PRESENCE OF SOLUBLE CD14 IN BOVINE AND EQUINE MILK

by

JARRED MATHEW WILLIAMS

Under the direction of Liliana Jaso-Friedmann

ABSTRACT

Soluble CD14 (sCD14) is a protein receptor specific for LPS, which circulates in the blood of many species, such as cows and horses. We showed in equine colostrum, and re-confirmed in bovine colostrum, a 48 kD isoform of sCD14. Colostral sCD14 concentrations were higher than serum sCD14 levels from lactating equine and bovine dams. Concentrations of the 48 kD isoform of sCD14 were very low in serum taken from foals and calves before nursing, but very high in serum from the same neonates 24 hours after ingestion of colostrums. Also, bovine mammary epithelial cells were cultured and identified, and the 56 and 50 kD isoforms of sCD14 were detected in the lysates. These findings indicate a role for sCD14 in bovine and equine colostrum, and neonatal immunity.

INDEX WORDS: Colostrum, Soluble CD14, Bovine Mammary Epithelial Cells, Endotoxin, Septicemia, Passive Immunity

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B.S., The University of Georgia, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2002

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August 2002

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the laboratories of Dr. Barton and Dr. Moore for funding this research. I would also like to thank the following people for all they have done for me over the past two years. Dr. James Moore and Dr. Michelle Barton for all their help in every step of my project, for providing me with outstanding opportunities towards my future, for their participation as committee members, and for all of their tireless help, support, and encouragement. My advisor, Dr. Liliana Jaso-Friedmann, for her guidance and willingness to teach. Dr. Thomas Krunkosky for all his expertise and assistance with the cell culture and participation as a committee member. Dr. Michel Vandenplas for his technical expertise and participation as a committee member. Dr. Carla Jarrett for all of her help with histology. Dr. David Hurley for his experimental suggestions and assistance in the research. Heath Cross, Markus Boenig, and Deanne King for their long, hard work and help in collecting the horse and cow samples. Natalie Norton and Cindy Huff for doing all the intangibles that made my life a thousand times easier. Brian McNeill, Dr. Katharina Lohmann, Dr. Margreet Wolfert, and Chris Brandon for all their support, friendship, patience, and senses of humor.

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INTRODUCTION

Cluster differentiation antigen 14 (CD14) is a bacterial pattern recognition receptor on the surface of mononuclear phagocytes and neutrophils (1). CD14 plays a pivotal role in the recognition of and cell activation induced by microbial cell wall components of Gram-negative and Gram-positive bacteria, most notably endotoxin (2). Endotoxin is a lipopolysaccharide (LPS) component of the outer cell membrane of Gram-negative bacteria and is essential for their survival. When LPS gains access to the bloodstream, it forms aggregates due to its amphipathic properties. An endotoxin transporter, lipopolysaccharide binding protein (LBP), extracts LPS monomers from these aggregates and delivers them to cells with membrane-bound CD14 receptors (3-5). The presence of CD14 allows the innate immune system to detect traces of LPS within the bloodstream, which leads to the synthesis and release of several proinflammatory mediators (6-8). The biologic actions of these proinflammatory mediators account for the biologic responses associated with endotoxemia. An important piece of evidence supporting CD14's role as a central component of the innate system is that CD14-deficient mice fail to mount a normal inflammatory response to LPS.

In addition to membrane-bound CD14, two soluble forms of CD14, sCD14□ (50 kD) and sCD14□ (56 kD), have been identified in normal plasma at concentrations of approximately 2-3 μg/mL (9-10). Soluble CD14 binds to whole bacteria and bacteria cell wall components, such as endotoxin, and mediates bacterial-

induced activation of cells that do not express membrane-bound CD14 (11-13). Recently, a third isoform of CD14 (48 kDa) was identified in the colostrum of cows and women (14). This sCD14 is present in colostrums at concentrations 25 times those in plasma, and is produced by mammary epithelial cells (14). The high concentrations of sCD14 in colostrum suggest that proteins other than colostral antibodies are important in conferring passive immunity, especially in species having a placental structure that prevents the transfer of large proteins directly to the fetus.

The aim of this study was to identify and quantify sCD14 in the colostrum of mares and cows, and to determine whether or not sCD14 persists in milk postpartum. Furthermore, serum concentrations of sCD14 were monitored in foals, calves and their dams over the course of 30 days postpartum.

Due to the reported role of mammary epithelial cells in the production of colostral sCD14, a second aim of this project was to harvest and culture a mammary epithelial cells. Ultimately, this established cell culture was used to identify the mechanisms and factors responsible for the production of sCD14 by mammary epithelial cells.

It is our hope that by studying sCD14 from its production, to its transfer and introduction into neonatal foals and calves, information can be gained that will not only contribute to the further understanding of the relationship between colostral sCD14 and innate immunity, but will also expand the potential for reducing the susceptibility of foals and calves to septicemia.

LITERATURE REVIEW

Endotoxemia

As normal structural components of the cell wall of Gram-negative bacteria, endotoxins are ubiquitous. Gram-negative bacteria can persist in many ways within mammals; they may circulate in the blood stream in their intact form (bacteremia), be confined to a localized infection, or be part of the endogenous bacterial flora of the gastrointestinal tract. Regardless of the scenario, endotoxin molecules are released in large numbers after bacterial cell death or, conversely, as normal by-products of bacterial growth (15). Common infectious conditions associated with endotoxemia in the horse include bacterial pneumonia and pleuropneumonia, endometritis, peritonitis, neonatal Gram-negative sepsis (septicemia or septicemia), and infectious colitis caused by bacteria such as Salmonella spp., that are not part of the normal intestinal flora (16).

The natural intestinal flora of horses consists mainly of Gram-negative, anaerobic bacteria. Thus, large amounts of endotoxin normally reside within the lumen of the equine intestinal tract, and small amounts of endotoxin are able to cross the intact mucosal barrier and reach the circulation (17). The entry of endogenous bacteria and/or bacterial products from the gastrointestinal tract into tissues and circulation is termed translocation (18). Translocation of small quantities of endotoxins is not life threatening, as the endotoxins are cleared from the plasma by the mononuclear phagocyte system in the liver. In order for endotoxin translocation to become a serious problem, large

amounts of endotoxin must cross the intestinal barrier and overwhelm the mononuclear phagocyte system, thus leading to unrestricted activation of the immune system (16).

In cases of acute gastrointestinal disease (colic) in horses, the function of the intestinal mucosal barrier is frequently impaired, rendering these animals prime candidates for development of endotoxemia. Gastrointestinal rupture, intestinal obstruction, severe inflammation (enteritis and colitis), and intraluminal acidosis from grain overload, are common conditions that cause increased permeability of the mucosal barrier (19-20). One study, however, reported no difference in plasma endotoxin concentrations between horses with different diseases, emphasizing the fact that many abdominal diseases can induce endotoxemia in the horse (21).

Although insults to the integrity of the intestinal barrier are quite common causes for endotoxemia in horses, it is important to note that these insults are not the only cause. Experimental studies involving various animal models have demonstrated that hypovolemic shock, burn injury, trauma, and malnutrition are other means of initiating an endotoxin-associated immune response (22-24). Furthermore, endotoxin also has the ability to cause bacterial translocation into mesenteric lymph nodes when administered intraperitoneally to mice (25). These findings help explain the pathogenesis of endotoxic shock in animals lacking evidence of an overwhelming bacterial infection.

Endotoxin

Endotoxin was first described as a toxin " closely attached to, and probably integral of, the bacterial body", that was believed to be distinct from the actively secreted, heat-labile and proteinacious bacterial exotoxins (26). Rather than a substance that is

internalized within the bacterium, as its name would imply, endotoxin is a structural component of an outer membranous coating of the cell wall of the bacterium (27). It is a heat-stable lipopolysaccharide (LPS) structure, and the terms endotoxin and LPS are used interchangeably.

Endotoxin is a major structural component of the cell wall of all Gram-negative bacteria. It makes up about 75% of the outer layer of the outer cell membrane, and is a key functional molecule for the bacterial outer membrane serving as a barrier against external agents (28). As Gram-negative bacteria undergo periods of rapid growth or when the bacterial wall disintegrates during death of the bacterium, endotoxin is released into the surroundings.

Structurally, the LPS molecule consists of 4 domains arranged according to their affinity for water. Three of the domains (inner core, outer core, and O-specific chain) represent the hydrophilic polysaccharide portion of the molecule, whereas the lipid A domain is hydrophobic. In aqueous solutions, LPS molecules form micellar aggregates because of the amphiphilic properties conferred on them by the four domains.

The outer portion of endotoxin is composed of a chain of repeating oligosaccharides, which determine the antigenic specificity of the bacterium (29). The O-specific chains (also called O-antigen polysaccharides or O-chains) are enormously variabe, thus creating a tremendous number of different O-specific polysaccharides that distinguish the various families and strains of *Salmonella, Escherichia coli, Proteus, Pseudomons*, and other Gram-negative bacteria (30). The oligosaccharide units are preformed and are added to a growing polymer chain. The polymer chain is attached to the last monosaccharide of the core, and serves as a continually growing anchor for the

attachment of the repeating oligosaccharide units (31). Upon interaction with a host's immune system, the O-specific chains serve as antigens for the production of specific antibodies (32)

The inner (lipid A proximal) and outer (O-chain-proximal) core oligosaccharides make up the last two hydrophilic portions of endotoxin. These portions are more conserved among different strains of Gram-negative bacteria than the O-specific chain, and are primarily composed of sugars (30). Specifically, the inner and outer core oligosaccharides are an arrangement of several monosaccharides (including glucose, galactose, and heptose), N-acetylglucosamine, phosphate, and ethanolamine linked to lipid A by the unique sugar KDO (3-deoxy-D-manno-oct-2-ulopyranosoic acid (33). The core portion of endotoxin is synthesized from the lipid A region "outward", thus, any failure to add the next sugar unit within the core region prevents synthesis of an intact complex (34). Synthesis of a minimal core structure is essential for the survival of bacteria (33), and the smallest naturally occurring LPS structure consists of lipid A and KDO (35).

The final domain of endotoxin is a lipid portion termed lipid A due to its solubility in acid. The lipid A portion anchors the LPS molecule in the bacterial outer membrane and is the toxic principle of LPS (36). Hydrophobic by nature, lipid A is oriented towards the interior of the bacterium where it links to the cell wall. The chemical makeup of lipid A is unique in nature, and its structure is highly conserved among Gram-negative bacteria (37). The common structure shared by lipid A molecules is a 1,4'-bisphosphorylated β1,6-linked D-glucosamine disaccharide backbone (lipid A backbone), which is acylated by up to 6 fatty acids (30).

CD14

Any antigenic determinant on a pathogenic microorganism has the potential to initiate an immune response within a given mammalian organism. An almost infinite number of receptor molecules (T-cell receptors and antibodies) exist in the mammalian immune system, to specifically recognize such antigens. Unfortunately, the unique diversity of this immune response takes too long upon initial exposure to the pathogen. The killing of pathogens by antibodies or effector T cells involves proliferation of antigen-specific T- and B- cell clones, a process which takes several days to weeks to complete. The precious time expended in mounting such a response could lead to irreversibly harmful or fatal results. Therefore, the immediate and often life-saving immune response against a pathogen relies on a more nonspecific recognition of antigens by a small number of pattern recognition receptors. These receptors are designed to target structures sharing a certain degree of similarity in a large number of types of strains of microorganisms (e.g. LPS, lipoteichoic acid, muramyldipeptide), but are not present on host tissues (38). Some of the pattern recognition receptors currently identified are the scavenger receptors, the family of Toll-like receptors, and CD14 (39).

The CD14 molecule is primarily expressed on monocytes and some macrophages, but has been identified on polymorphonuclear granulocytes (40-41). In mice, expression of CD14 mRNA has been documented in many tissues, specifically those having the potential for exposure to LPS, such as uterus, adipose tissue, and lung (42). Additionally, CD14 protein expression has been detected in tracheal epithelial cells and hepatocytes from LPS-treated mice (43-44).

Structurally, the CD14 protein is attached to the cell surface by a glycosyl-phosphatidyl-inositol (GPI) anchor and can be removed by incubation with phosphatidylinositol-specific phospholipase C (45-46). CD14 has four potential N-linked glycosylation sites and bears O-linked carbohydrates (47). Of the total molecular mass of the mature 53-kD glycoprotein, 20% is due to its carbohydrate content (9). Despite the fact that the total lack of N-linked carbohydrates is associated with diminished CD14 surface expression on transfected CHO cells, glycosylation is not required for the protein to be functional (47-48).

The primary sequence of CD14 has been determined in a number of different species, (e.g. human, horse, cow, rabbit, mouse and rat). The sequence of equine CD14 is quite homologous (approximately 70%) to its human counterpart, and to a lesser extent, to mouse, rat, cow, and rabbit (49-52). CD14 contains a number of leucine-rich repeats, which also occur in a number of phylogenetically distant proteins. These repeats are purported to mediate protein-protein interactions, however, no function has actually been shown (53).

CD14 was discovered to be a key receptor on the surface of monocytes required for the induction of an inflammatory response by trace amounts (pico- and nanomolar) of LPS (6-8). Recognition of LPS by CD14 is catalyzed by lipopolysaccharide-binding protein (LBP), an acute-phase serum reactant (54-57). The binding stoichiometry of LPS and CD14 has been reported to be 1:1, with a K_d value of 27nM, although under different experimental conditions, large complexes of LPS, LBP, and CD14 form (58-60).

CD14 is not only present in a membrane-bound form, but a substantial amount of CD14 exists as a soluble form (sCD14) in serum and other body fluids. As a free-

moving, soluble form, sCD14 has been suggested to inhibit LPS-induced activation of monocytes through direct competition with membrane-bound CD14 (mCD14) (61-63). However, neutralization of LPS is not sCD14's only function, as it also acts as a stimulatory soluble receptor, which facilitates LPS-induced activation of cells lacking mCD14 (12,64-67). In humans, sCD14 is present in serum at a concentration of 1-6 μg/mL, in a number of different isoform patterns (68). Initially, two forms were described, a high-molecular mass form of 53-56 kD, and a low-molecular mass form of 48-50 kD (9,69). Subsequently, the two isoforms have been identified in the supernatants of a human monocyte cell line (MonoMac-6) (13). Increased serum concentrations of CD14 have been reported in a number of clinical conditions in people, including chronic inflammatory diseases, polytrauma and burns, and septic patients with multiple-organ failure (70-76). In studies involving septic patients, serum sCD14 concentrations correlated with increased mortality and there was a predominance of the high-molecular-mass isoform (55kD) of sCD14 (77).

There have been many studies performed to elucidate the mechanisms involved in the generation of sCD14 and its isoforms. It was first reported that CD14 was released from the surface of monocytes after stimulation with various activating agents, including phorbol 12-myristate 13-acetate, interferon-γ, LPS, a calcium ionophore, and anti-CD14 antibodies. It was further demonstrated that the sCD14 naturally shed from the monocyte surface was smaller than mCD14 or the soluble form released by treatment with phosphatidylinositol-specific phospholipase C (78). Because the GPI anchor attachment is associated with the removal of a hydrophobic C-terminal signal peptide, sCD14 may escape post-translational modification and be directly secreted. The results of some

studies indicate that the absence of the eight C-terminal amino acids prevents mCD14 expression, but not the secretion of a high-molecular-mass form of sCD14 (10).

Lipopolysaccharide Binding Protein

Lipopolysaccharide binding protein (LBP) is another plasma protein having the ability to recognize and bind LPS. LBP occurs in high concentrations in serum of humans and other species (79-80). Initially, complexes of LBP and LPS were shown to be bound by mCD14 and this led to an enhanced cellular stimulation than that of LPS alone. The function of CD14 as an LPS receptor was first demonstrated when purified LBP became available, and experiments were performed showing it binding LBP and LPS complexes (54). Despite their roles as the two major endotoxin binding proteins, LBP and CD14 do not share sequence homology, and their mechanisms of release into the bloodstream are completely different. LBP, an acute-phase protein, is regulated transcriptionally, while CD14 is released mainly by the shedding of the cell-bound receptor molecule (78,81). However, similarities do exist between the two proteins. Both CD14 and LBP are present in similar quantities in serum, increase in concentration during sepsis, and have approximately the same molecular weight (82-84). Also, for the regulation of certain LPS-dependent effects, LBP clearly works in synergy with CD14.

Structurally, LBP is a glycosylated 58-kD protein synthesized by hepatocytes, and released into the bloodstream as part of the acute-phase response (79-81,85). In human serum, LBP exists at normal concentrations of 5-15 μg/L and increases up to 100-fold during the acute-phase response. Through analysis of their genes and genomic organization, it was determined that LBP belongs to a family of lipid-binding proteins,

which includes bactericidal/permeability increasing protein (BPI), phospholipid ester transfer protein (PLTP), and cholesterol ester transfer protein (CETP) (86-92). Like other proteins in this family, LBP also binds certain phospholipids (93-94).

Functionally, LBP binds to the lipid A part of LPS, and in low concentrations catalyzes its transfer to membrane-bound or soluble CD14 (3-5). The binding site is believed to consist of a particular pattern of charged amino acid residues, which also exists in BPI, and in an endotoxin-binding protein of the horseshoe crab (86,95-99). Besides binding to LPS and certain phospholipids, LBP also binds to the surface of whole bacteria and to LPS-coated erythrocytes. The result of such binding is an enhanced attachment of these particles to macrophages (100).

LBP concentrations increase dramatically during an acute-phase stimulation, such as trauma, the systemic inflammatory response syndrome (SIRS), or sepsis. The increase in LBP concentrations is caused by transcriptional activation of the LBP gene mediated by cytokines IL-1 and IL-6, either by IL-6 alone or synergistically with IL-1 (81,85,101-102). Transcriptional regulation of acute-phase proteins in the liver is due to the proximity of macrophages releasing proinflammatory cytokines to the hepatocytes that synthesize the proteins (103). The increase in LBP serum concentrations is often maximal by day 2-3 during the acute-phase response (81).

Mechanistically, LBP facilitates the interaction of LPS with CD14, while polymeric LPS binds weakly to leukocytes and fails to provoke much of a response at low concentrations. The addition of plasma, as a source of both LBP and CD14, dramatically accelerates the binding of LPS to cells. The presence of LBP allows cellular responses to be evoked at LPS concentrations of picogram/mL (2,105).

LPS monomers spontaneously diffuse from aggregates at a very slow rate in plasma, so effective recognition of endotoxin requires factors that facilitate the quick and efficient dispersal of LPS in plasma. LBP is one such factor, as it binds to LPS aggregates with high affinity, and facilitates the transfer of LPS monomers to cellular binding sites, such as CD14 (3,54,106-109). LPS monomers complexed to LBP have the ability to activate cells that express CD14 on their surface (83,110-117). Stimulation of cells lacking mCD14 requires the presence of sCD14, and LBP plays a much less pronounced role than in cells with mCD14. LBP also catalyzes the transport of LPS to lipoproteins or phospholipid vesicles directly, or by utilizing sCD14 as a possible transfer molecule (5,118). The binding of LPS by lipoprotein results in increased LPS clearance and decreased binding of LPS to cells. This leads to diminished production and release of proinflammatory cytokines (119-120). Thus, during endotoxemia LBP plays a dual role by enabling and inhibiting responses to LPS, as LBP catalytically transfers LPS to at least two destinations, CD14 and lipoproteins.

