host defense mechanisms, and can also provide a route for bacterial colonization of subepithelial tissues. Activity of eukaryotic cell tyrosine protein kinases, intact microfilaments and *de novo* eukaryotic protein synthesis were required for invasion of *S. dysgalactiae* into bovine mammary epithelial cells; a process that appeared to occur via receptor-mediated endocytosis. In contrast, *de novo* bacterial protein synthesis was not required for invasion of *S. dysgalactiae* into MAC-T cells.

Collectively, these studies provide insight towards a more fundamental understanding of early interactions between bovine mammary epithelial cells and *S. dysgalactiae* and should assist in further research directed to develop methods to minimize production losses associated with intramammary infections in dairy cows.

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LITERATURE REVIEW

Introduction

Bovine mastitis is an inflammation of the mammary gland in response to injury. Mastitis is one of the most significant limiting factors to profitable dairying throughout the world, and losses in the United States have been estimated to exceed 2 billion dollars annually (reviewed by DeGraves and Fetrow, 1993; Bramley et al., 1996). Estimates indicate that 10 to 11% of total yearly productive capacity is lost when decreased milk production, increased replacement cost, milk discarded due to antibiotic treatment, cost of antibiotics, veterinary fees and extra labor are considered. Although mastitis is a multietiological disease, most cases have bacterial etiology (Watts, 1988a). Several bacterial genera and species capable of causing mastitis are widespread in the environment of dairy cows and present a constant threat to the mammary gland (Schalm, et al. 1971).

Mastitis organisms have been categorized as contagious or environmental pathogens based on their distinct characteristics of distribution and interaction with the teat and teat duct. Contagious pathogens need to live and multiply on and 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 are a heterogeneous group of bacterial genera, species and strains. The most frequently isolated environmental pathogens are streptococci other than *S. agalactiae* (environmental streptococci) and

coliform bacteria (Smith et al., 1985; Oliver, 1988; Smith and Hogan, 1993). Among the environmental pathogens, *Streptococcus uberis* and *Streptococcus dysgalactiae* are the most prevalent, invading mammary glands as favorable conditions arise (Smith et al., 1985; Oliver, 1988, Aarestrup et al., 1995, Todhunter et al., 1995).

Current mastitis control programs were devised in the late 1960s and are based on hygiene including pre- and postmilking teat disinfection, antibiotic therapy and culling of chronically infected cows (Booth, 1975). Acceptance and application of these measures has led to considerable progress in controlling contagious mastitis pathogens. However, postmilking teat disinfection and antibiotic dry cow therapy have been less effective against environmental streptococci and ineffective against *Escherichia coli* (Smith et al., 1985). Therefore, it is not surprising that environmental mastitis has become a major problem in many well-managed dairy farms that have successfully controlled contagious pathogens (Oliver and Mitchell, 1984; Smith et al., 1985; Oliver, 1988; Todhunter et al., 1995). In these herds, environmental streptococci account for a significant number of both subclinical and clinical intramammary infections (IMI) (Oliver and Mitchel, 1984; Hogan et al., 1989; Todhunter et al., 1995). In spite of its high prevalence, little information is available about the epidemiology and factors that contribute to the virulence of environmental streptococci.

Contagious pathogens are associated primarily with subclinical mastitis, whereas environmental pathogens are associated primarily with clinical mastitis (Oliver and Mitchel, 1984; Smith and Hogan, 1993). A high proportion of environmental pathogen infections result in clinical mastitis (Bramley, 1990; Smith and Hogan, 1993), particularly in the case of *S. dysgalactiae* (Oliver et al., 1990; Todhunter et al., 1995), and are treated with antibiotics during lactation with variable efficacy (Guterbock et al., 1993). In addition, antibiotic dry cow therapy reduces the rate of IMI during the early stages of the nonlactating period but has little, if any, effect during the periparturient period (Smith et

al., 1985). This raises concerns about reduced efficacy of antibiotic therapy against environmental pathogens, and presence of undesirable residues originating from widespread, indiscriminate use of antibiotics during lactation and the nonlactating period that can become a public health hazard and a problem to the dairy industry (Huber, 1971). Furthermore, antibiotic treatment during lactation causes direct economic losses to the dairy producer due to discarding of contaminated milk (Reneau and Packard, 1991).

Consequently, alternative methods are necessary to make further progress in mastitis control and thus minimize direct and indirect costs associated with this disease. In addition to the required practices of good herd management and hygiene, alternative control measures should take into account manipulation of host defense mechanisms, assuming that some entry of bacteria into the mammary gland will occur during most lactations (Hill, 1986). The aim should be to achieve an efficient elimination of infection, accepting the fact that a self-limiting mastitis would be the inevitable consequence of natural challenge (Craven and Williams, 1985). 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 (Hill, 1986). Our current understanding of mechanisms of teat duct penetration by environmental pathogens as well as subsequent host-pathogen interactions that lead to establishment of IMI are very limited (Bramley, 1990; Hill, 1991). A more thorough understanding of how these interactions influence mammary secretory epithelial cell function could lead to methods that minimize production losses associated with mastitis in dairy cows.

Increased exposure of mammary glands to mastitis pathogens results from the interaction of both managerial and environmental factors that favor spread of bacteria and decrease natural resistance of the cow to infection (Schalm et al., 1971). Inflammation of the mammary gland is the response of mammary tissue to microbial invasion and toxins

produced by bacterial pathogens resulting in damaged secretory tissue, reduced milk synthesis and alterations in milk composition. Development of IMI and response of mammary tissue to presence of the invading mastitis pathogen(s) depend upon the rate of bacterial multiplication, type and concentration of bacterial virulence factors, as well as effectiveness of the cow's defense mechanisms (Schalm et al., 1971). Interaction of these factors will in turn determine the magnitude of reduced milk yield and alterations in milk composition. Cows affected with clinical mastitis show both a marked decrease in milk yield and macroscopic changes in milk composition. In contrast, milk from cows with subclinical mastitis, which is the most common form of the disease, has a normal macroscopic appearance, although yield is reduced and composition is altered (Oliver and Calvinho, 1995).

Most information on the influence of inflammation on mammary gland metabolism and milk composition has been derived from studies using an endotoxin mastitis model. Endotoxin elicits an inflammatory response similar to a bacterial infection but without complications associated with the presence of the organism, endangerment of the health of the animal or affecting the integrity of the mammary gland (Guidry et al., 1983). Local and systemic effects observed after intramammary infusion of endotoxin are considered to be the result of release of endogenous inflammatory mediators rather than direct toxicity (Lohuis et al., 1988b; Shuster et al., 1991). However, there is little information about host-pathogen interactions conducive to establishment of an IMI and events that take place prior to the development of the inflammatory response.

Infection of the mammary gland occurs when mastitis pathogens pass through the streak canal and into the teat cistern and subsequently become established in an area of secretory tissue. During bacterial multiplication, cytotoxigenic substances are produced which attract blood neutrophils, also known as polymorphonuclear leukocytes (PMN), into injured areas (van Miert, 1991). The interaction of bacterial products with

components of the normal mammary gland generates additional endogenous chemotactic factors that precede the PMN response (McKenzie and Anderson, 1981; Craven, 1983). Changes in milk composition observed as a consequence of mammary gland inflammation are likely due to factors including impaired cellular synthesis and secretion, cellular degeneration and paracellular transport of molecules from blood to milk and from milk to blood (Oliver and Calvinho, 1995). Several studies have shown that major components of milk synthesized specifically by mammary gland epithelial cells such as lactose, fat, and casein are decreased during inflammation (reviewed by Kitchen, 1981; Munro et al., 1984; Oliver and Calvinho, 1995). Bacterial adherence, multiplication and toxin production that favor survival of pathogens within the mammary gland, as well as the action of macrophages, chemotactic factors, inflammatory mediators and PMN influx have been associated with impaired cellular synthesis, impaired cellular secretion, cellular degeneration and cell death. However, both the primary deleterious factors that cause damage to secretory epithelium and the series of events that take place during bacterial invasion to the mammary gland are not completely understood (Oliver and Calvinho, 1995; Paape et al., 1995). Reduced milk production during mastitis is due to factors that can exert direct damage to secretory epithelial cells such as: **(1) production of bacterial factors (diffusible and non-diffusible), bacterial attachment and invasion, (2) PMN migration across the epithelium, (3) release of microbicidal products from actively phagocytosing or degenerating PMN, and/or (4) release of cytokines during the inflammatory response**. In addition, factors such as anorexia, hormonal changes and systemic effects of inflammatory mediators can alter synthetic activity of epithelial cells by interfering with substrate availability for milk synthesis and/or altering metabolic activity of milk producing cells by either reducing the concentration of a galactopoietic hormone or by increasing the concentration of inhibitory substances (Shuster et al., 1991).

Attempts have been made to dissociate these factors in order to assess individually their potential for damaging the mammary epithelium and decreasing milk secretion.

Production of bacterial factors, bacterial attachment and invasion

Potential virulence factors of *Streptococcus dysgalactiae.*

Streptococcus dysgalactiae isolated from bovine mastitis belongs to Lancefield serological group C (Garvie et al., 1983). In addition, taxonomic studies have classified organisms designated previously as *Streptococcus equisimilis* as well as group L and human group G as *S. dysgalactiae* (Farrow and Collins, 1984). A recent study, based on electrophoresis of cell wall proteins and physiological tests (Vandamme et al., 1996) proposed the name *S. dysgalactiae* subspecies *dysgalactiae* for strains of animal origin that belong to Lancefield serogroups C and L, and the name *S. dysgalactiae* subsp. *equisimilis* for human isolates that belong to Lancefield serogroups C and G. Group L streptococci, *S. equisimilis* and human group G streptococci are isolated infrequently from bovine mammary glands (Watts, 1988b). Bovine strains of *S. dysgalactiae* are a homogeneous group of non-hemolytic or α-hemolytic cocci that should therefore be distinguished from β-hemolytic strains of group L, human group G and *S. equisimilis* (Watts, 1988b).

Information regarding the epidemiology of *S. dysgalactiae* in dairy herds is scarce. This organism has characteristics of both a contagious and an environmental pathogen. *Streptococcus dysgalactiae* is isolated from infected mammary glands and teat injuries (Schalm et al., 1971) and is transmitted primarily during milking (Bramley and Dodd, 1984), which are characteristics of contagious pathogens. However, detection of several potential extramammary reservoirs such as cattle tonsils, mouth, and vagina

(Schalm et al., 1971; Cruz Colque et al., 1993), and occurrence of *S. dysgalactiae* IMI during the nonlactating period in herds with no previous history of *S. dysgalactiae* IMI (Bramley and Dodd, 1984) suggests that the organism also behaves as an environmental pathogen. In addition, *S. dysgalactiae* is significantly involved in the multietiological clinical entity referred to as summer mastitis which affects dry cows and heifers during summer months in northern Europe and Japan (Madsen et al., 1990; Pyörälä, 1995). This organism, among other causative agents, has been isolated from the common cattle fly *Hydrotaea irritans*, which appears to play a significant role in establishment and maintenance of bacterial contamination of teats of healthy cattle (Bramley et al., 1985; Madsen et al., 1991). *Streptococcus dysgalactiae* is considered to be the first bacterial species to colonize the bovine teat, subsequently providing a favorable environment for colonization by *Actinomyces pyogenes* and anaerobic bacteria such as *Peptostreptococcus indolicus* and *Fusobacterium necrophorum* (Pyörälä, 1995).

Small numbers of *S. dysgalactiae* when infused into the teat cistern are capable of causing IMI (Higgs et al., 1980). However, little is known about bacterial factors that contribute to establishment and persistence of *S. dysgalactiae* within the mammary gland. *Streptococcus dysgalactiae* isolates infused into bovine mammary glands showed differences in infectivity which did not correlate with small differences in growth rate in raw milk observed between these isolates (Higgs et al., 1980). *Streptococcus dysgalactiae* strains isolated from bovine IMI grew well in nonlactating mammary secretions as well as in milk obtained during late and early lactation. Differences in the ability to grow in these secretions were observed among strains (Oliver, 1991).

The ability to initiate growth *in vivo* and stably infect a host requires acquisition by the invading pathogen of virulence factors capable of neutralizing nonspecific mechanisms of the host's defense. These factors include structural components, toxins and enzymes that serve to overcome the otherwise effective nonspecific defensive

measures of the host (Brubaker, 1985). Some of these bacterial factors can exert a direct effect on stromal cells while others can thwart one or more host defense mechanisms to allow for survival and persistence of the pathogen in the invaded tissue (Woolcock, 1988). *Streptococcus dysgalactiae* isolated from bovine IMI is endowed with a number of cell-associated as well as extracellular potential virulence factors (Table 1). However, importance of these factors has not been determined.

Streptococcus dysgalactiae can interact with several plasma and extracellular host-derived proteins, such as IgG, albumin, fibronectin (Fn), fibrinogen, collagen, vitronectin, and α_2-macroglobulin (α_2-Ma). These interactions are mediated by bacterial surface proteins which, due to the high specificity and affinity of binding, are also termed receptors; although a signaling event following the binding has not been demonstrated (Rantamäki and Müller, 1995). *Streptococcus dysgalactiae* isolated from bovine IMI are capable of binding to IgG in a nonimmune fashion (Müller and Blobel, 1983a; Lämmler and Frede, 1989; Rantamäki and Müller, 1995). A receptor for the Fc component of IgG (FcR) was isolated from 8 of 26 culture supernatants of group C streptococcal strains, and the purified FcR had an estimated molecular weight of 78 KDa (Müller and Blobel, 1983a). Specificity of IgG binding reaction was confirmed through inhibition by prior addition of IgG or Fc-components of IgG to FcR but was not affected by prior addition of $F(ab)_2$-fragments. The strong precipitating activity observed with sera from human, horses, cattle, pigs, sheep, rabbits, and guinea pigs, but not from dogs, rats, and mice suggested that the isolated FcR represented an FcR type III (Müller and Blobel, 1983a). Lämmler and Frede (1989) showed that *S. dysgalactiae* isolated from bovine IMI interacted both with IgG and albumin. None of the strains evaluated bound albumin without simultaneously binding IgG. Binding activities for IgG and albumin were located on one protein molecule similar to protein G of group G streptococci while a second group of binding proteins with almost identical molecular weight interacted with IgG but

Table 1. Potential virulence factors of *Streptococcus dysgalactiae* isolated from bovine intramammary infections.

Potential factor	Reference
Cell-associated activity/protein	
IgG-binding	Müller and Blobel, 1983a
IgG/albumin-binding	Lämmler and Frede, 1989
IgG/α_2-Ma*-binding	Jonsson and Müller, 1994
IgG/α_2-Ma/albumin-binding	Jonsson et al., 1994
α_2-Ma-binding	Müller and Blobel, 1983b
Fibronectin-binding	Mamo et al., 1987; Lindgren et al., 1992
Fibrinogen-binding	Mamo et al., 1987; Traore et al., 1991
Vitronectin-binding	Filippsen et al., 1990
Collagen-binding	Mamo et al., 1987
Plasminogen-binding	Ullberg et al., 1989
Capsule	Matthews and Oliver, 1993
Extracellular	
Hyaluronidase	Sting et al., 1990
Fibrinolysin	Garvie et al., 1983; Vandamme et al., 1996

*α_2-macroglobulin

not with albumin (Lämmler and Frede, 1989). Binding of human ^{125}I-IgG to *S. dysgalactiae* could be almost completely inhibited by unlabeled human IgG, and partially inhibited by horse and bovine IgG (Lämmler and Frede, 1989).

A role for IgG-binding proteins as virulence factors has not been determined. These proteins may interfere with host defense mechanisms at sites where IgG is present in low concentrations by forming nonimmune complement-fixing complexes (Boyle, 1990). Using a mouse model of skin infection where the local concentration of IgG was low, an association between expression of IgG-binding proteins and infectivity of group A streptococci was observed (Raeder and Boyle, 1993).

Recently, the gene for a type-III Fc receptor from a strain of *S. dysgalactiae* isolated from bovine IMI (Sc1) has been cloned and sequenced (Jonsson and Müller, 1994). Five IgG-binding domains were found, rather than two or three as shown in other streptococcal type III FcR. Estimated molecular mass of this receptor based on amino acid residues after deletion of the predicted signal sequence was 69 kDa (Jonsson and Müller, 1994). In addition, a unique sequence upstream from the IgG-binding domain mediated binding to the "fast" form of α_2-Ma (Müller and Blobel, 1983b) instead of to albumin as seen for other FcR. Further work performed with another strain of *S. dysgalactiae* of bovine origin led to cloning and sequencing of a gene encoding for a trifunctional protein receptor that can bind α_2-Ma, albumin and IgG (protein MAG, Jonsson et al., 1994). Further work showed that the albumin-binding domain of *S. dysgalactiae* MAG bound efficiently to serum albumin from different animal species (Jonsson et al., 1995). Influence of the combination of binding activities on the pathogenesis of *S. dysgalactiae* remains to be elucidated.

Streptococcus dysgalactiae also binds to Fn (Myhre and Kuusela, 1983, Mamo et al., 1987), a high-molecular-weight glycoprotein found in soluble form in plasma and body fluids and in an insoluble form in connective tissue and basement membranes

(Yamada and Olden, 1978). Binding of bacteria to Fn has been implicated as a mechanism of bacterial adherence to host tissues. Two separate genes in a strain of *S. dysgalactiae* isolated from bovine mastitis coding for Fn-binding protein have been identified (Lindgren et al., 1992). Only one of the proteins coded by these genes was expressed under standard culture conditions. However, Southern-hybridization studies showed that both genes were present in 20 clinical isolates of *S. dysgalactiae* (Lindgren et al., 1993). Receptor activity was localized in repeated motifs of 32-37 amino acids in length which shared conserved features with Fn receptor of *Staph. aureus* and *S. pyogenes* (McGavin et al., 1993; Joh et al., 1994). In addition, presence of a previously unidentified ligand binding site in a Fn-binding receptor from *S. dysgalactiae* was demonstrated by Speziale et al. (1996). This binding site, termed Au, was contained within a 30-amino acid residue segment located N-terminal of the previously identified primary binding domains. A monoclonal antibody raised against the Au binding site only recognized the Au amino acid sequence upon binding of Au to Fn suggesting that these amino acid residues adopt a specific conformation on binding to Fn (Speziale et al., 1996). It has been speculated that conformational changes in the Au sequence induced by binding to Fn can favor bacterial adherence to host tissues (Speziale et al., 1996).

Strains of *S. dysgalactiae* from cattle bound to α_2-macroglobulin-trypsin (α_2-M-T) (Müller and Blobel, 1983b, Valentin-Weigand et al., 1990, Rantamäki and Müller, 1995). Alpha$_2$-macroglobulin is a plasma glycoprotein that can inhibit nearly all endoproteases by a unique trap mechanism. This protein has also been detected in mastitic milk as a result of increased vascular permeability, although local production by mammary epithelial cells or by macrophages migrating into the mammary gland cannot be ruled out (Rantamäki and Müller, 1992). Binding of α_2-M-T to *S. dysgalactiae* was specific and could be inhibited by homologous α_2-M-T and not by other proteins such as fibrinogen, Fn, IgG or albumin (Valentin-Weigand et al., 1990). Pretreatment of *S. dysgalactiae*

isolated from cattle with α_2-M-T led to a concentration-dependent inhibition in phagocytosis of this bacterium by bovine neutrophils, suggesting that binding of α_2-M-T to *S. dysgalactiae* might block structures on the streptococcal surface required for adherence to PMN during phagocytosis (Valentin-Weigand et al., 1990).

Streptococcus dysgalactiae from bovine IMI bound to vitronectin (Filippsen et al., 1990, Rantamäki and Müller, 1995). Vitronectin is a multifunctional plasma protein that plays a role in complement-dependent lysis, the coagulation system and cellular adherence (Hayman et al., 1983; Preissner et al., 1985; Kuby, 1994). Plasminogen has been found to bind to surface receptors on *S. dysgalactiae* (Ullberg et al., 1989); however, the significance of this finding is not clear. Evidence was presented by Traore et al. (1991) that *S. dysgalactiae* bound to fibrinogen which inhibited C3-fixation and subsequent phagocytic killing by PMN. Phagocytic killing was inhibited by fibrinogen beta and gamma-chains nearly to the same extent as by the intact fibrinogen molecule. Binding of M proteins of group A streptococci to fibrinogen or complement factor H have been considered as mechanisms to evade phagocytosis (Kehoe, 1994). Therefore, the fibrinogen binding component of *S. dysgalactiae* might represent a pathogenic factor similar to group A streptococcal M protein.

Among factors associated with bacterial virulence, capsules are considered to play a fundamental role during phases of microbial colonization, and resistance to phagocytosis and antimicrobials (Wilkinson et al., 1979; Pulliam et al., 1985; Dall et al., 1987). Presence of capsule, both by India ink staining and by scanning electron microscopy was detected in only five of 33 strains of *S. dysgalactiae* freshly isolated from bovine IMI; however, capsule expression was lost upon storage (Matthews and Oliver, 1993). Attempts to revive capsule expression in *S. dysgalactiae* strains isolated from bovine IMI found to be encapsulated upon primary culture using supplemented media were unsuccessful (Calvinho et al., 1996b). Expression of capsule in *S. uberis* has been

associated with resistance to phagocytosis by bovine mammary gland macrophages (Almeida and Oliver, 1993b). Further studies are needed to determine conditions of capsule expression by *S. dysgalactiae* as well as relative importance of the capsule.

Products released by pathogenic streptococci could favor establishment and persistence of IMI. *Streptococcus dysgalactiae* from bovine origin may produce a fibrinolysin for bovine but not for human fibrin (Garvie et al., 1983; Vandamme et al., 1996), distinct from streptokinase detected in other streptococci (Vandamme et al., 1996). Extracellular hyaluronidase, an enzyme that degrades hyaluronic acid, has been isolated and purified from *S. dysgalactiae* isolates from bovine IMI (Sting et al., 1990). However, the importance of this factor has not been evaluated. Since hyaluronic acid is an important intercellular component, it has been hypothesized that hyaluronidase could contribute to streptococci tissue-invasive properties (Sting et al., 1990). Conversely, hyaluronidase can remove hyaluronic acid capsules, thus rendering encapsulated organisms more sensitive to phagocytosis (Timoney, 1993).

Effects of bacterial determinants on mammary epithelial cells

Attempts have been made to determine effects of bacterial products considered as potential virulence factors on bovine mammary epithelial cells. Extracellular enzymes and toxins can act at different levels such as initial microtrauma, lysis of neutrophils, and terminal necrotic changes (Brubaker, 1985). However, since a multiplicity of factors are involved in establishing and maintaining an IMI, it is difficult to attribute any single component or biological activity to a particular area or level of attack (Anderson, 1983; Sutra and Poutrel, 1994). Many of these substances may be virulence determinants that promote dissemination *in vivo* causing changes conducive to progressive colonization of host tissues (Brubaker, 1985).

Several *in vitro* models have been used to study effects of bacterial determinants on mammary tissues and cells. Most of the experimental work has involved bacterial factors from *Staph. aureus* and *E. coli*. Explants prepared from mammary tissue of cows in early lactation showed tissue damage and decreased secretory activity, measured by increased N-acetyl-β-D- glucosaminidase (NAGase) activity and decreased amino acid incorporation, respectively, when treated with *Staph. aureus* α- and β-toxins, leukocidin and an *E. coli* culture filtrate (Capuco et al., 1985). Proliferation of a bovine mammary epithelial cell line, indicated by [^{3}H] thymidine incorporation, was inhibited by *E. coli* endotoxin and *Staph. aureus* α-toxin, but not by *Staph. aureus* β-toxin (Matthews et al., 1994a). A recent study by Cifrian et al. (1995) showed changes in cell permeability of secretory, ductal and teat cell monolayers incubated with staphylococcal α-toxin consistent with the pore formation mechanism of action of α-toxin observed in other mammalian cells. In addition, phase-contrast microscopy of monolayers exposed to α-toxin for 24 h showed weakened cell junctions and small gaps between cells (Cifrian et al., 1995). MacDonald et al. (1994) cocultured *Staph. aureus* strain Newbould 305 with bovine mammary epithelial cell monolayers and observed no significant decrease in transepithelial resistance indicating absence of damage to tight junctional complexes between adjacent cells.

There is only scarce information regarding effects of potential virulence factors of environmental streptococci on mammary epithelial cells. Hyaluronidase, an enzyme produced by *S. uberis* and *S. dysgalactiae* (Schaufuss et al., 1989; Sting et al., 1990), and hyaluronic acid capsule from *S. uberis* (Almeida and Oliver, 1993a), inhibited proliferation of mammary epithelial cells (Matthews et al., 1994a). In addition, lipoteichoic acid from group A streptococcus, considered to play a role in adherence of streptococci to mammalian cells and exert cytotoxic effects on mammalian cells (Simpson et al., 1982), inhibited proliferation of bovine primary epithelial cell

monolayers at 1, 8 and 16 μg/ml (Calvinho et al., 1996a). Inhibition of proliferation could be significant during the periparturient period when mammary glands undergo marked cellular, ultrastructural and biochemical changes. Conversely, streptokinase from group C streptococci showed no effect on proliferation of primary bovine mammary epithelial cell monolayers (Calvinho et al., 1996a). Among the environmental streptococci, *S. uberis* produces a protein capable of activating bovine plasminogen (Leigh, 1994), and streptokinases have been detected in group C pathogenic streptococci isolated from animals showing physical and antigenic similarities (McCoy et al., 1991). Streptokinase forms a strong complex with plasminogen causing its activation to plasmin which hydrolyses fibrin as well as connective tissue proteins. Although a direct effect of streptokinase on mammary epithelial cells was not observed, it has been hypothesized that plasmin may enhance the ability of streptococci to spread in host tissues and promote growth of cocci by making peptides and amino acids available for bacterial growth during the early stages of infection (Leigh, 1994).

Bacterial adherence and invasion

Mastitis pathogens may enter the teat cistern by propulsion, by growth, or by a combination of both mechanisms, which can take place in the presence or absence of machine milking (Anderson, 1983; Sutra and Poutrel, 1994). Mastitis pathogens are subject to the flushing action of milk that serves as an important physical barrier to invasion. It is possible, however, that invading bacteria may resist the flushing effect of milking by adhering to tissue linings and replicating at a rate to maintain or expand their population (Arp, 1988). Accordingly, specific adherence of mastitis pathogens to cells may be an important stage in the pathogenesis of mastitis prior to the establishment of an IMI (Frost et al., 1977; Wanasinghe, 1981).

Only a few *in vivo* studies showed adherence of mastitis bacterial pathogens to mammary tissues (Pattison, 1951; Gudding et al., 1984; Thomas et al., 1994). High bacterial inocula used to achieve infection and the need to sacrifice animals shortly after inoculation (Ofek and Doyle, 1994) are a few of the inherent difficulties involved in development and interpretation of these kind of studies. In addition, a large animal population is needed to provide statistical validation of the data (Ofek and Doyle, 1994). Early work reported adherence of *S. agalactiae* to ductular and secretory epithelium of the goat (Pattison, 1951). Following inoculation of mice mammary glands with *Staph. aureus* and subsequent suckling by the offspring, most organisms were associated with PMN rather than in direct contact with the alveolar epithelium (Anderson, 1978a). Conversely, evidence was provided of cocci attaching to ductal epithelium following inoculation of *Staph.* aureus into cow's mammary glands (Gudding et al., 1984). Following experimentally induced IMI in the cow, *S. uberis* adhered to the luminal surface of damaged ductular or secretory epithelium but not to large ducts or lactiferous sinuses (Thomas et al., 1994), providing evidence of adherence of this environmental streptococcus to epithelial cells *in vivo.*

Most studies on adherence of mastitis pathogens to mammary epithelium are based on *in vitro* model systems. Early work by Frost et al. (1977) showed that both contagious and environmental pathogens adhered to freshly isolated cells obtained from scrapings of mammary epithelium; showing increased adherence to large ducts than to lactiferous sinus cells. However, cell suspensions obtained from scrapings of mammary epithelium yielded a large percentage of damaged or effete cells which can alter bacterial adherence (Thomas et al., 1992). *Streptococcus dysgalactiae* of bovine origin adhered to highly viable bovine buccal mucosal cell suspensions (Valentin-Weigand et al., 1988). However, caution must be exercised in interpretation of adherence studies involving cell suspensions since the basolateral surface of cells, not exposed *in vivo* and considered to

have binding sites dissimilar from the apical surface, is exposed to the bacterial pathogen (Freter and Jones, 1983). In addition, exfoliated and scraped cells can also harbor normal microbiota, which may occupy sites under investigation (Ofek and Doyle, 1994).