Molecular Cell Signaling Pathways

The activation of LPS-responsive cells by endotoxin is the initial mechanism of cellular activation by LPS, and leads to an increase in expression of inflammatory mediators. Macrophages, the first immune cells to encounter endotoxin, respond to minute amounts, thus allowing for an extremely efficient method for removal of Gram-negative bacteria and free LPS from the circulation. The immune system responds to endotoxins because of the aforementioned LBP, which functions mainly to transfer LPS to endotoxin-responsive cells such as: mononuclear phagocytes, neutrophils, and

lymphocytes. This highly sensitive response to LPS was demonstrated in experiments using LBP "knock-out" mice, which are unable to control any Gram-negative bacterial infection and are rapidly overwhelmed (121). In cases of extremely high concentrations of LPS, however, cells can be activated in the absence of LBP (54). Besides its role as an LPS transporter, LBP also has opsonizing abilities (100). By being able to opsonize endotoxin, LBP is the crucial identification step in the phagocytosis of LPS by macrophages and neutrophils (122). Phagocytosis of LPS is extremely important, as it is an endotoxin elimination step that does not involve internal cellular signaling (123).

Aside from LBP, LPS receptors are extremely important in the effective response to endotoxin. The two LPS receptors of the most importance are CD14 and Toll-like receptor 4 (TLR4) (124). Like CD14, TLR4 is a pattern recognition receptor, that recognizes LPS as a pattern that is common to all Gram-negative bacteria (125). As mentioned in an earlier section, CD14 is anchored to the cell membrane by GPI anchor (46). Lacking a membrane spanning hydrophobic domain capable of directly transmitting a signal into the cell, CD14 must rely on another means of relaying its message (45). TLR4 does have a transmembrane domain and it, along with a co-receptor named MD-2, is the means by which CD14 is linked to the cytosolic environment (126). TLR4 got its name because of its homology, in its mammalian form, with the "Toll" receptor type in Drosophila. (127). This receptor is important for dorsoventral orientation and immune responses in the fly. To date a number of Toll-like receptors have been identified in mammalian species, i.e. TLR1-TLR9, but TLR4 seems to be the receptor most strongly associated with endotoxin and cell signaling (124). Its importance has

been reported in a number of experiments in which the mouse gene encoding for TLR4 is mutated or deleted, resulting in a LPS-hyporesponsive phenotype (128-129).

After monomeric LPS is bound by LBP and transferred to the CD14 receptor, a reaction occurs between CD14 and TLR4, in which the LPS signal is internalized. The result is an initiation of signaling events within the cell and the alteration of cellular metabolism. This sequlae of events represents cell activation. Signaling pathways are generally characterized by constant and ongoing phosphorylation and activation of enzymes, which typically culminate into the activation of transcription factors; these transcription factors are proteins that bind to DNA and promote activation of genes for certain inflammatory cytokines. The two pathways of particular importance to endotoxin-induced cell signaling are the mitogen activated protein kinase (MAPK) pathway, which activates the transcription factor AP-1, and the nuclear factor κB (NFκB) pathway, which activates transcription factor NFκB (130-131).

The NFκB pathway begins as the intracellular domain of TLR4 associates with the adapter protein MyD88 (myeloid differentiation factor 88) and recruits interleukin-1 associated kinase (IRAK) to the complex. IRAK activation is then followed sequentially by the activation of tumor necrosis factor receptor-associated factor (TRAF6), NFκB-inducing kinase (NIK), IκB-kinase (IKK), and culminates with the phosphorylation of IκB, an inhibitor protein complex that retains and inactivates NFκB in the cytoplasm. When IκB gets phosphorylated, it becomes ubiquinated and degraded, and NFκB is translocated to the nucleus where it exerts its activity (132). Structurally, NFkB is a dimeric protein complex with several isoforms, but the p65/p50 heterodimer has been recognized as the most important inducible complex in mammals (133). Many proteins,

whose genes contain promoter elements for NF□B, are of particular importance for the pathogenesis of septic shock; the most notable are cytokines, inducible nitric oxide synthase and cyclooxygenase-2 (130).

Three groups of the MAP kinase pathways are known to be of critical importance for LPS-induced signal transduction: extracellular signal-regulated kinase (ERK), c-JUN-terminal kinase (JNK), and p38. Signaling through any or all of these MAPK pathways could lead to the activation of several different transcription factors (i.e. AP-1), translation initiation factors, and cytosolic enzymes such as phospholipase A_2, or it could increase the expression of several different adhesion molecules on the cell surface (130). It is important to note that despite the functional independence each of the MAPK pathways possesses, they all most likely work simultaneously and synergistically. It has been reported that the activation of all three pathway groups together results in higher levels of TNF reporter gene expression, than that from each group individually (130,134).

Innate Immunity of the Foal

Septicemia is a rapidly progressive, often fatal systemic infection that affects foals less than 7 days of age. It is the most common cause of severe illness and death in newborn foals, and in one study of 160 foals under the age of 14 days, septicemia was the second most common problem diagnosed, behind inadequate transfer of maternal antibodies. Infections that occur after parturition are most likely in conjunction with inadequate colostral consumption, which is the primary mode of antibody transfer within the foal. Clinically, inadequate colostral antibody transfer is known as partial or complete failure of passive transfer (FPT).

The anatomy of a mare's placenta is markedly different from that of a human. Anatomically, a mare's placenta is epitheliochorial, meaning six layers of tissue separate the maternal and fetal circulations (135). The extensive configuration of the many layers of tissue effectively prevents any in utero transfer of maternal antibodies to the fetus; although, some viruses, such as equine herpes virus 1, and bacteria, can cross the placenta to infect the fetus. Consequently, the foal is born with no circulating IgG and only small amounts of its own IgM. Due to the latter, the fetus has some immunologic capacity to mount a response to infection in utero, as well as immediately postpartum (135). Despite the fact that the newborn foal is capable of mounting an immune response to antigenic stimulation, the response is only a primary one, and is accompanied by a prolonged lag time and low antibody production (135). Therefore, it is absolutely essential that the foal receive "immunologic assistance" from another source, primarily, the gastrointestinal absorption of colostrum from the dam. Without this assistance, the foal is left unprotected against the environmental microorganisms that it will encounter immediately at birth.

Colostrum is the first mammary secretion produced by the mare. It contains high concentrations of IgG and IgG(T), and smaller amounts of IgM and IgA (136). Unfortunately, the obvious immunological benefits of colostrum are not always available to the foal. The foal's gastrointestinal tract allows for the absorption of the high molecular-weight immunoglobulins for a relatively short period immediately after birth. During this period, there is a special adaptation in the cells of the intestinal lining that allow antibodies to cross the mucosal barrier and enter the bloodstream (137). The peak absorption is believed to be within the first 8 hours of life, with absorptive ability

declining to zero by the end of the first full day as the aforementioned mucosal barrier shuts down (138). Studies have shown that between 2.9% and 24% of all foals have some degree of FPT (139). With the potential for FPT being so high, several reasons have been identified as the culprits, including, failure of the mare to produce antibody-rich colostrum, prepartum lactation, insufficient intestinal absorption, or foal weakness at birth, preventing adequate nursing.

The amount of colostrum a foal should receive is quite variable depending on the density of antibodies present within it. Normal concentrations of mare's colostral IgG range from 4000 to 6100 mg/dL. Based on this concentration, ingestion of 1 to 2 liters of colostrum will increase the foal's antibody concentrations to at least 800-1000 mg/dL (136). Although there had been considerable controversy concerning the concentration of antibody necessary for protecting the foal, the majority of septic foals have IgG concentrations below 800 mg/dL (140). Conversely, it should also be noted that not all foals with low immunoglobulin concentrations will become septic.

While the anatomical or developmental factors mentioned earlier are important reasons for the occurrence of FPT, management factors can also influence the incidence of disease greatly. Foals raised in unsanitary conditions may be at a higher risk of developing disease, according to studies that suggest that lower environmental temperatures and the amount of solar radiation affect colostral and foal serum IgG concentrations (141-142). The age of the dam has also been shown as quite important, as 71% of foals from mares younger than 15 years of age had serum IgG concentrations greater than 800 mg/dL, compared to only 45% of foals from mares older than 15 years (142). Other risk factors include the health and condition of the mare, difficulty of

parturition, the presence of new pathogens in the environment against which the mare has not produced antibodies, and the gestational age of the foal, as premature and postmature foals are at a greater risk of having FPT(142). Ultimately, though, most factors associated with the potential for septicemia or infection in the foal are due to the partial or complete failure of colostral antibody transfer.

Septicemia

Management of equine neonates with sepsis is one of the most difficult problems for equine veterinarians to handle. Septicemia is a systemic disease involving the presence of pathogenic microorganisms and/or their toxins in the blood (143). The classic example of sepsis, and the topic of this discussion, is that of disseminated Gram-negative bacterial infections, but it should be pointed out that a similar septic syndrome occurs in patients with gram-positive bacterial infections, viral infections, trauma, hypovolemia, hemorrhage, and immunologic and drug reactions (144-147). However, bacterial infection is the most common and drastic cause of sepsis, as it accounts for 33% of foal mortality (148). This is because sepsis represents a systemic inflammatory response to the infection or injury; therefore, it can rapidly progress to irreversible septic shock. Once a foal is in shock, making the diagnosis is not difficult. The true challenge is to recognize the sick foal prior to it entering the shock stage. The clinical manifestations of sepsis vary, as the severity of infection in the neonate can range from a focal area of infection, such as an umbilical abscess, to multi-organ involvement.

The initial abnormalities associated with clinical sepsis result from a nonspecific innate inflammatory response termed the systemic inflammatory response syndrome

(SIRS), which is not necessarily associated with a bacterial infection (149-150). SIRS is a common terminal phase of the inflammatory response characterized by global activation of multiple proinflammatory pathways (145). It is defined by the presence of two or more of the following abnormalities: fever or hypothermia (temperature greater 39.2ºC or less than 37.2ºC), tachycardia (heart rate greater than 120 beats/minute), tachypnea (respiratory rate greater than 36 breaths/min) or hypocapnea (partial pressure of arterial CO_2 less than 32mm Hg), leukocytosis or leukopenia (leukocyte count greater than 12,500 or less than 4000 cells/μl), or an increased number of immature forms of granulocytes (greater than 10% bands) (151-152). Because many different stimuli can induce a SIRS response, and the manifestations of it are the same as those used to define sepsis, SIRS due to infection has become the new definition of sepsis (153). Shock, from the changes associated with SIRS, can result in hypoperfusion and organ dysfunction (termed multiorgan dysfunction syndrome) such that homeostasis cannot be maintained without intervention (146,150-151). Generally, this syndrome begins with cardiovascular dysfunction and leads to dysfunction of the respiratory, hepatic, gastrointestinal, renal, cardiac, and neurologic systems (146,154).

A foal can be infected through a variety of ways, for example: the blood of a septic dam, an infected placenta, contamination of the umbilical stump, ingestion, inspiration, or wounds (143). Regardless of the route, infections are most commonly associated with FPT in combination with exposure to environmental organisms (143). The bacteria most often involved with neonatal sepsis are Gram-negative organisms. Of the many bacterial isolates obtained from the foals with sepsis, *Escherichia coli* is the most predominant, followed in no particular order by *Klebsiella* spp, *Enterobacter* spp,

Actinobacillus spp, *Pseudomonas* spp, *Citrobacter* spp, *Actinobacter* spp, and *Salmonella* spp and *Enterococcus*(135,155). Of the gram-positive species isolated, *Streptococcus* spp and *Staphylococcus* spp are the most frequent (156).

Gram-positive bacteria contain several immune-reactive cell wall components, including peptidoglycans (PGs), lipoteichoic acids (LTAs), enterotoxins, and superantigenic exotoxins (149,157-159). Although bacterial infections, and their constitutive components, may be responsible for the initiation of an inflammatory response, the inflammatory process itself is a result of the production of endogenous mediators.

Endotoxin is removed from circulation through the mononuclear phagocyte system, as LBP recognizes LPS and presents it to CD14, which also binds PG and LTA (149,157,160). These cells then become activated as a result of interactions between CD14 and TLR4. Various inflammatory cytokines then are released, which mediate the hematologic abnormalities that characterize septic shock. One of the important inflammatory pathways initiated by the interaction of LPS and macrophages involves the activation of a membrane-bound enzyme called phospholipase A_2 (143). The function of this enzyme is to cleave arachidonic acid from the cellular membrane, which is then acted upon by two other major enzymes, cyclooxygenase and lipoxygenase. The products of cyclooxygenase activity are prostacyclin, thromboxane, $PGF_{\square}$, and PGE_2. Lipoxygenase activity results in the production of the leukotrienes (20). Together with inducible nitric oxide synthase, these enzymes constitute some of the more important proinflammatory enzymes responsible for synthesis of inflammatory mediators. Among other recognized mediators are the aforementioned inflammatory cytokines (e.g., TNF-$\square$, IL-1, IL-6) and

adhesion molecules (e.g., selectins, intracellular adhesion molecules) (161). Transcription of many of the genes encoding for these mediators and enzymes depends on the transcription factor NF-κB (162-164).

The initial changes that occur in an inflammatory response are primarily the result of local vasodilation and increased vascular permeability caused by the effects of mediators, like histamine, serotonin, eicosaniods, cytokines, platelet activating factor, or fibrin degradation products, released by the injured or infected cell. Ultimately, the systemic manifestations of inflammation are fever, lethargy, malaise, loss of appetite, and cachexia, and are due primarily to the release of TNF-α and IL-1 (165). These clinical signs are often the markers of the detrimental effects of an overwhelming immune response and septicemia.

Mammary Glands

As the primary locale for colostrum and milk production, the mammary gland plays an important role in the synthesis of colostral sCD14. A complete anatomical and physiological understanding of the entire gland is therefore a vital step in identifying the source of CD14 and why it is present in colostrum.

The mammary glands (mammae) are modified sudoriferous (sweat) glands. As an embryo, mammals begin to develop the mammary gland along a line on each side of the abdominal wall parallel to the midline, known as the "milk line". In domestic animals, such as the horse or cow, only the inguinal mammary glands develop, and of these, the most caudal group, either a pair (horse) or four (cow) glands, develops (166). Due to the availability of mammary tissue from the cow, bovine tissue will be the anatomical subject of this review.

The mammary gland or udder of the cow consists of four quarters, with each quarter being its own entity. The skin of the udder is completely covered in fine hair, except for the teat, which is hairless. The right and left halves each consist of a cranial (front) and a caudal (hind) quarter, each representing the four isolated quarters. As far as blood supply, nerve supply, and suspension are concerned, the two halves are almost completely independent. This separation is marked by a longitudinal furrow, the intermammary groove, and occasionally by a transverse furrow between the quarters of each half. As a testament to their independence, one half of the udder can be removed surgically without damaging the other half. Within each half, each quarter is also mostly separate with respect to glandular tissue and the duct system; each quarter has tissue and ductile structures that intermingle with the other, yet they both retain their own identity, anatomically. Thus, all the milk from one teat is produced by the glandular tissue of its respective quarter (166).

The epithelial tissue (parenchyma) of the mammary gland resembles lung tissue, with alveoli and duct systems stemming from them. The alveoli are the chief structures where milk production occurs. The various orders of ducts continuously converge to form larger ducts, which eventually drain into a larger basin, the lactiferous sinus. The lactiferous sinus is technically divided into two cavities, a larger one called the pars glandularis (gland cistern) and a smaller one called the pars papillaris (teat cistern). The term lactiferous sinus is synonymous with either cistern (166).

Within each quarter, the gland cistern is the cavity located above the base of the teat and is continuous with the teat cistern, with the exception of a circular ridge containing a vein and some smooth muscle fibers. The wall of the empty cistern (gland

and teat) contains numerous overlapping longitudinal and circular folds that are obliterated through expansion of the wall when the teat is full of milk. There also may be pockets within the wall of the teat cistern. The teat cistern is continuous with the exterior of the teat through a narrow opening in the end of the teat, the streak canal, which opens at the ostium papillae. The streak canal, 8.5 mm in length, consists of epithelial folds that project inward from its wall, leaving only a star-shaped opening. At the junction of the teat cistern and streak canal, the epithelium lining the teat is arranged in a group of radial folds called the rosette of Furstenberg. Of the folds that make up the rosette of Furstenberg, about 8 are regarded as primary, while the rest are secondary, all of which are expanded during milking by pressure from milk in the teat cistern. A sphincter composed of circular smooth muscle fibers surrounds the streak canal at the end of the teat. This sphincter controls the ability of the cow to secrete milk; a "hard milker" represents a cow with a particularly tight sphincter, while a "leaky" cow describes a cow with an extremely loose sphincter (166).

The mammary gland is classified as a compound tubulo-alveolar gland, consisting of a stroma (connective tissue framework), parenchyma (epithelial portion), ducts, vessels, and nerves. The surface of the teat is covered with stratified squamous epithelium, which runs all the way down through the streak canal as the same type of epithelium. Surrounding the streak canal are many smooth muscle fibers, most of which are arranged in a circular (annular) fashion to form a sphincter. The rest of the muscle fibers, not involved in sphincter construction, are arranged longitudinally parallel to the lumen of the streak canal (166).

At the junction of the streak canal and the teat cistern, where the rosette of Furstenberg is located, the epithelial lining changes from stratified squamous to stratified columnar epithelium that is usually two cells thick. This stratified columnar epithelium lines both the teat and gland cistern, as well as the larger lactiferous ducts. As the ducts branch and get smaller into the parenchyma, the epithelial lining changes again; first, to simple columnar, and then, to secretory epithelium in the alveoli. These secretory epithelial cells are of particular interest for the expression of sCD14.

The mammary gland is different from most other exocrine glands in that the secretory portion is not limited to the terminations of the smallest ducts, but rather, milk-secreting structures empty directly into the larger ducts and then into the gland and teat cisterns. The ducts are classified in increasing size as intralobular, interlobular, intralobar, and interlobar. A group of alveoli surrounded by a connective tissue septum form a more or less distinct unit called a lobule. The alveoli making up the lobule empty into the intralobular ducts, which, in turn, drain into a central collecting space, from which the interlobular ducts emerge. A group of lobules within a connective tissue compartment form a lobe. Within each lobe, interlobular ducts unite to form a single intralobar duct, which is called an interlobar duct as soon as it emerges from the lobe. The interlobar duct may enter the gland cistern directly or indirectly by joining one or more interlobar ducts first. Many of these ducts have numerous dilations that act as collecting spaces for milk, much like the lactiferous sinus (166). A diagram of this anatomical configuration can be seen following this chapter in figure 1.1 (167).

The alveoli and ducts are surrounded by contractile cells called myoepithelial cells, or basket cells. As their name implies, these cells form a basket-like covering

around the epithelial cells throughout the alveoli and ducts. The myoepithelial cells resemble smooth muscle fibers, and actually contract or squeeze milk out of cells and down through the ducts during "let-down". In addition to the epithelial parenchyma and the myoepithelial cells, the mammary gland is made up of a stroma of white fibrous connective tissue and yellow elastic connective tissue. Blood vessels, lymph vessels, and nerves run throughout the stroma, and a network of capillaries surround the ducts and alveoli. The complex configuration of vessels and nerves allow for proper cellular stimulation and activation, thus leading to the secretion of milk (166).

Lactogenesis (Regulation of Milk Secretion)

The term lactogenesis is used to describe the initiation of milk secretion, when the mammary glands have reached a functional degree of development. The process of lactogenesis at parturition encompasses three steps: hormonal control involved in the initiation of milk secretion, milk secretion by the epithelial cells of the alveolus, and removal of milk stored in the mammary gland (168).

The concentrations of estrogen and progesterone, maintained by the ovaries and placenta during pregnancy, stimulate the development of the mammary glands, while at the same time inhibiting lactogenesis. At parturition, these concentrations change, and lactogenesis occurs. During pregnancy, blood levels of progesterone, estradiol, adrenal steroids, and placental lactogen are relatively high, but prolactin levels are low. The result is little milk secretion, but actively growing mammary glands. After parturition prolactin increases while progesterone, estradiol, and adrenal steroid concentrations are low. With the decrease in concentration of circulating ovarian and placental steroids, progesterone, and placental estrogen, comes the release of prolactin from the

adenohypophysis by acting on the hypothalamus, which otherwise inhibits the release of prolactin. Prolactin, somatotropin, and the adrenal corticoids are the essential hormones for the initiation of lactation (169).

Epithelial alveolar cells of the lactating mammary gland synthesize and secrete milk proteins (casein, serum albumin, alpha-lactalbumin, beta-lactalbumin, immunoglobulins, and glycoproteins), fats, and lactose. At approximately midgestation, the epithelial alveolar cells begin to secrete quantities of the different milk products, which begin accumulating in the mammary gland as precolostrum. This stage of epithelial secretion is regarded as lactogenesis I, and is a prerequisite for lactogenesis II, which is the secretion of colostrum and milk at parturition (168).

During lactogenesis I, the appearance of lactose in the precolostrum is important, as it indicates that the intracellular apparatus for milk synthesis is functionally differentiated; in mares this occurs at approximately 15 days before foaling, and in cows, approximately 10 days before calving. Throughout this period of late pregnancy, the mammary gland remains unmilked, and tight junctions are maintained as "leaky" to keep the epithelial paracellular pathway open and the ducts permeable. Transfers between extracellular fluid and precolostrum are possible, and the precolostrum contains some large molecules such as immunoglobulins, or possibly sCD14. The aqueous phase of the precolostrum has fluctuating concentrations of chloride, sodium, potassium, and lactose. Also, fat globules, protein granules, desquamated epithelial cells, and leukocytes accumulate in the lumen of the alveoli. With the removal of the precolostrum through milking, the tight junctions are no longer "leaky", and the paracellular pathways in the alveolar epithelium and duct system are closed (168). Lactogenesis II usually begins

before parturition, when the mammary gland first releases colostrum, and continues into the release of normal milk (168). It represents any stage of lactation associated with the removal of milk stored in the mammary gland.

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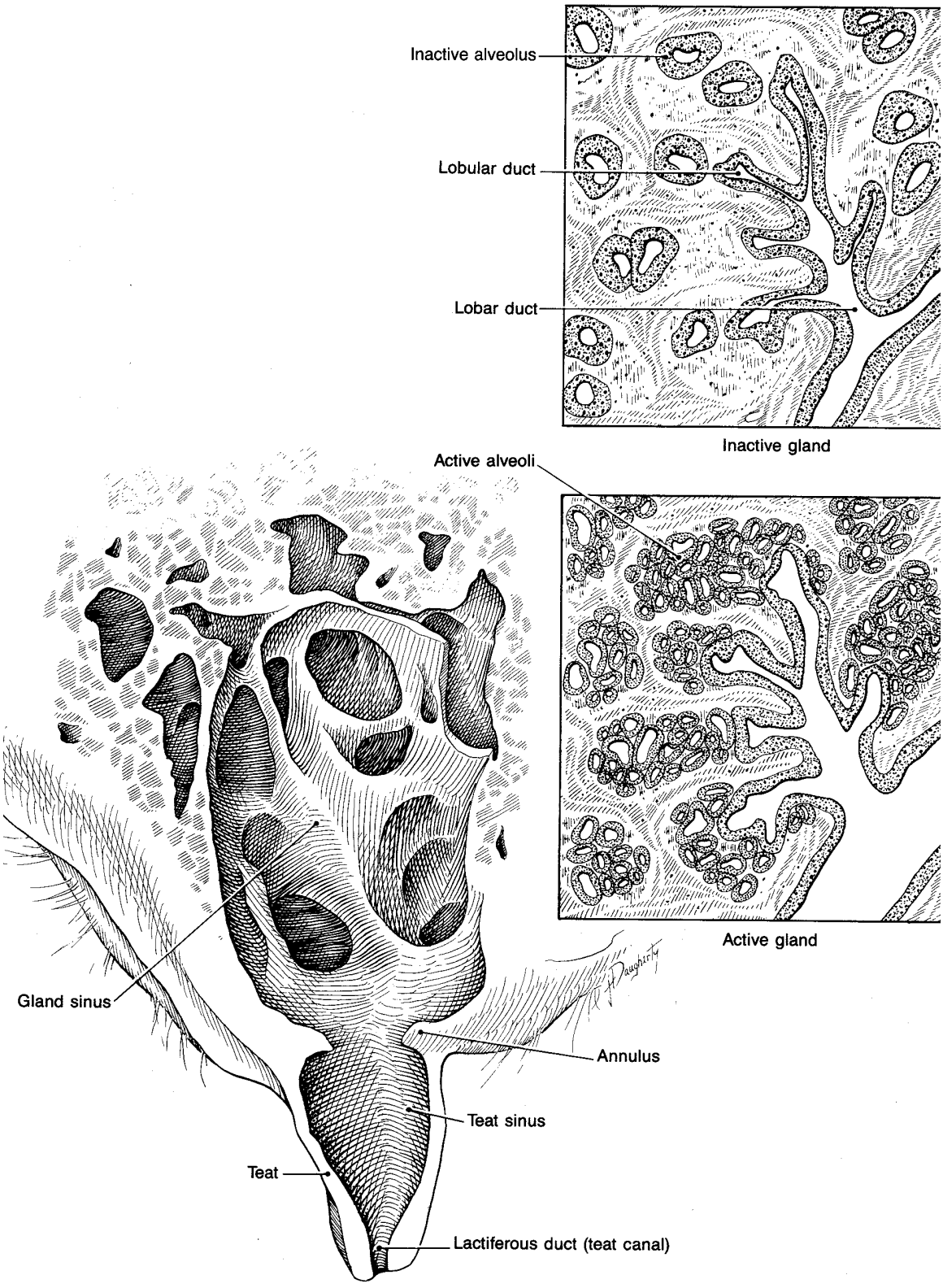
Figure 1.1 **Bovine Mammary Gland**. Diagram of the internal anatomy of an inactive and active mammary gland. Included in the diagram are the lobar and lobular ducts, as well as an enlarged view of the gland sinus. Figure obtained from the 3rd edition of Applied Veterinary Histology.


Inactive alveolus
Lobular duct
Lobar duct
Inactive gland
Active alveoli
Active gland
Gland sinus
Annulus
Teat sinus
Teat
Lactiferous duct (teat canal)

CHAPTER 1

SOLUBLE CD14 IS PRESENT IN BOVINE AND EQUINE MILK AND IS PASSIVELY TRANSFERRED TO FOALS AND CALVES

Introduction

Immediately after the birth of foals or calves, the once sterile fetal intestine becomes colonized with many types of bacteria (1), including endotoxin (lipopolysaccharide; LPS) -containing Gram-negative bacteria. These bacteria have been incriminated as a major contributor to the pathogenesis of a variety of immune and inflammatory neonatal conditions, such as enterocolitis and septicemia (2). The association between endotoxemia and many of the life-threatening complications encountered in equine practice, is due in large part to the immune response of the neonate, rather than a direct toxic effect of the LPS.

LPS, a component of the outer cell wall of Gram-negative bacteria, is among the most potent proinflammatory substances known, initiating the production of multiple host-derived inflammatory mediators, including cytokines (e.g., TNF), arachidonic acid metabolites, and tissue factor (3,4). LPS causes these effects after binding to cluster differentiation antigen 14 (CD14) on mononuclear phagocytes, or to soluble CD14 in plasma and then to cells lacking CD14 (5-8). Soluble CD14 in plasma arises primarily from mononuclear phagocytes, which shed or secrete CD14 into the circulation (9). It has, however, recently been demonstrated that human mammary epithelial cells also

secrete a soluble isoform of CD14 into milk and that concentrations of this CD14 are 20 times higher in colostrums, compared to serum CD14 (10). The milk CD14 isoform differs from isoforms in serum by size and glycosylation profile.

We were interested in determining whether soluble CD14 exists in equine and bovine milk, and whether concentrations of milk CD14 change after parturition. Consequently, we collected milk and serum samples from cows and horses at specific intervals after parturition, and analyzed these samples for sCD14 using a western blot technique.

Based on clinical case studies, approximately 33% of foals that die do so as a result of bacterial infections, with Gram-negative septicemia being an important cause for this mortality (11-15). Furthermore, failure of passive transfer of immunity by ingestion of colostrum, the first secretion of milk presented to the foal, has been positively correlated with sepsis (16,17). These facts led us to wonder whether sCD14 in colostrum was absorbed by the newborn foal or calf. To study this, we collected serum samples from newborn foals and calves before and after ingestion of colostrum, and analyzed these samples for sCD14 using a western blot technique. The foals and calves used for this project were the progeny of the mares and cows being sampled. Consequently, all milk and colostrum samples were collected at the same time the serum samples were obtained from the neonates. Additional serum samples were obtained from the foals for up to 30 days after parturition.

Materials and Methods

Reagents

Leupeptin, PMSF, Tris (hydroxymethyl)-aminomethane (Tris), glycine, lauryl sulfate, sodium orthovanidate ($NaVO_4$), aprotinin, sodium flouride (NaF), β-mercaptoethanol (BME), Ponceau stain, Tween 20, bovine serum albumin (BSA), phosphate buffered saline (PBS), and ethanol were purchased from Sigma Chemical Company (St. Louis, MO). Methanol was purchased from Fisher Scientific (Norcross, GA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). Dura SuperSignal® detection reagent was purchased from Pierce (Rockford, IL). Fluor-S Multi-Imager, Bradford solution, 10% polyacrylamide Criterion gels, Criterion Cell gel system, and Trans-blot transfer cell were purchased from Bio-Rad (Hercules, CA). Immobilized rProtein A was purchased from RepliGen (Cambridge, MA). Mouse anti-human CD14 antibody (Tuk-4) was purchased from CALTAG Laboratories (Burlingame, CA). Sheep anti-mouse IgG HRP-linked whole antibody was purchased from Amersham (Piscataway, NJ).

Animals

Bovine

Cows and calves were provided by the University of Georgia, Dairy Collection Program at the Coastal Plains Experiment Station (Tifton, GA). Parturition by 11 clinically normal cows was attended, and each newborn calf was immediately bled via the jugular vein prior to suckling. Approximately 2-3 mL of blood was collected. Cows

were bled via the jugular vein within the first few hours postpartum, and a similar volume of blood was collected. A sample of colostrum (3-5 mL) was taken immediately after blood collection, just before the calf was allowed to suckle. Colostrum was then collected and bottle-fed to the calf. After approximately 24 hours, the calves were again bled via the jugular vein. After 24 hours postpartum, the calves are fed from a pooled milk supply, so correlations between CD14 concentrations in the milk of the dam and her calf could not be determined after that time. Therefore, calves were only sampled at birth and 24 hours postpartum. Serum and milk samples were obtained from the dams on days 1, 3, 7, 14, 21, and 28 postpartum. A complete calving history for each cow/calf is provided in tables 1-11 in the Appendix, along with a listing of any medications given to a cow or calf during the sampling period.

Equine

Mares and foals were provided by the University of Georgia, Equine Breeding Program at the Smithonia Barn (Winterville, GA). Parturition by 10 clinically normal mares was attended, and each newborn foal was immediately bled via the jugular vein prior to suckling. Approximately 2-3 mL of blood was collected. The umbilical stump was treated with chlorhexidine within 30-60 minutes after the foal stood for the first time. Dams were bled via the jugular vein (2-3 mL) within 60 minutes postpartum. Colostrum samples (10-15 mL) were collected immediately after blood collection, just before suckling was allowed. Mares and their foals were allowed to bond during the foal's teat-seeking period. Most foals were given assistance in locating the teat. Thereafter, the

foals were allowed to suckle without interruption. Assistance was provided until the foal had ingested an acceptable amount of colostrum (approx. 2 liters) or appeared capable of nursing without human intervention. In one instance, a nasogastric tube was used to administer colostrum to the newborn foal. A detailed foaling history is provided for each mare and foal in tables 12-21 in the Appendix.

Each mare was monitored until passage of the placenta. In some instances, oxytocin was administered to facilitate this process (see tables 12-21). All placentas were examined and antibiotics were administered to one foal at increased risk of sepsis (see tables 12-21). Foals and dams were bled via the jugular vein at 4 different time intervals after parturition: 2-3 days, 6-8 days, 13-15 days, and 27-29 days. Milk samples were also obtained at the same time points. Data for each mare and foal are provided in tables 12-21.

A gelded horse was bled via the jugular vein, and his serum used as a universal standard sample in every western blot analysis. The gelding was a donation horse from the University of Georgia College of Veterinary Medicine.

Detection of Birth

Equine

Equine parturition was detected with the Foalert monitoring system. One to two weeks prior to due date, a Foalert transmitter was sutured into the vulva of each mare. The entire vulvar surface was cleaned and surgically prepared with chlorhexidene scrub- and alcohol-soaked gauze. Approximately 5-8 mL of lidocaine was injected into each side of the vulva to anesthetize the region. Sterile precautions and technique were used to

yield the cleanest surgical procedure possible. Two sutures (1", proline) were placed in the left labia approximately 5 centimeters deep, and 1/3 of the way from the top and the bottom of the vulva. The sutures exited the vulvar area approximately 4-5 centimeters to the left of the vaginal opening. Two additional sutures, of the same size, were placed in the center of the right labia. The Foalert transmitter was fixed to the left labia, and the "tripwire" magnet was fixed to the right labia. Upon completion of the surgical procedure, the magnet was placed in the transmitter, and the Foalert was activated. A diagram of the installation of the Foalert transmitter is provided in figure 2.1.

The mares were maintained on pasture, within 300 meters of the Foalert signal-receiving antenna, for the remainder of their term. Upon initiation of foaling, the "tripwire" magnet disengaged from the Foalert transmitter. The Foalert transmitter then sent out a signal, which was received by the antenna and processed by a Foalert receiver box. The Foalert receiver box then activated an automatic dialer, which contacted the investigators via telephone.

Bovine

Parturition in the cows was detected through constant visual monitoring. No electronic devices or signalers were used.

Immunoprecipitation

The blood samples were centrifuged at maximal speed for 10 minutes; serum was collected and stored in small aliquots at –70º C. Each milk sample was centrifuged at 3,500 rpm for 10 minutes, and the fatty layer was removed from the top. The milk

samples also were divided into aliquots and stored at –70º C. Once an entire sample set was complete, every sample was diluted 1:30 (except for colostrum samples from the time of birth, which were diluted 1:60) with Goodie buffer protease inhibitor cocktail: PMSF, leupeptin, Tris(hydroxymethyl)-aminomethane (Tris), sodium orthovanidate ($NaVO_4$), aprotinin, sodium flouride (NaF), and β-mercaptoethanol (BME). A 50/50 slurry of Protein A beads mixed with 1X PBS and 0.02% sodium azide was added to the diluted serum, colostrums, and milk samples to remove IgG. The immunoprecipitating samples were maintained at 4º C and rotated on a rocker for 12 hours. After a thorough immunoprecipation, the samples were centrifuged at 10,000 rpm for 1 minute, and the supernatant was saved.

Protein Determination

The amount of total protein in the immunoprecipitated samples was determined by Bradford protein determination. Approximately 10 μl of each sample was added to 5 mL of Bradford solution, and total protein was measured by spectrophotometer (Therm spectromic Genesys 5). The concentrations of protein in the serum and milk samples are listed in the foaling and calving history tables (tables 1-21).

Western Blot Analysis

Approximately 1 μg of total protein for each sample was diluted 1:2 with Laemmli reducing sample buffer, boiled for 5 minutes, loaded onto pre-casted 10% polyacrylamide Criterion gels, and electrophoresed in the presence of SDS running buffer (lauryl sulfate, Tris, and glycine) in the Criterion Cell Gel system. Proteins were then

transferred to nitrocellulose membranes in transfer buffer (48 mM Tris, 39mM glycine, and 20% methanol) overnight at 0.1 Amps using a Trans-blot transfer cell.

Blots were stained briefly with Ponceau Stain to label lanes and molecular markers, and washed with TBS containing 0.1% Tween 20 (TTBS). Membranes were then incubated in 5% BSA at room temperature on a rocker for 4 hours to prevent non-specific binding. The membranes were then drained of the blocking agent, and exposed to a 1:10,000 dilution of the Tuk-4 antibody overnight at 4ºC on a rocker. After incubation with the primary antibody, the membranes were washed 3 times for 10 minutes each with the TTBS solution, and then mixed with an anti-mouse IgG-horse radish peroxidase conjugated antibody for 1-2 hours on a rocker at room temperature. Finally, the blots were washed another 6 times for 10 minutes each with the TTBS solution and detected with Dura SuperSignal detection reagent, using the Fluor-S Multi-Imager and the Quantity One computer software.

Densitometry

The intensity of each band on the western blot was measured based on the total number of saturated pixels within a defined area. The densimetric analysis was performed using the Quantity One computer software (BioRad).

Statistics

All P-values were determined by a T-test. Regressional analysis of the dam serum samples was performed by the Prism statistics program.

Results

Detection of sCD14 in Bovine and Equine Colostrum. Western blot analysis, using a mouse anti-human CD14-specific antibody (Tuk-4), of cow and horse colostrum samples collected at the time of birth showed a strong single 48 kD polypeptide band (Figure 2.2). The western blot also detected very low amounts of the typical sCD14 doublet, 56 kD and 50 kD, present in serum samples. These results were constant throughout all the bovine and equine colostrum samples; figure 2.2 is representative data from one horse and one cow.

Semi-quantification of sCD14 in Bovine and Equine Samples. Quantification was not possible due to reasons presented in the discussion section. Consequently, concentrations of sCD14 in the samples were assigned a value based on the total number of saturated pixels in a standardized area. Variability among the western blots was compensated for by including a universal standard on every blot. The total number of saturated pixels in each band of sCD14 in the universal standard was averaged within each gel. Then, the total number of saturated pixels in each band of every sample was normalized based on the variance between the value for the universal standard on that given blot and the average value for all of the blots. The result thus provides a semi-quantitative assessment of sCD14 based on the number of saturated pixels rather than protein concentration.

Concentrations of the 48 kD Isoform of sCD14 in Bovine and Equine Colostrum. Average total saturated pixel numbers for the 48 kD isoform of sCD14 in

colostrum samples from 10 horses and 11 cows were 2605 and 1334, respectively. The average relative concentrations of the 48 kD isoform were decreased in all milk samples collected more than 2 days postpartum. In both species, concentrations of the 48 kD isoform of sCD14 in milk declined to very low values by day 2 postpartum and remained at low values throughout the sampling period (Figure 2.3). Concentrations of the 48 kD isoform of sCD14 in colostrum were significantly greater than the concentrations of each of the milk samples (Cow: n=11, $P < 0.0001$) (Horse: n=10, $P < 0.0001$).

All colostrum and milk samples contained small amounts of two slower migrating isoforms, ~56 and 50 kD in size, similar to serum sCD14□ and sCD14□, respectively. These isoforms were present in low concentrations in the milk/colostrum samples. Taken together, sCD14 exists as a complex of three different isoforms. The banding complex is clearly evident in a representative western blot analysis of colostrum and milk from one cow (Figure 2.4) and one horse (Figure 2.5).

Two smaller isoforms were also identified on the western blot analysis by the Tuk-4 antibody (Figures 2.4 and 2.5). These isoforms were present in colostrum from both species. The larger of these isoforms was present in very low concentrations in the colostrum. In contrast, the relative amount of this isoform was significantly increased, compared to colostrums, in every milk sample collected postpartum (Cow: n=11, P=0.0064) (Horse: n=10, P=0.0076). The smaller isoform was in very low concentrations in all colostrum and milk samples, for both species, regardless of sample time.