Thomas et al. (1992) found no evidence of adherence of mastitis pathogens to epithelial cells using mammary gland explants. Rather, bacteria appeared to have a preferential tropism for exposed connective tissue at the periphery of cultures. Tissue explants expose subepithelial matrix components not exposed *in vivo* which are considered to be highly susceptible to bacterial adherence (Paape et al., 1995), thus making interpretation of host-pathogen interactions difficult.

The mechanism by which environmental streptococci adhere to surfaces is not understood. *Streptococcus dysgalactiae* and *S. uberis*, although in varying degrees, bound to Fn, fibrinogen, collagen, and laminin (Mamo et al., 1987; Valentin-Weigand et al., 1988; Lindgren et al., 1992; Almeida et al., 1996b). *Streptococcus dysgalactiae* was found to interact with a 210-kDa C-terminal fragment of Fn. However, binding involved the host attachment domain of Fn and therefore was not considered to mediate streptococcal adherence to oral bovine epithelial cells (Valentin-Weigand et al., 1988). In addition, Fn only weakly inhibited adherence of *S. dysgalactiae* to epithelial cells (Valentin-Weigand et al., 1988). Conversely, Lindgren et al. (1992) found that *S. dysgalactiae* strain S2 isolated from bovine milk bound to a site in the amino-terminal region of Fn, confirming previous findings of Mamo et al. (1987). *Streptococcus dysgalactiae* was also shown to specifically bind bovine S protein (vitronectin) through a region that did not involve the cell-binding domain of the protein, leading to the speculation that S protein can mediate adherence of *S. dysgalactiae* to host cells (Filippsen et al., 1990).

Adherence of several bacterial species to host cells has been related to bacterial surface hydrophobicity (SH) (Rosenberg and Kjelleberg, 1989; Courtney et al., 1990).

Streptococcus dysgalactiae strains showed extensive variation in expression of SH after growing in culture medium supplemented with whey, skim milk, lactose and casein (Calvinho et al., 1996b). A hydrophilic strain of *S. dysgalactiae* did not adhere as well to mammary epithelial cell monolayers as a hydrophobic strain. Trypsin treatment significantly reduced adherence of this hydrophobic strain to mammary epithelial cells while adherence of a hydrophilic strain remained unaltered (Calvinho et al., 1996b). Mamo et al. (1987) found no correlation between expression of SH and binding to Fn among *S. dysgalactiae* isolated from bovine IMI. Biological differences in expression of SH by bacteria is not clear, however, it may be related to changes in the environment and may reflect an adaptive response to allow bacterial survival during the early stages of infection (Courtney et al., 1990).

About 50% of strains of *S. uberis* possess a capsule (Matthews et al., 1994d), and the major component appeared to be hyaluronic acid (Almeida and Oliver, 1993a). The role of *S. uberis* capsule in adherence to mammary epithelial cells is not understood. However, an encapsulated strain of *S. uberis* adhered better to bovine mammary epithelial cell monolayers than a nonencapsulated strain (Almeida et al., 1996b). Furthermore, preincubation of mammary epithelial cells with lipoteichoic acid and/or treatment of *S. uberis* with antibodies directed against the carboxyl-terminal half of type 24M protein reduced adherence of both encapsulated and nonencapsulated strains, suggesting that lipoteichoic acid and M-like protein may play a role in adherence of *S. uberis* to bovine mammary epithelial cells (Almeida et al., 1996b). Lipoteichoic acids and M-like proteins have been detected in several streptococcal species (Fischer, 1988; Fischetti, 1989), which suggests that these molecules may also be present in other mastitis-causing streptococci such as *S. dysgalactiae*.

Bacterial invasion into mammary epithelial cells is a potentially important feature in the pathogenesis of some mastitis pathogens. Bacterial invasion could result in

protection from host defense mechanisms and action of antimicrobial agents and may affect secretory cell function. In addition, an infection route from the lumen into subepithelial tissues may be provided (Chandler et al., 1980; Falkow et al., 1992; Galán, 1994). On the other hand, phagocytosis by mammary epithelial cells could be a host protective mechanism against bacterial infection (Brooker, 1983). Detection of mastitis pathogens within mammary epithelial cells and evidence for intracellular location of mastitis pathogens during the establishment of IMI have been contradictory. Early work of Pattison and Smith (1953) showed that cocci penetrated through the alveolar epithelium and smaller ducts to reach interacinar tissue following experimental infection of goat mammary glands with *S. dysgalactiae*. Observations on experimental *S. agalactiae* mastitis in the mouse showed cocci in close relation with secretory cells bordering the acinar lumen as well as cocci within vacuoles in cells identified as secretory acinar cells (Chandler, 1970). In addition, cocci were observed below the basal membrane of a secretory cell suggesting penetration to the *lamina propria* (Chandler, 1970). Intracellular location of *S. agalactiae*, *Staph. aureus, Staphylococcus epidermidis* and *E. coli* in mammary secretory cells was further confirmed in a mouse mastitis model (Chandler and Anger, 1977; Anderson, 1978b). Following experimental infection of the mouse mammary gland, bacteria were seen within phagolysosomes in secretory epithelial cells and changes in internalized bacteria suggested enzymatic digestion by the cell (Chandler et al., 1980). Thomas et al. (1994) observed cocci penetrating the secretory epithelium 24 h after inoculation of *S. uberis* into the mammary gland of cows. Conversely, following *Staph. aureus* experimental infection of bovine mammary glands, Nickerson and Heald (1982) observed cocci either in neutrophils or macrophages but never within epithelial cells. The nature of discrepancies between studies regarding intracellular location of cocci may be due to animal species used as well as different bacterial strains, dosage and sampling times after bacterial inoculation. During naturally

occurring staphylococcal and streptococcal IMI in cattle, secretory cells showed various pathological changes, but cocci were never observed in ultrathin sections (Chandler and Reid, 1973). In addition, Sordillo et al. (1989) seldom observed cocci proximal to parenchymal tissue or internalized within phagocytes during naturally occurring *Staph. aureus* infections in preparturient cows. However, due to the nature of these studies, duration of infection and therefore the precise stage in the infectious process could not be ascertained.

Different pathogenic organisms have developed various strategies to gain access into host cells, thereby avoiding, subverting or nullifying host protective mechanisms (reviewed by Galán, 1994). Bacterial invasion of eukaryotic cells usually involves utilization of host cell functions by invading pathogens which exploit existing signal transduction pathways to trigger cytoskeletal rearrangement and bacterial uptake (Rosenshine et al., 1994). Many of these pathways involve protein kinases as well as Ca^{2+} fluxes to transmit signals. Although attachment, invasion and disruption of host cell functions by a pathogenic organism can be considered to be genetically separate events, they represent individual steps of a process that will lead to persistence of the organism within the host (Bliska et al., 1993). Therefore, to understand bacterial invasion, the role of components and functions of the eukaryotic cell need to be scrutinized. Only limited information is available about bacterial and eukaryotic cell mechanisms that take place during early host-pathogen interactions that lead to invasion of mammary epithelial cells.

In vitro studies showed that *S. uberis*, *S. dysgalactiae* and *Staph. aureus* invaded mammary epithelial cell monolayers (Matthews et al., 1994b; Almeida and Oliver, 1995; Almeida et al., 1996a). Microfilaments, but not microtubules, were found to be necessary for bacterial entry into eukaryotic cells (Matthews et al., 1994b; Almeida and Oliver, 1995; Almeida et al., 1996a). Active involvement of the host cytoskeleton in bacterial invasion suggests that invasive bacteria, upon contact with a eukaryotic cell receptor,

generate a signal which induces the host cell to internalize them (Rosenshine et al., 1992; Bliska et al., 1993). The mechanisms by which a receptor transmits a signal to the eukaryotic cell cytoskeleton is not understood. However, some mammalian cell responses associated with receptor stimulation by bacteria, such as tyrosine phosphorylation, Ca^{2+} increase or cell activation have been characterized (Bliska et al., 1993).

Streptococcus uberis was observed within vacuoles in the cytoplasm for up to 8 h following infection of mammary epithelial cells, and by 24 h numerous bacteria were free in the cytoplasm and many appeared to replicate. However, no morphological changes indicative of cell damage were observed as a consequence of bacterial invasion (Matthews et al., 1994b). Following invasion of *S. dysgalactiae* into bovine mammary epithelial cells that appeared to occur by an endocytosis mechanism, no net intracellular growth of the organism was detected. Coculture of mammary epithelial cells with *S. dysgalactiae* did not appear to affect cell viability. However, lactate dehydrogenase activity increased in coculture supernatants suggesting that some cellular damage was induced by bacterial invasion (Almeida and Oliver, 1995). Preliminary studies showed changes in mammary epithelial cell gene expression in response to experimental infection by *S. agalactiae*. Expression of α-lactalbumin mRNA was inhibited while αS_1 casein mRNA was reduced slightly. In addition, expression of testosterone-repressed prostate mucin-2 mRNA, associated with apoptosis, increased during mastitis (Kotolski et al., 1993).

In vitro studies showed that *S. uberis* expressed several proteins during coculture with mammary epithelial cells that were not detected in bacterial cells cultured alone or in mammary epithelial cells cultured alone (Matthews et al., 1994c). Detection of these proteins via Western blot analysis with bovine serum obtained from cows following experimental or natural infection by *S. uberis* suggests that *S. uberis* may be expressing proteins in *in vitro* coculture that are possibly involved in the pathogenesis of IMI. Recent

studies demonstrated that protein and nucleic acid synthesis both in bacteria and mammary epithelial cells were required for invasion of *Staph. aureus* into mammary epithelial cells, indicating that prokaryotic and eukaryotic cell functions were involved in the invasion process. In addition, inhibition of receptor-mediated endocytosis decreased invasion of *Staph. aureus* into mammary epithelial cells suggesting that this can be a mechanism used by this bacterial species for entry into bovine mammary epithelial cells (Almeida et al., 1997).

Neutrophil migration across the epithelium

Macrophages are the predominant cell type in secretions from uninfected mammary glands of lactating cows (Lee et al., 1980). However, during IMI, the number of PMN in lacteal secretions increases dramatically. Neutrophils migrate from blood across the endothelial barrier towards chemoattractants generated at the site of injury. Chemoattractants bind to specific receptors on the PMN plasma membrane leading to changes in cell shape that favor subsequent diapedesis and migration into the inflamed area (Paape et al., 1991, Downey, 1994). At the infection site, the role of PMN is to ingest and kill invading mastitis pathogens in the presence of IgG_2 and IgM which recognize bacteria through Fab region and bind to PMN via Fc receptors. Neutrophils also bind bacteria by non-immunological recognition involving hydrophobic properties of cell surfaces, and nonspecific glycosylated receptors that recognize lectins on target organisms (Paape et al., 1991; Smith, 1994). Neutrophils rapidly enter mammary parenchyma and subsequently enter into milk stored in alveoli of mammary glands undergoing inflammatory reactions. Migration of neutrophils to the mammary gland due to chemotactic factors generated during inflammation continues for a variable period even

after disappearance of clinical signs. Research has been directed towards study of both the process of PMN migration into the mammary gland and the effect of PMN migration on the mammary secretory epithelial cell.

Harmon and Heald (1982) found that PMN did not pass freely into all alveoli during experimentally induced subclinical *Staph. aureus* mastitis in cows. Rather, PMN accumulated in the perivascular space outside alveoli that showed no damage from the infection, and between the basal portion of adjacent epithelial cells of alveoli that showed signs of damage. Migrating PMNs were associated frequently with gaps in epithelial cells left by cell lysis or sloughing of portions of the epithelial lining. No evidence was found that PMN traversed intact tight junctions between epithelial cells.

Once PMN reached the lumen, macrophages and lymphocytes infiltrated the parenchymal tissue displacing the basal secretory cell plasma membrane from the underlying basal lamina, which was found to be associated with epithelial cell damage during both naturally occurring and experimentally induced *Staph. aureus* mastitis (Nickerson and Heald, 1982; Sordillo et al., 1989). Increased numbers of leukocytes within the alveolar lumen were often associated with nonactive alveolar epithelium. In addition, milk components were shown to be present under the basal plasma membrane (Sordillo et al., 1989).

Considering the importance of PMN in the defense of the bovine mammary gland, the influence of *S. dysgalactiae* and *S. agalactiae* culture supernatants on various functions of bovine PMN such as attachment to endothelium, locomotion, ingestion of bacteria and oxidative metabolism was studied (Lambot et al., 1992). Only a slight reduction on phagocytosis kinetics was observed, suggesting that these mastitis pathogens did not produce *in vitro* factors that could hinder PMN activities. MacDonald et al. (1994) and Lin et al. (1995) assessed the effect of PMN migration through a mammary epithelial cell line monolayer toward a medium containing *Staph. aureus* and found no

evidence of disruption of tight junctions or damage to epithelial cells. This indicated a close association between PMN and epithelial cells during diapedesis with subsequent rapid resealing of tight junctional complexes. However, the mechanism by which PMN cause a transient dissolution of junctional complexes is not understood. Human recombinant tumor necrosis factor (TNF)-α has been shown to cause a rapidly reversible dose-dependent effect on the tight junctional region of kidney epithelial cells, lowering resistance and potential difference, and increasing solute flow between cells and across the epithelium (Mullin and Snock, 1990).

Release of microbicidal products from actively phagocytosing or degenerating PMN

Once phagocytosis is initiated, PMN microbicidal mechanisms consist of a combination of oxidative and enzymatic processes (Weiss, 1989; Paape et al., 1991). The former is known as the oxidative burst resulting in production of cytotoxic reactive oxygen species mediated by a membrane-associated enzyme complex, and the later, called degranulation, corresponds to release of enzyme-containing granules into the phagosome or phagocytic vacuole to form a phagolysosome (Weiss, 1989; Paape et al., 1991). Generation and release of reactive oxygen species occur when PMN are triggered by proinflammatory signals, and almost simultaneously granules fuse with the plasma membrane and discharge their contents both into the extracellular medium and into the phagosome (Weiss, 1989).

Host tissue damage by neutrophils can occur through premature activation during migration, release of microbicidal products during the killing process, removal of infected or damaged cells during tissue remodeling or failure to terminate an acute inflammatory response (Smith, 1994). Both reactive oxygen species and proteolytic enzymes of PMN

are considered to play a role in cytotoxicity and tissue destruction *in vivo*. Reactive oxygen species exert a direct effect or destroy the antiproteinase shield that prevents PMN proteolytic enzymes to attack and degrade host tissues, while proteolytic enzymes of PMN can exert a direct effect once the antiproteinase shield has been subverted. Proteolytic enzymes of neutrophil origin and oxidatively inactivated protease inhibitors have been detected in fluids isolated from inflammatory sites (Weiss, 1989).

In an attempt to determine the effect of PMN phagocytic function on neighboring cells, Capuco et al. (1986) cocultured intact, lysed and phagocytosing PMN with mammary tissue explants. The majority of PMN-treated explants showed gross swelling of the cell cytoplasm, cell sloughing of the basement membrane, nuclear pyknosis and vacuolation. In addition, increased NAGase activity was observed. However, rates of total protein and fatty acid synthesis were not decreased by coincubation with PMN.

Release of cytokines during the inflammatory response

Pathophysiological responses to exogenous and endogenous insult are a complex series of host reactions directed to prevent tissue damage, isolate and destroy the infective organism and activate tissue repair processes. Regardless of the initiating factor, macrophages are considered to be the cells that elicit this cascade of complex reactions known as the acute phase response, through release of a broad spectrum of mediators (van Miert, 1991; Baumann and Gauldie, 1994). Activated macrophages release cytokines which induce several clinical, hematological, blood biochemical and immunological responses, acting in concert with hormones derived from the pituitary, adrenal cortex and pancreas (Baumann and Gauldie, 1994). Among these cytokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF) are regarded as early inflammatory mediators that have

pleiotropic activity acting both locally and distally to initiate the acute phase response (Baumann and Gauldie, 1994). At the reactive site, IL-1 and TNF act on stromal cells to cause release of a secondary wave of cytokines which increases the homeostatic signal. As a consequence, local tissues generate highly chemotactic molecules for PMN and mononuclear cells which in turn release their own cytokines after migrating to target tissue (van Miert, 1991, Baumann and Gauldie, 1994). In addition, macrophages provide costimulatory signals for activation of T cells that result in production of IL-2, IL-4, IL-5 and interferon (IFN)-γ all of which are involved in regulation of adaptive responses (Campos et al., 1993).

The influence of cytokines on mammary epithelial cell function during inflammation is not fully understood. Following intramammary administration of *E. coli* endotoxin in the cow both systemic and local changes occur (Lohuis et al., 1988a;b; Shuster et al., 1991). Among the local changes, decreased milk production and altered milk composition have been detected (Lohuis et al., 1988b; Shuster et al., 1991). These changes are mediated by cytokines originating from the inflamed mammary gland either acting locally or after being absorbed systemically (Lohuis et al., 1988a;b). The decline in milk production is considered to be the result of both systemic and local stimuli. Shuster and Harmon (1991) induced a partial refractory state to endotoxin in lactating cows, characterized by a lack of response to cytokines, observing a recovery of milk production and fat composition, thus providing evidence to support the concept of a cytokine-mediated systemic mechanism for suppression of milk production. In addition, local suppression may result from a delayed response to locally produced inflammatory mediators or to effects of leukocytosis (Shuster et al., 1991).

Attempts have been made to assess effects of cytokines both in lactating and involuting mammary glands. Cows infused intramammarily with a single dose of recombinant bovine (rBo) interleukin-2 (IL-2) showed neither gross milk abnormalities

nor swelling of infused quarters. However, somatic cell counts and bovine serum albumin of quarters treated with 100 μg of rBo IL-2 increased significantly 12 h following infusion; the former remained elevated during a 48 h trial period while the latter decreased to pretreatment levels at 36 h post infusion (Sordillo et al., 1991). Mammary quarters infused with three doses of 100 mg of rBo IL-2 showed clinical signs of inflammation, while those infused with three doses of 25 or 100 μg showed increased serum albumin and decreased lactose concentration in milk, indicating increased permeability of the blood-milk barrier and disruption of cellular integrity (Sordillo et al., 1991). Involuting mammary glands infused with rBo IL-1 or IL-2 showed an increase in the number of somatic cells in milk; the former elicited mainly a neutrophil response and the latter a mononuclear cell response (Nickerson et al., 1993). Both lactating and involuting mammary glands infused with rBo IL-2 showed a significant reduction in alveolar luminal area and increase in interalveolar stromal area indicating acceleration of involution (Sordillo et al., 1991, Nickerson et al., 1992; 1993). Regarding composition of milk, no significant changes were observed following infusion of rBoIL-1 and rBoIL-2 during early involution of bovine mammary glands, indicating lack of adverse influence on mammary gland function (Rejman et al., 1995).

An *in vitro* study assessed the effect of rBoIL-1 and rBoIL-2 on mammary epithelial cell proliferation and showed that rBoIL-1b decreased proliferation of mammary epithelial cells in a dose dependent manner, while rBoIL-2 had no effect on proliferation of this cell line (Rejman et al., 1993). A recent study showed that IL-1 receptor antagonist (ra) can prevent IL-1 bioactivity in milk after endotoxin infusion. However, no effect was observed on local inflammation, systemic reaction or productive performance, leading to the speculation that either IL-1 is not the sole critical mediator of inflammation during endotoxin-induced mastitis or that intramammary infusion does not

deliver IL-1ra to the appropriate site of interaction of IL-1 with its receptor (Shuster and Kehrli, 1995).

Tumor necrosis factor is involved in early pathophysiological alterations after exposure to endotoxin (Remick et al., 1990). Binding of lipopolysaccharide (LPS)-LPS binding protein complex to CD14 receptors on monocytes, macrophages and PMN induced release of TNF-α which appeared to be responsible for induction of other endogenous mediators (van Miert, 1991). Infusion of TNF-α into the mammary gland of mice induced a rapid influx of PMN (Sanchez et al., 1994). Increased TNF-α was detected in milk and blood of cows after intramammary inoculation of *E. coli* or endotoxin (Sordillo and Peel, 1992; Shuster and Kehrli, 1995) and was associated with an unfavorable outcome of the disease (Sordillo and Peel, 1992). A ten-fold higher TNF-α concentration was observed in milk compared with serum suggesting that TNF-α is probably absorbed from the mammary gland rather than systemically induced following endotoxin absorption. Prophylactic administration of rBoIFN-γ prior to experimental challenge was associated with down-regulation of TNF-α production during acute *E. coli* mastitis (Sordillo and Peel, 1992). In addition, a recent *in vitro* study showed that priming of mammary gland macrophages with rBoIFN-γ following LPS stimulation down-regulated production of TNF-α by these cells. However, macrophage priming with rBoIFN-γ before LPS stimulation had no effect on TNF-α levels (Pighetti and Sordillo, 1994) emphasizing that complex interactions of multiple mediator cascades rather than a single critical factor are likely to determine the final outcome of the inflammatory process (van Miert, 1991). These studies have contributed to define the fundamental role that cytokines play during inflammation. However, knowledge of mechanisms by which these mediators can affect mammary epithelial cell function is still limited.

Summary and Conclusions

Environmental mastitis pathogens have become increasingly important during the last decade, particularly in well-managed dairy herds applying measures to control contagious mastitis pathogens. Among environmental mastitis pathogens, *S. dysgalactiae* is one of the most frequently isolated organisms. However, mechanisms involved in the transmission and pathogenesis of IMI caused by *S. dysgalactiae* are not understood.

Changes in milk composition and reduced milk yield are consequences of inflammation due to bacterial infection of bovine mammary glands. Host-pathogen interactions lead to an inflammatory response and subsequent impairment of mammary epithelial cell functions. However, there is only limited information on the series of events that take place during establishment of IMI and the primary deleterious factors that cause damage to the secretory epithelium. Host and bacterial factors have been dissociated to evaluate their potential for damaging the mammary epithelium and decreasing milk production. Effects of components of the inflammatory response and bacterial factors on mammary epithelial cell integrity have been studied using different *in vitro* models. This contributed to the present understanding of some pathogenic mechanisms involved in IMI caused by *Staph. aureus* and *E. coli*. However, information on mechanisms involved in establishment and persistence or IMI caused by environmental streptococci is scarce.

During the last decade, several potential virulence factors of *S. dysgalactiae* have been identified; yet, the relative importance of these factors in the pathogenesis of IMI is not known. Adherence to and invasion of mammary epithelial cells by mastitis pathogens are considered to be important pathogenic mechanisms that lead to establishment of IMI. However, there is only limited information on the role of bacterial and epithelial cell factors during these early host-pathogen interactions. This strongly points out the need for

a more fundamental understanding of bacterial components and mammary epithelial cell functions during infection with mastitis pathogens. Characterization of host-pathogen interactions that take place during the early stages of mammary gland infection by mastitis pathogens will enhance our understanding of pathogenesis of IMI which may contribute to development of methods to minimize production losses associated with IMI of dairy cows.

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CHAPTER II

FACTORS INFLUENCING ADHERENCE OF STREPTOCOCCUS DYSGALACTIAE TO BOVINE MAMMARY EPITHELIAL CELL MONOLAYERS

Abstract

Streptococcus dysgalactiae accounts for a significant number of intramammary infections during lactation and during the nonlactating period. In spite of its high prevalence, little information is available about factors than contribute to the virulence of *S. dysgalactiae*. Specific adherence to mammary epithelial cells may be an important stage in the pathogenesis of mastitis prior to the establishment of an intramammary infection. Adherence of streptococcal mastitis pathogens to epithelial cells has been studied using different *in vitro* models. However, limitations of these systems have led to the use of intact mammary epithelial cell monolayers to scrutinize early host-pathogen interactions. In this study, factors that affected adherence of *S. dysgalactiae* to monolayers of primary bovine mammary epithelial cells and to a bovine mammary epithelial transformed cell line were evaluated. Adherence of *S. dysgalactiae* was affected by both the mammary epithelial cell type and bacterial strain used. Cell culture medium was as effective as several blocking agents to decrease adventitious adherence of *S. dysgalactiae* to plastic. Bacterial adherence was mediated by saturable cell receptors, and reduction of bacterial adherence following cell fixation indicated that cell surface proteins played a major role in this process. Delineation of bacterial and epithelial cell factors influencing adherence of *S. dysgalactiae* to mammary epithelial cell monolayers resulted in development of an *in vitro* method for quantifying bacterial adherence which will be useful in subsequent studies to characterize mechanisms involved in the adherence process.

Introduction

Environmental mastitis has become a major problem in many well-managed dairy herds that have successfully controlled contagious mastitis pathogens (Oliver, 1988; Todhunter et al., 1995). In these herds, environmental streptococci account for a significant number of both subclinical and clinical mastitis (Hogan et al., 1989; Todhunter et al., 1995). However, little information is available about the epidemiology and factors that contribute to the virulence of environmental streptococci.

In animals in which the target tissue is not normally colonized by bacteria, initial colonization and later persistence usually involves bacterial adherence to tissues. This is especially important in organs in which mechanical clearance is a primary host defense mechanism (Costerton et al., 1981). Mastitis pathogens are subject to the flushing action of milk that acts as an important physical barrier to invasion. It is possible, however, that invading bacteria may resist the flushing effects of milk by adhering to tissue linings and replicating at a rate to maintain or expand their population (Arp, 1988; Ali-Vehmar and Sandholm, 1995). Accordingly, specific adherence of mastitis pathogens to mammary epithelial cells is likely an important stage in the pathogenesis of mastitis prior to the establishment of an intramammary infection (IMI) during lactation (Frost et al., 1977).

Adherence of streptococcal mastitis pathogens to epithelial cells has been studied using different *in vitro* models. Bovine mammary epithelial cells and bovine buccal mucosal cells in suspension were used to show adherence of *Streptococcus dysgalactiae* and *Streptococcus uberis* to epithelial cells (Frost et al., 1977; Wanasinghe, 1981; Valentin-Weigand et al., 1988). Thomas et al. (1992) used mammary gland explant cultures to study adherence, tropism and cytopathogenicity of mastitis pathogens for mammary gland tissue. Limitations of these methods such as decreased viability of scraped cells (Thomas et al., 1992), exposure of the basolateral surface in cells in

suspension (Freter and Jones, 1983), and exposure of subepithelial matrix components in tissue explants (Paape et al., 1995) have led to use of mammary epithelial cell monolayers to study early interactions between mastitis pathogens and host cells (Paape et al., 1995). Recent studies in our laboratory examined the influence of bacterial factors from *S. dysgalactiae* and *S. uberis* on adherence to cell monolayers of a transformed mammary epithelial cell line (Almeida et al., 1996; Calvinho et al., 1996). The objective of this study was to characterize factors that influence specific adherence of *S. dysgalactiae* isolated from bovine IMI to bovine mammary epithelial cell monolayers.

Materials and Methods

Mammary epithelial cells

Cells used in these experiments were an established transformed mammary epithelial cell line (MAC-T; Huynh et al., 1991), obtained from J.D. Turner, McGill University, Quebec, Canada and primary bovine mammary epithelial cells obtained from A.J. Guidry, USDA, Beltsville, MD. MAC-T cells were produced by transfecting primary bovine mammary alveolar cells with SV-40 large T-antigen (Huynh et al., 1991). Primary bovine mammary epithelial cells were collected from secretory tissue of lactating Holstein-Friesian cows and identified previously as fully differentiated mammary secretory cells based on presence of major milk proteins and staining for cytokeratin (Cifrian et al., 1994).

Cell culture medium

Cell culture medium (CCM) contained 40% RPMI 1640 (Sigma Chemical Co., St. Louis, MO), 40% Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Inc., Grand Island, NY), 10% fetal bovine serum (FBS; Bio Whittaker, Inc., Walkersville, MD), 1 mM sodium pyruvate (Sigma Chemical, Co.), 2 mM L-glutamine (Bio Whittaker, Inc.) and 40 mM HEPES buffer (Sigma Chemical Co.). The following supplements were added to the medium: bovine insulin (5μg/ml; Sigma Chemical Co.), hydrocortisone (1μg/ml; Sigma Chemical Co.), and bovine prolactin (1μg/ml; kindly provided by USDA, Animal Hormone Program, Beltsville, MD) (Cifrian et al., 1994).