Changes in Concentration of the 48 kD Isoform of sCD14 In Bovine and Equine Serum Postpartum. Western blot analysis using the Tuk-4 antibody of equine

and bovine dam serum samples taken during the first month postpartum documented an increase in the relative concentration of the 48 kD isoform of sCD14 over time. The average total saturated pixel numbers for the 48 kD isoform of sCD14 in serum samples of 10 horses and 11 cows taken at the time of birth were 226 and 238, respectively. Which are approximately equal to that of the gelding used as the universal standard, 293. The average relative concentrations of the 48 kD isoform increased postpartum, except for the last sample date for horses. On that day, the average saturated pixel number decreased from 733 to 583 and did not follow a linear increase (Figure 2.6). Overall, the relative concentration of the 48 kD isoform significantly increased from the serum samples taken at parturition as compared to those at the last sampling date for the cow ($n=11$, $P=0.001$, $r^2=0.9824$), but did not increase as significantly for the horse over those same two points, ($n=10$, $P=0.2768$, $r^2=0.3694$).

As occurred with the milk samples, all of the serum samples also contained the two slower migrating isoforms, ~56 and 50 kD. These isoforms, however, were present in low concentrations. Thus, equine and bovine sera contain a sCD14 complex consisting of three isoforms. This complex is clearly evident in a representative western blot analysis of serum from one cow (Figure 2.7) and one horse (Figure 2.8).

Presence of the 48 kD Isoform of sCD14 in Foal and Calf Serum After Ingestion of Colostrum. Western blot analysis using the Tuk-4 antibody of foal and calf serum samples collected pre- and post-suckle, documented an increase in the relative concentration of the 48 kD isoform of sCD14. The average total saturated pixel numbers for the 48 kD isoform of sCD14 in pre-suckle serum samples of 10 foals and 11 calves

were 51 and 178, respectively (Figure 2.9). Average values for the 24-hour post-suckle serum samples (foals=938 and calves=956) were significantly higher (calves: n=11, $P < 0.0001$) (foals: n=10, $P = 0.0080$).

The values of the 48 kD isoform of sCD14 in the 24-hour post-suckle serum samples for foals remained significantly high throughout the entire first month postpartum (P=0.0074), despite decreasing over that same period of time (Day 2=938 versus Day 27-29=433) (Figure 2.9).

Like their dams, all the foal and calf serum samples possessed the two slower migrating isoforms, ~56 and 50 kD. These isoforms, however, were present in low concentrations. Thus, the sCD14 complex described in adult serum is contained in post-suckle neonatal serum. This complex is evident in a representative western blot analysis of serum from one calf (Figure 2.10) and one foal (Figure 2.11).

Discussion

Quantifying sCD14 from Equine and Bovine Samples

Originally, the goal of this study was to quantify sCD14 in serum, colostrum, and milk samples from cows and horses. Ideally, ELISAs specific for bovine and equine CD14 should have been used. Unfortunately, commercially available ELISA kits did not exist and there was insufficient time and resources available to develop one. Consequently, we attempted to quantify the sCD14 in our samples by Western blot analysis and densitometry. It was our intent to use an anti-CD14 antibody to detect sCD14, and then compare that signal against a standard curve of sCD14. We encountered several difficulties executing this technique.

The first challenge was to identify an antibody that cross-reacted with bovine and equine CD14, as well as with the CD14 protein we would use for the standard curve. We originally selected MY4, a monoclonal anti-human CD14 antibody (10). After the first few attempts, it appeared that MY4 was reacting not only with the CD14 within the cow and horse samples, but also, non-specifically with the heavy and light chains of IgG. Upon further investigation with a negative control, we determined that the secondary antibody, a sheep anti-mouse IgG, was cross-reacting with the IgG in the horse and cow samples. In an attempt to alleviate this problem, we switched to a polyclonal rabbit anti-equine CD14 antibody, and a goat anti-rabbit IgG secondary antibody. The polyclonal antibody reacted with the bovine and equine samples, and the anti-rabbit IgG secondary antibody did not cross-react with the equine and bovine IgG.

Once the correct antibody was established, we then performed experiments to optimize the conditions for the standard curve. Our first task was to decide which CD14 protein we would use in the standard curve, as we had a commercial source of human recombinant CD14 (hurCD14), and an equine recombinant CD14 (eqrCD14) expressed in insect cells in our laboratory. Initially, we chose to use the eqrCD14 because the primary antibody was directed against equine CD14. Not surprisingly, the polyclonal rabbit anti-equine antibody reacted with the eqrCD14 protein, thus providing an acceptable CD14 standard curve. We then performed several western blot analyses using an entire sampling set from one horse and the standard curve. As the concentration of sCD14 in equine serum had not been determined previously, we needed to establish the sensitivity of our assay. Unfortunately, our results were unrealistically high. Serum from normal, healthy humans contains 1-6 μg/mL; our results suggested that serum from a normal,

healthy horse contains 2-3 mg/mL. Because a 1000-fold difference in the concentration of sCD14 between human and equine serum was unrealistic, we sought to check the accuracy of our assay by performing a western blot analysis using human serum. We obtained human serum from a healthy adult and compared it against a standard curve of human recombinant CD14. Unfortunately, the polyclonal rabbit anti-equine CD14 did not react well with the hurCD14, making it impossible to check the accuracy of our assay using human serum. As a result, we switched to a third primary antibody, BCA-60.

BCA-60, like MY4, is a monoclonal mouse anti-human CD14 antibody, necessitating that a sheep anti-mouse IgG secondary antibody be used in the assay. The BCA-60 cross-reacted with the equine IgG, and did not react with the hurCD14. In fact, the western blots of the hurCD14 detected by BCA-60 had CD14-specific bands, which were the inverse of bands. At the areas one would expect to see a dark band indicating CD14, all that could be seen was the absence of a band, or a "ghost" band. Furthermore, the ghost bands existed in a dose-dependent manner, increasing in size as the concentration of hurCD14 increased in the standard curve.

In an attempt to eliminate cross-reactivity with the sheep anti-mouse IgG secondary antibody, we decided to immunoprecipitate the IgG from the equine and bovine samples. We did this, and then performed western blot analyses of the immunoprecipitated samples and hurCD14 using three antibodies: MY4, BCA-60, and Tuk-4, a third anti-human CD14 antibody. The results were encouraging in that the sheep anti-mouse IgG secondary antibody no longer cross-reacted with the equine IgG. Unfortunately, none of the three antibodies reacted with the hurCD14, thus eliminating our ability to quantify.

In light of these findings, we concluded that our best approach would be to develop a technique that would allow semi-quantification of sCD14. This technique, which was based on the relative concentration of saturated pixels of sCD14 bands as detected by western blot analysis, provided semi-quantitative data because concentrations of sCD14 were expressed as total number of saturated pixels per standard area, rather than total amount of protein per standard volume.

After several additional experiments, we determined that the sCD14 within the equine and bovine samples was detected most specifically by western blot analysis using the Tuk-4 antibody. The method for determining the relative amounts of saturated pixels from bands specific for sCD14 is described in the results section of this chapter. The samples from all 10 horses and 11 cows were processed similarly and the results are discussed below.

sCD14 in Equine and Bovine Samples

The results of this study document the presence of the 56 and 50 kD isoforms of soluble CD14, and high concentrations of the isoform 48 kD in size, in bovine and equine colostrum, and relatively lower concentrations of all the isoforms of sCD14 in milk from both species. Furthermore, all 3 isoforms exist in the serum of mares and cows, and the 48 kD isoform increases in concentration postpartum. Finally, the 48 kD isoform is not present in serum from foals and calves before they ingest colostrum, but is present in their serum after they are allowed to nurse. The larger isoforms of sCD14 are present in all neonatal serum samples both pre- and post-suckle, suggesting a source of sCD14

production for the 56 and 50 kD sized isoforms before parturition, but not for the 48 kD isoform of sCD14.

Colostrum and Milk

Based on the results of this study, bovine and equine colostrum contain all three isoforms of sCD14, with the predominant one being the 48 kD isoform. The primary role of colostrum in both of these species is to provide a means for transferring immunity, from the dam to the neonate, as large proteins cannot traverse the placenta of the cow or horse. Knowing this, we surmise that sCD14, a receptor involved in the innate recognition of bacterial toxins, exists in high concentrations in the colostrum to enhance development and/or function of the neonatal immune system.

Newborn foals and calves are able to absorb large colostral proteins, such as IgG and sCD14, during the first 12-24 hours of life, with maximum absorption occurring by approximately 8 hours. After the first 12-24 hours postpartum, the neonate is unable to absorb these proteins, and all ingested proteins are processed through the gastrointestinal tract. Not coincidentally, the period when large immunoproteins can be absorbed terminates as the mammary gland ceases to produce colostrum. After the first 24 hours, the mammary secretions become less protein rich, and are considered milk. In short, colostrum contains the large immunoproteins at the only time the neonate can take advantage of them. Milk with large amounts of IgG, or sCD14 for that matter, would be a waste. Therefore, it was not surprising to see the concentration of the 48 kD isoform of sCD14 decrease substantially in the bovine and equine milk samples (figure 2.3).

In contrast, we did not expect to identify two smaller molecular weight bands in the colostrum and milk samples that bound the anti-CD14 antibody. Because the Tuk-4

antibody specifically recognized these two distinct bands in the absence of other non-specific bands, we speculate they are directly related to sCD14. These bands could represent either two new, smaller isoforms that exist in equine and bovine milk and colostrum, or degradation products of sCD14. Support for the contention that at least one of these bands represents a degradation product, is the fact that the band is present in very low concentrations in the colostrum, where the concentration of the 48 kD isoform of sCD14 is high. The same small band is present in much higher concentrations in the milk samples, where the 48 kD isoform of sCD14 exists in very low concentrations (figures 2.4 and 2.5). It is reasonable to speculate that the lower bands, or at least one of them, are degradation products of the 48 kD isoform of sCD14 and can not be absorbed by the neonate. It is not possible to determine the identity of these bands until further experiments can be done with the milk and colostrum samples. For example, the sCD14 could be immunoprecipitated from the colostrum and milk samples and then separated out onto a polyacrylamide gel. The sCD14 bands could then be transferred to a membrane, stained, cut out, and sequenced. The sequence could then be compared to the sequence of known bovine sCD14 to search for partial sequence matches.

Maternal Serum

We expected to identify sCD14 in the maternal serum samples, as sCD14 exists in the serum of other mammalian species. We did not expect to identify an increase in the concentration of the 48 kD isoform of sCD14 in the bovine and equine dam serum samples over time after parturition (figure 2.6). These results may suggest that the 48 kD isoform of sCD14 is transported from the mare's and cow's bloodstream into their colostrum. This would contradict previously reported findings that mammary epithelial

cells produce the 48 kD isoform of sCD14. It is important to note that sCD14 in the colostrum is present in very high concentrations, much higher than those in the maternal serum. Furthermore, there is a much larger difference in the concentration of the 48 kD isoform of sCD14 between colostrum and milk, than there is between maternal serum samples taken on different days postpartum.

Taken together, these findings suggest that sCD14 in colostrum may also arise from a source other than mammary epithelial cells, which may be a major contributor of the 48 kD isoform of sCD14, as a transformed human mammary epithelial cell line (MCF-7) has been shown to produce it (10). This isoform increases in concentration by nearly three times in the dam's bloodstream long after production of colostrum has ceased. Furthermore, the 48 kD isoform was present in the serum of the gelding used for the universal standard at approximately the same concentration of that found in the serum of the dams at parturition, providing further evidence for a source other than mammary epithelium.

Discovering the second source of the 48 kD isoform of sCD14 could be a very difficult task. One possible method could be to test horses and cows that have a Gram-negative bacterial infection for the 48 kD isoform of sCD14 in their bloodstream. If concentrations of the isoform are increased, as compared to average concentrations in the bloodstream of healthy animals, tests could be done to recognize which cells are present and/or elevated due to the infection. Those cells could be targeted and tested as potential producers of the 48 kD isoform of sCD14, as well as any tissue-fixed cells in the infected region.

Neonatal Serum

The 48 kD isoform of sCD14 was not detected in any of the pre-suckle blood samples from either the foals or calves. In contrast, the 48 kD isoform was present in all the 24 hour, post-suckle serum samples, and in all of the subsequent serum samples taken from the foals. Thus, ingestion of colostrum rich in sCD14 resulted in detectable serum concentrations of the protein.

These findings suggest that there is transfer of the 48 kD isoform of sCD14 to the neonate after ingestion of colostrum. An informative follow-up study to this one would involve evaluation of serum samples from foals or calves with failure of passive transfer, as definded by serum IgG concentrations <800 mg/dL. This could definitively demonstrate the importance of colostrum in providing the proteins required for modulating local and adaptive immune responses.

In conclusion, the findings here indicate that the 48 kD isoform of sCD14 is present in large quantities in colostrum of horses and cows, and is transferred to the newborn within 24 hours of ingestion. These findings provide the basis for future studies designed to more fully characterize the role of sCD14 and other immunoproteins in the development of the innate immune system.

Potential Roles of sCD14

There are several possible roles for the 48 kD isoform of sCD14: (i) neutralization of endotoxin, (ii) opsonization of bacteria, (iii) activation of an inflammatory response if LPS and bacteria are present, and (iv) activation of B cells. Despite the high circulating concentrations of sCD14 in the healthy neonates in this study, clinical evidence of

activation of an inflammatory response was lacking (i.e., evidence of the biological activites of cytokines). This lack of an obvious systemic inflammatory response, in light of the presence of bacteria and sCD14 within the neonate's intestinal tract, could be due to two processes. First, the 48 kD isoform of sCD14 could lose its bioactivity during transit through the newborn's stomach and proximal intestine; this has been proposed by Labeta et al (10). That hypothesis suggests that the large amounts of sCD14 protein in the colostrum are necessary to compensate for such losses. The sCD14 may help the newborn cope with the vast intestinal microbial inoculum by initiating local intestinal innate and adaptive immune responses that have been modulated in such a way to avoid excessive immune and inflammatory reactions (10).

Alternatively, the 48 kD isoform of sCD14 could function differently than the 50 and 56 kD isoforms of sCD14. Rather than initiating an immune response, the 48 kD isoform could bind and neutralize endotoxin in the GI tract or in circulation, thereby competing with the other two isoforms that can bind LPS and elicit a response. For example, recombinant sCD14 can inhibit the oxidative burst response of isolated human mononuclear cells (19) and LPS-induced TNF release in whole blood (20). Furthermore, high doses of recombinant sCD14 protect mice from LPS-induced lethality (21,22). Like recombinant sCD14, the high concentration of the 48 kD isoform of sCD14 in colostrum could allow the neonate to compensate for vast microbial colonization occurring within its gut, or protect it from excessive upregulation of the inflammatory response if septicemia occurs.

Secondly, colostral sCD14 may be to add bacterial opsonization. In an attempt to reduce the amount of bacteria circulating within the neonate, large amounts of sCD14 in

colostrum could opsonize the pathogen. Thus, the colostral sCD14 assists the developing immune system of a neonate in recognizing and targeting harmful bacteria for elimination.

A third role for colostral sCD14 could be to initiate an inflammatory response to endotoxin or bacteria. In one study, it was shown that colostrum-fed foals experienced a significantly higher increase in serum IL-6 concentrations when infused with endotoxin than colostrum-deprived foals infused with the same amount of LPS (23). The colostrum-fed foals of that study could have experienced higher concentrations of IL-6 because colostral sCD14 recognized the infused endotoxin and initiated an inflammatory response culminating in the release of larger concentrations of cytokines. Alternatively, the colostrum-deprived foals could have released lower concentrations of IL-6 because they did not have access to the colostral sCD14 possibly capable of initiating a greater inflammatory response. Assuming that colostral sCD14 is an inflammatory response initiator, deprivation of colostrum and colostral sCD14 would appear to be a beneficial role. However, it should not be overlooked that the aforementioned study infused the foals with endotoxin, which without an inflammatory response is relatively harmless, and not Gram-negative bacteria containing endotoxin, which can be harmful without an inflammatory response. Colostrum-deprived foals would not possess an adequate defense against the bacteria without colostral sCD14 to recognize and initiate the necessary inflammatory response.

Lastly, in a recent study, it was reported that human and bovine colostrum and milk enriched in sCD14 induced B cell growth and differentiation (18). Our findings further demonstrate the presence of sCD14 in colostrum and milk from horses and cows.

A potentially informative follow-up study would involve testing various fractions of colostrum for their ability to promote B cell growth and differentiation in blood collected from foals or calves with failure of passive transfer. Commercially available sCD14 and IgG would be used as controls. This could help to reinforce the notion of sCD14 within colostrum as a valuable inducer of humoral immunity.

In conclusion, this study confirms the presence of 48 kD isoform of sCD14 in bovine colostrum. It is the first to report that the same isoform is present in the colostrum of horses. It is also the first documentation of the transfer of the 48 kD isoform of sCD14 from the dam to the neonate following ingestion of colostrum. Furthermore, this study is the first to document increased concentrations of the 48 kD isoform in the dam's serum post partum. Lastly, this study is the first to report that the concentration of the 48 kd isoform of the sCD14 is 2-3 times greater than that of adults and that it decreases over the first month post partum to average adult levels.

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Figure 2.1 **Foalert Detection of Foaling**. Diagram of exact Foalert transmitter placement. The Foalert transmitter was sutured into the left side of the vulva, and the tripwire magnet was sutured into the right side. The magnet connected in the transmitter to activate the system. A breach in the connection between the magnet and transmitter immediately activated a signal that notified all study investigators of a foaling. Figure obtained at Foalert.com.

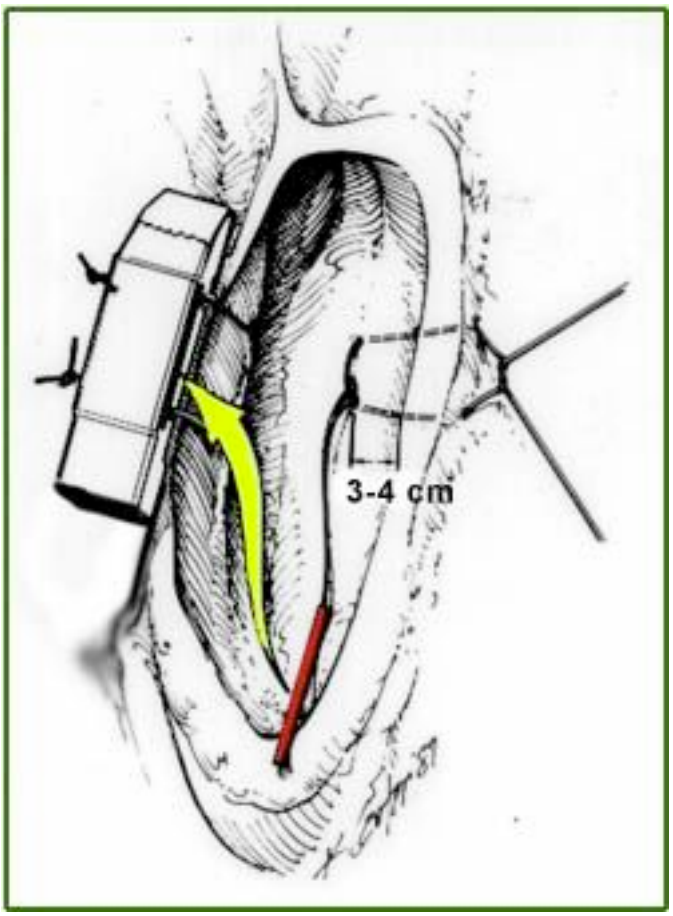

3-4 cm

Figure 2.2 **Presence of 48 kD isoform of Soluble CD14 in Bovine and Equine Colostrum**. Western blot analysis of colostrum samples from cows and mares. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is 1 μg of total protein of bovine colostrum with IgG removed by immunoprecipitation. Lane 2 is 1 μg of total protein of equine colostrum with IgG removed by immunoprecipitation. Each isoform of sCD14 is labeled according to molecular weight.