Immunocytochemistry

Primary cells were further characterized as epithelial on the basis of specific staining for cytokeratin by a two-stage technique (Gibson et al., 1991). Cultured cells were plated at $< 10^5$ cells/chamber on 4-well polystyrene tissue culture chambers (Nunc, Inc., Naperville, IL) and incubated at 37° C in 5% CO_2 until confluent growth was reached. The cell culture was rinsed twice with Hanks balanced salt solution (HBSS, Gibco BRL) and fixed with Bouin's solution for 15 min at room temperature with rocking. Bouin's solution was removed and cells were rinsed with 70% ethanol and phosphate buffered saline (PBS, pH 7.6). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water and 2% normal goat serum in PBS was added as a protein block followed by incubation for 20 min at room temperature with rocking.

Excess liquid was shaken off. Primary mouse monoclonal antibodies to human cytokeratin and porcine vimentin (Dako Corporation, Carpinteria, CA) were added, followed by incubation for 30 to 60 min at room temperature with rocking. A goat anti-mouse antibody was used as the second antibody for cytokeratin and vimentin (HistoGen, BioGenex, San Ramon, CA). A peroxidase-anti-peroxidase kit (HistoGen, BioGenex) was used to complete the reaction using 3-amino-9-ethylcarbazole as the chromogen. Slides were counter stained with Mayer's hematoxylin and mounted with aqueous mounting medium (BioGenex). Presence of a reddish-brown color indicated a positive protein identification. Bovine fibroblasts and HeLa cells, provided by J.A. MacCabe, The University of Tennessee, Knoxville were used as positive and negative controls for cytokeratin and vimentin staining. Non-immune normal mouse serum (NMS) was used as a negative control for primary antibody. Mammary epithelial cell cultures were tested for the presence of mycoplasma contamination using a commercial test (Gibco, BRL).

Polymerase chain reaction amplification

To further characterize bovine primary cells, a DNA fragment between nucleotides 10,592 to 11,466 of the bovine κ-casein gene was amplified by polymerase chain reaction (PCR). A typical 50 µl PCR was performed as described by Pinder et al. (1991). DNA from bovine leukocytes and MAC-T cells were used as positive controls while DNA from HeLa cells and from a strain of *S. dysgalactiae* isolated from a cow with mastitis were used as negative controls. Amplified products of κ-casein PCR were separated by electrophoresis in 2% agarose (J.T. Baker Inc., Phillisburg, NJ) at 100 V for

2.45 h. Gels were stained with ethidium bromide (1μg/ml), and DNA was visualized by UV transillumination and photographed with type 55 Polaroid film.

Bacteria

Streptococcus dysgalactiae isolates used in these studies were identified by conventional biochemical tests and Rapid Strep identification system (bioMérieux, Vitek Inc., Hazelwood, MO). Organisms were serotyped using a streptococcal agglutination system (Streptex, Wellcome Diagnostics, Research Triangle Park, NC) containing A, B, C, D, F and G group-specific antibodies. Organisms were characterized further by PCR-based DNA fingerprinting (Jayarao and Oliver, 1994). Two strains of *S. dysgalactiae* were selected according to cluster analysis of PCR-based DNA fingerprint patterns among the population of *S. dysgalactiae* isolated from two dairy herds. *Streptococcus dysgalactiae* strain UT516 was isolated from a cow with subclinical mastitis and *S. dysgalactiae* strain UT519 was isolated from a cow with clinical mastitis. A strain of *Streptococcus pyogenes* (adherent) and *Escherichia coli* DH5α (Gibco, BRL, Bethesda, MD) (nonadherent) were used as controls.

Growth and preparation of bacteria

Bacteria were activated from frozen stocks (-80°C) by culture in skim milk (Difco Laboratories, Detroit, MI) for 18 h at 37°C. After activation, bacteria were subcultured in Todd Hewitt Broth (THB, Difco Laboratories) and incubated for 15 h at 37°C. Culture density was determined by measuring optical density at 400 nm, and counting colony forming units (CFU) by plating appropriate dilutions on brain heart infusion agar (BHIA, Becton Dickinson Microbiology Systems, Cockeysville, MD). Bacterial purity was determined by culture on blood agar.

Assay of blocking agents

Ability of blocking agents and CCM to reduce adventitious adherence of *S. dysgalactiae* to plastic was evaluated. Agents included 0.5% bovine serum albumin (BSA, Sigma Chemical Co.), 2 mg/ml hemoglobin (Sigma Chemical Co.), and 0.01% of detergent Tween 20 (Sigma Chemical Co.). Each agent was dissolved in PBS at the indicated concentrations, 100 µl were added to triplicate wells and plates were incubated at 37° C for 1 h. *Streptococcus dysgalactiae* strains UT516 and UT519 were grown in THB for 16 h at 37° C, harvested, rinsed twice and resuspended in PBS. Appropriate dilutions of bacterial suspensions were prepared to yield an approximate concentration of 10^7 CFU/ml and 100 µl of each suspension were added to each well. Following incubation at 4° C for 1 h, plates were washed three times with 5% nonfat dry milk in PBS and then three times with PBS. After each washing, plates were vortex-shaken for 10 sec to remove non-adherent bacteria. Adherent bacteria were released by addition of 25 µl of 0.5% Triton X-100 (Sigma Chemical Co.) for 10 min. Sterile saline (75 µl) was added and each sample was mixed vigorously. Adherent bacteria were quantified by plating appropriate dilutions on BHIA (Miles et al., 1938). Experiments were performed in triplicate and repeated at least twice. Results were expressed as percentage of the initial number of bacteria added to wells.

Adherence assay

Confluent monolayers of mammary epithelial cells in 96-well tissue culture plates were rinsed twice with CCM. Bacteria were harvested, rinsed twice and resuspended in PBS. To disrupt chains and clusters, bacterial suspensions were sonicated using a 2.4 mm microprobe tip for 6 sec at setting 10 of an ultrasonic cell disrupter (Microson Ultrasonic Cell Disrupter, Model MS-50, Heat Systems Ultrasonics, Inc., Farmingdale, NY). These settings yielded the highest number of CFU without affecting bacterial viability (data not

shown). Appropriate dilutions of bacterial suspensions were prepared and 100 µl of each suspension were added to each well. Experiments were performed in triplicate and repeated at least twice. Number of mammary epithelial cells per well was determined by counting in a hemocytometer to further estimate the bacteria:epithelial cell ratio used in each experiment. Control wells without epithelial cells were prepared in a similar manner to quantify nonspecific bacterial adherence to plastic. Plates were centrifuged for 10 min at 500 x g in a plate spinner and subsequently incubated for 1 h at 4° C. This temperature was used to prevent intracellular invasion (Almeida and Oliver, 1995). Wells were aspirated and washed three times with 5% nonfat dry milk in PBS and then three times with PBS. After each addition of buffer, the plate was vortex-shaken for 10 sec to remove non-adherent bacteria (Tamura et al., 1994). Adherent bacteria were released by addition of 25 µl of 0.5% Triton X-100 for 10 min. Sterile saline (75 µl) was added and each sample was mixed vigorously. Adherent bacteria were quantified by plating appropriate dilutions on BHIA. Specific adherence to mammary epithelial cells was expressed as the total number of CFU minus the number of CFU adherent to wells without cells.

Cell fixation

Primary bovine mammary epithelial cell monolayers were grown to confluence in 96-well tissue culture plates, washed once with PBS and fixed for 10 min in 0.5% electron microscopy-grade glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS. Cell monolayers were washed twice with PBS, treated for 5 min with 0.1 M glycine (Sigma Chemical Co.) in PBS and washed three times with 5% nonfat milk in PBS and three times with PBS (Tamura et al., 1994). Adherence of *S. dysgalactiae* strain UT516 to fixed and unfixed cells was performed simultaneously at 4°C.

Statistical analysis

Data on adherence of bacteria to plastic were expressed as a percentage of the original inoculum. Efficacy of blocking agents was compared by the mixed procedure of SAS. Means were separated by Fisher's least significant differences test (SAS 1994). Adherence of *S. dysgalactiae* strain UT516 to fixed and unfixed cell monolayers was compared by Student's *t* test.

Results

Characterization of mammary epithelial cells

Primary bovine mammary epithelial cells formed confluent cobblestone-like monolayers, and stained positive for cytokeratin and vimentin while MAC-T cells stained positive for cytokeratin only (Figure 1). Staining was not observed when primary antibodies to cytokeratin and vimentin were replaced by non-immune NMS. PCR resulted in amplification of a 874 basepair (bp) DNA fragment of bovine κ-casein from primary bovine mammary epithelial cells, MAC-T cells and bovine leukocytes. No DNA fragments consistent with these sizes were detected from HeLa cell or *S. dysgalactiae* DNA (Figure 2).

Assay of blocking agents

Various agents were evaluated for their ability to block nonspecific binding of *S. dysgalactiae* strains UT516 and UT519 to plastic. Percentage blocking achieved with

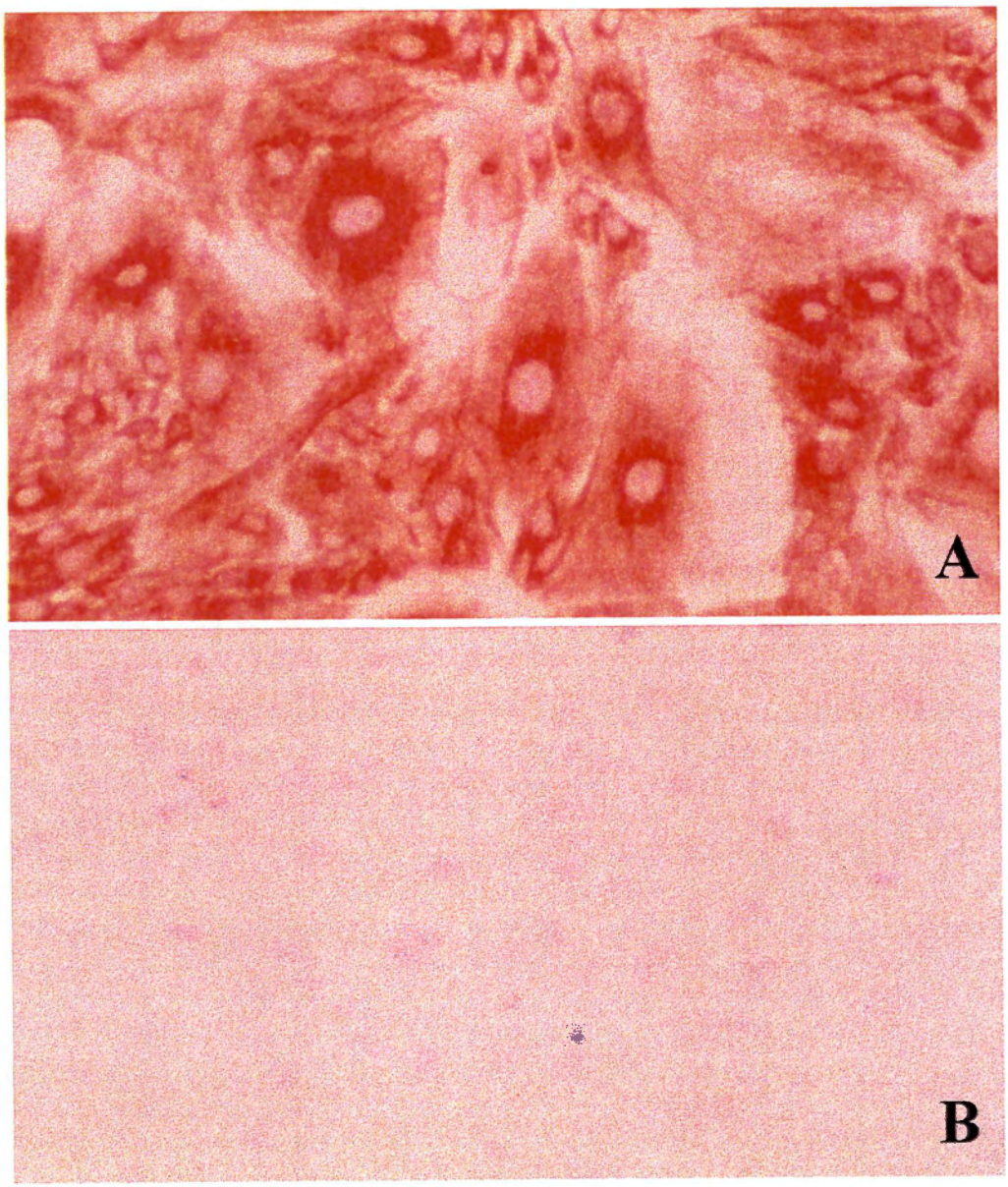

Figure 1. Cytokeratin immunostaining of primary bovine mammary epithelial cells monolayer. A: monolayer stained using mouse monoclonal antibodies to cytokeratin displayed positive diffuse staining. B: negative control using of normal mouse serum instead of primary antibodies.

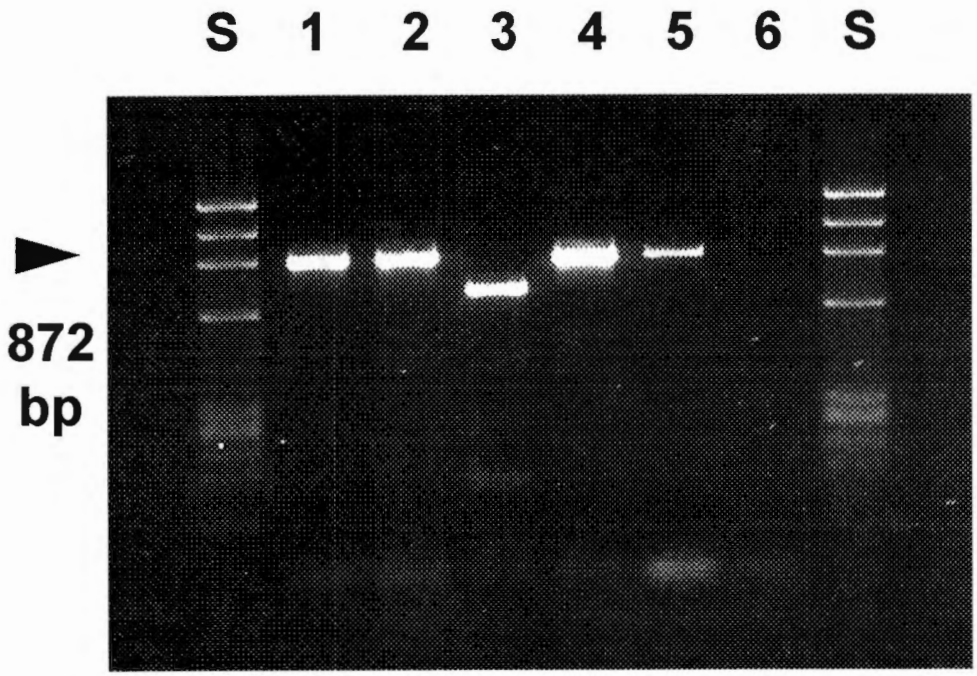

Figure 2. Polymerase chain reaction amplification of a 874 basepair DNA fragment of bovine κ-casein. Lanes 1 and 2: primary bovine mammary epithelial cells; lane 3: HeLa cells; lane 4: MAC-T cells; lane 5: bovine leukocytes and lane 6: *S. dysgalactiae*. Lanes S: *Hae*III digested ΦX174 DNA restriction fragment size markers (in basepairs).

these agents was compared to blocking with CCM. Significant differences were detected between blocking with BSA and CCM for both bacterial strains tested ($P<0.05$). None of the agents evaluated was as effective as CCM alone to block nonspecific binding to plastic (Table 2).

Adherence of *S. dysgalactiae* to mammary epithelial cells

Streptococcus dysgalactiae strains UT516 and UT519 adhered to both primary bovine mammary epithelial cells and to MAC-T cells. Since the process of bacterial adherence to a substrate follows the rules of equilibria (Ofek and Doyle, 1994), the extent of adherence of *S. dysgalactiae* strains UT516 and UT519 to mammary epithelial cells was tested using several different bacterial densities keeping the number of mammary epithelial cells constant. Mean number of confluent primary bovine mammary epithelial cells per well was 11,570 (SD 2,756) while mean number of MAC-T cells per well was 44,534 (SD 9,355). Number of adherent bacteria was measured as a function of bacteria added to the cell monolayer. Adherence of *S. dysgalactiae* strains UT516 and UT519 to mammary epithelial cells was proportional to the number of bacterial cells added until a saturation point was reached. This allowed selection of inocula sizes below saturating bacterial densities. Results of three separate experiments of adherence of *S. dysgalactiae* strain UT516 to primary bovine mammary epithelial cells are presented in Figure 3. Since adherence assays were performed with confluent epithelial cell monolayers to minimize nonspecific adherence of bacteria to unoccupied plastic space, subtracting adherence of bacteria to wells without epithelial cells necessarily led to overestimation of nonspecific

Table 2. Blocking of nonspecific binding of *Streptococcus dysgalactiae* strains UT516 and UT519 to plastic by various agents.

	% Inhibition of binding ± SEM			
Blocking agent	*S. dysgalactiae* UT516		*S. dysgalactiae* UT519	
Cell culture medium	(n=15)	99.59 ± 0.17[a]	(n=15)	99.14 ± 0.26[a]
BSA (0.5%)	(n=9)	90.02 ± 4.97[b]	(n=9)	96.19 ± 1.56[b]
Hemoglobin (2 mg/ml)	(n=15)	95.78 ± 1.14[a]	(n=9)	98.22 ± 1.06[a]
Tween 20 (0.01%)	(n=6)	97.85 ± 0.15[a]	(n=9)	97.94 ± 0.38[a]

[a, b] Different letters within a column indicate significant differences ($P<0.05$).

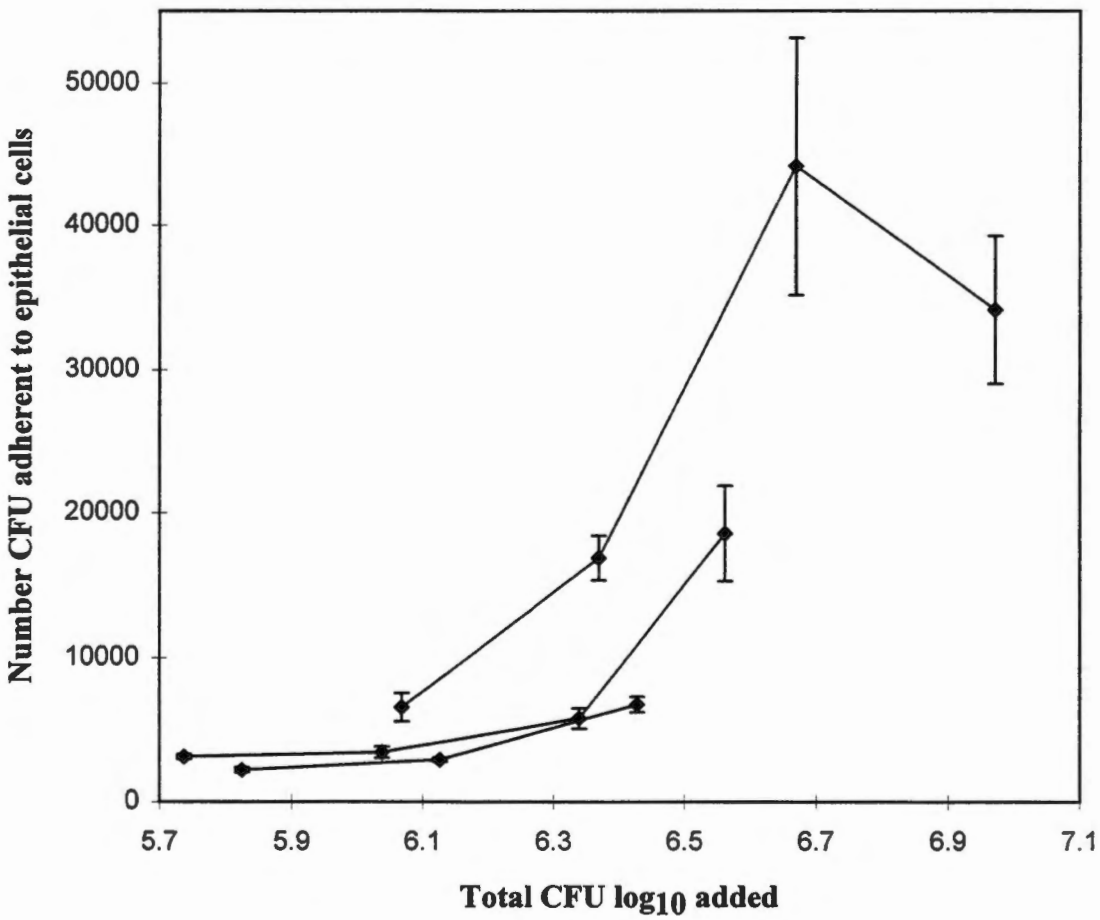

Figure 3. Binding of *Streptococcus dysgalactiae* strain UT516 to primary bovine mammary epithelial cell monolayers. Increasing bacterial numbers were added to mammary epithelial cell monolayers. Data represent results of three separate experiments. Error bars (representing SEM) were omitted if bars were smaller than symbols.

bacterial binding to plastic (Ofek et al., 1986). Therefore, differences in viable counts between wells containing epithelial cells and those without cells indicated only specific bacterial adherence to cells. To further illustrate this phenomenon, higher bacterial densities were added to cell monolayers and both adherence to cells and to plastic were measured as a function of total number of bacteria added. At high bacterial densities, adherence to plastic was higher than adherence to cells (Figure 4). Based on these results, bacteria:epithelial cell ratios of <1500:1 were used in subsequent adherence experiments. Percent adherence of *S. dysgalactiae* strains UT516 at several bacteria:epithelial cell ratios below 1500:1 averaged 0.45% (SEM 0.039) and 2.85% (SEM 0.33) to primary cells and MAC-T cells, respectively. Percent adherence of *S. dysgalactiae* UT519 to MAC-T cells averaged 3.72% (SEM 0.57).

Effect of cell fixation on adherence

Adherence of *S. dysgalactiae* strain UT516 to fixed and unfixed primary bovine mammary epithelial cell monolayers was measured to evaluate whether glutaraldehyde fixation modified determinants on the epithelial cell important for bacterial adherence. The experiment was performed in triplicate wells and repeated three times. Adherence of *S. dysgalactiae* strain UT516 to fixed cell monolayers was reduced more than 90% compared to adherence to unfixed cell monolayers ($P<0.05$).

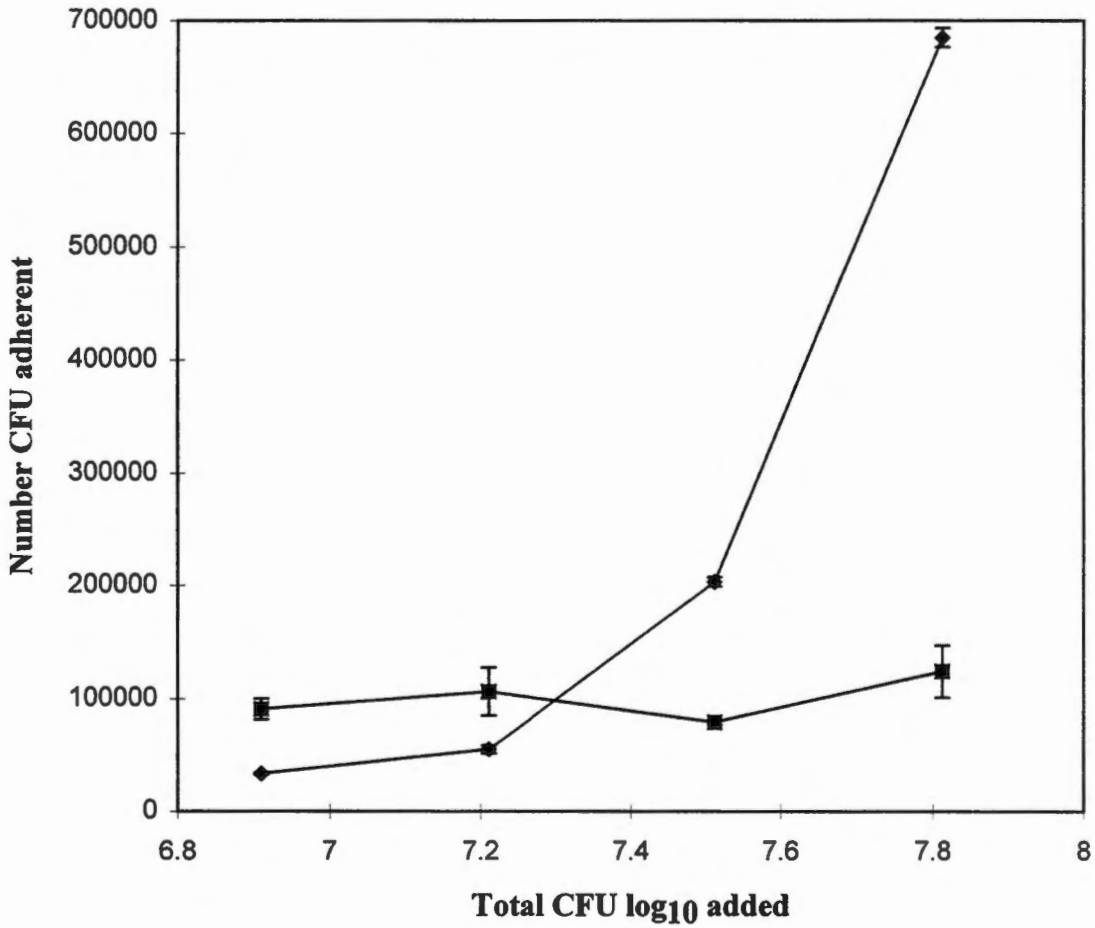

Figure 4. Binding of *Streptococcus dysgalactiae* strain UT516 to primary bovine mammary epithelial cells and to plastic. Increasing bacterial numbers were added to wells with and without mammary epithelial cell monolayers. Squares represent adherence to mammary epithelial cells and diamonds represent adherence to plastic. Error bars (representing SEM) were omitted if bars were smaller than symbols.

Discussion

Adherence of *S. dysgalactiae* to bovine mammary epithelial cells has been studied using different *in vitro* models. *Streptococcus dysgalactiae* adhered to cells in suspension obtained from scrapings of teat sinus, lactiferous sinus and major ducts from recently slaughtered cows (Frost et al., 1977, Wanasinghe, 1981). However, this method was found to yield a high percentage of damaged cells which can alter bacterial adherence (Thomas et al., 1992). In addition, the basolateral cell surface that is not exposed *in vivo*, is exposed in cells in suspension (Freter and Jones, 1983), and can harbor normal microbiota that may occupy sites under investigation (Ofek and Doyle, 1994). *Streptococcus dysgalactiae* did not appear to adhere to epithelial cells in mammary explants, but showed a preferential tropism for the exposed connective tissue in the explant periphery (Thomas et al., 1992). Mammary explants offer the advantage that target cells are in place in relation to other supporting tissues (Thomas et al., 1992); however, subepithelial matrix components highly susceptible to bacterial adherence are exposed, thus making interpretation of host-pathogen interactions difficult (Paape et al., 1995). Use of intact mammary epithelial cell monolayers has minimized some of the limitations exhibited by the former methods. Cell monolayers allow for direct observation of pathogen/host cell interface and do not exhibit subepithelial matrix components that are not exposed under *in vivo* conditions (Cifrian et al., 1994; Paape et al., 1995).