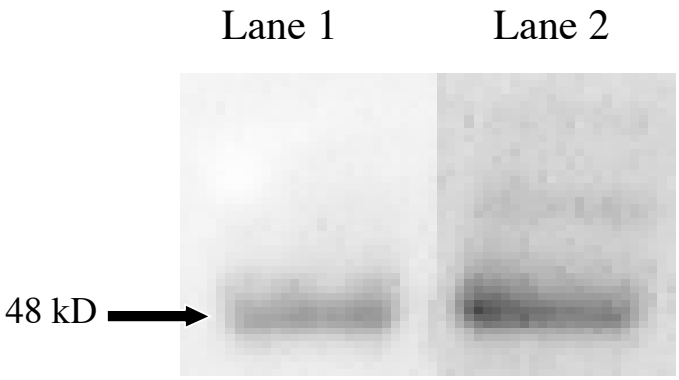

Lane 1
Lane 2
48 kD

Figure 2.3 **Changes in sCD14 in Colostrum and Milk Samples of all Equine and Bovine Study Participants**. Averages of the relative concentrations of the 48 kD isoform of sCD14 from colostrum and milk samples of 10 horses and 11 cows taken at the time of birth. The concentrations were determined by densitometry of a western blot analysis of equine and bovine colostrum and milk samples collected during the first month postpartum. The average relative concentrations of the isoform decreased as time postpartum elapsed.

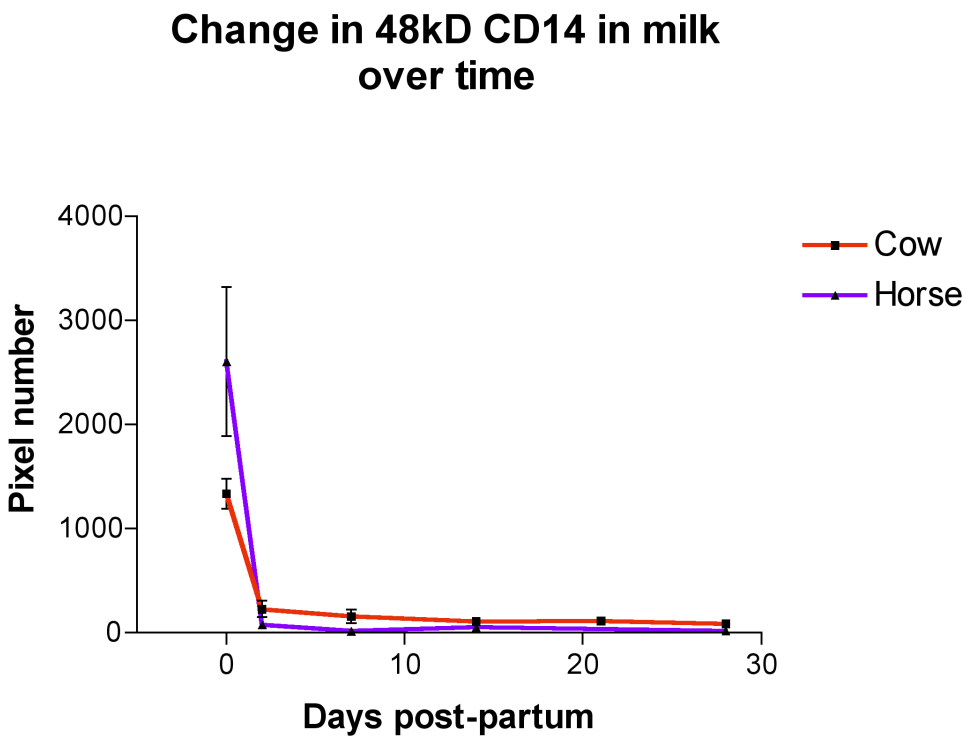

Change in 48kD CD14 in milk over time
Pixel number
4000
3000
2000
1000
0
0
10
20
30
Days post-partum
Cow
Horse

Figure 2.4 **Changes in Soluble CD14 in Bovine Colostrum and Milk Over 28 Days Postpartum**. Western blot analysis of colostrum and milk samples from cows. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is bovine colostrum at parturition; lanes 2-6 are bovine milk at 2, 7, 14, 21, and 28 days postpartum. Each isoform of sCD14 is labeled according to molecular weight.

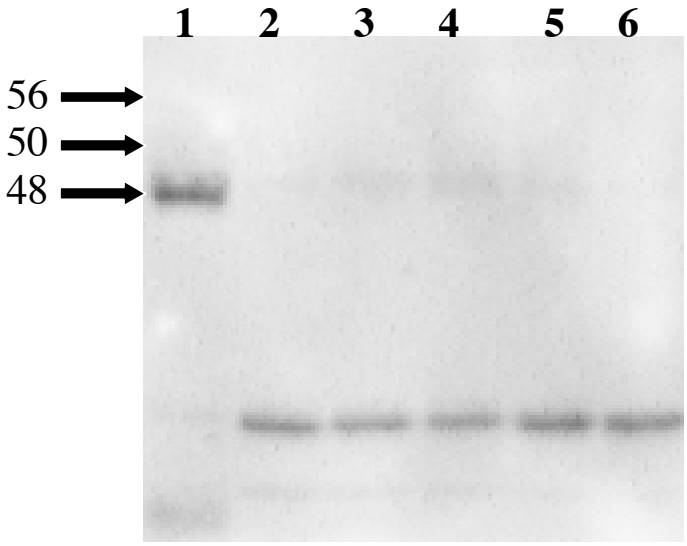

1
2
3
4
5
6
56
50
48

Figure 2.5 **Changes in Soluble CD14 in Equine Colostrum and Milk Over 29 Days Postpartum**. Western blot analysis of colostrum and milk samples from horses. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is equine colostrum at parturition; lanes 2-5 are equine milk after 2-3, 6-8, 13-15, and 27-29 days postpartum. Each isoform of sCD14 is labeled according to molecular weight.

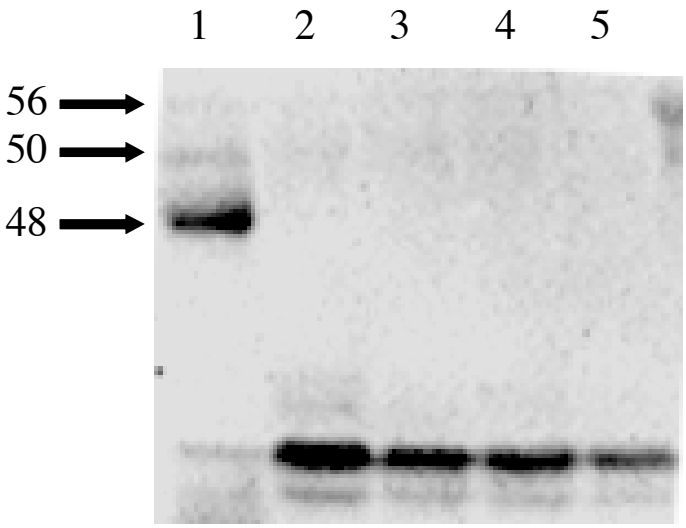

1
2
3
4
5
56
50
48

Figure 2.6 **Changes in sCD14 in Serum Samples of all Equine and Bovine Study Participants**. Averages of the relative concentrations of the 48 kD isoform of sCD14 from serum samples of 10 horses and 11 cows taken at the time of birth. The concentrations were determined by densitometry of a western blot analysis of equine and bovine serum samples collected during the first month postpartum. The average relative concentrations of the isoform increased as time postpartum elapsed.

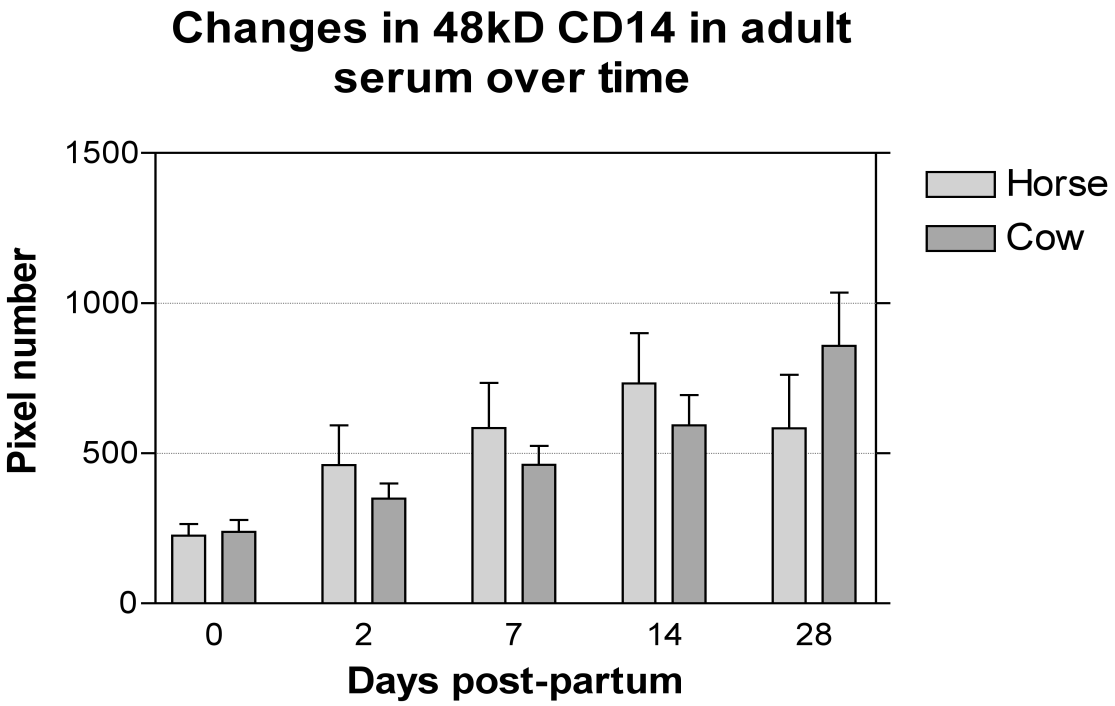

Changes in 48kD CD14 in adult serum over time
Pixel number
1500
1000
500
0
0
2
7
14
28
Days post-partum
Horse
Cow

Figure 2.7 **Changes in Soluble CD14 in Bovine Serum Over 28 Days Postpartum**. Western blot analysis of serum samples from cows. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is bovine serum at parturition; lanes 2-6 are bovine serum after 2, 7, 14, 21, and 28 days postpartum. Each isoform of sCD14 is labeled according to molecular weight.

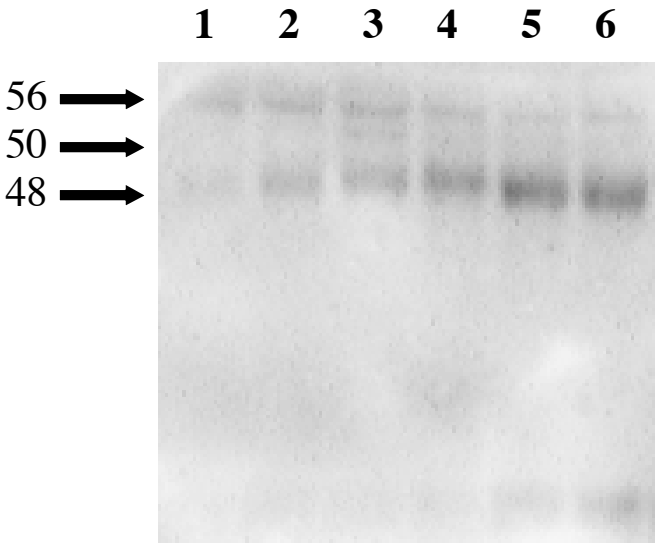

1 2 3 4 5 6
56
50
48

Figure 2.8 **Changes in Soluble CD14 in Equine Serum Over 29 Days Postpartum**. Western Analysis of serum samples from horses. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is equine serum at parturition; lanes 2-5 are equine serum after 2-3, 6-8, 13-15, and 27-29 days postpartum. Each isoform of sCD14 is labeled according to molecular weight.

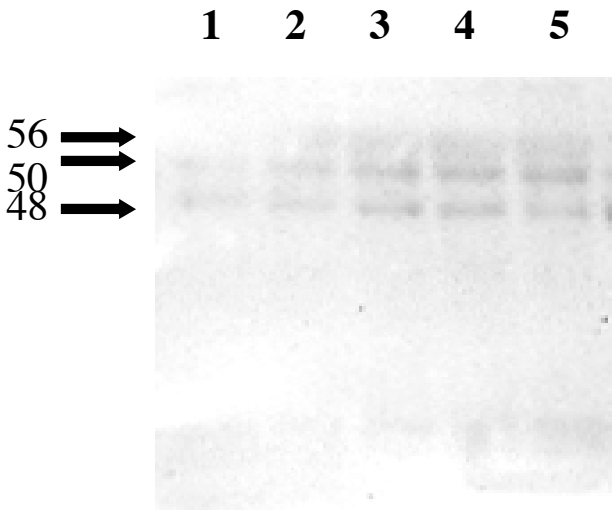

1
2
3
4
5
56
50
48

Figure 2.9 **Changes in sCD14 in Serum Samples of all Foal and Calf Study Participants**. Averages of the relative concentrations of the 48 kD isoform of sCD14 from serum samples of 10 foals and 11 calves taken at the time of birth. The concentrations were determined by densitometry of a western blot analysis of foal and calf serum samples collected during the first month and the first 24 hours postpartum, respectively. The average relative concentrations of the isoform were very low at the time of birth and rose dramatically after 24 hours postpartum.

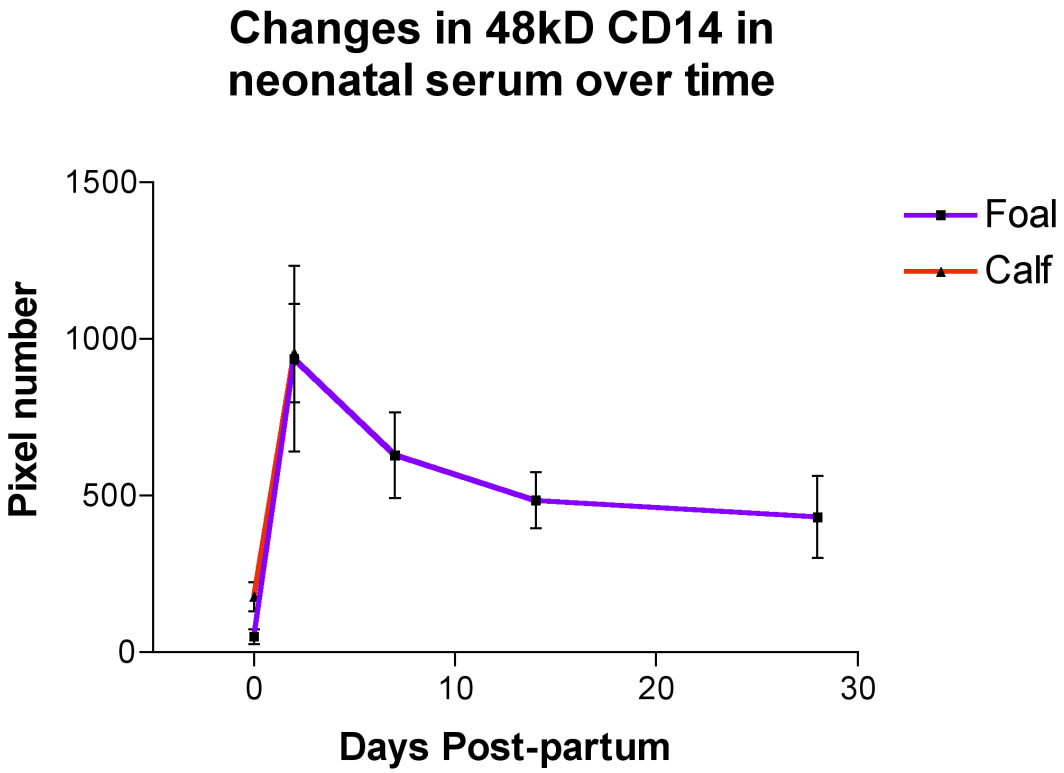
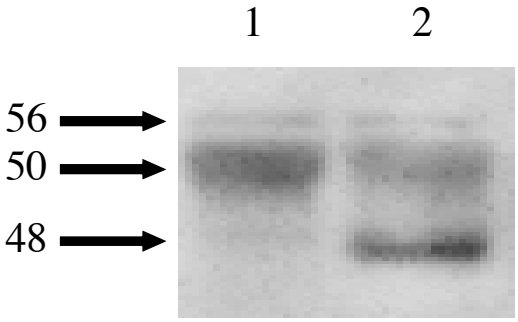

Changes in 48kD CD14 in neonatal serum over time
Pixel number
1500
1000
500
0
0
10
20
30
Days Post-partum
Foal
Calf

Figure 2.10 Appearance of Soluble CD14 in Calf Serum after 24 Hours Postpartum. Western blot analysis of serum samples from calves. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is calf serum at parturition and before nursing was allowed; lane 2 is calf serum after 24 hours postpartum and suckling. Each isoform of sCD14 is labeled according to molecular weight.


1
2
56
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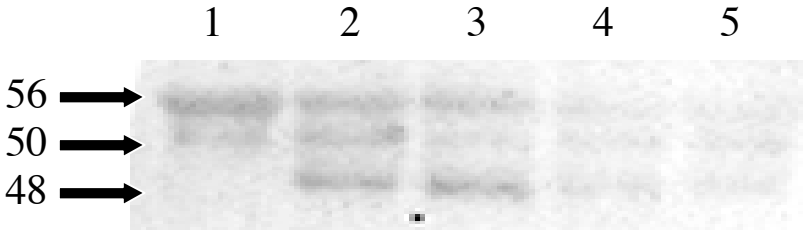
48

Figure 2.11 Appearance of Soluble CD14 in Foal Serum after 29 Days Postpartum. Western blot analysis of serum samples from foals. Samples were tested for sCD14 with Tuk-4, a monoclonal anti-human CD14 antibody. Lane 1 is foal serum at parturition and before nursing was allowed; lane 2-5 is foal serum after 2-3, 6-8, 13-15, and 27-29 days postpartum and suckling. Each isoform of sCD14 is labeled according to molecular weight.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CHAPTER 2

HARVEST AND CULTURE OF BOVINE MAMMARY EPITHELIAL CELLS

Introduction

When the neonate emerges from the mother, its intestine is no longer protected from environmental bacteria and pathogens. Consequently, the once sterile environment is heavily colonized by a variety of organisms, including *Escherichia coli* and *Streptococci (1)*. If the exposure to bacteria is overwhelming, several immune and inflammatory conditions can arise. Therefore, it has been evolutionarily imperative for the coordination of a local innate and adaptive immune response in the maintenance of gut homeostasis (2).

The bovine and equine placentas consist of six layers of tissue. This organization is called epitheliochorial, which differs from the human placenta, which is comprised of just three layers of tissue. The bovine and equine placentas have three layers of fetal tissue and three layers of maternal tissue. Because of the extra three layers of maternal tissue, blood vessels from the dam or cow never come in contact with the fetal tissue of the placenta. Such a configuration effectively prevents any *in utero* transfer of maternal antibodies to the equine or bovine fetus (3), whereas in the human, maternal blood vessels come into direct contact with the fetal tissue and passive transfer of antibodies and large proteins occurs across the placenta. Consequently, foals and calves are born lacking circulating IgG and with only small amounts of their own IgM (3). In contrast,

human babies are born with much larger amounts of antibodies. The result of the small antibody content in foals and calves is a very weak immune response to antigenic stimulation. To compensate for this deficiency, the colostrum of mares and cows contains many factors that help provide an immunologic boost to the neonoate. Protection by colostrum has been ascribed to maternal immunocompetent cells, immunoglobulins, immune reactive peptides, anti-infectious oligosaccharides, growth factors, cytokines, lysozyme, lactoferrin, and complement components (4,5). Failure to consume an adequate amount of colostrum correlates directly with partial or complete failure of passive transfer for these immuno-naïve calves and foals (6,7).