Primary bovine mammary cells used in this study were characterized as epithelial on the basis of morphology and specific staining for cytokeratin, which is a distinct characteristic of epithelial cells (Cooper et al., 1985). These cells, however, also expressed vimentin immunoreactivity, which has been reported in cultured epithelial cells (Munson et al., 1988; Nisolle et al., 1995). Although a double-staining procedure was not used, staining for both intermediate filaments was performed simultaneously, cells in

each preparation had the same morphology and all cells stained for both intermediate filaments ruling out the possibility of contamination. In addition, primary bovine mammary epithelial cells were further characterized based on the presence of DNA coding for a fragment of bovine κ-casein. Primary cultures of bovine mammary epithelial cells and a transformed bovine mammary epithelial cell line have been used as an *in vitro* model to study cytotoxicity as well as adherence of bacterial pathogens to epithelial cells. Primary cultures of mammary epithelial cells, although placed in an unnatural environment, are considered to resemble more closely actual cells *in vivo* (Cifrian *et al.*, 1995; Olmsted and Norcross, 1992); while transformed mammary epithelial cells offer the advantage of homogeneity of target cells used in each experiment, although the process of transformation might affect expression of surface receptors (Goldschmidt and Panos, 1984; Olmsted and Norcross, 1992).

Quantification of adherent bacteria to a defined area using a single bacterial density in triplicate is used commonly in adherence experiments (Ofek and Doyle, 1994). However, it is important to determine the extent of bacterial adherence to a substrate since for most types of adherence studies the use of saturating cell densities is not recommended (Ofek and Doyle, 1994). In this study, adherence of *S. dysgalactiae* to intact monolayers of both primary bovine mammary epithelial cells and a transformed cell line was shown. In agreement with previous *in vitro* studies using different methodologies, adherence to cells was saturable at high bacterial densities indicating that cells had a limited numbers of sites where bacteria could bind (Ofek and Doyle, 1994). In addition, results from the present study identified bacterial densities not approaching saturation for use in subsequent studies designed to evaluate effects of various adherence inhibitors (Ofek and Doyle, 1994).

An incubation temperature of 37° C has been associated with a higher degree of bacterial adherence to ovine mammary epithelial cells (Amorena et al., 1990). However,

previous studies performed in our laboratory demonstrated that *S. dysgalactiae* invades epithelial cells in intact monolayers at 37° C (Almeida and Oliver, 1995). Attempts were made to provide a test system in which bacteria did not invade mammary epithelial cells other than using low temperature. Use of glutaraldehyde-fixed cell monolayers has been reported as a method to prevent bacterial invasion into cultured cells (Kusters et al., 1993; Tamura et al., 1994). The effect of glutaraldehyde fixation of mammary epithelial cells on adherence of *S. dysgalactiae* strain UT516 was evaluated. A significant decrease in adherence to glutaraldehyde-fixed cells compared to unfixed cells indicated that cross-linking between adjacent proteins that takes place during fixation with aldehydes affected bacterial binding to mammary epithelial cells. Therefore, a non-permissive temperature of 4°C (Tamura et al., 1994) was used throughout this study to more accurately assess bacterial adherence to mammary epithelial cells.

Percent adherence of *S. dysgalactiae* strain UT516 was higher to MAC-T cells than to primary bovine mammary epithelial cells. This cannot be attributed to the higher number of cells per well in MAC-T monolayers since similar bacterial:epithelial cell ratios were taken into consideration for comparisons. Differences in binding affinity of groups A and B streptococci between freshly obtained and transformed cell lines have been observed and have been considered to reflect distinct availability of receptors in either cell type (Goldschmidt and Panos, 1984; Courtney et al., 1992).

In conclusion, adherence of *S. dysgalactiae* isolated from bovine IMI to mammary epithelial cells was affected by the mammary epithelial cell type used. Measurement of the extent of adherence to mammary epithelial cells strongly indicated that the process was mediated by saturable cell receptors, and reduction of bacterial adherence following cell fixation indicated that cell surface proteins played a major role in this process. In addition, determination of bacterial and cell factors influencing adherence of *S. dysgalactiae* to mammary epithelial cell monolayers resulted in development of an *in*

vitro method for quantifying bacterial adherence which can be used in subsequent experiments to characterize mechanisms involved in this process.

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CHAPTER III

CHARACTERIZATION OF MECHANISMS INVOLVED IN ADHERENCE OF STREPTOCOCCUS DYSGALACTIAE TO BOVINE MAMMARY EPITHELIAL CELLS

Abstract

Specific adherence of *Streptococcus dysgalactiae* to mammary epithelial cells may be an important stage in the pathogenesis of mastitis prior to the establishment of intramammary infection; however, mechanisms by which this organism adheres to host surfaces are not understood. In this study, presence of two bacterial factors, lipoteichoic acid (LTA) and M-like protein, potentially involved in adherence of *S. dysgalactiae* to mammary epithelial cells, was detected in strains of *S. dysgalactiae* isolated from bovine intramammary infections. Role of these factors, as well as enzymatic modification of the bacterial surface, on adherence of *S. dysgalactiae* to mammary epithelial cells was evaluated. Results indicated that bacterial surface proteins played a major role in adherence of *S. dysgalactiae* to mammary epithelial cells. However, use of antibodies directed against streptococcal M24 protein did not affect adherence of *S. dysgalactiae* to mammary epithelial cells. LTA appeared to play a minor role in adherence of one strain of *S. dysgalactiae* to mammary epithelial cells but had no effect on another strain of *S. dysgalactiae*. Additional research on identification and isolation of *S. dysgalactiae* surface structures involved in adherence to mammary epithelial cells should be conducted to determine their role in establishment and progression of intramammary infection in dairy cows.

Introduction

Mastitis caused by environmental pathogens is a major problem that affects many well-managed dairy herds that apply a program to control contagious mastitis pathogens (Oliver, 1988; Todhunter et al., 1995). Among the environmental pathogens, *Streptococcus dysgalactiae* is isolated frequently from intramammary infections (IMI) during lactation and during the nonlactating period (Oliver, 1988; Oliver et al., 1990). In spite of its high prevalence, little is known about the epidemiology and factors that contribute to the virulence of *S. dysgalactiae*.

Bacterial adherence to tissues is considered to be an important step in the pathogenesis of infections in which mechanical clearance by host fluids is a primary host defense mechanism (Costerton et al., 1981). Mastitis pathogens are subject to the flushing action of milk that acts as an important physical barrier to invasion. It is possible, however, that invading bacteria may resist the flushing effects of milk by adhering to tissue linings and replicating at a rate to maintain or expand their population (Arp, 1988; Ali-Vehmar and Sandholm, 1995).

Streptococcus dysgalactiae of bovine origin adhered to freshly isolated cells obtained from scrapings of mammary epithelium (Frost et al., 1977), and bovine buccal mucosal cell suspensions (Valentin-Weigand et al., 1988). However, mechanisms by which *S. dysgalactiae* adheres to host surfaces are not understood. *Streptococcus dysgalactiae* bound to fibronectin (Fn), fibrinogen, collagen, laminin, and vitronectin (Mamo et al., 1987; Valentin-Weigand et al., 1988; Filippsen et al., 1990; Lindgren et al., 1992). The organism interacted with a 210-kDa C-terminal fragment of Fn. However, binding involved the host attachment domain of Fn and therefore was not considered to mediate streptococcal adherence to oral bovine epithelial cells (Valentin-Weigand et al., 1988). Conversely, Lindgren et al. (1992) found that *S. dysgalactiae* strain S2 isolated

from bovine milk bound to a site in the amino-terminal region of Fn, confirming previous findings of Mamo et al. (1987). *Streptococcus dysgalactiae* was also shown to specifically bind bovine S protein (vitronectin) through a region that did not involve the cell-binding domain of the protein, leading to the speculation that S protein can mediate adherence of *S. dysgalactiae* to host cells (Filippsen et al., 1990).

Bacterial surface hydrophobicity has also been related to adherence of several bacterial species to host cells (Rosenberg and Kjelleberg, 1989; Courtney et al., 1990). A strain of *S. dysgalactiae* expressing low surface hydrophobicity did not adhere as well to mammary epithelial cell monolayers as a hydrophobic strain (Calvinho et al., 1996b). Trypsin treatment significantly reduced adherence of the hydrophobic strain to mammary epithelial cells whereas adherence of the hydrophilic strain remained unaltered (Calvinho et al., 1996b).

Adherence of *Streptococcus pyogenes* (Group A) to epithelial cells has been proposed to follow a two-step kinetic model involving lipoteichoic acid (LTA) and M-protein (Courtney et al., 1992). In addition, LTA is considered to be associated with adherence of *Streptococcus agalactiae* (Group B) to epithelial cells (Nealon and Mattingly, 1984; Teti et al., 1987). Recent studies in our laboratory showed that preincubation of mammary epithelial cell monolayers with LTA and/or treatment of *Streptococcus uberis* with antibodies directed against the carboxy-terminal of type 24 M-protein reduced adherence of both encapsulated and nonencapsulated strains of *S. uberis* to mammary epithelial cells (Almeida et al., 1996). Thus, LTA and M-protein may be two important streptococcal virulence determinants involved in adherence to epithelial cells. However, additional research is needed to substantiate this hypothesis.

In the present study, experiments were conducted to characterize mechanisms involved in adherence of *S. dysgalactiae* isolated from bovine IMI to mammary epithelial cell cultures. Objectives were to detect the presence of bacterial factors potentially

involved in adherence to epithelial cells in strains of *S. dysgalactiae* isolated from bovine IMI, and to evaluate the role of these factors in bacterial adherence to bovine mammary epithelial cells *in vitro*.

Materials and Methods

Mammary epithelial cells

Cells used in these experiments were an established transformed mammary epithelial cell line (MAC-T; Huynh, et al., 1991) obtained from J.D. Turner, McGill University, Quebec, Canada and primary bovine mammary epithelial cells obtained from A.J. Guidry, USDA, Beltsville, MD. Primary bovine mammary epithelial cells were collected from secretory tissue of lactating Holstein-Friesian cows and reported previously as fully differentiated mammary secretory cells based on presence of major milk proteins and staining for cytokeratin (Cifrian et al., 1994). Bovine mammary epithelial cells were further characterized by specific staining for cytokeratin and vimentin, and polymerase chain reaction (PCR) amplification of a DNA fragment between nucleotides 10,592 to 11,466 of the bovine κ-casein gene as described previously in chapter II. Cell cultures were tested for mycoplasma contamination using a commercial test (Gibco, BRL, Life Technologies, Inc., Grand Island, NY).

Cell culture medium

Cell culture medium (CCM) contained 40% RPMI 1640 (Sigma Chemical Co., St. Louis, MO), 40% Dulbecco's modified Eagle's medium (DMEM; Gibco BRL), 10% fetal bovine serum (FBS; Bio Whittaker, Inc., Walkersville, MD), 1 mM sodium pyruvate (Sigma Chemical, Co.), 2 mM L-glutamine (Bio Whittaker, Inc.) and 40 mM HEPES

buffer (Sigma Chemical Co.). The following supplements were added to the medium: bovine insulin (5μg/ml; Sigma Chemical Co.), hydrocortisone (1μg/ml; Sigma Chemical Co.), and bovine prolactin (1μg/ml; USDA, Animal Hormone Program, Beltsville, MD) (Cifrian et al., 1994).

Bacteria

Streptococcus dysgalactiae strains used in these studies were identified by conventional biochemical tests and Rapid Strep identification system (bioMérieux Vitek, Inc., Hazelwood, MO). Organisms were serotyped using a streptococcal agglutination system (Streptex, Wellcome Diagnostics, Research Triangle Park, NC) containing A, B, C, D, F and G group-specific antibodies. Organisms were characterized further by PCR-based DNA fingerprinting (Jayarao and Oliver, 1994). Four strains of *S. dysgalactiae* were selected according to cluster analysis of PCR-based DNA fingerprint patterns among the population of *S. dysgalactiae* isolated from two dairy herds. *Streptococcus dysgalactiae* strains UT516 and UT857 were isolated from cows with subclinical mastitis and *S. dysgalactiae* strains UT519 and UT265 were isolated from cows with clinical mastitis. A strain of *Streptococcus pyogenes* (adherent) and *Escherichia coli* DH5α (Gibco, BRL, Bethesda, MD) (nonadherent) were used as controls.

Growth and preparation of bacteria

Bacteria were activated from frozen stocks (-80° C) by culture in skim milk (Difco Laboratories, Detroit, MI) for 18 h at 37° C. After activation, bacteria were subcultured in Todd Hewitt Broth (THB, Difco Laboratories) and incubated for 15 h at 37° C. Culture density was determined by measuring optical density at 400 nm, and counting colony forming units (CFU) by plating appropriate dilutions on brain heart

infusion agar (BHIA, Becton Dickinson Microbiology Systems, Cockeysville, MD). Bacterial purity was determined by culture on blood agar.

Detection of M-like protein and lipoteichoic acid by SDS-PAGE and immunoblot analysis

Bacterial proteins were solubilized by adding 100µl of reducing buffer (62.5mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate, 5% β-mercaptoethanol) to 10 mg wet weight of bacteria. This suspension was heated at 95° C for 2 min and unsolubilized material was removed by centrifugation for 5 min at 14,000 x g. Proteins were separated by SDS-PAGE in a 10% separating gel (Laemmli, 1970), transferred electrophoretically to nitrocellulose (Towbin et al., 1979) using an electroblotting system (MilliBlot-SDE, Millipore Corporation, Bedford, MA) and detected by rabbit polyclonal antibodies raised against the distal 180 amino acids of the amino-terminal half region of *S. pyogenes* M24 protein. Polyclonal antibodies to M24 protein were provided by M.S. Bronze, The University of Tennessee, Memphis.

Nitrocellulose membranes were blocked with 0.1% gelatin and 0.05% bovine gamma globulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.05% Tween 20 (Sigma Chemical Co.) in PBS, pH 7.3. Membranes were washed three times with wash buffer (WB, 0.05% Tween 20 in PBS, pH 7.3), blots were developed with peroxidase-conjugated second antibody ($F(ab')_2$ fragment donkey anti-rabbit IgG; (Jackson ImmunoResearch Laboratories) and stained with 4-chloro-1-naphthol (Bio-Rad Laboratories, Hercules, CA). Use of normal rabbit serum instead of anti-M24 and omission of the first antibody were used as negative controls. To detect presence of IgG-binding activity in strains of *S.dysgalactiae,* a biotin-conjugated bovine IgG Fc fragment (Jackson ImmunoResearch Laboratories) was used instead of the first antibody, and an

avidin-peroxidase conjugate (Molecular Probes, Inc., Eugene, OR) was used instead of the second antibody.

Detection of LTA in strains of *S. dysgalactiae* was performed using a dot-blot technique (Towbin et al., 1979). Bacterial suspensions containing approximately 1 x 10^{11} CFU/ml in PBS were applied to a 0.22 µm pore size nitrocellulose (Bio-Rad Laboratories) and blocked with blocking buffer (0.1% gelatin, 0.05% bovine gamma globulin (Jackson ImmunoResearch Laboratories), 1% goat serum (Sigma Chemical Co.) in 0.05% Tween in PBS, pH 7.3. Membranes were washed three times with WB and incubated with a 1:500 dilution of murine IgM monoclonal antibody (Mab) directed against the polyglycerophosphate backbone of *S. pyogenes* LTA at room temperature overnight. Anti-LTA Mab were obtained from J.J. Drabick, Walter Reed Army Institute of Research, Washington, DC. Membranes were washed and incubated with peroxidase-conjugated second antibody ($F(ab')_2$ fragment goat anti-mouse IgM, µ chain specific (Jackson ImmunoResearch Laboratories) and stained as mentioned for Western blotting. Lipoteichoic acid (Sigma Chemical Co.) and *S. pyogenes* were used as positive controls. Omission of first antibody, normal mouse serum instead of first antibody and a reference strain of *Salmonella typhimurium* (14028) from the American Type Culture Collection (ATCC), Rockville, MD were used as negative controls. A reference strain of *S. dysgalactiae* (ATCC 27957) was included in this experiment.

Adherence assay

Confluent monolayers of mammary epithelial cells in 96-well tissue culture plates were rinsed twice with CCM. Bacteria were harvested, rinsed twice and resuspended in PBS. To disrupt chains and clusters, bacterial suspensions were sonicated using a 2.4 mm microprobe tip for 6 sec at setting 10 of an ultrasonic cell disrupter (Microson Ultrasonic Cell Disrupter, Model MS-50, Heat Systems Ultrasonics, Inc., Farmingdale, NY). These

settings yielded the highest number of CFU without affecting bacterial viability (data not shown). Appropriate dilutions of bacterial suspensions were prepared and 100 µl of each suspension were added to each well. Experiments were performed in triplicate and repeated at least twice. Number of mammary epithelial cells per well was determined by counting in a hemocytometer to further estimate the bacteria:epithelial cell ratio used in each experiment. Control wells without epithelial cells were prepared in a similar manner to quantify nonspecific bacterial adherence to plastic. Plates were centrifuged for 10 min at 500 x g in a plate spinner and subsequently incubated for 1 h at 4° C. This temperature was used to prevent intracellular invasion (Almeida and Oliver, 1995). Wells were aspirated and washed three times with 5% nonfat dry milk in PBS and then three times with PBS. After each addition of buffer, the plate was vortex-shaken for 10 sec to remove non-adherent bacteria (Tamura et al., 1994). Adherent bacteria were released by addition of 25 µl of 0.5% Triton X-100 (Sigma Chemical Co.) for 10 min. Sterile saline (75 µl) was added and each sample was mixed vigorously. Adherent bacteria were quantified by plating appropriate dilutions on BHIA (Miles et al., 1938). Specific adherence to mammary epithelial cells was expressed as the total number of CFU minus the number of CFU adherent to wells without cells.

Modification of bacterial surface

Approximately 5 x 10^8 bacteria per ml were incubated at 37° C for 1 h with or without 1.2 mg of trypsin (Gibco BRL) per ml in PBS pH 7.4, and in 50 µg per ml of pepsin (Sigma Chemical Co.) in KH_2 PO_4-Na_2HPO_4, 0.067 M buffer at pH 5.8. To stop enzymatic reactions, 1 mg/ml of soybean trypsin inhibitor (Sigma Chemical Co) was added to trypsin incubation and 7.5% $NaHCO_3$ to pepsin incubation to raise the pH to 7.4. After incubation, bacteria were washed two times with PBS. Viable bacterial counts were performed before and after treatment to determine if treatment affected viability. The

adherence assay was carried out concurrently with treated and nontreated bacteria. Following enzymatic treatment, bacterial proteins were solubilized and separated by 10% SDS-PAGE as described above. Gels were silver stained (Oakley et al., 1980), and M-like protein was detected by Western blotting as described previously.

Treatment with LTA and antibodies

MAC-T cells were pretreated with increasing concentrations of LTA (100 to 500 μg/ml) for 30 min at 37 C°. Following treatment, cells were washed twice with PBS and bacterial adherence to MAC-T cells was measured by the protocol described previously. Cells treated with PBS were used as positive control for adherence. Since in a previous study it was determined that LTA inhibited proliferation of bovine mammary epithelial cells *in vitro* (Calvinho et al., 1996a), effect of LTA on mammary epithelial cell viability was assessed by trypan blue exclusion following incubation of cells for 30 min at 37° C with 100 to 500 μg/ml of LTA. Triplicate wells were used for each LTA concentration. Mammary epithelial cells incubated with PBS were used as control for viability. Bacterial suspensions were treated with murine IgM Mab directed against the polyglycerophosphate backbone of *S. pyogenes* LTA and with rabbit polyclonal antibodies directed against the distal 180 amino acids of the amino-terminal half region of type M24 protein for 1 h at 37° C with gentle shaking. Three dilutions of Mab against LTA were tested (1:100, 1:500 and 1:1,000), while two dilutions of anti-M24 were tested, (1:100 and 1:500). Following incubation, bacteria were washed twice with PBS and adherence assay was performed as described previously. Bacteria suspended in PBS or treated with normal mouse serum or normal rabbit serum were used as negative controls for anti-LTA and anti-M antibodies, respectively.

Statistical analysis

Adherence data were expressed as a percentage of the original inoculum. Effect of pretreating mammary epithelial cells with LTA on bacterial adherence was compared by the mixed procedure of SAS. Means were separated by Fisher's least significant differences test (SAS 1994). Comparisons between adherence of nontreated bacteria and bacteria treated with enzymes and antibodies were analyzed by Student's *t* test.

Results

Detection of M-like protein and LTA

All four strains of *S. dysgalactiae* evaluated cross-reacted with anti-M24 antibodies showing a specific band distinct from bands corresponding to IgG-binding activity and nonspecific components present in normal rabbit serum. This band had an approximate MW of 35 kDa (Figures 5 and 6). All strains of *S. dysgalactiae* strains evaluated, including the reference strain (ATCC 27957), cross-reacted with Mab directed against the polyglycerophosphate backbone of *S. pyogenes*. No reactivity was observed for *Salmonella typhimurium* or when primary antibody was omitted or replaced by normal mouse serum (Figure 7).

Effect of modification of bacterial surface on adherence

Influence of enzymatic treatment on bacterial surface proteins was assessed by SDS-PAGE and effect of enzymatic treatment on detection of M-protein was assessed by Western blotting. Several protein bands were no longer observed following trypsin and pepsin treatment (Figure 8). Immunoreactivity to anti-M24 serum was reduced greatly following trypsin treatment, but unaffected following pepsin treatment of both *S.*

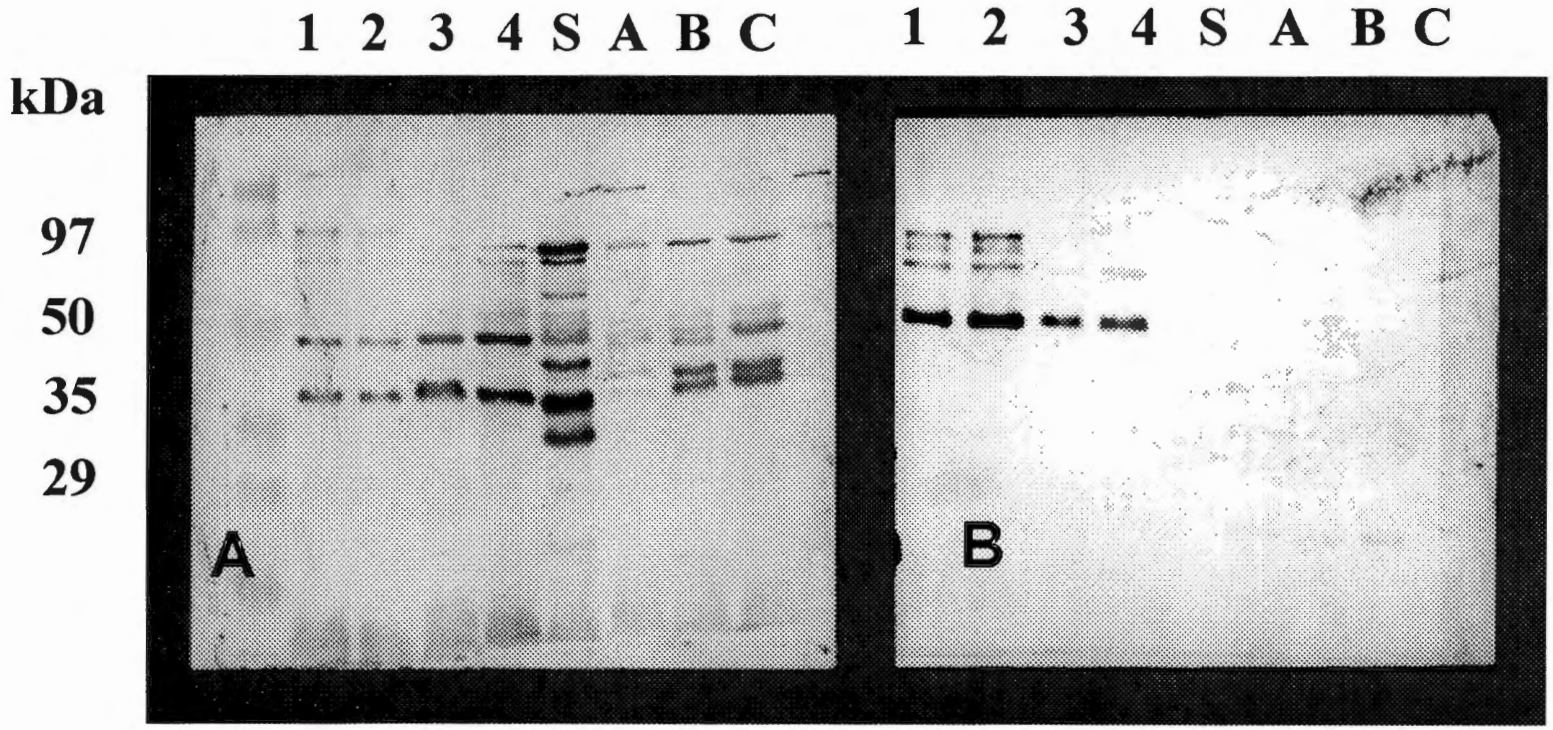

Figure 5. Detection of M-like protein by Western blot. Lanes 1-4: *Streptococcus dysgalactiae* strains UT516, UT519, UT857 and UT265; Lane 5: *Streptococcus pyogenes*, Lanes A, B and C: *Streptococcus uberis*. A: use of anti-M24 as primary antibody. A specific band of approximate 35 kDa was detected. B: use of biotinylated bovine IgG Fc instead of primary antibody.

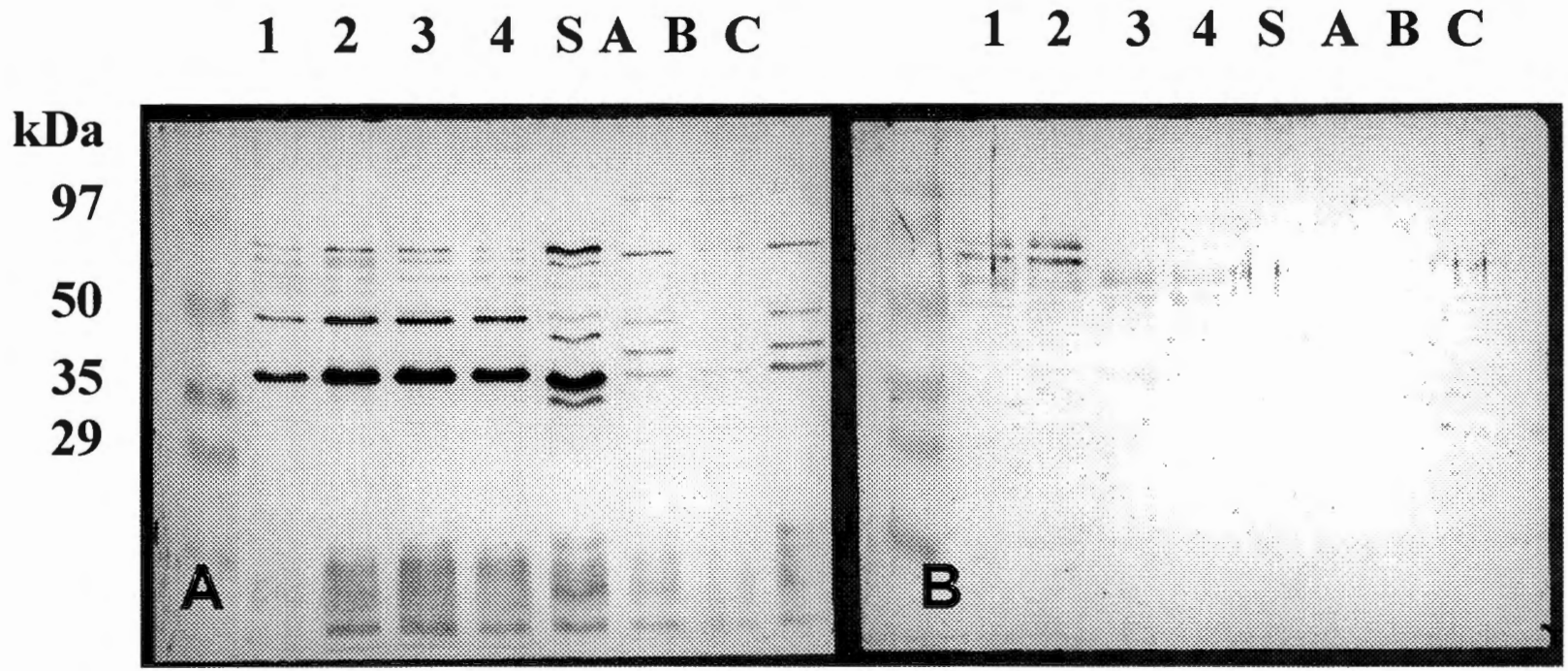

Figure 6. Detection of M-like protein by Western blot using normal rabbit serum as control. Lanes 1-4: *Streptococcus dysgalactiae* strains UT516, UT519, UT857 and UT265; Lane 5: *Streptococcus pyogenes*, Lanes A, B and C: *Streptococcus uberis*. A: use of anti-M24 as primary antibody. A specific band of approximate 35 kDa was detected. B: use of normal rabbit serum instead of primary antibody.