It was recently reported that innate recognition of bacteria in human milk is facilitated by milk-derived soluble CD14 (8). We suspected that the derivation of sCD14 in colostrum was attributed to cells involved in milk secretion rather than those of the immune system. This led us to study bovine mammary epithelial cells *in vitro*.

Mammary epithelial cells can be divided into two, functionally distinct categories. Mammary secretory epithelial cells actively secrete the components that make up colostrum: fats, proteins, peptides, vitamins, enzymes, hormones, growth factors, cytokines, minerals, nucleotides, and immunoglobulins (9). The secretory epithelium somehow obtains or manufactures each of these components, and release them into the alveolar ducts of the mammary gland. The second type of mammary epithelial cell is the myoepithelial cell. As the name implies, these cells resemble smooth muscle fibers, and have a contractile function (10). Myoepithelial cells are also known as basket cells because they form a basket-like covering around the secretory epithelial cells (10). As

the myoepithelial cells contract, they physically expel the milk, or milk factors, out of the cells and down through the ducts of the mammary tissue (10).

It is unknown why sCD14 exists in colostrum, or how it gets there. It was the aim of this project to establish a bovine mammary epithelial cell culture, to provide the basis for future studies to investigate the regulation of sCD14 production. Such studies have attractive research possibilities for exploring the metabolic control of cellular activities by providing a means of disengaging the functional processes of the secretory cells from the metabolic effects of the dam. In order for this to be useful, however, the cell cultures should be able to duplicate known functions of the intact tissue. This is not an easy task, as primary cultures are associated with a number of potential problems or differences to that of the natural occurring tissue.

A successful primary culture must first be established as a healthy and viable cellular system within an environment mimicking endogenous conditions. A primary culture must also be proven to be a culture of the correct cell type. This project seeks to establish a healthy bovine mammary epithelial cell culture, identify and characterize the contents of the culture, and investigate the presence sCD14.

Materials and Methods

Mammary Glands

Lactating bovine mammary glands were obtained from Shapiro's, an abattoir within the relative proximity of the University of Georgia (Augusta, GA). Donation cows were of the beef variety, although information pertaining to the exact type, age, or length of lactation was unavailable. Animals were transported to the abattoir directly from the

farm, and euthanized by captive bolt followed by exanguination. Fresh mammary glands were immediately obtained through removal of the entire udder, thus maintaining the conformation of the mammary gland and the integrity of the internal epithelial tissue, before further dissection.

Reagents

Permanox culture chamber slides were purchased from VWR (Suwanee, GA). MEGM bullet-kit was purchased from Biowhittaker (Walkersville, MD). Trypsin-(ethylenedinitrilo)-tetraacetic acid (EDTA), PMSF, leupeptin, tris (hydroxymethyl)-aminomethane (Tris), glycine, lauryl sulfate, sodium orthovanidate ($NaVO_4$), aprotinin, sodium flouride (NaF), β-mercaptoethanol (BME), cell dissociation sieve/tissue grinder kit, Ponceau stain, Tween 20, mouse anti-cytokeratin (CK8.12), mouse anti-vimentin (Cy3)(V9), rabbit anti-mouse (FITC-conjugated), epidermal growth factor (EGF), bovine serum albumin (BSA), human transferrin, isoproterenol, insulin, Cholera toxin, hydrocortisone, Penicillin/Streptomycin, phosphate buffered saline (PBS), ethanol, Type I crude collagenase, and hyaluronidase were purchased from Sigma Chemical Company (St. Louis, MO). DMEM-F12, 1:1 mixure with L-glutamine, 15mM HEPES buffer and 3.151g glucose/L, methanol, DMEM with 4.5g glucose and glutamine, and mouse anti-human CD14 antibody (MY4) were purchased from Fisher Scientific (Norcross, GA). Slowfade® light anti-fade kit was purchased from Molecular Probes, Inc. (Eugene, OR). Lab Pak Nitex filter membranes were purchased from Sefar America, Inc. (Kansas City, MO). Rat-tail collagen, type I was purchased from Becton-Dickinson (Bedford, MA). fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Fungizone and

Gentamicin were purchased from Life Technologies (Rockville, MD). Cytofunnel® disposable sample chambers and Cytospin 2 were purchased from Shandon (Sewickley, PA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). Dura SuperSignal® detection reagent was purchased from Pierce (Rockford, IL). Fluor-S Multi-Imager, Bradford solution, 10% polyacrylamide Criterion gels, Criterion Cell gel system, and Trans-blot transfer cell were purchased from Bio-Rad (Hercules, CA). Sheep anti-mouse IgG HRP-linked whole antibody was purchased from Amersham (Piscataway, NJ).

Preparation of Tissue

Whole udders from two cows were obtained and an initial, crude dissection isolated a section of epithelial tissue deep within each udder. Sections were immediately dipped in 70% ethanol, washed two times in 0.01 M phosphate buffered solution (PBS)(1X) with 10 μg/mL fungizone (2X) and 200 U/mL penicillin/streptomycin (pen/strep)(2X), and rinsed in DMEM-F12 with L-glutamine, 15mM HEPES buffer, 3.151g glucose/L, 2X fungizone and 100 U/mL pen/strep (1X), until milk was no longer seen in the wash. Samples were then placed on a sterile surface and cut into smaller sections approximately 2.5 cm X 2.5 cm X 7.5 cm in size. A separate small tissue sample was excised from each cow and immediately placed in formalin. Tissue obtained from the smaller sectioning was again washed 2 times in the antibiotic PBS solution, rinsed twice in DMEM-F12 solution with antibiotics, and immersed in the same media solution in 250 mL Erlenmeyer flasks on ice for the trip back to Athens, GA.

Rectangular sections approximately 7.5 cm^3 in size, were removed from Erlenmeyer flasks under sterile conditions within a culture hood and placed in a sterile petri dish with sterile forceps. Each side of tissue section was excised with a sterile scalpel and discarded. Remaining tissue was washed 2 times in sterile PBS with 2X fungizone and antibiotics, another 2 times in sterile DMEM-F12 media supplemented with 1X pen/strep and fungizone, 10% FCS, 10μg/ml insulin, 5ng/ml EGF, 0.1μg/ml hydrocortisone, and 1ng/ml Cholera toxin, and placed in a new, sterile petri dish. Tissue was then minced very finely with sterile scissors, placed in a sterile 15 ml conical tube, and mixed with enough of the DMEM-F12 media, with supplements, to fill the conical tube. Tissue and media solution were vortexed and centrifuged at 500g for 10 minutes. Supernatant was discarded and replaced with new media, and tissue was re-vortexed and re-centrifuged under identical conditions. Tissue was then ready for enzymatic digestion.

Tissue Digestion

Approximately 5 ml of minced tissue from each cow remained after the final centrifugation was complete. These samples were divided in half and placed in 15 ml conical tubes. Efforts were expended to keep sample sizes less than 1/3 the total volume of the conical tube. The minced tissue was then mixed with an enzymatic solution containing 1500 units of hyaluronidase, 3000 units of collagenase, and approximately 10 mls of media. Each of the 2 samples per cow were placed on a rotating rocker in a 37° C incubator. One sample from each cow was allowed to digest for 7-8 hours, while the other sample underwent an overnight digestion of approximately 15 hours. Upon completion of the respective digestions, cells were centrifuged at 500g, and the enzymatic

solution was decanted. The pellets were then washed 4 times with media, under identical conditions: 12 ml media, 10 second vortex, 10 minute centrifugation at 500g and decant of the washing media. Finally, the cells were re-suspended in 5 ml of media.

Filtration

Prior to filtration, a double-mesh filtration system was constructed using sterile technique. A 150μ Nitex filter membrane (Sefar America) was placed on top of a 50 mesh screen (Sigma) sealed in a filtration cup (Sigma). The cup complex was then placed within a funnel on a ring stand. The ring stand held the funnel high enough from the hood surface to allow for a 50ml conical tube to be held under the funnel's lowest point. The filters were pre-wetted with DMEM-F12 cell media to prevent surface adhesion. The re-suspended cell solution was drawn in and out of a pasteur pipette to aid in the dispersion of the remaining pieces of large tissue, before the solution was poured through the filtration system. The top filter effectively caught most tissue debris, but also many undissociated cell clumps. The filters were rinsed with approximately 40 ml of the DMEM-F12 media, and filtrate was collected in a 50 ml conical tube. The remaining tissue caught within the Nytex mesh was saved and re-suspended in 40 ml cell media. All cells were washed twice by centrifuging at 500 g for 10 minutes in fresh media.

Following the final wash, cells were re-suspended in 8 ml of DMEM-F12 media and plated on 12-well plates, one ml per well, thus dividing one plate for the filtrate and filter samples from each cow. The remaining 2 ml were divided into 1 ml for western blot analysis and 1 ml to culture on Permanox culture chamber slides (VWR) for immunohistochemistry. Cells were examined at each stage of the procedure with a

phase-contrast microscope, and the final cells were tested for viability by the trypan-blue exclusion technique. After an 8-12 hour incubation at standard incubator conditions, 37ºC and 5% CO_2, media was removed from each well and re-plated on rat-tail collagen pre-coated 12 well plates. The original plates were given new media and all plates were then treated equally. The media was changed every 24-48 hours depending on color and pH of the media.

Upon confluent growth of cells, media was decanted, each well was rinsed with PBS, and 1 ml of 1X Trypsan-EDTA (Sigma) was added. Trypsan-EDTA was allowed to disrupt cell to cell and cell to surface connections for 15 minutes in the incubator. Following trypsanization, cells were harvested into a 15 ml conical tube containing ~10 ml DMEM-F:12 media with 10% Fetal Calf Serum. Cells were pelleted following centrifugation at 500g for 10 minutes, re-suspended in fresh media, and re-plated. Following first doubling, all cells were plated on collagen-less 12 and 6 well plates. Original plates were also rinsed with DMEM-F:12 media and fed with fresh media. All cells were kept under standard incubator conditions. A flow diagram of cell harvest and digestion steps is shown in figure 3.1.

Techniques for Monitoring Contamination

As this culture was a primary cell culture, contamination was a very serious concern and was monitored accordingly. All cell plates were carefully monitored with the naked eye for cloudy coloration, floating debris, or very quick changes in media color or pH. To test for latent or slow developing infections, a second technique was employed. Media from a well was extracted and centrifuged at 500g for 10 minutes. The

pellet was then re-suspended in 500μl cell media. Fifty microliters of 5% BSA, followed by 100μl of the cell solution were loaded into a Cytofunnel® disposable sample chamber (Shandon) and spun onto a glass slide after 3 minutes at 1500 rpm in the Cytospin 2 (Shandon). Cell samples were then fixed and stained on the slide by a Diffquick stain, and observed for contamination by a phase-contrast microscope.

Histological Techniques

Briefly, samples of bovine mammary gland tissue were collected and fixed in 10% formalin. Tissues were processed routinely in a Leica TP 1050 automated tissue processor. Tissues were then embedded in a Tissue-Tek tissue embedding console system. Thin sections (~3 μm) were made on a Leitz 1512 rotary microtome and floated out on a Tissue Prep floatation bath. Sections were mounted on Superfrost® Plus microscope slides (Fisher). Sections were stained with a Hematoxylin and Eosin method on a Leica XY automated stainer and coverglassed on Leica CY automated coverslipper.

Immunohistochemistry

Cells cultured in the Permanox culture chamber slides (VWR) were used for immunohistochemistry. Media from these slides was aspirated from the cells and washed with warm PBS (37ºC) three times for 5 minutes. The cells were then fixed in pre-cooled 100% methanol (approx. –20ºC) for 10 minutes. The methanol was immediately washed off with PBS during another three, 5 minute washes. Due to the potential of non-specific binding by the immunofluourescent antibodies, the cells were blocked with 3% BSA for 30 minutes at room temperature. Upon completion of the blocking step, the cells were

incubated with a primary antibody cocktail consisting of a 1:50 dilution of mouse anti-cytokeratin (CK8.12)(Sigma) and a 1:200 dilution of mouse anti-vimentin (Cy3)(V9)(Sigma) mixed in a 3% BSA solution. The CK8.12 antibody is specific for cytokeratins 13 and 16, both of which are found in mammary epithelial and myoepithelial cells. The V9 antibody is specific against vimentin, a known component of both myoepithelial cells and fibroblasts (12-14).

The cells remained incubated with the cocktail for 30-60 minutes at room temperature in a humidified chamber. Following exposure to the primary antibodies, the cells were thoroughly washed in PBS for three times, at 10 minutes at wash. After an appropriate wash, the cells were incubated for 30-60 minutes with a 1:200 dilution of the secondary antibody, rabbit anti-mouse (FITC-conjugated)(Sigma), in a 3% BSA solution. The cells were, again, incubated at room temperature in a humidified chamber. Washes following the secondary antibody incubation were identical to those after the exposure to the primary antibody solution. Finally, the cells were coated with a glycerol based mounting media, Slowfade® light anti-fade kit (Molecular Probes), to prevent drying, and viewed under a fluorescent microscope.

Western Blot Analysis

Cells were centrifuged at 10,000 rpm for 1 minute in a microcentrifuge and re-suspended in a Goodie buffer protease inhibitor cocktail: 1 mM PMSF, 10 μg/mL leupeptin,20 mM tris/Cl (hydroxymethyl)-aminomethane (Tris) pH=7.5, 1mM sodium orthovanidate ($NaVO_4$), 5 μg/mL aprotinin, 100 μM sodium flouride (NaF), and 50mM β-mercaptoethanol (BME). Cells were sonicated in the lysis buffer for 20 seconds total,

two-10 second sonications, and re-centrifuged at 10,000 rpm for 1 minute. The supernatant was collected, and the total amount of protein was determined by Bradford protein determination (Bio-Rad) and measured by spectrophotometer (Therm spectromic Genesys 5). Cell supernatant was then diluted 1:2 with Laemmli reducing sample buffer, boiled for 5 minutes, loaded onto pre-casted 10% polyacrylamide Criterion gels (Bio-Rad), and electrophoresed in the presence of SDS running buffer (Lauryl sulfate, Tris, and Glycine) (SIGMA) in the Criterion Cell Gel system (Bio-Rad). Proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell) in transfer buffer (48 mM Tris, 39mM glycine, and 20% methanol) overnight at 0.1 Amps using a Trans-blot transfer cell (Bio-Rad).

Blots were stained briefly with Ponceau Stain (SIGMA) to label lanes and molecular markers, and washed with TBS containing 0.1% Tween 20 (TTBS)(SIGMA). Membranes were then incubated at 4º C on a rocker with 5% BSA for 6-8 hours to block against non-specific binding. The membranes were then drained of the blocking agent, and exposed to a 1:2000 dilution of the MY-4 mAb overnight at 4ºC on a rocker. Following incubation with the primary antibody, the membranes were washed 3 times for 10 minutes each with the TTBS solution, and then mixed with an anti-mouse IgG-Horse radish peroxidase conjugated antibody for 2 hours on a rocker at room temperature. Finally, the blots were washed another 6 times for 10 minutes with the TTBS solution and detected with Dura SuperSignal detection reagent (Pierce) using the Fluor-S Multi-Imager (BioRad) and the Quantity One computer software.

Results

Bovine mammary tissue secretes milk components prior to digestion. A section of tissue embedded in paraffin and stained with Hematoxylin and Eosin, displayed the ductal structure of the mammary gland (Figure 3.2). Mammary epithelial cells surround secreted lipid and proteinacious material. Included within the secreted product are stained immune cells, such as leukocytes.

Length of enzymatic digestion of Tissue. Minced sections of bovine mammary tissue were incubated with equal amounts of collagenase and hyaluronidase. Due to differences in each cow, the length of time necessary to fully digest the tissue varied. Mammary tissues from older cows were more fibrotic and demanded up to 16-18 hours of enzymatic digestion. Mammary tissue from younger cows generally had "softer" or more digestible tissue. These tissues were digested within one day, 2-8 hours. Tissue digestion was determined visually. Full digestion represents a reduction in size from small, minced pieces to finely ground bits of tissue.

Large clumps of cells enhance the growth of cells in culture. Filters ranging from 150-250 microns separated digested cells from cellular debris and clumps. Individual cells, separated by filtration, represented a small yield after 24 hours in culture (approximately 30% of field under 10X magnification on a phase contrast microscope). Cell cultures reached confluence (95-100% growth) in 8 days. Large cell clumps and cell debris caught in filters represented a cellular population of high yield after 24 hours in population (approximately 70-80% of field under 10X magnification on a phase contrast

microscope). Cell cultures reached confluence (95-100% growth) in approximately 3-4 days.

Cultured mammary cells secrete milk components. Cells were grown to confluence and passaged 2 times while in culture for 26 days. The cells were then fixed with methanol and examined under phase contrast microscopy (Figure 3.3). The micrograph reveals the cultured cells were secreting a lipid-like substance. The cells also appeared to be epithelial-like and conform to the ductal structure common to bovine mammary tissue.

Primary cell cultures are bovine mammary epithelial and myoepithelial cells. Cells grown to confluence and passaged twice while in culture for 26 days, were fixed with methanol. Fixed cells were stained with a FITC-labeled anti-cytokeratin antibody and a Cy3-labeled anti-vimentin antibody, and viewed with fluorescent microscopy. When veiwing cells that were stained with FITC-labeled anti-cytokeratin, the majority of the cells (~95%) positively stained for cytokeratin (Figure 3.4). When examining the cells stained with both cytokeratin and vimentin together, a large portion of the cells (~75%) stained exclusively for the cytokeratin, representing the secretory epithelial cells (appearing green), a smaller portion of the cells (~20%) stained positively for the cytokeratin and vimentin, representing myoepithelial cells (appearing yellowish-orange in color), and a very small amount of cells (~<5%) stained only for vimentin, representing fibroblasts (appearing red) (Figure 3.5). Also cultured and stained were MAC-T cells, a pure bovine mammary epithelial cell line. When stained with the cytokeratin and

vimentin together, the cells stained exclusively for the cytokeratin, as was expected, and had a slightly red background due to non-specific binding of the vimentin. The MAC-T cells were a control that allowed us to detect any non-specific binding of the vimentin in our primary cell culture.

Primary cell culture lysates contain soluble CD14. Western blot analysis using an anti-CD14-specific antibody of lysates from bovine mammary tissue showed the doublet of soluble CD14 (56 kD) and CD14 (50 kD) polypeptides (Figure 3.6). The single 48 kD isoform of sCD14, characteristic of bovine colostrum, was not present. Western blot analysis using an anti-CD14-specifec antibody of lysates from a primary culture of bovine mammary epithelial cells grown for 26 days following 18 hours of exposure to the digestion enzymes showed the doublet of sCD14 and sCD14 (Figure 3.6). The 48 kD isoform of sCD14 was not present in any of the lysates of cells.

Discussion

Bovine Mammary Epithelial Cell Culture

Here, we demonstrated that a primary culture of bovine mammary epithelial cells could be harvested and cultured *in vitro*. Establishing a primary culture, however, involves creating a general protocol and making numerous adjustments in order to troubleshoot any problems. The protocol for harvesting bovine mammary epithelial cells was a modification Pitelka *et al* and Burwen *et al* (10 and 11). While these protocols were acceptable, there were several steps that were modified.

Digestion

Previous studies described the digestion of mouse mammary tissue, which varies very little between mice. Bovine mammary tissue, however, is subject to a great deal of variability because of the extreme stress put on the tissue by the demand for milk. Older cows or cows with greater experience being milked, tend to have mammary tissues adapted to a higher usage. The adaptation, is for the tissues to become "tougher" or more fibrotic. Fibrotic tissues are more difficult to digest with enzymes and a much longer enzyme digestion time is essential for proper dissociation of the cells present within the tissue. Because the previous studies did not have to account for glandular differences based on fibrosis, we sought to modify the protocol accordingly.

As a means of establishing an ideal time to digest bovine mammary tissue, we incubated bovine mammary tissue, of varying degrees of fibrosis, with equal amounts of digestive enzymes for different lengths of time. Upon completion of each incubation time, we determined the level of digestion through a visual evaluation and its ability to pass through a 5 mL pipet. Any sample unable to pass through a pipet at all, or just barely, was considered under-digested. Any sample that could easily or adequately pass through the pipet was considered fully digested by our standards.

After sampling 6 different bovine mammary glands, with differing levels of fibrosis, and incubating each of them with digestive enzymes for varying lengths of time (2,4,8,10,16, and 18 hours), we determined that it was not possible to establish an exact digestion time for our protocol. Some samples were fully digested after 2 hours, as opposed to other samples that took nearly 18 hours to digest. More interestingly, while fibrosis was generally the reason for extended digestion times, it was not possible to

roughly approximate the digestion time based on the level of fibrosis. This led us to conclude that because of varying degrees of fibrosis among the tissues, each sample collected had to be visually monitored and tested throughout the duration of enzymatic digestion, and proper digestion of the tissue should be determined accordingly.

Filtration

Our initial protocol called for the use of two differently sized mesh filters to filter out cell debris and attempt to separate different structures based on size. The original filters were 150 and 50 microns in size. We used these filters for our initial attempt at filtration, however they were not effective. The pore sizes were much too small for the digested sample. Rather than attempting to digest the sample down to a filterable level, which would risk over-digesting the cells and killing them, larger filters were obtained. The new filters were 250 and 150 microns in size, and were able to filter out the majority of cell debris and large cell clumps. The large cell clumps, present on top of the 250 micron filter, were saved and treated the same as the filtrate. Ultimately, cells that remained in large clumps and were not filtered, grew the most efficiently and were the healthiest. One reason for this may be that the individual cells may become over-digested and damaged. In contrast, large cell clumps were able to keep their cellular integrity and health through protection from each other, while still becoming digested from the cellular matrix of the mammary tissue.

The three major cell types in the culture were epithelial cells, myoepithelial cells, and fibroblasts. Fibroblasts adhere well to numerous surfaces, i.e. plastic, and themselves, while it takes the epithelial and myoepithelial cells much longer to adhere.

Also, cultured fibroblasts grow and expand at a more rapid rate, and insufficient removal of them will cause a targeted cell culture to be overgrown. As mentioned above, the original protocol used the smaller filters to help separate organoids from individual cells, but they may have also helped separate fibroblasts from the epithelial cells, as they are different sizes. Again, our protocol had to be changed to use the larger filters, which were not efficient at separating out cell types based on size. To compensate for changing to the larger filters, the cells were initially plated onto plastic wells for 12-24 hours. Due to fibroblasts' quick adherence ability, the majority of them stuck to the plastic. After 24 hours, the media containing cells still in solution was re-plated on plates coated with collagen. The collagen provided a more natural and desirable surface for the remaining cells in population, which included a majority of epithelial and myoepithelial cells, with few fibroblasts, which did not adhere to the original plastic surface. Following cellular growth to confluence on the collagen, the cells were trypsinized. After 5 minutes, the Trypsin-EDTA solution was removed, as were the most readily detachable cells, the fibroblasts. Thus allowing for a final selection step for epithelial and myoepithelial cells. More Trypsin-EDTA was added, and the rest of the cells were passaged and plated onto plastic wells.

Establishing Cellular Characteristics

Prior to digesting the various cell populations, a section of the mammary tissue was embedded in paraffin and stained. This provided a positive control and a representative sample of tissue that was digested and cultured. The section showed the ductal structure of the mammary gland and the alignment of mammary epithelial cells

around ducts of secreted milk components, such as lipids, proteins, and white blood cells. The section also provided a visual image of the matrix targeted for digestion by the collagenase and hyaluronidase. The collagenase breaks up the collagen fibers holding the cells in place and the hyaluronidase catalyzes the hydrolysis of endo-N-acetylhexosaminic bonds of hyaluronate and chondroitin sulfate A and C (but not B), which also help form the matrix holding the cells structurally in place.

Following digestion, cultures grown and passaged for several weeks were examined histologically. The cells examined under a phase contrast microscope were epithelial-like and contained numerous granules similar to that which is seen *in vivo*. When compared to the reference section, the epithelial-like cells appeared to form circular regions around what appeared to be ductal stuctures.

Identifying Cell Populations

Examining the cells under a phase contrast microscope allowed us to visualize a population of cells structurally similar to a reference set of bovine mammary epithelial cells. However, it was necessary to specifically identify the population of cells cultured by immunohistochemisty. As mentioned earlier, the three cell populations present before digestion, and, consequently, most likely to be present in our culture, were lumenal epithelial cells, myoepithelial cells, and fibroblasts. Epithelial and myoepithelial cells are the most prominent cells and are similar in that they both contain a number of cytokeratin proteins, most notably for our study, cytokeratin 16. Myoepithelial cells, because they have contractile fibers, also contain vimentin, which is also present in fibroblasts.

Additionally, fibroblasts do not contain cytokeratin. Having two common proteins among three cell types allowed us to distinguish each population.

Epithelial Cells

The antibody specific for cytokeratin 16 was FITC-labeled, which means it gave off a green color when examined with a fluorescent microscope. After staining our cultured cells with the anti-cytokeratin antibody, and the appropriate secondary antibody, green fluorescence was detected on a fluorescent microscope. Of the cultured cells, 95% were epithelial and/or myoepithelial cells. The lumenal epithelial and myoepithelial cells appeared to form in a circular formation characteristic of their natural ductal structure.

Myoepithelial Cells

An anti-vimentin antibody was used in conjunction with the anti-cytokeratin antibody to visually separate the lumenal epithelial cell population from the myoepithelial cell population. The anti-vimentin antibody was Cy3-conjugated; meaning, it fluoresced in the red spectrum when examined with fluorescent microscopy. As described earlier, myoepithelial cells contain both cytokeratin and vimentin, so when incubated with the labeled antibodies against each protein, both green and red fluorescence will be emitted. Depending on the concentration of each protein within the myoepithelial cell and the efficiency of the binding of the antibody, yellow or orange shades of color represented the presence of myoepithelial cells. The yellow and orange coloration denoted the spectrum of fluorescence created from the mixing of green and red emissions.

In order to visualize the double-staining of the cells, multiple exposures with different filters were taken of the same section of culture on a fluorescent microscope. Areas of yellowish and orangish color predominated on the outside of green color. The green color was the pure lumenal epithelial cell populations and the cells outside of them, emitting yellow and orange color, were the myoepithelial cell populations. This arrangement appears anatomically correct. In natural mammary tissue, myoepithelial cells, also called basket cells, grow around lumenal epithelial cells and hold them like a basket would its contents. The contractile fibers of myoepithelial cells squeeze the lumenal epithelial cells and express the milk components from them into central lobes encircled by the epithelial cells.

Fibroblasts

The Cy3-labeled anti-vimentin antibody was also used to identify pure fibroblast populations. Histologically examining cells positive for Cy3-labeled anti-vimentin antibody by fluorescent microscopy emits red color, and represents the fibroblast population. There was very little red seen on the micrograph (<5%), suggesting that the cultured population was mostly pure epithelial and myoepithelial. Due the aggressive growth of fibroblasts and the frequency of their existence in mammary tissue, it is highly unrealistic to expect total elimination of fibroblasts from a primary culture. Plating the samples on plastic for 12-24 hours was successful for fibroblast removal. This is a reasonable conclusion because, had the fibroblasts not been adequately removed in that step, it is most likely that a population of fibroblasts would have overtaken all other cell populations by the 26th day in culture.

Cellular Activity

The final step of this study determined that lysates of the epithelial cells originally harvested contained sCD14. In addition, lysates from cells digested for three different lengths of time still contained sCD14. Western blot analysis of lysates from bovine mammary epithelial cells harvested from a bovine udder, but not yet digested, showed the presence of the 56 kD and 50 kD isoforms of CD14. It did not show the presence, however, of the 48 kD isoform commonly found in the colostrum of cows.

Initially, the absence of the 48 kD isoform was surprising, considering mammary epithelial cells secrete this polypeptide and the cows the cells were excised from were actively lactating (8). However, after reviewing the results, we determined these results to be quite appropriate. In the first study of the thesis, we demonstrated that after 2-3 days post-partum, levels of the 48 kD isoform of sCD14 drop dramatically in bovine and equine milk, and the protein is barely detectable. It was unknown how long the cows we sampled had been lactating, but it was extremely unlikely that they were brought to slaughter the same day they birthed. The most likely scenario, is that they were many weeks post-partum and their calves had already been weaned. So, after considering that levels of the 48 kD isoform of sCD14 drop off dramatically after the first few days post-birth, and the cows we sampled were most likely many days past giving birth, we determined it was likely and expected that the isoform was not detected in the cell lysates.

The same results were seen in cells that had been digested for different lengths of time and then grown for 26 days. These cells were cultures of the tissue from the same cows described above. Therefore, it was not surprising to see that the 48 kD isoform of

sCD14 was not present in the lysates. We also determined that the lengths of digestions we used for our cells did not affect the sCD14 in the lysates. This is important because it helps to establish parameters for how long we can digest the cells in the future, without affecting cellular function.

Finally, it was extremely encouraging to discover that we could grow a primary cell culture, and that after a month in culture, levels of sCD14 could still be detected. By establishing a bovine mammary epithelial cell culture that retained its functional abilities to express CD14, we opened the door for future studies. The next step is to explore the mechanisms behind expression of sCD14 by bovine mammary epithelial cells. Clearly, different factors, such as hormones or growth supplements, affect expression of the sCD14. With this cell culture, studies can be done to determine which factors can regulate sCD14. In the future, the ultimate goal is to determine the signaling mechanisms regulating sCD14 expression in mammary epithelial cells. Regulation of sCD14 expression in mammary epithelial cells, coupled with a greater understanding of the role of sCD14 in colostrum and innate immunity of the neonate, can hopefully lead to a reduction in illness caused by endotoxin or bacterial infection in foals and calves.

In conclusion, this study reported the culture of bovine mammary epithelial cells and myoepithelial cells. It is the first to report the absolute distinction between bovine mammary epithelial cells and myoepithelial cells using immunohistochemisty. This study is the first to document expression of sCD14 from a primary culture of secretory epithelial cells, as all other documentation refers to secretion of sCD14 by a transformed human mammary epithelial cell line.

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Figure 3.1 **Flow diagram of all the cell harvest and digestion steps.** Flow diagram represents an organized overview of the cell harvest and culture technique used with the bovine mammary epithelial cells.

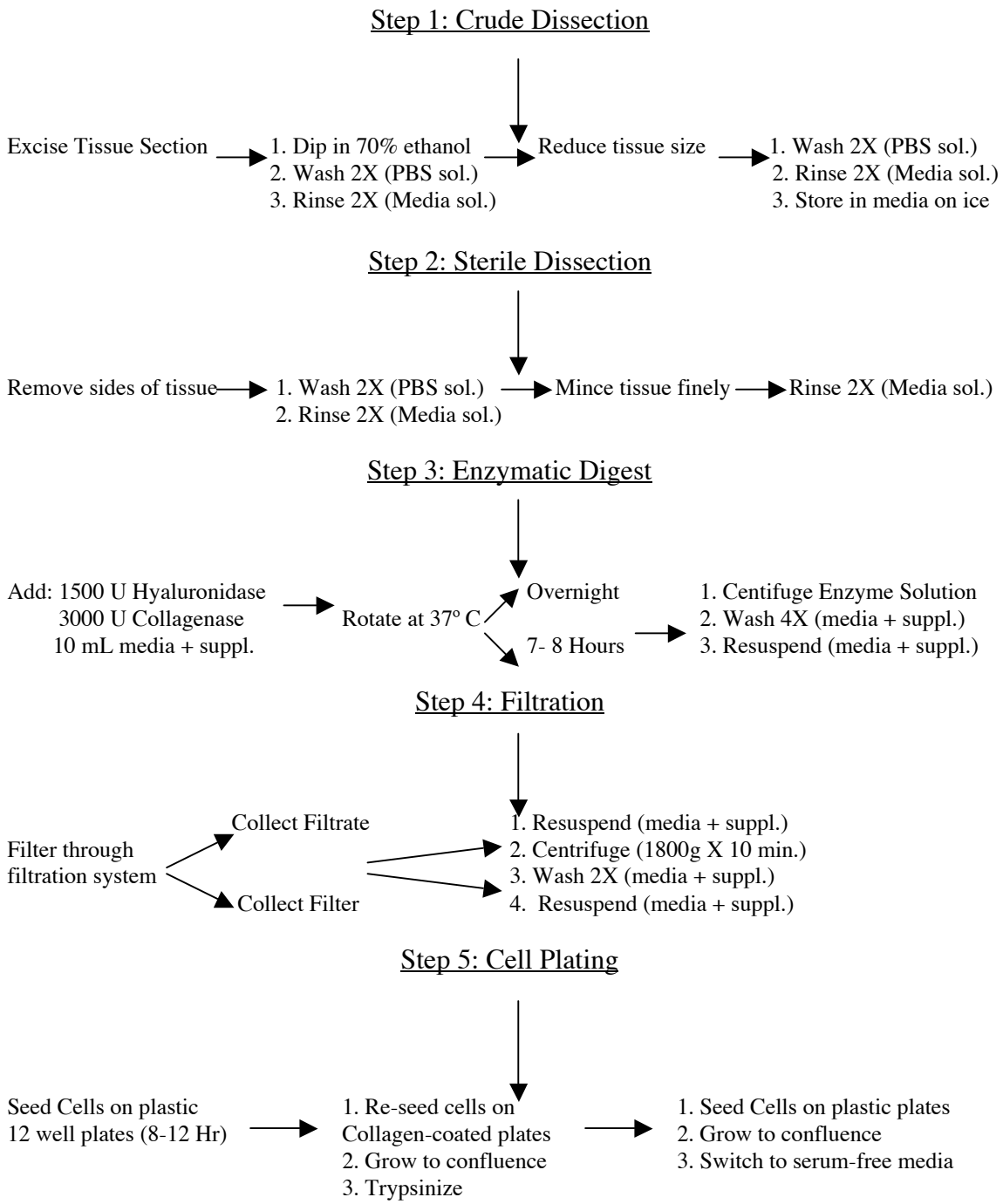

Step 1: Crude Dissection
Excise Tissue Section
1. Dip in 70% ethanol
2. Wash 2X (PBS sol.)
3. Rinse 2X (Media sol.)
Reduce tissue size
1. Wash 2X (PBS sol.)
2. Rinse 2X (Media sol.)
3. Store in media on ice
Step 2: Sterile Dissection
Remove sides of tissue
1. Wash 2X (PBS sol.)
2. Rinse 2X (Media sol.)
Mince tissue finely
Rinse 2X (Media sol.)
Step 3: Enzymatic Digest
Add: 1500 U Hyaluronidase
3000 U Collagenase
10 mL media + suppl.
Rotate at 37° C
Overnight
7- 8 Hours
1. Centifuge Enzyme Solution
2. Wash 4X (media + suppl.)
3. Resuspend (media + suppl.)
Step 4: Filtration
Filter through
filtration system
Collect Filtrate
Collect Filter
1. Resuspend (media + suppl.)
2. Centrifuge (1800g X 10 min.)
3. Wash 2X (media + suppl.)
4. Resuspend (media + suppl.)
Step 5: Cell Plating
Seed Cells on plastic
12 well plates (8-12 Hr)
1. Re-seed cells on
Collagen-coated plates
2. Grow to confluence
3. Trypsinize
1. Seed Cells on plastic plates
2. Grow to confluence
3. Switch to serum-free media

Figure 3.2 **Bovine Mammary Tissue**. Paraffin embedded mammary tissue isolated during lactation. Tissue section stained with H and E. Viewed under a phase contrast microscope (400X). Micrograph represents ductal structure of mammary tissue. Labeled areas: Mammary epithelial cells (A), Secreted lipid material (B), Secreted proteinacious material (C), and Lymphocyte (D).

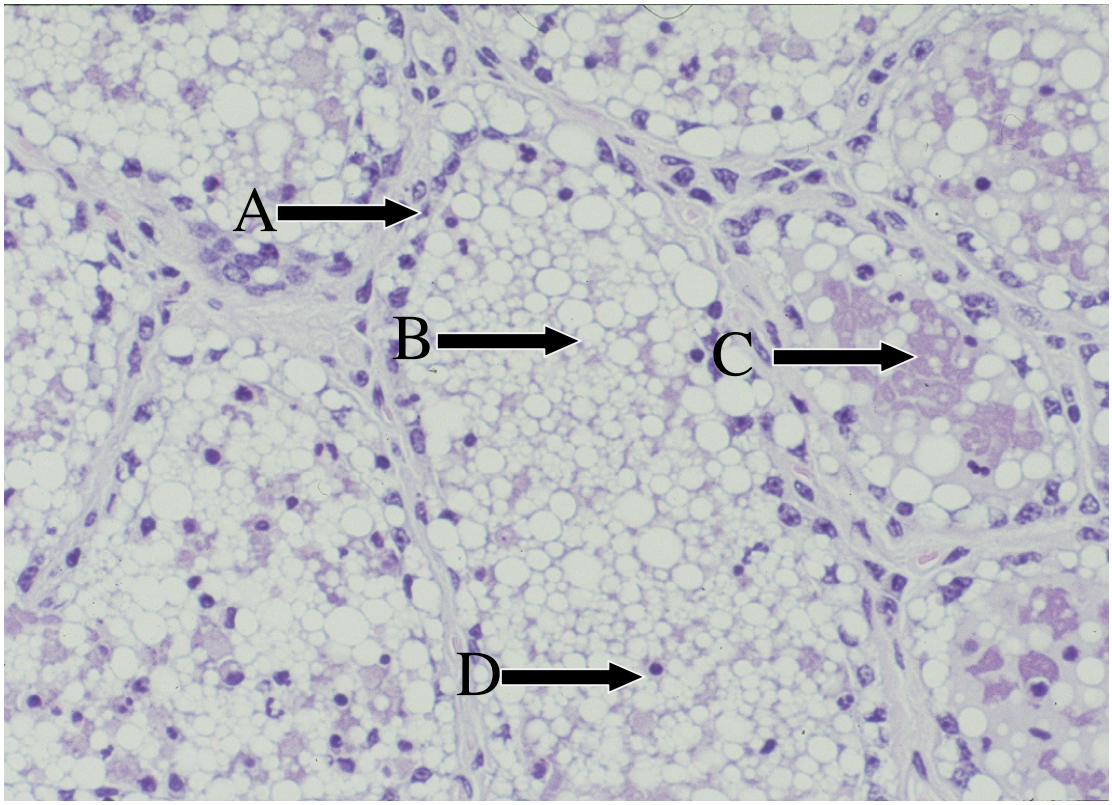

A
B
C
D

Figure 3.3 **Cultured Bovine Mammary Cells.** Cells grown for 26 days and fixed in methanol. Viewed under a phase contrast microscope (400X). Cells appear to be epithelial-like and growing into natural ductal formation. Epithelial-like cells (A) appear to be secreting lipid and proteinacious-like material (B).