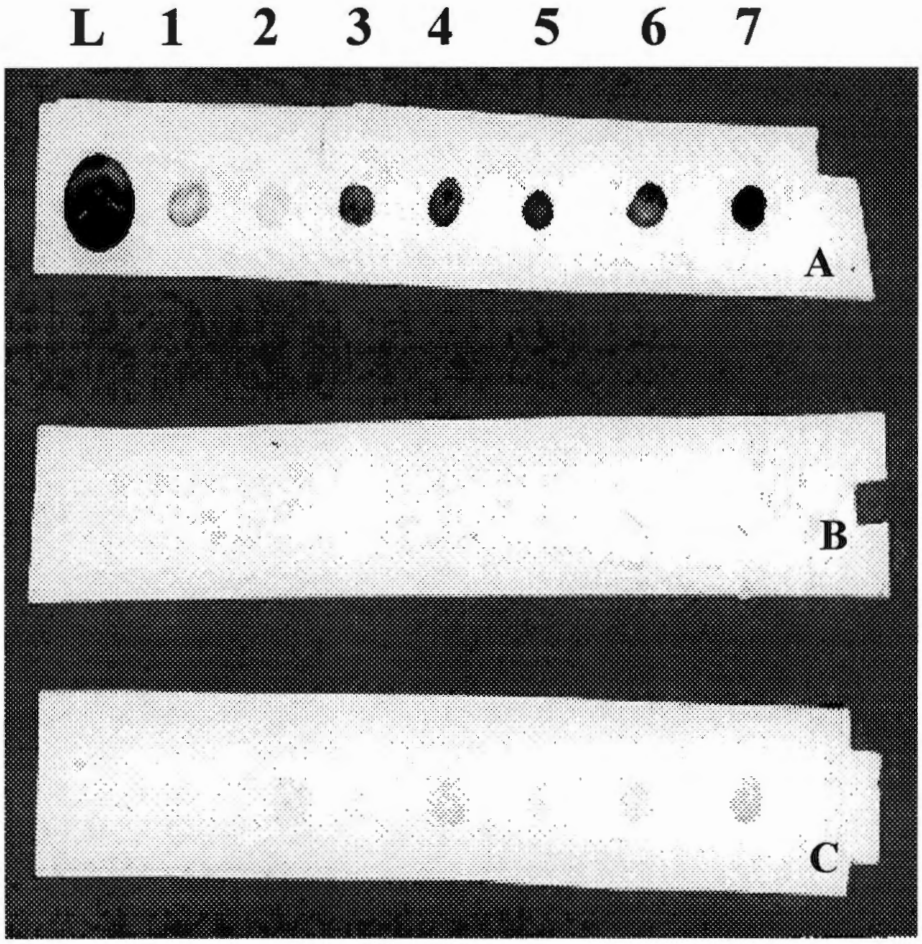

Figure 7. Detection of polyglycerophosphate backbone of LTA by dot blot. L: LTA from *Streptococcus pyogenes*; 1: *Streptococcus pyogenes;* 2: *Salmonella typhimurium* (ATCC 14028); 3: *Streptococcus dysgalactiae* strain UT516; 4: *S. dysgalactiae* strain UT519; 5: *S. dysgalactiae* strain UT857; 6: *S. dysgalactiae* strain UT265; 7: *S. dysgalactiae* (ATCC 27957). A: use of anti-LTA as primary antibody; B: use of normal mouse serum instead of primary antibody; C: omission of primary antibody.

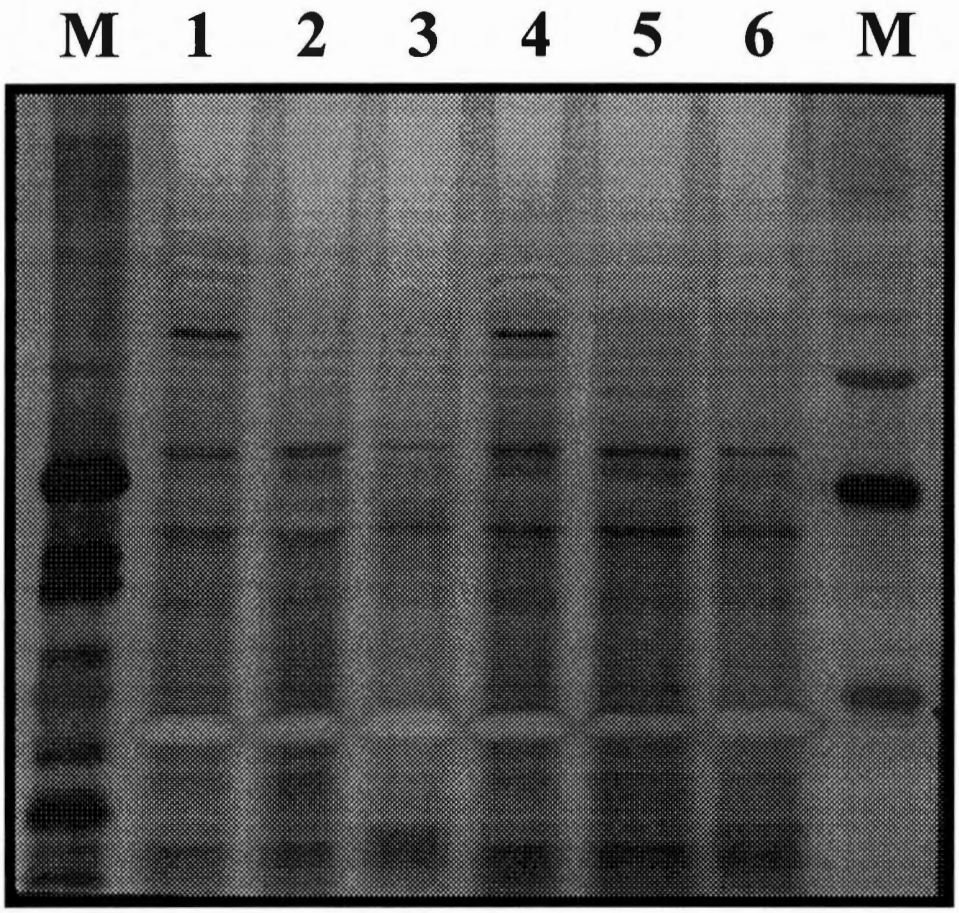

Figure 8. Separation of *Streptococcus dysgalactiae* strains UT516 and UT519 extracted cell wall proteins by 10% SDS-PAGE following enzymatic treatment with trypsin and pepsin. MWM: molecular weight markers; Lane 1: *S. dysgalactiae* strain UT516 nontreated; Lane 2: *S.* dysgalactiae strain UT516 pepsin-treated; Lane 3: *S.* dysgalactiae strain UT516 trypsin-treated; Lane 4: *S. dysgalactiae* strain UT519 nontreated; Lane 5: *S. dysgalactiae* strain UT519 pepsin-treated; Lane 6: *S. dysgalactiae* strain UT519 trypsin-treated.

dysgalactiae strains UT516 and UT519 extracted cell wall proteins. In this experiment, immunoreactivity to M protein was detected as two bands with an approximate molecular weight of 35 kDa rather than as a single band as in the previous experiment. These bands were specific as determined by use of normal rabbit serum instead of primary antibody to detect nonspecific nonimmune binding (Figure 9). Table 3 shows percent reduction of adherence of *S. dysgalactiae* strains UT516 and UT519 to mammary epithelial cells following enzymatic treatment. Trypsin treatment decreased adherence of *S. dysgalactiae* strains to primary bovine mammary epithelial cells ($P<0.05$) and to MAC-T cells ($P<0.01$). Following pepsin treatment, adherence of *S. dysgalactiae* strain UT516 to primary bovine mammary epithelial cells decreased ($P<0.01$), while adherence of *S. dysgalactiae* strains UT516 and UT519 to MAC-T cells was not affected.

Effect of LTA and antibodies on adherence

Preincubation of MAC-T cells for 30 min with increasing concentrations of LTA had no effect on cell viability, as determined by trypan blue exclusion. Preincubation of MAC-T cells with increasing concentrations of LTA did not affect adherence of *S. dysgalactiae* strain UT516 to MAC-T cells, however, adherence of *S. dysgalactiae* strain UT519 was inhibited significantly ($P<0.005$) at the two highest LTA concentrations (Table 4). *Streptococcus dysgalactiae* strains UT516 and UT519 were treated with Mab against the polyglycerophosphate backbone of *S. pyogenes* LTA at three different concentrations. Adherence of Mab-treated bacteria was compared to adherence of NMS-treated bacteria, since adherence of nontreated bacteria suspended in PBS was

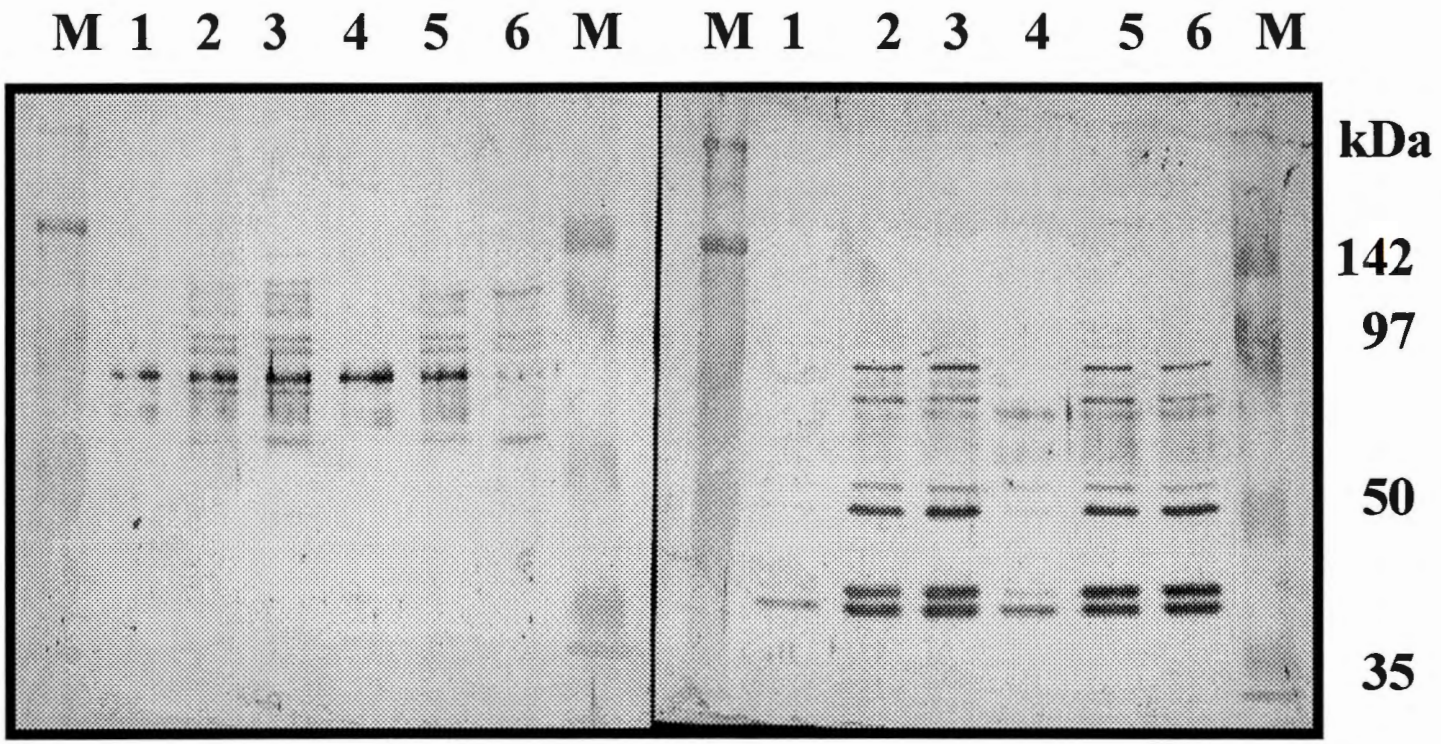

Figure 9. Immunoblot of M-like protein following enzymatic treatment with trypsin and pepsin of *Streptococcus dysgalactiae* strains UT516 and UT519. Lane 1: pre-stained molecular weight markers (MWM); Lane 2: *S. dysgalactiae* strain UT519 trypsin-treated; Lane 3: *S. dysgalactiae* strain UT519 pepsin-treated; Lane 4: *S. dysgalactiae* strain UT519 nontreated; Lane 5: *S. dysgalactiae* strain UT516 trypsin-treated; Lane 6: *S. dysgalactiae* strain UT516 pepsin-treated; Lane 7: *S. dysgalactiae* strain UT516 nontreated; Lane 8: pre-stained MWM. Bands of approximate 35 kDa indicate specific immunoreactivity against anti-M24 antibody.

Table 3. Adherence of *Streptococcus dysgalactiae* strains UT516 and UT519 to bovine mammary epithelial cells following enzymatic treatment[1].

		% Reduction in adherence	
	Primary cells	MAC-T cells	MAC-T cells
Treatment/Strain	UT516	UT516	UT519
Trypsin (1.2 mg/ml)	98.3 ± 0.4*	94.56 ± 1.6*	93.15 ± 2.01*
Pepsin (50µg/ml)	66.3 ± 7.7*	ND[2]	ND[2]

[1]Data are expressed as percent decrease in adherence ± SEM as compared to nontreated control. Data are means of 9 observations.

[2] ND: no decrease.

* Different from nontreated control ($P<0.05$).

Table 4. Adherence of *Streptococcus dysgalactiae* strains UT516 and UT519 to MAC-T cells following pretreatment of MAC-T cells with LTA[1].

	% Adherence to MAC-T cells *S. dysgalactiae*	
LTA (µg/ml)	UT516	UT519
0	6.48 ± 0.80[a]	7.06 ± 0.50[a]
100	7.02 ± 0.56[a]	4.99 ± 1.37[a]
200	7.65 ± 0.95[a]	5.30 ± 0.9[a]
300	8.27 ± 0.96[a]	5.08 ± 0.92[a]
400	8.45 ± 1.79[a]	3.30 ± 0.56[b]
500	5.82 ± 1.01[a]	3.57 ± 0.54[b]

[1]Data are expressed as percent adherence ± SEM, and are means of 6 observations.

[a, b] Different letters in the same row indicate that differences were significant ($P<0.005$).

consistently lower than that of serum-treated bacteria. Treatment with anti-LTA Mab did not affect bacterial adherence to MAC-T cells (Table 5). Pretreatment of bacteria with rabbit polyclonal antibodies directed against the distal 180 amino acids of the amino-terminal half region of *S. pyogenes* type M24 protein did not affect adherence to MAC-T cells (Table 6).

Discussion

Streptococcus dysgalactiae is endowed with several potential virulence factors that can contribute to its establishment and persistence within the mammary gland. However, there is only scarce information on the importance of these factors. In this study, presence of *S. dysgalactiae* surface components was detected and attempts were made to determine the role of these components in adherence to mammary epithelial cells. Lipoteichoic acids are high molecular weight amphiphilic molecules found both as cellular components and secretory products of *Streptococcus* species and other Gram-positive bacteria. The predominant type of LTA is a 1,3-linked polyglycerophosphate chain attached by a phosphodiester bond to bacterial membrane lipids (Fischer, 1988). LTA has been detected in streptococci of various serological groups (Wicken and Knox, 1975; Miörner et al., 1983; Goldschmidt and Panos, 1984). Among group C streptococci, an LTA-like molecule was extracted from *Streptococcus equi* (Srivastava and Barnum, 1983). This molecule inhibited a specific reaction between purified LTA from *S. pyogenes* and anti-LTA rabbit serum indicating antigenic similarities with *S. pyogenes* LTA. In the present study, all strains of *S. dysgalactiae* evaluated reacted specifically with Mab raised against the polyglycerophosphate backbone of *S. pyogenes* LTA indicating

Table 5. Effect of monoclonal antibodies (Mab) against LTA polyglycerophosphate backbone on adherence of *Streptococcus dysgalactiae* to MAC-T cells[1].

	% Adherence to MAC-T cells			
	S. dysgalactiae UT516		*S. dysgalactiae* UT519	
Dilution	Mab	NMS	Mab	NMS
1:100	4.07 ± 0.54	3.22 ± 0.49	4.04 ± 1.37	3.26 ± 0.57
1:500	3.88 ± 0.80	2.03 ± 0.45	4.70 ± 0.68	4.08 ± 0.73
1:1000	2.78 ± 0.23	4.60 ± 0.68	2.95 ± 0.51	3.78 ± 0.35

[1]Data represent means of 6 observations and are expressed as means ± SEM. Adherence of each strain to MAC-T cells was compared within each dilution by Student's *t* test; bacteria treated with normal mouse serum (NMS) were considered as control. No significant differences were observed.

Table 6. Effect of *Streptococcus pyogenes* M24 antibodies on adherence of *Streptococcus dysgalactiae* to MAC-T cells[1].

	% Adherence to MAC-T cells			
	S. dysgalactiae UT516		*S. dysgalactiae* UT519	
Dilution	anti-M24	NRS	anti-M24	NRS
1:100	0.58 ± 0.16	1.21 ± 0.46	2.87 ± 0.38	2.37 ± 0.42
1:500	0.64 ± 0.25	0.34 ± 0.14	NT[2]	NT[2]

[1]Data represent means of 6 observations and are expressed as means ± SEM. Adherence of each strain to MAC-T cells was compared within each dilution by Student's *t* test; bacteria treated with normal rabbit serum (NRS) were considered as control. No significant differences were observed.

[2]NT: not tested.

that an antigenically related structure is present on the surface of *S. dysgalactiae*. This finding agrees with previous studies that showed that LTAs of many Gram-positive bacteria were able to cause extensive serological cross-reactions (Knox and Wicken, 1973; Jackson et al., 1984) and confirms the broad reactivity of the antibody used (Drabick et al., 1993).

LTA is involved in adherence of streptococci to epithelial cells. Hasty et al. (1992) proposed that adherence of streptococci to epithelial cells is mediated by multiple adhesins that probably function in distinct kinetic steps. LTA appears to be important in a first step involving weak hydrophobic interactions with fatty acid-binding domains present on membranes of many different cell types (reviewed by Hasty et al., 1992). Group A streptococci can bind to epithelial cells by a mechanism mediated by LTA and M-protein (Courtney et al., 1992). However, other studies suggested that bacterial proteins through binding to Fn, rather than LTA, played a major role in adherence of these organisms to epithelial cells (Caparon et al., 1991; Talay et al., 1991). The nature of these discrepancies can be explained, in part, by dependence of molecular mechanisms employed by group A streptococci to attach to host cells on both the type of cell and the bacterial strain used in adherence studies (Courtney et al., 1992). In addition, inhibition studies preincubating epithelial cells with varying concentrations of LTA have yielded contradictory results regarding the role of LTA of group B streptococci in adherence to epithelial cells (Nealon and Mattingly, 1984; Teti et al., 1987; Tamura et al., 1994). Little information has been published on the possible role of LTA from streptococci isolated from bovine IMI on adherence to mammary epithelial cells.

Previous studies performed in our laboratory have shown a decrease in adherence of *S. uberis* to MAC-T cells following cell preincubation with 100 μg/ml of *S. pyogenes* LTA (Almeida et al., 1996). In the present study, LTA from *S. pyogenes* significantly inhibited adherence of *S. dysgalactiae* strain UT519 only at the two highest

concentrations evaluated (400 and 500 μg/ml). Although binding of Mab against the LTA polyglycerophosphate backbone was demonstrated on strains of *S. dysgalactiae*, use of this antibody in an attempt to block possible binding sites did not inhibit adherence of *S. dysgalactiae* to MAC-T cells. These data suggest that LTA plays only a minor role in adherence of *S. dysgalactiae* strain UT519 to epithelial cells, and is apparently not involved in adherence of *S. dysgalactiae* strain UT516 in the experimental model used. Studies performed with group B streptococci showed that Mab directed against the polyglycerophosphate backbone of LTA (Jackson et al., 1984) inhibited adherence of these organisms to vaginal epithelial cells (Teti et al., 1987). Regarding group C streptococci, Srivastava and Barnum (1983) observed no inhibition of *S. equi* adherence to horse tongue and cheek cells following cell treatment with purified *S. pyogenes* LTA. In addition, polyclonal antibodies raised against purified *S. pyogenes* LTA had no effect on adherence of *S. equi* to horse epithelial cells (Srivastava and Barnum, 1983).

Group A streptococcal M-proteins have been studied extensively since 1928, and have been shown to be important virulence factors and highly protective antigens (Fischetti, 1989; Kehoe, 1994). M-proteins or M-like proteins have been detected in groups B, C, E and G streptococci based either on serological cross-reactivity with antisera raised against M-proteins from group A streptococci or on ability of organisms to resist phagocytosis (Fischetti, 1989). Traore et al. (1991) found that preincubation of *S. dysgalactiae* with fibrinogen significantly reduced their phagocytic killing by neutrophils. This inhibition was abolished following enzymatic treatment known to destroy streptococcal binding sites for fibrinogen, suggesting that the fibrinogen binding component of *S. dysgalactiae* might represent a factor similar to M-protein of *S. pyogenes* (Traore et al., 1991).

In the present study, all strains of *S. dysgalactiae* evaluated cross-reacted with antibodies raised against a partially purified pepsin extract of *S. pyogenes* M24 protein.

Since IgG-binding proteins, known to be present in *S. dysgalactiae* (Müller and Blobel, 1983; Lämmler and Frede, 1989; Rantamäki and Müller, 1995), can have structural homology with M- and M-like proteins (Kehoe, 1994), specificity of the reaction was confirmed by including normal rabbit serum and bovine IgG Fc as controls. Specific bands of approximately 35 kDa and distinct from bands corresponding to normal rabbit serum and bovine IgG Fc binding activity were detected in all strains evaluated. These data demonstrated that binding of antibodies was not mediated by Fc receptors. Purified pepsin extracted M24 protein from *S. pyogenes* yielded a single band of 33.5 kDa (Beachey et al, 1977), while partially purified extracts of streptococcal group G yielded three major bands with molecular weights between 31 and 45 kDa (Bisno et al., 1987).

M-protein of *S. pyogenes* is one of the adhesins that mediate adherence of group A streptococci to certain types of host cells (Hasty et al., 1992; Courtney et al., 1994). Purified pepsin-extracted M-protein has been shown to inhibit adherence of group A streptococci to HEp-2 cells, and M^- strains showed reduced adherence to HEp-2 cells compared to M^+ strains (Courtney et al., 1992; 1994). In this study, no decrease in adherence to MAC-T cells was observed following treatment of *S. dysgalactiae* strains with M24 antibody which was used in an attempt to block possible binding sites of the detected M-like protein to epithelial cells. These data suggest that the identified structure does not appear to be involved in adherence of *S. dysgalactiae* to MAC-T cells. Previous studies from our laboratory showed that antibodies directed against the carboxy-terminal half of M24 protein reduced adherence of *S. uberis* strains to MAC-T cells (Almeida et al., 1996). Mild pepsin treatment, known to remove the amino-terminal half of M-protein of group A streptococci (Beachey and Ofek, 1976; Wädstrom et al., 1984), extracted a number of proteins as observed by SDS-PAGE, but did not affect immunoreactivity to M-protein. Conversely, following trypsin treatment, immunoreactivity to M-protein detected by Western blot was reduced greatly, indicating sensitivity of these antigens to trypsin

activity. This suggests that components extracted by mild pepsin treatment in *S. pyogenes* (Pep M) which was used to generate the antibody used in this study could not be extracted from the cell wall of *S. dysgalactiae* by the same mild treatment. In addition, mild pepsin treatment, known to reduce adherence of *S. pyogenes* to HEp-2 cell monolayers (Grabovskaya et al., 1980), significantly reduced adherence of *S. dysgalactiae* strain UT516 to primary bovine mammary epithelial cells, while adherence of *S. dysgalactiae* strains UT516 and UT519 to MAC-T cells remained unaffected. These data indicate that reduced adherence of *S. dysgalactiae* strain UT516 to primary epithelial cell cultures cannot be attributed to absence of the detected M-like protein. In addition, differences in adherence to MAC-T and primary bovine mammary epithelial cells of *S. dysgalactiae* strain UT516 following pepsin treatment suggests that these cell types differed in expression of receptors involved in bacterial adherence. Attachment of streptococci to host cells depends not only on the type of cell used in adherence studies, but also on the donor of a given cell type (Goldschmidt and Panos, 1984; Courtney et al., 1992; Tamura et al., 1994). Further studies are needed to extract and isolate native M-proteins from *S. dysgalactiae* and delineate their role in pathogenesis of bovine IMI.

Trypsin treatment reduced adherence of *S. dysgalactiae* strains to both cell types used in this study by more than 90%, suggesting a major role of surface proteins as adhesins or accessory molecules to adhesins. This agrees with previous results from our laboratory showing both decreased bacterial surface hydrophobicity and adherence to MAC-T cells following trypsin treatment of a hydrophobic strain of *S. dysgalactiae* (Calvinho et al., 1996b). In addition, trypsin treatment decreased binding of *S. dysgalactiae* isolated from bovine IMI to Fn (Mamo et al., 1987) and vitronectin (Filippsen et al., 1990) which may be involved in adherence of this organism to mammary epithelial cells.

In conclusion, results of this study showed that adherence of *S. dysgalactiae* isolated from bovine IMI to mammary epithelial cells was affected by both the cell type and bacterial strain used. Enzymatic treatment indicated that bacterial surface proteins played a major role in this process. In addition, molecules antigenically related to LTA and M-protein of *S. pyogenes* are present in *S. dysgalactiae* isolated from bovine IMI. However, only LTA appeared to play a minor role in adherence of *S. dysgalactiae* strain UT519 to MAC-T cells. Further research directed toward isolation of surface structures detected in this study should be conducted to determine their role in establishment and progression of *S. dysgalactiae* IMI in dairy cows.

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CHAPTER IV

INVASION AND PERSISTENCE OF STREPTOCOCCUS DYSGALACTIAE WITHIN BOVINE MAMMARY EPITHELIAL CELLS

Abstract

Little is known about bacterial and host factors that contribute to establishment and persistence of intramammary infection by *Streptococcus dysgalactiae. Streptococcus dysgalactiae* adheres to bovine mammary epithelial cells and to extracellular matrix proteins *in vitro*, and invades bovine mammary epithelial cells in culture; all of which can be potentially important pathogenic mechanisms. The objective of this study was to characterize mechanisms involved in invasion of *S. dysgalactiae* into bovine mammary epithelial cells. Studies were conducted to determine whether *S. dysgalactiae* invaded mammary epithelial cell cultures in a dose-dependent manner and whether mammary epithelial cells harboring different numbers of *S. dysgalactiae* for varying periods of time were damaged. Results demonstrated that bacterial invasion increased with inoculum size; however, number of intracellular bacteria was not proportional to inoculum size indicating that a finite number of organisms are capable of invading epithelial cells. No net increase of intracellular organisms was detected at any bacterial density evaluated; however, *S. dysgalactiae* remained viable throughout the period of evaluation. In addition, *S. dysgalactiae* did not appear to cause cell injury at any bacterial density and time point evaluated. These data suggest that *S. dysgalactiae* can survive within mammary epithelial cells for extended periods of time without losing viability or damaging the eukaryotic cell. This feature may be associated with development of persistent infection, protection of organisms from antimicrobial drugs and host defense

mechanisms, and can also provide a route for bacterial colonization of subepithelial tissues.

Introduction

Bacterial adherence to mammary epithelial cell surfaces and entry into mammary epithelial cells may be important pathogenic mechanisms of some mastitis pathogens. Invasion of bacteria into epithelial cells could result in protection from both host defense mechanisms and action of antimicrobial agents, and may also affect mammary secretory cell function. In addition, an infection route from the lumen into subepithelial tissues may be provided (Chandler et al., 1980; Falkow et al., 1992; Galán, 1994). Early work of Pattison and Smith (1953) showed that cocci penetrated through the alveolar epithelium and smaller ducts to reach interacinar tissue following experimental infection of goat mammary glands with *S. dysgalactiae*. Observations on experimental *Streptococcus agalactiae* mastitis in mice showed cocci in close association with secretory cells bordering the acinar lumen as well as within vacuoles in cells identified as secretory acinar cells (Chandler, 1970). In addition, cocci were observed below the basal membrane of a secretory cell suggesting penetration to the *lamina propria* (Chandler, 1970). Further studies confirmed the intracellular location of *S. agalactiae*, *Staphylococcus aureus* and *Escherichia coli* within phagolysosomes of mammary secretory cells in a mouse mastitis model, and described changes in internalized bacteria that suggested enzymatic digestion by epithelial cells (Chandler et al., 1980). Thomas et al. (1994) observed cocci penetrating the secretory epithelium 24 h after inoculation of *Streptococcus uberis* into the mammary gland of cows.

Streptococcus dysgalactiae is a frequent cause of mastitis in dairy cows. However, little is known about both bacterial and host factors that contribute to establishment and persistence of intramammary infection (IMI) by this organism. *Streptococcus dysgalactiae* adheres to bovine mammary epithelial cells and to extracellular matrix proteins *in vitro* (Frost et al., 1977; Mamo et al., 1987; Filippsen et al., 1990; Calvinho et al., 1996), and invades bovine mammary epithelial cells in culture (Almeida and Oliver, 1995). Microfilaments but not microtubules were found to be necessary for bacterial entry into eukaryotic cells which appeared to occur by an endocytosis mechanism. Coculture of a transformed mammary epithelial cell line with *S. dysgalactiae* did not appear to affect cell viability. However, lactate dehydrogenase activity increased in coculture supernatants suggesting that some cellular damage was induced following bacterial invasion (Almeida and Oliver, 1995).

The objective of this study was to further characterize mechanisms involved in invasion of *S. dysgalactiae* into bovine mammary epithelial cells. Specifically, studies were conducted to determine if *S. dysgalactiae* invaded mammary epithelial cell cultures in a dose-dependent manner and whether mammary epithelial cells harboring different numbers of *S. dysgalactiae* for extended periods of time were damaged.

Materials and Methods

Mammary epithelial cells

An established transformed mammary epithelial cell line (MAC-T; Huynh, et al., 1991) obtained from J.D. Turner (McGill University, Quebec, Canada) was used in these experiments. The number of epithelial cells per well was estimated by counting in a hemocytometer to determine the bacteria:epithelial cell ratio used during invasion

experiments. Cell cultures were tested for the presence of mycoplasma contamination using a commercial test (Gibco, BRL).

Cell culture medium

Cell culture medium (CCM) contained 40% RPMI 1640 (Sigma Chemical Co., St. Louis, MO), 40% Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Inc., Grand Island, NY), 10% fetal bovine serum (FBS; Bio Whittaker, Inc., Walkersville, MD), 1 mM sodium pyruvate (Sigma Chemical, Co.), 2 mM L-glutamine (Bio Whittaker, Inc.) and 40 mM HEPES buffer (Sigma Chemical Co.). The following supplements were added to the medium: 5μg/ml of bovine insulin (Sigma Chemical Co.), 1μg/ml of hydrocortisone (Sigma Chemical Co.), and 1μg/ml of bovine prolactin kindly provided by USDA, Animal Hormone Program, Beltsville, MD (Cifrian et al., 1994).

Bacterial strains and culturing

Two strains of *S. dysgalactiae* were selected according to cluster analysis of PCR-based DNA fingerprint patterns among the population of *S. dysgalactiae* isolated from two dairy herds (Jayarao and Oliver, 1994). *Streptococcus dysgalactiae* strain UT516 was isolated from a case of subclinical mastitis and *S. dysgalactiae* strain UT519 was isolated from a case of clinical mastitis. A noninvasive organism, *Escherichia coli* DH5α (Gibco, BRL, Bethesda, MD) was used as a negative control and *Salmonella typhimurium* 14028 (American Type Culture Collection; Rockville, MD) was used as a positive control. Bacteria were activated from frozen skim milk stocks (-80° C) by inoculating skim milk and incubating for 18 h at 37° C. After activation, bacteria were grown in Todd Hewitt broth (THB, Gibco BRL, Life Technologies, Inc., Grand Island, NY) overnight at 37° C to mid logarithmic phase ($\approx 10^8$ colony forming units (CFU)/ml). Control strains were

grown in LB broth (Gibco BRL, Life Technologies, Inc.). On the day of experimentation, bacteria were harvested by centrifugation, washed twice in PBS and resuspended in PBS. Bacterial concentration and purity were determined by plate count on brain heart infusion agar (BHIA, Becton Dickinson Microbiology Systems, Cockeysville, MD) and blood agar, respectively.

Invasion assay

The invasion assay was performed using standard *in vitro* invasion methodology involving incubation of bacteria with epithelial cells followed by selective killing of extracellular bacteria by gentamicin and penicillin G and release of internalized bacteria by lysis of mammary epithelial cells with detergent (Rubens et al., 1992). Mammary cells were grown to confluence in 24-well tissue culture plates, inoculated with varying concentrations of *S. dysgalactiae* and centrifuged at 600 x g for 5 min. Appropriate dilutions were performed to obtain varying numbers of organisms per epithelial cell. Cocultures were incubated for 2 h at 37° C in 5% CO_2:95% air (invasion incubation). Following invasion incubation, supernatants of wells were removed, replaced with CCM containing gentamicin (100 µg/ml, Sigma Chemical Co.) and penicillin G (5µg/ml, Sigma Chemical Co.), and incubated for 2 h at 37° C (antibiotic incubation). Control strains were incubated with CCM containing 100 µg/ml of gentamicin. Effectiveness of antibiotics in killing extracellular bacteria was monitored by culturing supernatants from infected cell monolayers treated with antibiotics. After removing media containing antibiotics, monolayers were washed three times with PBS, treated with 0.025% trypsin, and further lysed with Triton X-100 (Amersham, Arlington Heights, IL) at a final concentration of 0.1% (vol/vol) in PBS. Cell lysates were transferred to 1.5 ml Eppendorf tubes and vortexed vigorously. Lysates were diluted serially 10-fold, plated on BHIA, and incubated overnight at 37° C to determine CFU/ml. Bacterial inocula were cultured for 2

h in cell growth medium at 37° C, and CFU/ml were determined. Percentage of invasive bacteria was calculated by dividing the number of CFU/ml of intracellular bacteria by the number of CFU/ml of bacteria in the inoculum x 100. A previous study showed that invasion varied on a daily basis, but experimental results from control and test strains were reproducible relative to each other (Elsinghorst and Kopecko, 1992). Therefore, strain invasiveness was only compared at the same bacterial density and within the same day. Experiments were repeated three times and data from average values of triplicate wells in a single experiment representative of values of three replicates were presented (Elsinghorst and Kopecko, 1992).

Assessment of possible intracellular replication of *S. dysgalactiae*

To determine possible intracellular replication of *S. dysgalactiae* ingested by epithelial cells, the standard invasion assay was modified by extending the length of the antibiotic incubation over several hours (Elsinghorst, 1994). Mammary epithelial cells were cocultured with *S. dysgalactiae* for 2 h, supernatants were removed by aspiration, and monolayers were washed three times with PBS. Monolayers were then incubated an additional 2, 6 or 12 h with CCM containing penicillin G and gentamicin. As gentamicin may slowly penetrate the eukaryotic membrane, after 2 h of incubation the concentration of gentamicin was decreased four-fold (Elsinghorst, 1994). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gentamicin for *S. dysgalactiae* UT516 and UT519 were 1.25 μg/ml and 2.5 μg/ml, respectively; while MIC and MBC of penicillin for both strains of *S. dysgalactiae* was 1.25 μg/ml as determined by standard methodology (Baron and Finegold, 1990). Monolayers were washed three times with PBS and lysed with trypsin and Triton X-100; CFU/ml of internalized bacteria were determined in cell lysates at each incubation time as described for the invasion assay.

Assessment of viability of mammary epithelial cells harboring ingested *S. dysgalactiae*

Two methods were performed to assess the viability of mammary epithelial cells harboring ingested *S. dysgalactiae*. The first was a fluorescent method that determined intracellular esterase activity and plasma membrane integrity (Live/dead EukoLight™ Molecular Probes, Inc., Eugene, OR). Cultures of bovine mammary epithelial cells were grown to confluence on 4-well polystyrene tissue culture chambers (Nunc, Inc., Naperville, IL). Epithelial cell cultures were allowed to ingest *S. dysgalactiae* at three bacterial densities for 2 h, followed by application of antibiotic treatment. Incubation of epithelial cells harboring intracellular *S. dysgalactiae* continued for 2, 6 or 12 h, and epithelial cell monolayers were assayed at these time intervals for viability. Prior to the assay, cells were washed gently with Dulbecco's PBS (D-PBS) to remove or dilute serum esterase activity generally present in serum-supplemented growth media. Reagents (150 µl) containing 2 µM calcein AM in anhydrous dimethyl sulfoxide (DMSO) and 4 µM ethidium homodimer (EthD-1) in DMSO/H_2O 1:4 (v/v) were added and cells were incubated for 45 min at room temperature. Following incubation, 10 µl of reagents were added to a clean coverslip and mounted. Labeled cells were viewed with a fluorescence microscope (50-W vapor mercury lamp, exciter 490 nm, dichroic mirror 510 nm, and barrier 520 nm). Live cells produced an intense uniform green color due to retention of polyanionic calcein while dead cells produced a bright red color due to EthD-1 penetration into cells with damaged membranes and binding to nucleic acids undergoing a 40 x enhancement of fluorescence. One chamber in each slide remained untreated as a control of cell viability. Epithelial cells in 20 fields were examined for each bacterial density and time point, and percent viability was determined as number of live cells/total number of cells x 100.

A colorimetric assay that determined activity of mitochondrial dehydrogenases was the second method used to assess viability of mammary epithelial cells harboring ingested *S. dysgalactiae*. Live cells reduce 3(4,5-dimethyl-thiazoyl-2-yl)2,5-dephenyltetrazolium bromide (MTT, Sigma Chemical Co.) by mitochondrial dehydrogenases to a purple formazan crystalline form and this amount is proportional to the number of viable cells (Lattime et al., 1988). Fifty µl of MTT at a concentration of 5 mg/ml in PBS were added to wells without washing or removing supernatant and cells were incubated for 4 h at 37° C. Supernatants were discarded by turning the plate over and blotting fluid, and 250 µl of acidified isopropanol (1.5 ml HCL 12 N in 500 ml 2-propanol) and 50 µl of 3% sodium dodecyl sulfate (SDS) were added. Plates were incubated for 30 min on a rotating plate to allow complete crystal dissolution and were read on an automated microplate reader (Model EL311SL, Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 570 nm. Equal volumes of isopropanol and 3% SDS were added to wells as a blank. Cell viability was determined as: % live cells = $(O.D._{\text{treated cells}}/O.D._{\text{control cells}}) \times 100$. Control wells consisted of supernatants harvested from wells with media alone, and from wells with nontreated epithelial cells. Since bacteria can reduce MTT (Gomez-Flores et al., 1995), the contribution of bacterial MTT reduction to overall MTT reduction of cells harboring bacteria was estimated. MTT reduction of bacterial suspensions of similar and higher CFU to those recovered from cells was determined in a concurrent experiment.

Transmission electron microscopy

MAC-T cell monolayers grown on cell culture plastic chamber slides (Nunc Inc.) were inoculated with *S. dysgalactiae* at three different bacterial densities and the invasion assay was performed as described previously. At 2, 6, or 12 h of antibiotic incubation, monolayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at room

temperature. Monolayers were washed three times with 0.1 M cacodylate buffer (pH 7.2) for 15 min. Postfixation was carried out in 1% osmium tetroxide (Sigma Chemical Co.), and samples were dehydrated through a graded alcohol series. Monolayers were embedded in Epon 812 resin (Polysciences, Werrington, PA), thin sectioned along the transverse plane, and stained with uranyl acetate and lead citrate. Ultrathin sections were examined by transmission electron microscopy (TEM) with a Hitachi H600 electron microscope.

Statistical analysis

Each condition in the invasion assay was performed in triplicate and experiments were repeated three times. All data obtained were expressed as mean ± standard error of the mean (SEM). Invasiveness of strains of *S. dysgalactiae* was compared to noninvasive and to invasive controls by Student's *t* test. For intracellular replication, number of CFU obtained at different time points for each strain and bacterial density were compared by the mixed procedure of SAS. Means were separated by Fisher's least significant differences test (SAS 1994). Viability of cells harboring different bacterial densities was compared with nontreated cells at each time point by Student's *t* test.

Results

Invasion of *S. dysgalactiae* into MAC-T cells

Both *S. dysgalactiae* UT516 and UT519 were significantly more invasive than the noninvasive control strain *E. coli* DH5α ($P<0.05$) and less invasive than *S. typhimurium* 14028 ($P<0.005$). No significant differences in invasiveness were detected between strains of *S. dysgalactiae* UT516 and UT519 (Figure 10). Mean number of MAC-T cells

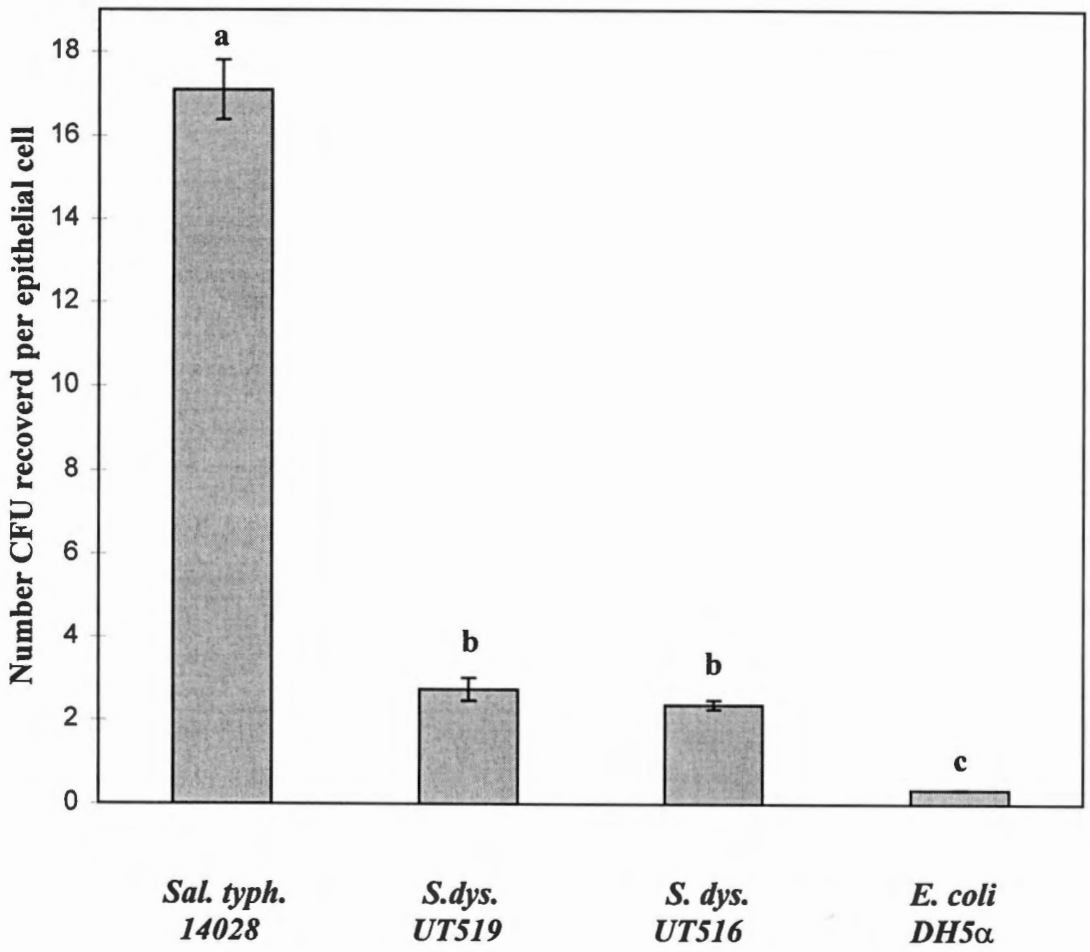

Figure 10. Invasion of MAC-T cells by *Streptococcus dysgalactiae* strains UT516 and UT519 compared with invasion by *Salmonella typhimurium* ATCC 14028 and noninvasive *Escherichia coli* DH5α. Different letters indicate significant differences ($P<0.05$).

per well was 1.37 x 10^5 (SEM 2 x 10^4). Bacterial inoculum size used to infect cell monolayers affected the number of organisms recovered from cell lysates. Doubling dilutions from an initial bacterial suspension were used to infect cell monolayers to achieve different bacteria:epithelial cell ratios, keeping the number of epithelial cells constant. Number of CFU recovered from cell lysates was proportional to the bacterial inoculum until a saturation point was reached, indicating that a finite number of organisms could enter epithelial cells (Table 7).

Assessment of possible intracellular replication of *S. dysgalactiae*

Following an invasion incubation period of 2 h, MAC-T cells harboring *S. dysgalactiae* at three different bacterial densities were incubated for 12 h in the presence of antibiotics. Number of intracellular CFU recovered at 2 h were compared with those recovered at 6 and 12 h for each bacterial density evaluated. For both strains of *S. dysgalactiae,* similar number of intracellular CFU were obtained at each time point (Figures 11 and 12). These results indicate that *S. dysgalactiae* remained viable within MAC-T cells, but probably did not replicate.

Assessment of viability of mammary epithelial cells harboring ingested *S. dysgalactiae*

MAC-T cells harboring *S. dysgalactiae* showed no decrease in viability as compared with nontreated controls; rather, percent reduction of MTT was higher for cells harboring bacteria. Contribution of bacterial MTT reduction to total MTT reduction of cells harboring bacteria was estimated to be 5.18% (SEM 0.32). Figures 13 and 14 show viability assessment of MAC-T cells harboring three different bacterial densities at 6 and 12 h of incubation in the presence of antibiotics. In addition, no decrease in viability was detected at any bacterial density or time point as determined by calcein AM and EthD-1

Table 7. Effect of inoculum size on invasion of *Streptococcus dysgalactiae* into MAC-T cell monolayers[1]

S. dysgalactiae UT516			*S. dysgalactiae* UT519		
Inoculum log_{10}	Bacteria:cell ratio	% invasion ± SEM	Inoculum log_{10}	Bacteria:cell ratio	% invasion ± SEM
6.94	56:1	0.13 ± 0.05	6.73	40:1	1.63 ± 0.37
7.25	112:1	0.08 ± 0.01	7.03	80:1	1.72 ± 0.38
7.55	224:1	0.10 ± 0.006	7.33	170:1	1.11 ± 0.1
7.85	450:1	0.13 ± 0.02	7.63	350:1	1.11 ± 0.02
8.15	900:1	0.18 ± 0.006	7.93	700:1	0.38 ± 0.06
8.45	1800:1	0.08 ± 0.006	8.23	1400:1	0.32 ± 0.06

[1]Experiments were repeated in triplicate. Data were obtained from triplicate wells of a representative experiment. Percent invasion is expressed as number of intracellular CFU/number of CFU in inoculum x 100. Results for both strains correspond to experiments performed on different days.

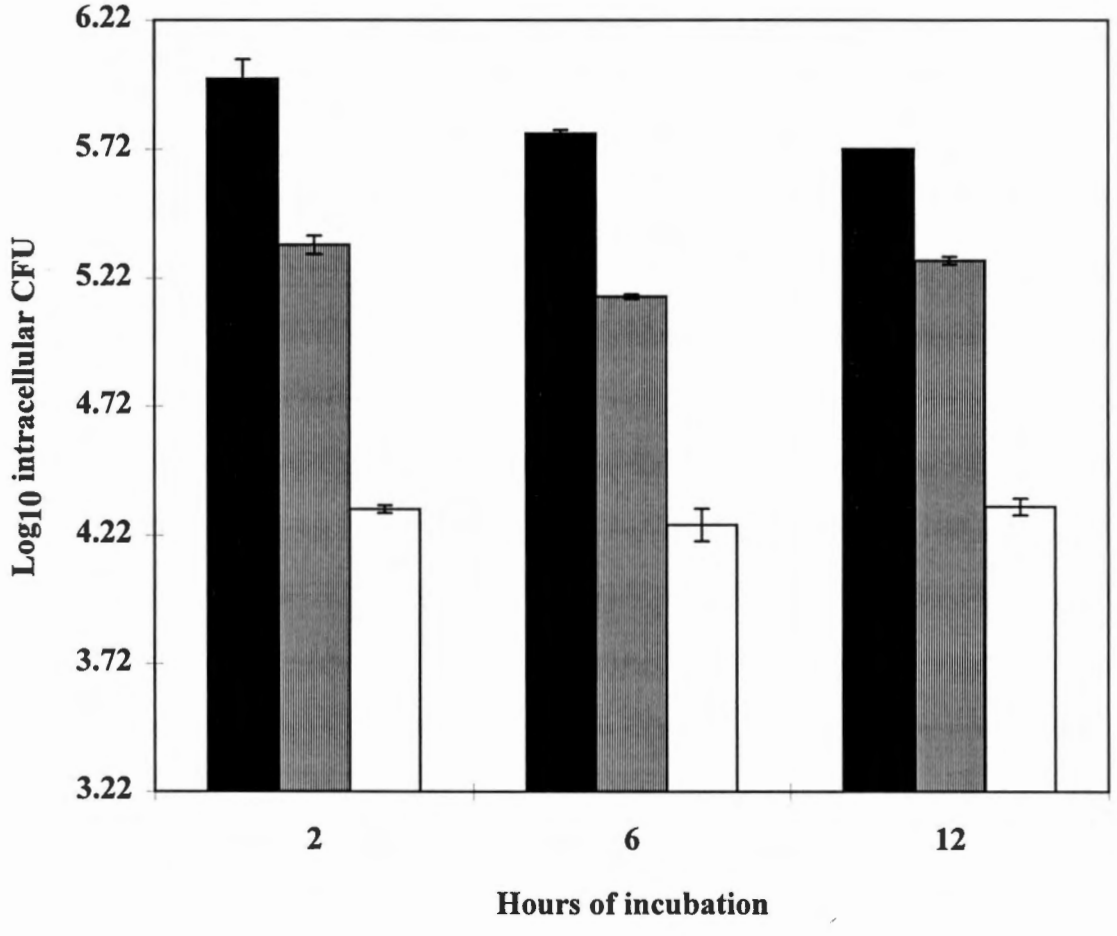

Figure 11. Number of intracellular *S. dysgalactiae* strain UT516 in MAC-T cell cultures during a period of 12 h. Experiment was repeated in triplicate. Data were obtained from triplicate wells of a representative experiment. References: bacteria:epithelial cell ratios: (solid bars) 650:1; (striped bars) 130:1; (open bars) 40:1.

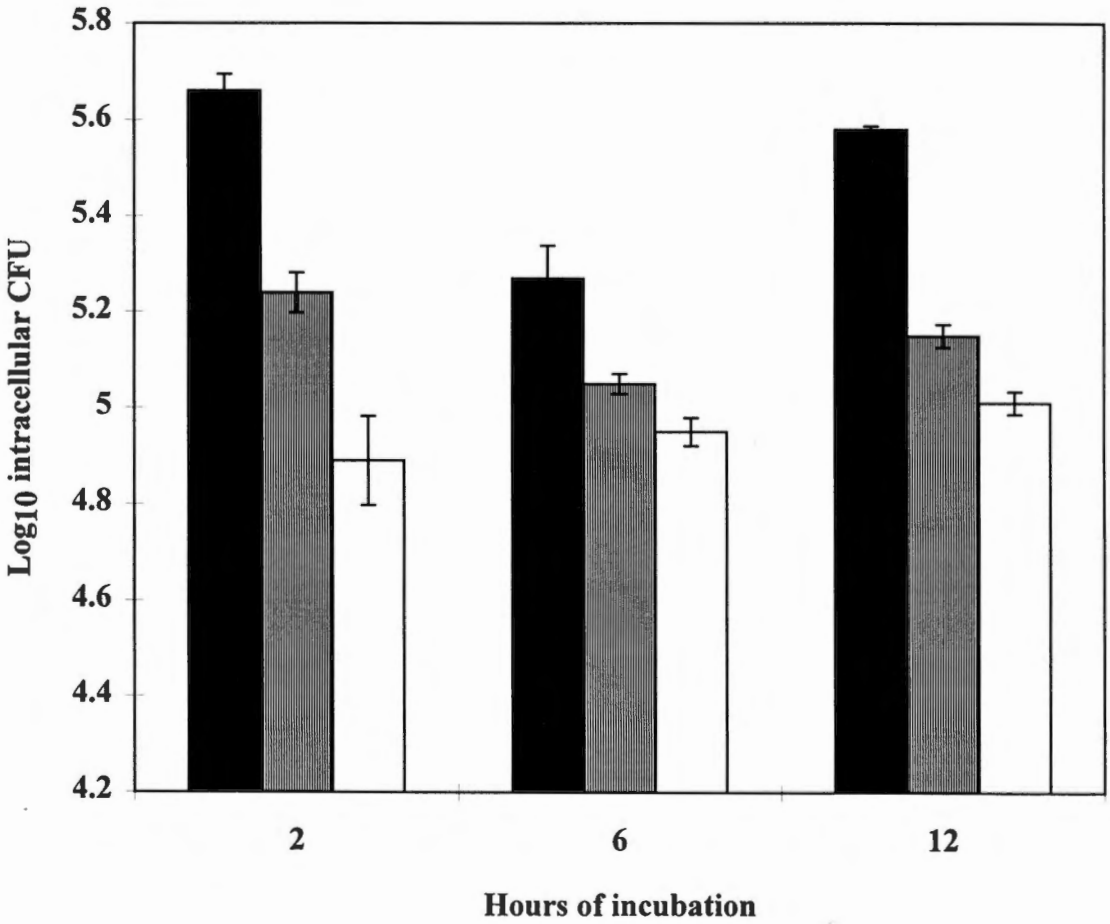

Figure 12. Number of intracellular *S. dysgalactiae* strain UT519 in MAC-T cell cultures during a period of 12 h. Experiment was repeated in triplicate. Data were obtained from triplicate wells of a representative experiment. References: bacteria:epithelial cell ratios: (solid bars) 250:1; (striped bars) 75:1; (open bars) 25:1.

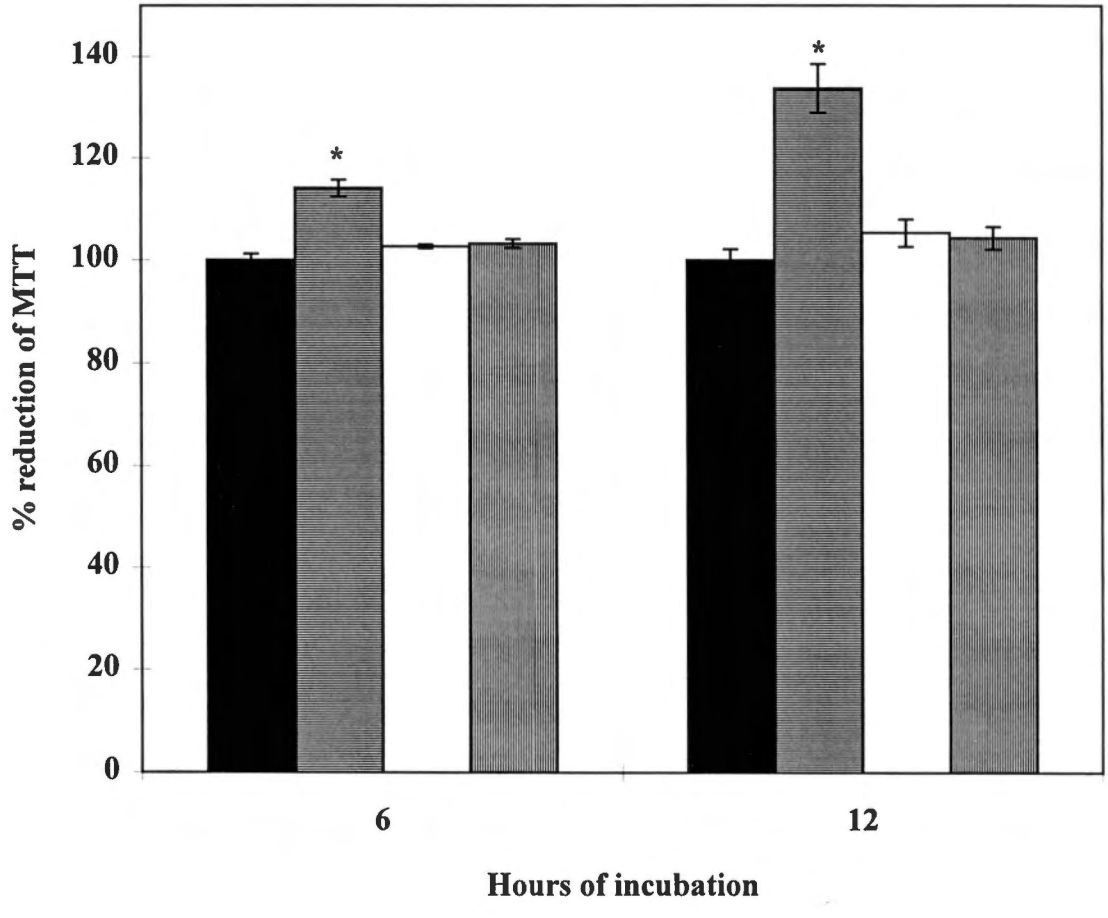

Figure 13. Viability of MAC-T cells harboring *S.dysgalactiae* UT516 at three different bacterial densities. References: bacteria:epithelial cell ratios: (solid bars) nontreated control, (horizontally striped bars) 2,000:1, (open bars) 500:1, (vertically striped bars) 70:1.

*Significant differences compared with nontreated control ($P<0.05$).

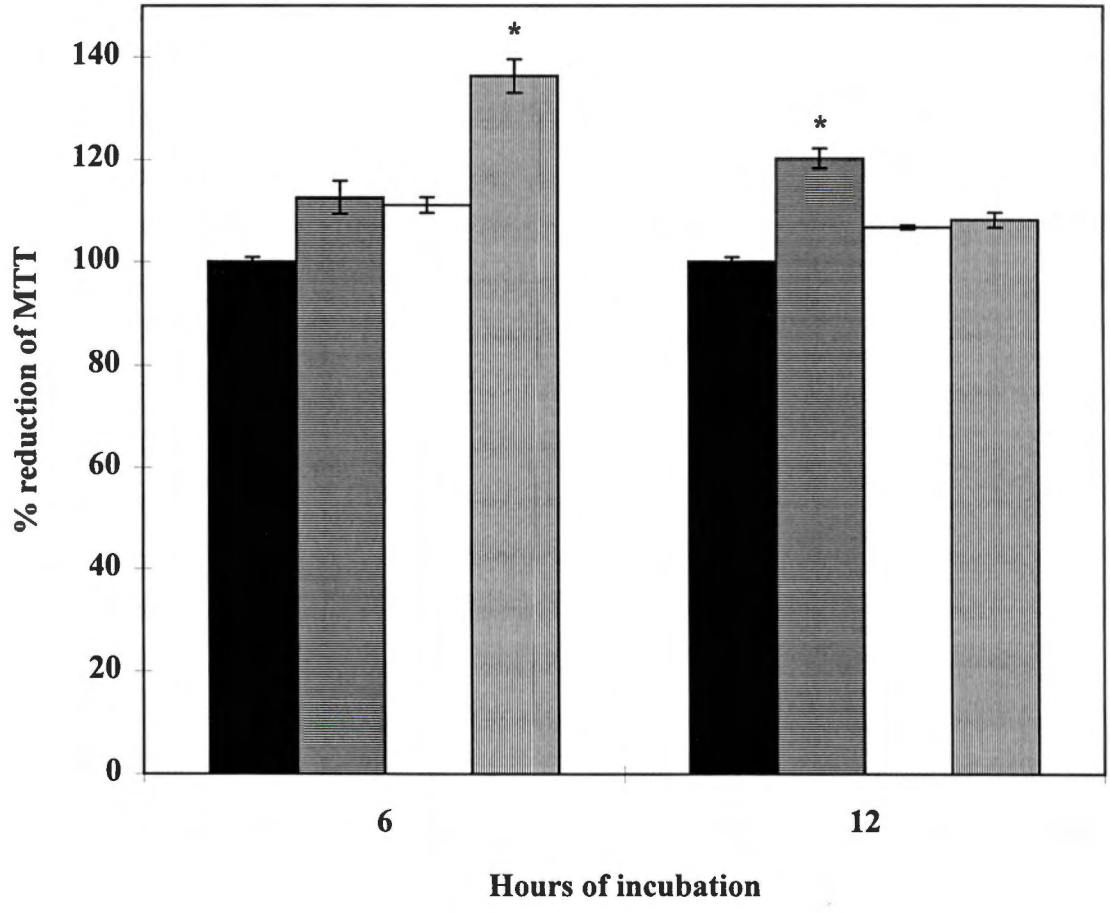

Figure 14. Viability of MAC-T cells harboring *S.dysgalactiae* UT519 at three different bacterial densities. References: bacteria:epithelial cell ratios: (solid bars) nontreated control, (horizontally striped bars) 250:1, (open bars) 75:1, (vertically striped bars) 30:1. *Significant differences compared with nontreated control ($P<0.05$).

staining of cells harboring *S. dysgalactiae* (Figure 15).

Transmission electron microscopy

Transmission electron microscopy was performed on equivalent monolayers inoculated with three different bacterial densities after 2h of invasion incubation and at 2, 6 and 12 h of antibiotic incubation (Figure 16). At 2h of invasion incubation, bacteria were observed both interacting with the eukaryotic cell membrane and within vacuoles inside MAC-T cells. Pseudopod-like elongations appearing to surround and engulf bacteria were observed. Intracellular bacteria were observed within membrane-bound vacuoles containing single coccus or groups of cocci. No morphological changes indicative of cell injury were observed for up to 12 h. Cocci were seen within vacuoles at every sampling time. Some cocci appeared to be replicating within vacuoles; and some vacuoles contained up to 15 cocci. However, most cells contained two or three cocci per vacuole. Bacteria were not seen within the epithelial cell nucleus or free in the cytoplasm.

Discussion

Streptococcus dysgalactiae isolated from bovine IMI invaded mammary epithelial cells *in vitro* in a time-dependent fashion (Almeida and Oliver, 1995). Results of this study demonstrated that as the inoculum increased, more intracellular CFU were recovered from MAC-T monolayers until a saturation point was reached. Thereafter, this number was not proportional to inoculum size, indicating that a finite number of organisms were capable of invading epithelial cells. This agrees with previous observations on invasion of group B streptococci into respiratory epithelial cells (Rubens et al., 1992), and may reflect a limited number of receptors per cell, a limited proportion

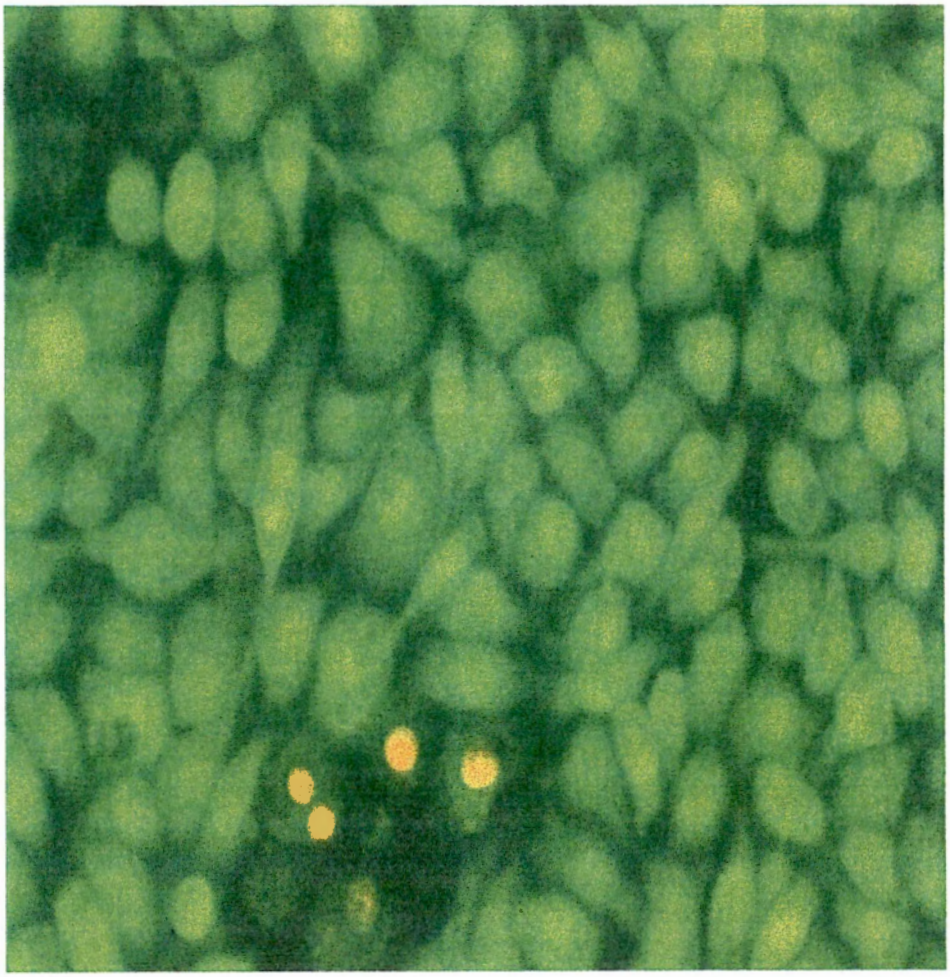

Figure 15. MAC-T cell monolayers harboring *S. dysgalactiae* UT519 for 12 h stained with calcein AM and EthD-1 (x 200). No decrease in viability was observed compared with nontreated control.

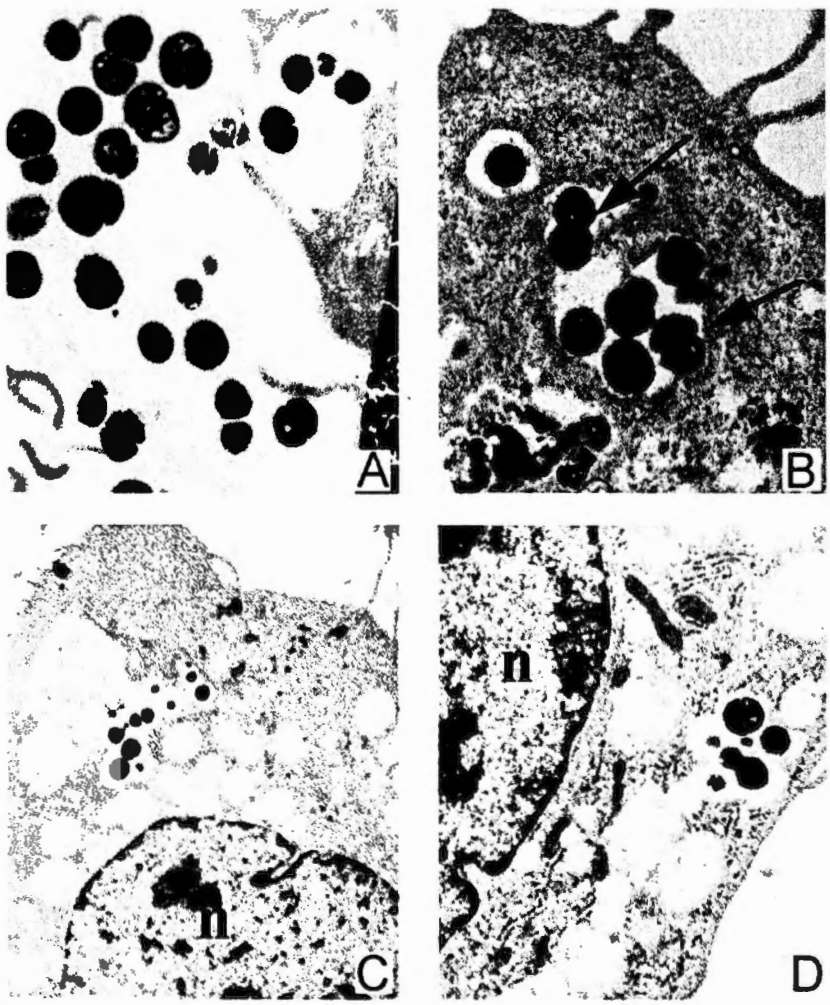

Figure 16. Electron micrographs of *Streptococcus dysgalactiae* within MAC-T cells. A: Cocci in the extracellular medium and contacting MAC-T cells (x 5,000); B: membrane bound vacuoles containing single coccus and several cocci after 2 h of invasion incubation. Some cocci appeared to be replicating (arrows) (x 15,000). C: Cocci contained in vacuole after 2 h of antibiotic incubation (x 5,000). D: Cocci in membrane bound vacuole after 12 h of antibiotic incubation (x 12,000). n: nucleus.

of the cell population involved in endocytosis, and/or a limited proportion of the bacterial population within the inoculum able to stimulate endocytosis by epithelial cells (Detilleux et al., 1990; Rubens et al., 1992).

Once internalized, some invasive bacteria are capable of replicating within eukaryotic cells (Finlay and Falkow, 1989). Previous observations showed that *S. dysgalactiae* did not appear to replicate inside MAC-T cells over a period of 6 h in the presence of antibiotics added to cell culture medium (Almeida and Oliver, 1995). In this study, MAC-T cells harboring *S. dysgalactiae* at three different bacterial densities were incubated for 12 h in the presence of antibiotics. No net increase of intracellular organisms was detected at any bacterial density evaluated, although *S. dysgalactiae* remained viable throughout this period. These results confirmed previous observations on intracellular survival of *S. dysgalactiae* within MAC-T cells (Almeida and Oliver, 1995), and agreed with similar findings on intracellular replication of group B streptococci in respiratory and endothelial cells through a time course of 8 h (Rubens et al., 1992; Gibson et al., 1993). However, since this methodology cannot distinguish between bacterial turnover within the eukaryotic cell and static maintenance of bacterial numbers (Elsinghorst, 1994), and considering that intracellular replication can occur beyond time periods evaluated in these studies, the possibility of intracellular bacterial replication during extended time periods cannot be ruled out. In addition, TEM showed cocci replicating in the extracellular medium after 2h of invasion incubation which correlated with increased number of CFU recovered in bacterial supernatants compared with the initial inoculum. Although some replicating cocci were observed by TEM within mammary epithelial cells at 2, 6 and 12 h of antibiotic incubation, it could not be determined by this method if cocci were replicating within mammary epithelial cells or were ingested by mammary epithelial cells during the process of replication in the extracellular medium and remained in this state throughout the observation period.

Internalized bacteria can cause damage to the eukaryotic cell (Vann and Proctor, 1987; Gibson et al., 1993). However, *S. dysgalactiae* did not appear to cause cell injury at any bacterial density and time point as evaluated by two different methods. In addition, no morphological changes suggesting cell injury were observed by TEM. An increase in MTT reduction at some bacterial densities and time points was observed; in most cases this increase corresponded to cells harboring the highest bacterial densities. However, contribution of bacterial MTT reduction to MTT reduction of cells harboring *S. dysgalactiae* was estimated to be approximately 5%. Therefore, increased MTT reduction in cells harboring *S. dysgalactiae* can only be partly attributed to bacterial MTT reduction and may reflect higher metabolic activity of invaded cells compared with noninvaded control cells. Previous studies showed that coculture of MAC-T cells with *S. dysgalactiae* for 2 and 4 h did not affect cell viability; however, increased lactate dehydrogenase activity in cell culture medium was detected suggesting induction of cell membrane damage as invading bacteria increased in number (Almeida and Oliver, 1995). Differences between studies may be due to the time frame during the invasion process when cell damage was evaluated (invasion incubation vs. antibiotic incubation), and possible differences in virulence between strains of *S. dysgalactiae* used in each study.

Results of this study and from previous research (Almeida and Oliver, 1995) indicated that *S. dysgalactiae* invaded bovine mammary epithelial cells *in vitro*. Results of earlier *in vivo* studies (Chandler et al., 1980) as well as recent *in vitro* studies (Matthews et al., 1994; Almeida et al., 1996) strongly suggest that bacterial entry into mammary epithelial cells may be a common feature among mastitis pathogens. A high proportion of *S. dysgalactiae* IMI result in clinical mastitis (Oliver et al., 1990; Todhunter et al., 1995) which are characterized by persistence of infection and poor response to antibiotic therapy (Guterbock et al., 1993). Adherence of *S. dysgalactiae* to mammary epithelial cell surfaces is most likely a prerequisite for invasion into epithelial cells.

However, there is only scarce information on bacterial and host structures involved in this process as well as in postadherence phenomena (Filippsen et al., 1990; Almeida and Oliver, 1995; Calvinho et al., 1996). Potential importance of invasion of mammary epithelial cells *in vivo* is not clear. From a bacterial standpoint, it can favor bacterial persistence by affording protection from host defense factors as well as antibiotics, and can provide a route to subepithelial tissues (Chandler et al., 1980). Early work showed that *S. dysgalactiae* penetrated through the epithelium of the alveoli and smaller ducts to reach interacinar tissues in experimental mastitis in goats (Pattison and Smith, 1953). Further studies using polarized cells would be needed to substantiate this hypothesis. Conversely, *in vivo* studies addressed the phagocytic potential of mammary epithelial cells, suggesting enzymatic digestion of bacteria by epithelial cells harboring mastitis pathogens (Chandler et al., 1980) which could thus favor host defense mechanisms (Brooker, 1983). This, however, was not observed in the present study.

In conclusion, *S. dysgalactiae* can survive within mammary epithelial cells for extended periods of time without losing viability or damaging the eukaryotic cell. These *in vitro* findings may be associated with persistence of IMI. Protection of *S. dysgalactiae* from host defense mechanisms and antibiotic drugs can be a contributing factor to the poor response to antibiotic therapy observed in mastitis caused by this organism and can favor colonization of deeper tissues.

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CHAPTER V

CHARACTERIZATION OF MECHANISMS INVOLVED IN UPTAKE OF STREPTOCOCCUS DYSGALACTIAE BY BOVINE MAMMARY EPITHELIAL CELLS

Abstract

Little is known about bacterial and host factors that contribute to establishment and persistence of intramammary infection by *Streptococcus dysgalactiae*. This organism adheres to bovine mammary epithelial cells and to extracellular matrix proteins *in vitro*, and invades bovine mammary epithelial cells in culture; all of which can be potentially important pathogenic mechanisms. The objective of this study was to characterize eukaryotic and prokaryotic cellular functions required for invasion of *S. dysgalactiae* into bovine mammary epithelial cells. Bovine mammary epithelial cells were pretreated with inhibitors of protein kinase activity, actin polymerization and receptor-mediated endocytosis. In addition, mammary epithelial cells and *S. dysgalactiae* were pretreated with inhibitors of protein synthesis. Results showed that activity of tyrosine protein kinases, intact microfilaments and *de novo* eukaryotic protein synthesis were required for invasion of *S. dysgalactiae* into bovine mammary epithelial cells; a process that appeared to occur via receptor-mediated endocytosis. In contrast, *de novo* bacterial protein synthesis was not required for invasion of *S. dysgalactiae* into MAC-T cells. These studies provide insight into bacterial and cellular mechanisms involved in early host-pathogen interactions, putting into perspective the role of mammary epithelial cells in development and establishment of intramammary infections by *S. dysgalactiae*.

Introduction

Entry of bacteria into eukaryotic cells is preceded by bacterial binding to cell surfaces; however, many bacterial species are able to bind to cell surfaces but do not invade eukaryotic cells (Isberg, 1991). Pathogenic organisms have developed various strategies to access host cells, thereby avoiding, subverting, or nullifying host protective mechanisms (Falkow et al., 1992; Galán, 1994). Bacterial invasion into eukaryotic cells usually involves utilization of host cell functions by invading pathogens which exploit existing signal transduction pathways to trigger cytoskeletal rearrangement and bacterial uptake (Rosenshine et al., 1994). Many of these pathways involve protein kinases as well as Ca^{2+} fluxes to transmit signals. Although attachment, entry and disruption of host cell functions by a pathogenic organism can be considered to be genetically separate events, they represent individual steps of a process that will lead to persistence of the organism within the host (Bliska et al., 1993). Therefore, to understand bacterial invasion, the role of components and functions of the eukaryotic cell need to be delineated.

Recent *in vitro* studies demonstrated that bacterial invasion of mammary epithelial cells appeared to be a common feature among mastitis pathogens (Matthews et al., 1994; Almeida and Oliver, 1995; Almeida et al., 1996). However, little information is available about mechanisms used by mastitis pathogens to induce internalization into host cells and to alter cell functions during attachment and internalization. *Streptococcus dysgalactiae* invades bovine mammary epithelial cells in culture in a time-dependent fashion (Almeida and Oliver, 1995). Intact microfilaments were necessary for bacterial entry into eukaryotic cells, which appeared to occur by an endocytosis mechanism. Coculture of mammary epithelial cells with *S. dysgalactiae* (Almeida and Oliver, 1995), and survival of *S. dysgalactiae* within mammary epithelial cells for varying periods of time, as described in Chapter IV, did not affect cell viability.

The objective of this study was to characterize mechanisms involved in uptake of *S. dysgalactiae* by bovine mammary epithelial cells. Studies were conducted to determine the effect of inhibitors of eukaryotic and prokaryotic functions on invasion of mammary epithelial cells by *S. dysgalactiae.*

Materials and Methods

Mammary epithelial cells

An established transformed mammary epithelial cell line (MAC-T; Huynh, et al., 1991) and HeLa cells were used in these experiments. MAC-T cells were obtained from J.D. Turner (McGill University, Quebec, Canada), and HeLa cells were obtained from J.A. MacCabe (The University of Tennessee, Knoxville). The number of epithelial cells per well was estimated by counting in a hemocytometer to determine the bacterial:epithelial cell ratio used during invasion experiments. Cell cultures were tested for the presence of mycoplasma contamination using a commercial test (Gibco, BRL, Life Technologies Inc., Grand Island, NY).

Cell culture medium

Cell culture medium (CCM) for MAC-T cells contained 40% RPMI 1640 (Sigma Chemical Co., St. Louis, MO), 40% Dulbecco's modified Eagle's medium (DMEM; Gibco BRL), 10% fetal bovine serum (FBS; Bio Whittaker, Inc., Walkersville, MD), 1 mM sodium pyruvate (Sigma Chemical, Co.), 2 mM L-glutamine (Bio Whittaker, Inc.) and 40 mM HEPES buffer (Sigma Chemical Co.). The following supplements were added to the medium: bovine insulin (5μg/ml; Sigma Chemical Co.), hydrocortisone (1μg/ml; Sigma Chemical Co.), and bovine prolactin (1μg/ml; kindly provided by USDA,

Animal Hormone Program, Beltsville, MD) (Cifrian et al., 1994). HeLa cells were cultured in minimum essential medium (MEM, Gibco, BRL) with 10% fetal bovine serum (FBS, Bio Whittaker, Inc., Walkersville, MD).

Bacterial strains and culturing

Two strains of *S. dysgalactiae* were selected according to cluster analysis of PCR-based DNA fingerprint patterns among the population of *S. dysgalactiae* isolated from two dairy herds (Jayarao and Oliver, 1994). *Streptococcus dysgalactiae* strain UT516 was isolated from a subclinical case of mastitis, and strain UT519 was isolated from a clinical case of mastitis. A noninvasive organism, *Escherichia coli* DH5α (Gibco, BRL, Bethesda, MD) was used as a negative control. *Yersinia enterocolitica* strain UT133 obtained from the Department of Food Science and Technology, The University of Tennessee, Knoxville and *Salmonella typhimurium* strain 14028 (American Type Culture Collection; (ATCC), Rockville, MD) were used as controls for inhibition of epithelial cell and bacterial functions. *Streptococcus dysgalactiae* strains were activated from frozen skim milk stocks (-80° C) by inoculating skim milk and incubating for 18 h at 37° C. After activation bacteria were grown in Todd Hewitt broth (THB, BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) overnight at 37° C to midlogarithmic phase [$\approx 10^8$ colony forming units (CFU)/ml]. *Salmonella typhimurium* and *Y. enterocolitica* were grown overnight in LB broth (Gibco, BRL) at 37°C, the latter in an atmosphere of 5% CO_2. On the day of experimentation, bacteria were harvested by centrifugation, washed twice in PBS and resuspended in PBS. Bacterial concentration and purity were determined by plate count on brain heart infusion agar (BHIA, Becton Dickinson Microbiology Systems, Cockeysville, MD) and blood agar, respectively.

Invasion assay

The invasion assay was performed using standard *in vitro* invasion methodology involving incubation of bacteria with epithelial cells followed by selective killing of extracellular bacteria by gentamicin and penicillin G and release of internalized bacteria by lysis of mammary epithelial cells with detergent (Rubens et al., 1992). Mammary cells were grown to confluence in 24-well tissue culture plates, inoculated with approximately 1 x 10^7 CFU/ml of *S. dysgalactiae* and centrifuged at 600 x g for 5 min. Cocultures were incubated for 2 h at 37° C in 5% CO_2:95% air and this was referred to as invasion incubation. Following invasion incubation, supernatants of wells were removed, monolayers were washed three times with PBS and CCM containing gentamicin (100 μg/ml, Sigma Chemical Co.) and penicillin G (5μg/ml, Sigma Chemical Co.) was added. Plates were incubated for an additional 2 h at 37° C and this was referred to as antibiotic incubation. Effectiveness of antibiotics in killing extracellular bacteria was monitored by culturing supernatants from infected cell monolayers treated with antibiotics. After removing media containing antibiotics, monolayers were washed three times with PBS, treated with 0.025% trypsin, and further lysed with Triton X-100 (Amersham, Arlington Heights, IL) at a final concentration of 0.1% (vol/vol) in PBS. Cell lysates were transferred to 1.5 ml Eppendorf tubes and vortexed vigorously. Lysates were diluted serially 10-fold, plated on BHIA, and incubated overnight at 37° C to determine CFU/ml. Bacterial growth during the assay was monitored by culturing bacteria in CCM for 2 h at 37° C in wells without epithelial cells and number of CFU/ml was determined. Percentage of invasive bacteria was calculated by dividing the number (CFU/ml) of intracellular bacteria by the number (CFU/ml) of bacteria in the inoculum x 100. Experiments were repeated at least twice and data from average values of triplicate wells in a single experiment were presented (Elsinghorst and Kopecko, 1992). Invasion of HeLa cells followed the method described by Rosenshine et al. (1992). HeLa cells were

cocultured with bacteria for 90 min (invasion incubation) and incubated in MEM containing 100μg/ml of gentamicin for an additional 90 min (antibiotic incubation).

Pretreatment of MAC-T cells with protein kinases inhibitors

All inhibitors were dissolved in dimethyl sulfoxide and stock solutions were divided into aliquots and stored at -80° C. Prior to use, protein kinase (PK) inhibitors were diluted in CCM, filter sterilized and then added to cultured epithelial cells. The following inhibitors were evaluated: 1μM staurosporine (Sigma Chemical Co.); 200 μM genistein (Sigma Chemical Co.); and 1000 μM tyrphostin (Sigma Chemical Co). Mammary epithelial cells were pretreated for 1 h with PK inhibitors. Epithelial cell and bacterial viability in the presence and absence of inhibitors were evaluated. Cells were cultured in the presence or absence of an inhibitor for 6 h and viability of treated cells was determined by trypan blue exclusion and compared with that of nontreated cells. Fresh overnight bacterial cultures (10 μl aliquots) were inoculated into 1 ml of CCM with or without an inhibitor. Cultures were incubated at 37° C in 5% CO_2/balance air for 2 and 4 h and number of CFU from treated and untreated bacteria were compared. Activity of PK inhibitors was established by determining their effect on invasion of *Y. enterocolitica* and *S. typhimurium* into HeLa cells (Rosenshine et al., 1992). Effects of PK inhibitors on invasion of *Y. enterocolitica* into MAC-T cells were evaluated to determine if this strain could be used as a control for PK inhibitor activity.

Pretreatment of MAC-T cells with inhibitors of actin polymerization and receptor-mediated endocytosis

MAC-T cell monolayers were preincubated for 1 h with the following inhibitors of actin polymerization: 0.5 μg/ml of cytochalasin B (CB, Sigma Chemical Co.) and 0.5 μg/ml of cytochalasin D (CD, Sigma Chemical Co.). MAC-T cell monolayers were

preincubated for 1 h with 5 μM, 50 μM and 500 μM of monodansylcadaverine (Sigma Chemical Co.) which is an inhibitor of receptor-mediated endocytosis (Pastan and Willingham, 1981). Prior to use, inhibitors were diluted in CCM, filter sterilized and then added to cultured epithelial cells. Effect of inhibitors on epithelial and bacterial cell viability was evaluated as described previously.

Fluorescent labeling of F-actin

To visualize F-actin, MAC-T cells were grown to confluency on 4-well polystyrene tissue culture chambers (Nunc, Inc., Naperville, IL) and treated with 0.5 μg/ml of CB and 0.5 μg/ml of CD for 1 h. Nontreated cells were used as a control. Actin filaments were visualized by staining with BODIPY® FL-phallacidin (Molecular Probes Inc., Eugene, OR). MAC-T cells were fixed for 10 min with 3.7% formaldehyde in PBS, washed, and treated with 0.1% Triton X-100 in PBS for 5 min. Cells were washed three times with PBS and incubated with 0.15 μM FL-phallacidin for 30 min at room temperature with constant agitation in the dark. After washing three times with PBS, slides were mounted in a 1:1 solution of PBS-glycerol and viewed with a fluorescence microscope (50-W vapor mercury lamp, exciter 505 nm, dichroic mirror 510 nm, and barrier 520 nm).

Adherence assay

Effects of CD on actin can influence bacterial adherence to eukaryotic cells (Ewanowich and Pepler, 1990). Therefore, effect of CD on adherence of *S. dysgalactiae* strain UT516 to MAC-T cells was evaluated by pretreating MAC-T cell monolayers with 0.5 μg/ml of CD for 1 h. Adherence assay was carried out at 4°C as described in Chapter II to avoid bacterial internalization.

Pretreatment with inhibitors of protein synthesis

MAC-T cells were pretreated with 50 μg/ml of cycloheximide (Sigma Chemical Co.) and bacteria were treated with chloramphenicol (Sigma Chemical Co.). Cycloheximide concentration was selected based on previous results that showed a 95% reduction of protein synthesis by MAC-T cells treated with 50 μg/ml (Almeida et al., 1997). Chloramphenicol concentrations of 10 and 15 μg/ml were selected based on a preliminary experiment to detect antibiotic concentrations that inhibited *S. dysgalactiae* growth without affecting bacterial viability. Prior to use, protein synthesis inhibitors were diluted in CCM and filter sterilized. Cycloheximide was then added to cultured epithelial cells and appropriate bacterial suspensions were added to CCM containing chloramphenicol. Epithelial cells and bacteria were pretreated for 30 min and inhibitors were present throughout the invasion assay. Plate counts were performed from supernatants of both *S. dysgalactiae* strains at every chloramphenicol concentration at the end of the invasion incubation period to monitor for bacterial growth. Inhibition of bacterial protein synthesis decreased invasion of *S. typhimurium* into epithelial cells (MacBeth and Lee, 1993). Therefore, chloramphenicol concentrations of 20 and 50 μg/ml that inhibited *S. typhimurium* strain ATCC 14028 growth without affecting bacterial viability were selected and this strain was included as a control. Effect of chloramphenicol and cycloheximide on cell viability was determined by trypan blue exclusion as described previously.

Statistical analysis

Each condition in the invasion assay was performed in triplicate and experiments were repeated at least twice. All data obtained were expressed as mean ± standard error of the mean (SEM). Data obtained for each condition were compared with untreated controls by Student's *t* test.

Results

Effect of protein kinase inhibitors

To confirm that PK inhibitors were influencing bacterial uptake rather than blocking other cell functions, effects of these inhibitors on invasion of two invasive bacteria, *Y. enterocolitica* and *S. typhimurium*, into HeLa cells was evaluated (Rosenshine et al., 1992). Invasion of *Y. enterocolitica* into HeLa cells was decreased by these inhibitors while invasion of *S. typhimurium* was not affected (data not shown). Since a similar effect was observed following invasion of *Y. enterocolitica* into MAC-T cells, this organism was included as a positive control thereafter. Staurosporine (1 μM) decreased invasion of *Y. enterocolitica* ($0.05<P<0.06$) but did not affect invasion of strains of *S. dysgalactiae* UT516 and UT519 into MAC-T cells. Genistein (200 μM) and tyrphostin (1000 μM) reduced invasion of *Y. enterocolitica* and *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells (Table 8). Neither bacterial nor epithelial cell viability were affected by PK inhibitor concentrations used.

Effect of inhibitors of actin polymerization and receptor-mediated endocytosis

Cytochalasins B and D promoted changes in the distribution of F-actin in MAC-T cells (Figure 17). Stress fibers composed of F-actin were observed in untreated cells. MAC-T cells treated with CB maintained a cellular architecture similar to that observed in untreated cells, but showed less stress fibers than untreated cells. MAC-T cells treated with CD showed a cellular morphology different from that of untreated cells. A marked cellular rounding was observed under the inverted microscope and by fluorescent staining stress fibers were no longer observed, rather, actin bundles were visualized at the periphery of cells. Pretreatment of MAC-T cells with CB and CD inhibited invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells ($P< 0.005$) (Table 9). Pretreatment of MAC-T cells with CD at 0.5 μg/ml caused a decrease of 80% on

Table 8. Effect of protein kinase inhibitors on invasion of *Streptococcus dysgalactiae* into MAC-T cells[1].

Inhibitor	Percent invasion *S. dysgalactiae* UT516	*S. dysgalactiae* UT519	*Y. enterocolitica* UT113
Staurosporine (1 μM)	ND[2]	ND[2]	59.3 ± 2.2
Genistein (200 μM)	53.7 ± 15.2	7.7 ± 5.3	29.0* ± 2.5
Tyrphostin (1000 μM)	6.6* ± 8.4	56.6* ± 14.0	10* ± 4.5

[1]Untreated controls were considered as 100% invasion for comparison purposes. Values are expressed as percentage of untreated controls and are the mean ± standard error of the mean of triplicate experiments.

[2]No decrease compared to untreated control.

*Indicates significant differences compared to untreated control ($P<0.05$).

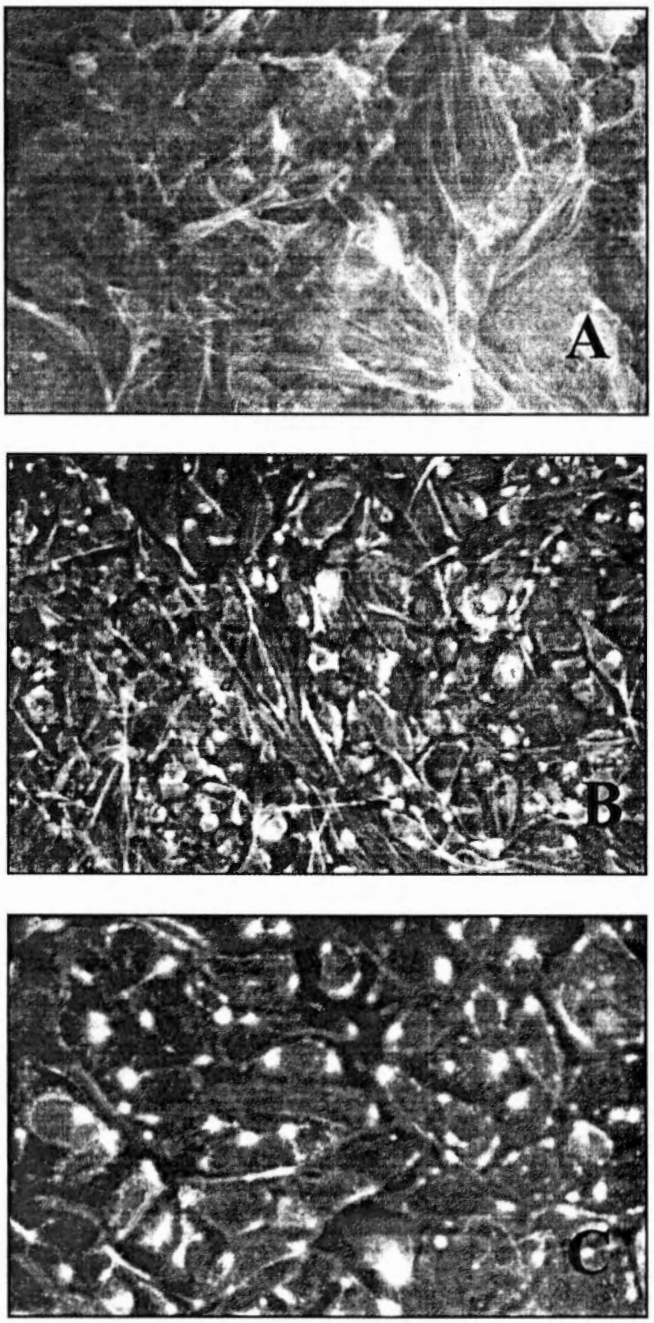

Figure 17. Fluorescent labeling of F-actin in MAC-T cell monolayers. A: untreated control; B: cell monolayers treated with 0.5μg/ml of cytochalasin B for 1 h at 37°C and C: cell monolayers treated with 0.5μg/ml of cytochalasin D for 1 h at 37°C. (x 200).

Table 9. Effect of inhibitors of actin polymerization and receptor-mediated endocytosis on invasion of *Streptococcus dysgalactiae* into MAC-T cells[1].

Inhibitor	Percent invasion	
	S. dysgalactiae UT516	*S. dysgalactiae* UT519
Cytochalasin B (0.5 μg/ml)	15.9* ± 3.6	6.0* ± 0.7
Cytochalasin D (0.5 μg/ml)	6.6* ± 1.2	5.0* ± 0.5
Monodansylcadaverine (5 μM)	ND[2]	78.1 ± 1.1
Monodansylcadaverine (50 μM)	21.0* ± 5.2	16.9* ± 1.9
Monodansylcadaverine (500 μM)	1.2* ± 0.4	0.5* ± 0.2

[1]Untreated controls were considered as 100% invasion for comparison purposes. Values are expressed as percentage of untreated controls and are the mean ± standard error of the mean of triplicate experiments.

[2]No decrease compared to untreated control.

*Indicates significant differences compared to untreated control ($P<0.05$).

adherence of *S. dysgalactiae* strain UT516 to MAC-T cells ($0.01<P<0.05$).

Pretreatment of MAC-T with monodansylcadaverine at three different concentrations caused a dose-dependent inhibition of invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells (Table 9). Inhibitors did not affect epithelial or bacterial cell viability at the concentrations used.

Effect of inhibitors of protein synthesis

Treatment of both strains of *S. dysgalactiae* with chloramphenicol did not affect invasion of these organisms into MAC-T cells (Table 10). Untreated bacteria replicated in CCM supernatants, while the number of chloramphenicol-treated bacteria detected at the end of the invasion incubation period was similar to those in the initial inoculum. Treatment of *S. typhimurium* with chloramphenicol decreased ($P<0.01$) invasion of this organism into MAC-T cells (Table 10). Pretreatment of MAC-T cells with cycloheximide decreased ($P<0.01$) invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells (Figure 18). No differences between strains were detected. Inhibitors did not affect epithelial or bacterial cell viability at the concentrations used.

Discussion

Intracellular location of bovine mastitis pathogens has been shown using *in vivo* (Chandler and Anger, 1977; Chandler et al., 1980) and *in vitro* models (Matthews et al., 1994; Almeida and Oliver, 1995; Almeida et al., 1996). *In vitro* invasion models have provided a useful tool to evaluate both bacterial and cellular functions involved in early host-pathogen interactions.

Invasive bacteria have developed mechanisms to access host cells by exploiting

Table 10. Effect of chloramphenicol on invasion of *Streptococcus dysgalactiae* into MAC-T cells[1].

Strain/treatment	Log_{10} CFU supernatants	Log_{10} intracellular CFU
S. dysgalactiae UT516		
Untreated	8.29	5.59 ± 0.40
Chloramphenicol 10 μg/ml	7.50	5.73 ± 0.68
Chloramphenicol 15 μg/ml	7.42	5.73 ± 0.20
S. dysgalactiae UT519		
Untreated	8.05	5.63 ± 0.33
Chloramphenicol 10 μg/ml	7.12	5.69 ± 0.17
Chloramphenicol 15 μg/ml	7.32	5.77 ± 0.43
S. typhimurium ATCC 14028		
Untreated	8.45	5.84 ± 0.10
Chloramphenicol 20 μg/ml	7.22	4.96* ± 0.47
Chloramphenicol 50 μg/ml	7.32	4.76* ± 0.09

[1]Log_{10} CFU in initial inoculum were 7.4 for *S. dysgalactiae* strain UT516, 7.6 for *S. dysgalactiae* strain UT519 and 7.8 for *Salmonella typhimurium* strain 14028. Data are the mean ± standard error of the mean of triplicate experiments.

*Indicates significant differences compared to untreated control ($P<0.05$).

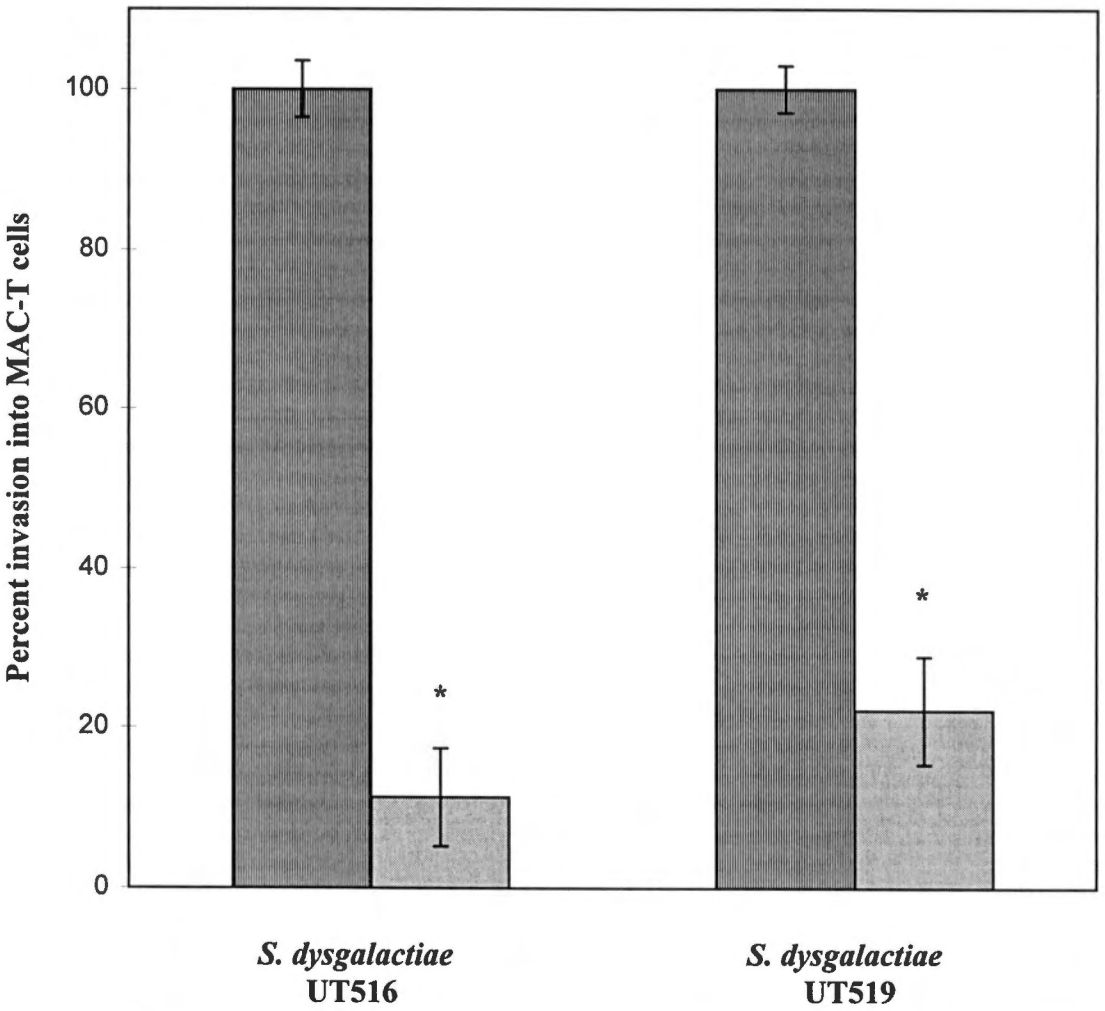

Figure 18. Effect of pretreatment of MAC-T cells with cycloheximide on invasion of *Streptococcus dysgalactiae* strains UT516 and UT519 into MAC-T cells. Untreated MAC-T cells (striped bars), MAC-T cells treated with cycloheximide (solid bars). Untreated controls were considered as 100% invasion for comparison purposes. Error bars indicate standard error of the mean of triplicate experiments.

*Indicates significant differences compared to untreated control ($P<0.05$).

existing host signal transduction pathways to trigger cytoskeletal rearrangements and bacterial uptake (Rosenshine et al., 1994). Protein kinases are involved in regulation of many cellular processes critical for growth, differentiation and tumor promotion by transducing signals generated by cell surface receptors to the cell nucleus (Tamaoki and Nakano, 1990). These enzymes catalyze phosphorylation of serine, threonine and tyrosine residues upon receiving a signal at the cell surface thus activating proteins that may in turn affect uptake of bacteria into eukaryotic cells (Galán, 1994; Rosenshine et al., 1994). Therefore, if PK activity is necessary for transducing a bacterial uptake signal, inhibitors of these enzymes should also inhibit bacterial invasion (Rosenshine et al., 1992).

In this study, PK inhibitors were used to determine whether invasion of *S. dysgalactiae* into MAC-T cells involved a host PK. Staurosporine did not affect invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells while genistein and tyrphostin decreased invasion of these organisms into MAC-T cells. Staurosporine is a potent inhibitor of protein kinase C (PKC), cAMP-dependent PK (PKA), and a less potent inhibitor of protein tyrosine kinase (PTK) activity of growth factor receptors such as epidermal growth factor and insulin and insulin-like growth factor (Tamaoki and Nakano, 1990). Genistein and tyrphostin are highly specific PTK inhibitors (Akiyama et al., 1987; Gazit et al., 1989). Genistein blocks binding of ATP to the enzyme, whereas tyrphostin competes with the tyrosine binding residue of the kinase (Rosenshine et al., 1994). Results of the present study suggest that PTK plays a significant role in uptake of *S. dysgalactiae* by MAC-T cells whereas PKC and PKA do not appear to be involved in this process. Tyrosine phosphorylation plays an essential role in signal transduction through integrins, a large family of α/β heterodimeric transmembrane protein receptors expressed by a variety of cell types (Hynes, 1992) that are known to interact either directly or indirectly with bacterial pathogens (Isberg, 1991; Falkow et al., 1992; Bliska et al., 1993). Results of Chapters II and IV showed that adherence and invasion of *S.*

dysgalactiae to MAC-T cells was mediated by saturable cell receptors, and reduction of bacterial adherence following cell fixation indicated that cell surface proteins played a major role in this process. Further work is needed to determine the nature of eukaryotic cell receptors involved in early interactions with *S. dysgalactiae*.

Invasion of several bacterial pathogens into nonprofessional phagocytes involves cytoskeletal actin microfilaments (Bernardini et al., 1989; Finlay and Falkow, 1989; Ewanowich and Peppler, 1990). Upon bacterial adherence to the cell membrane, transient actin polymerization proximal to the site of bacterial entry is associated with formation of a phagocytic vesicle enclosing the organism (Finlay and Falkow, 1989). The stimuli and signaling pathways that control rearrangement of F-actin are poorly understood (Cossart et al., 1996); however, phosphorylation of integrins has been associated with transduction of signals from the cell membrane to the actin network via intermediate proteins such as talin, vinculin, and α-actinin (Isberg, 1991; 1990; Bliska et al., 1993). Uptake of most bacteria into eukaryotic cells can be blocked by use of cytochalasins which bind to actin to modify its polymerization (Rosenshine et al., 1994). Recent studies in our laboratory showed that pretreatment of MAC-T cells with cytochalasins B and D caused a dose-dependent inhibition of *S. dysgalactiae* invasion into MAC-T cells (Almeida and Oliver, 1995). In the present study, decreased invasion of *S. dysgalactiae* into MAC-T cells following pretreatment of MAC-T cells with CB and CD was also observed. Morphological changes in actin filaments viewed by fluorescent staining of MAC-T cells treated with CB and CD substantiated these findings; CD caused marked disruption of actin filaments, while CB-treated MAC-T cells showed less pronounced changes. This agrees with previous observations on effects of CB and CD on HeLa cells (Ewanowich and Peppler, 1990). Marked morphological changes on MAC-T cell monolayers following treatment with CD correlated with a higher inhibition of invasion of *S. dysgalactiae* strain UT516 into MAC-T cells pretreated with CD. However, invasion

rates of *S. dysgalactiae* strain UT519 into MAC-T cells were similar for CB- and CD-treated cells.

Effects of CD on actin filaments can alter eukaryotic cell surface proteins to the extent that adherence of bacteria to cells can be affected (Ewanowich and Peppler, 1990). However, morphological changes caused by CD did not necessarily lead to impairment of bacterial adherence to eukaryotic cells (Guzman et al, 1994; Grassmé et al., 1996). To determine whether CD was affecting adherence to or uptake of *S. dysgalactiae* by MAC-T cells, the influence of pretreating MAC-T cells with CD on adherence of *S. dysgalactiae* UT516 to MAC-T cells was evaluated. Adherence of *S. dysgalactiae* strain UT516 to MAC-T cells decreased significantly following CD treatment. This correlated with morphological changes observed in MAC-T cells following CD treatment, suggesting that disruption of actin architecture also affected the normal external morphology of the cell. Therefore, decreased numbers of invasive bacteria following pretreatment of MAC-T cells with CD cannot be attributed solely to disruption of microfilament function.

Coated pits are the site of entry for many hormones and protein ligands that enter cells via receptor-mediated endocytosis. This process involves binding of ligands to specific membrane receptors, association of ligand-receptor complexes with clathrin-coated regions of the plasma membrane, and endocytosis of these complexes into cytoplasmic vesicles (Schlegel et al., 1982). Monodansylcadaverine, a transglutaminase inhibitor, has been used as an effective inhibitor of receptor-mediated endocytosis (Pastan and Willingham, 1981, Sreenivasan et al., 1993; Almeida et al., 1997). Transglutaminase is considered to cross-link cellular receptors to intracellular proteins leading to stabilization of ligand-receptor complexes in coated pits (Pastan and Willingham, 1981). Recent electron microscopy studies of MAC-T cells invaded by *S. dysgalactiae* showed accumulation of electron-dense aggregates underlying the cell membrane and formation

of clathrin-coated pit-like structures at the cell surface area where MAC-T cells and bacteria interacted (Almeida and Oliver, 1995). In the present study, a dose-dependent inhibition of invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells pretreated with monodansylcadaverine was observed. Collectively, results of this study and previous research (Almeida and Oliver, 1995) strongly suggest that *S. dysgalactiae* enters MAC-T cells through a receptor-mediated endocytosis mechanism.

Inhibitors of *de novo* bacterial protein synthesis did not affect invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells, while invasion of *S. typhimurium* ATCC 14028 was decreased significantly. MAC-T cells ingested similar numbers of untreated or chloramphenicol-treated *S. dysgalactiae* although untreated bacteria were replicating in cell culture medium supernatants. This confirms previous observations showing that a finite number of bacteria could be ingested by MAC-T cells (Chapter IV). These results suggest that bacterial protein(s) involved in uptake of *S. dysgalactiae* by MAC-T cells were already present on the bacterial surface and were synthesized constitutively under growth conditions used in this study. Invasive organisms such as *Y. enterocolitica* do not require *de novo* protein synthesis for invasion into eukaryotic cells (Finlay and Falkow, 1989). In contrast, requirement for *de novo* protein synthesis for invasion of eukaryotic cells has been reported for several bacteria (MacBeth and Lee, 1993; Sreenivasan et al., 1993) including *Staph. aureus* isolated from bovine intramammary infections (Almeida et al., 1997).

Inhibition of eukaryotic cell protein synthesis resulted in significant inhibition of invasion of *S. dysgalactiae* strains UT516 and UT519 into MAC-T cells, indicating that metabolically active eukaryotic cells were required for uptake of *S. dysgalactiae*. This agrees with similar requirements for a number of bacterial pathogens (Finlay and Falkow, 1989; Sreenivasan et al., 1993; Guzman et al., 1994) including *Staph. aureus* isolated from bovine intramammary infections (Almeida et al., 1997). In contrast, *Salmonella*

does not require *de novo* eukaryotic protein synthesis for invasion (Finlay et al., 1989). Previous electron microscopy studies showed presence of glutaraldehyde-killed bacteria within mammary secretory cells following inoculation of these organisms into mice mammary glands, suggesting that bacterial viability was not a prerequisite for invasion of mammary epithelial cells (Chandler et al., 1980).

In conclusion, these studies demonstrated that two strains of *S. dysgalactiae* isolated from bovine IMI invaded mammary epithelial cells. Involvement of host kinases, intact microfilaments and *de novo* eukaryotic protein synthesis were required for the invasion process; a process that appeared to occur by a receptor-mediated endocytosis mechanism. In contrast, *de novo* bacterial protein synthesis was not required for invasion of *S. dysgalactiae* into MAC-T cells. These data provide a foundation towards a more fundamental understanding of early interactions between bovine mammary epithelial cells and *S. dysgalactiae* and should assist in further research directed to define bacterial ligands and eukaryotic cell receptors involved in these early interactions.

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VITA

Luis Fernando Calvinho was born in San Isidro, Province of Buenos Aires, Argentina on October 13, 1955. In December 1972, he graduated from Instituto Martín y Omar in Buenos Aires province and entered the College of Veterinary Medicine of the University of Buenos Aires in March, 1974. In February 1981, he graduated as a Veterinarian and entered the University of La Plata to purse graduate studies on Bacteriology, receiving a degree in Clinical Bacteriology in 1986. In January 1988, he entered University College Dublin, Ireland and received a Master of Veterinary Medicine degree in 1990. He entered the University of Tennessee, Knoxville and began studies towards the Doctor of Philosophy degree with a major in Animal Science in the Fall of 1993.

In November 1983, he obtained a scholarship from the Institute of Agricultural Technology of Argentina to do research and bacteriological diagnosis of diseases of dairy cattle in the Experiment Station of Rafaela, Province of Santa Fe, Argentina. In 1987, he was appointed as staff researcher at the Institute of Agricultural Technology with dedication on bovine mastitis research and extension, and partial dedication on bacteriological diagnosis. In 1990, he was appointed as Lecturer at the College of Agriculture and Veterinary Medicine of Esperanza, Universidad del Litoral, Argentina, in the Department of Infectious Diseases, and in 1991 was appointed as Coordinator of the Program Physiology and Pathology at the Institute of Agricultural Technology. In 1992 and 1993, he served as an advisor to the Research Center of Veterinary Sciences of the Institute of Agricultural Technology, Castelar. The author is a member of the Veterinary

Medical Association of Argentina, the Argentine Association of Laboratory Diagnostic Veterinarians, the National Mastitis Council, and the Honor Society of Agriculture-Gamma Sigma Delta. The author was awarded a grant by The Graduate School of the University of Tennessee to present results of his research at a scientific meeting, and has published numerous abstracts and articles in peer-reviewed scientific journals.

The author married Patricia I. Nuñez Abrego in December 1982 and has two children, Francisco and Sofía. He will return to his former positions as a researcher and lecturer at the Experiment Station of Rafaela and College of Agriculture and Veterinary Medicine of Esperanza.