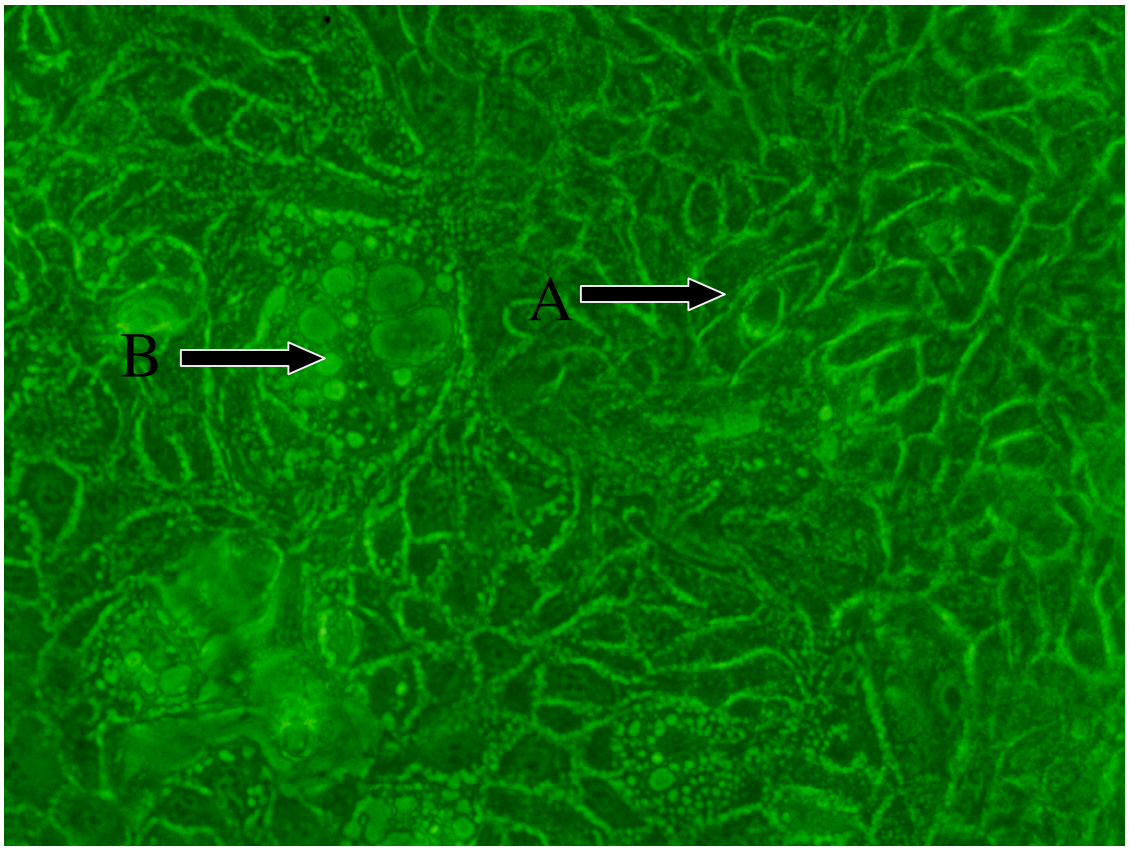

A
B

Figure 3.4 **Bovine Mammary Epithelial and Myoepithelial Cells**. Cells grown for 26 days and fixed in methanol. Cells stained for cytokeratin 16 and vimentin. Viewed under fluorescent microscope (400X). Green fluorescent staining represents positive binding to cytokeratin 16 (A), a component of mammary epithelial and myoepithelial cells.

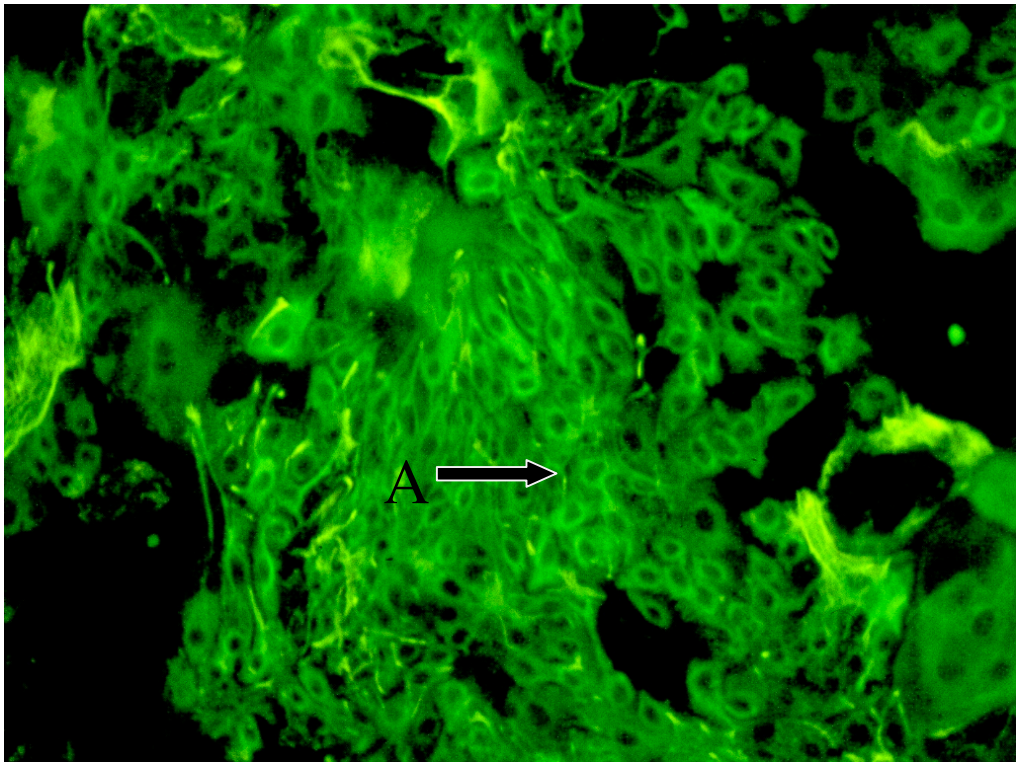

A

Figure 3.5 **Bovine Mammary Epithelial and Myoepithelial Cells**. Cells grown for 26 days and fixed in methanol. Cells stained for cytokeratin 16 and vimentin. Viewed under a fluorescent microscope (400X). Green fluorescence displays positive binding to cytokeratin 16, and represents pure mammary epithelial cell population (A). Yellow to orange fluorescence displays binding to cytokeratin 16 and vimentin, and represents pure mammary myoepithelial cell population (B). Red fluorescence displays binding to vimentin, and represents a fibroblast population (C).

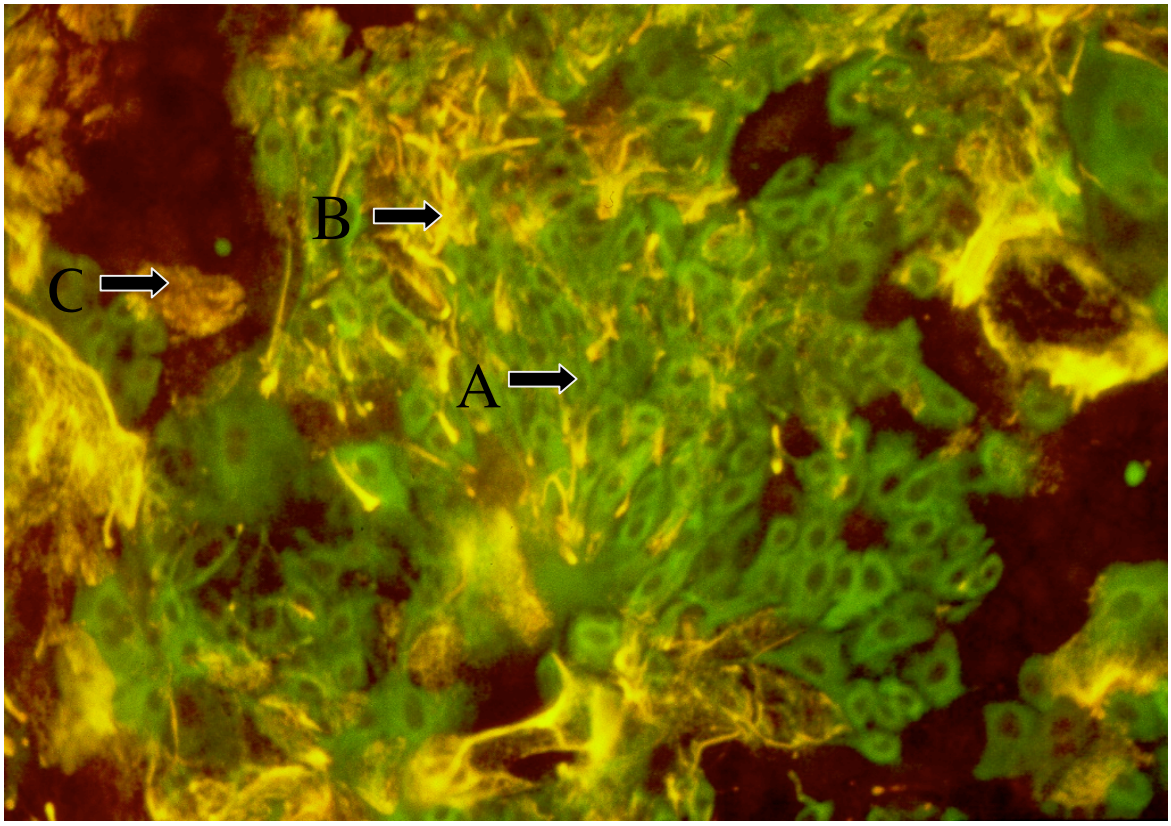
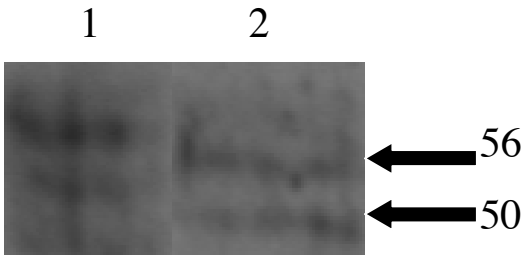

B
C
A

Figure 3.6 **Presence of Soluble CD14 in Mammary Tissue and Primary Epithelial Cell Culture**. Western blot analysis of lysates from mammary tissue and primary mammary epithelial cells cultured for 26 days after being digested for 18 hours. Samples were tested for sCD14 with MY4, a monoclonal anti-human CD14 antibody. Lane 1 is lysates from pre-digested mammary tissue. Lanes 2 is lysates from cultured primary mammary epithelial cells digested for 18 hours.


1
2
56
50

APPENDIX

Tables 1-21. Profiles of all cows and horses included in the study. Included are all the sampling dates, medications, and protein determinations for each animal.

Table 1: Cow 3310

Pregnancy Information

Calving Date
4/22/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
4/22/01	4/23/01	4/28/01	5/5/01	5/12/01	5/19/01

Notes

4/22: Received Calcium Selenium Gel, Oxytocin
5/2-5/3: All quarters of udder treated with Today
5/8: Left rear hoof received trim
5/8-5/10: Received LA 200

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	365	10.95
	2	1/30	383	11.51
	7	1/30	374	11.23
	14	1/30	365	10.95
	21	1/30	345	10.35
	28	1/30	311	9.35
Cow Colostrum	1	1/60	479	28.76
	2	1/30	244	7.33
	7	1/30	203	6.09
	14	1/30	158	4.75
	21	1/30	103	3.11
	28	1/30	114	3.42
Calf Serum	1	1/30	290	8.70
	2	1/30	286	8.59

Table 2: Cow 3704

Pregnancy Information

Calving Date
4/25/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
4/25/01	4/26/01	5/1/01	5/8/01	5/15/01	5/22/01

Notes

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	277	8.31
	2	1/30	500	15.00
	7	1/30	304	9.13
	14	1/30	299	8.97
	21	1/30	337	10.12
	28	1/30	273	8.20
Cow Colostrum	1	1/60	315	18.93
	2	1/30	162	4.86
	7	1/30	100	3.00
	14	1/30	93	2.79
	21	1/30	124	3.74
	28	1/30	93	2.79
Calf Serum	1	1/30	255	7.66
	2	1/30	255	7.66

Table 3: Cow 3719

Pregnancy Information

Calving Date
3/27/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
3/27/01	3/28/01	4/2/01	4/9/01	4/16/01	4/23/01

Notes

3/27: Received Naquasone, Calcium Selenium Gel, Oxytocin
3/28: Received Propylene Glycol, Naxcel
3/29-3/31: Received Today
3/30: Received LA 200
4/1: Left and right rear udder treated with Today
4/12: Received Lutalyse

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	443	13.30
	2	1/30	408	12.24
	7	1/30	411	12.35
	14	1/30	464	13.92
	21	1/30	441	13.25
	28	1/30	387	11.62
Cow Colostrum	1	1/60	346	20.81
	2	1/30	264	7.93
	7	1/30	137	4.11
	14	1/30	98	2.95
	21	1/30	82	2.47
	28	1/30	98	2.95
Calf Serum	1	1/30	260	7.82
	2	1/30	321	9.63

Table 4: Cow 4508

Pregnancy Information

Calving Date
4/17/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14
4/17/01	4/18/01	4/23/01	4/30/01

Notes

4/18-4/20: Received Today
4/21-4/25: Received Lutalyse, Penicillin
4/24-4/25: Received LA 200, Betadine flush
4/27-4/29: Received LA 200, Penicillin, Lutalyse
4/30: Received Penicillin flush
5/1: Received Propylene glycol, Rumen fluid
5/3: Received Propylene glycol, Resorb, Betadine flush
Only sampled through day 14

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	437	13.13
	2	1/30	361	10.84
	7	1/30	460	13.81
	14	1/30	402	12.07
Cow Colostrum	1	1/60	400	24.03
	2	1/30	230	6.90
	7	1/30	253	7.61
	14	1/30	190	5.72
Calf Serum	1	1/30	210	6.31
	2	1/30	286	8.59

Table 5: Cow 4519

Pregnancy Information

Calving Date
2/26/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
2/26/01	2/27/01	3/4/01	3/11/01	3/18/01	3/25/01

Notes

2/26: Received Calcium Selenium Gel
3/15-3/17: Received Lutalyse

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	348	10.46
	2	1/30	352	10.57
	7	1/30	275	8.26
	14	1/30	330	9.90
	21	1/30	363	10.90
	28	1/30	333	10.01
Cow Colostrum	1	1/60	389	23.36
	2	1/30	288	8.64
	7	1/30	63	1.90
	14	1/30	72	2.16
	21	1/30	271	8.15
	28	1/30	253	7.61
Calf Serum	1	1/30	232	6.96
	2	1/30	398	11.96

Table 6: Cow 5603

Pregnancy Information

Calving Date
3/12/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
3/12/01	3/13/01	3/18/01	3/25/01	4/1/01	4/8/01

Notes

3/12: Low yields of colostrum (2.5 quarts)
4/3: Recorded temperature of 104º F
4/3-4/5: Received Naxcel

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	271	8.15
	2	1/30	271	8.15
	7	1/30	288	8.64
	14	1/30	322	9.68
	21	1/30	345	10.35
	28	1/30	365	10.95
Cow Colostrum	1	1/60	411	24.70
	2	1/30	317	9.52
	7	1/30	255	7.66
	14	1/30	82	2.47
	21	1/30	101	3.05
	28	1/30	94	2.84
Calf Serum	1	1/30	259	7.77
	2	1/30	313	9.41

Table 7: Cow 5610

Pregnancy Information

Calving Date
2/22/02

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
2/22/01	2/23/01	2/28/01	3/7/01	3/14/01	3/21/01

Notes

2/22: Received Calcium Selenium Gel
2/22-2/25: Received Oxytocin
3/2-3/4, 3/6-3/8, 3/10-3/12: Received Lutalyse
3/5-3/9: Received Naxcel

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	293	8.81
	2	1/30	299	8.97
	7	1/30	328	9.85
	14	1/30	365	10.95
	21	1/30	380	11.40
	28	1/30	363	10.90
Cow Colostrum	1	1/60	306	18.38
	2	1/30	124	3.74
	7	1/30	101	3.05
	14	1/30	73	2.21
	21	1/30	98	2.95
	28	1/30	89	2.68
Calf Serum	1	1/30	313	9.41
	2	1/30	288	8.64

Table 8: Cow 5617

Pregnancy Information

Calving Date
4/25/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
4/25/01	4/26/01	5/1/01	5/8/01	5/15/01	5/22/01

Notes

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	374	11.23
	2	1/30	326	9.79
	7	1/30	382	11.46
	14	1/30	335	10.07
	21	1/30	383	11.51
	28	1/30	345	10.35
Cow Colostrum	1	1/60	203	12.19
	2	1/30	194	5.82
	7	1/30	226	6.79
	14	1/30	108	3.26
	21	1/30	65	1.95
	28	1/30	135	4.06
Calf Serum	1	1/30	248	7.44
	2	1/30	304	9.13

Table 9: Cow 5627

Pregnancy Information

Calving Date
3/5/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14
3/5/01	3/6/01	3/11/01	3/18/01

Notes

3/6: Calf Died
3/7-3/9: Received Today (Treatment for Mastitis)
3/14-3/16: Received Predef
3/14-3/16, 3/20-3/22: Received Naxcel
3/19: Received Dextrose IV, Dexamethasone
3/19-3/21: Received Lutalyse
Did not sample after day 14 and 24 hour post-suckle calf serum

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	288	8.64
	2	1/30	302	9.08
	7	1/30	310	9.30
	14	1/30	306	9.19
Cow Colostrum	1	1/60	422	25.37
	2	1/30	201	6.04
	7	1/30	163	4.91
	14	1/30	66	2.00
Calf Serum	1	1/30	268	8.04

Table 10: Cow 601

Pregnancy Information

Calving Date
3/21/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14
3/21/01	3/22/01	3/27/01	4/3/01

Notes

3/24: Retained placenta removed
3/29: Received Propylene Glycol
4/2: Received Vitamin B, Naxcel, Propylene Glycol
4/3: Received Propylene Glycol, Lutalyse, Naxcel, Betadine Flush
4/4: Received Propylene Glycol, Naxcel, Lutalyse, Predef
4/8: Died
Only sampled through day 14

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	348	10.46
	2	1/30	332	9.96
	7	1/30	310	9.30
	14	1/30	313	9.41
Cow Colostrum	1	1/60	346	20.81
	2	1/30	162	4.86
	7	1/30	163	4.91
	14	1/30	96	2.89
Calf Serum	1	1/30	317	9.52
	2	1/30	365	10.95

Table 11: Cow 888

Pregnancy Information

Calving Date
3/25/01

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	24 Hour	Day 7	Day 14	Day 21	Day 28
3/25/01	3/26/01	3/31/01	4/7/01	4/14/01	4/21/01

Notes

3/28: Received Propylene Glycol
4/7-4/9: Received Lutalyse

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Cow Serum	1	1/30	330	9.90
	2	1/30	280	8.42
	7	1/30	332	9.96
	14	1/30	374	11.23
	21	1/30	350	10.51
	28	1/30	367	11.01
Cow Colostrum	1	1/60	500	30.01
	2	1/30	167	5.02
	7	1/30	146	4.38
	14	1/30	98	2.95
	21	1/30	100	3.00
	28	1/30	94	2.84
Calf Serum	1	1/30	241	7.23
	2	1/30	357	10.73

Table 12: 110 Hanks Carli Tari

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
4/10/00	3/16/01	3/20/01 8PM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 3/30/01	(3) 3/23/01	(8) 3/28/01	(13) 4/2/01	(27) 4/16/01

Notes

Day 1 mare serum is not recorded because tube broke in the centrifuge

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	NOT	RECORDED	
	3	1/30	249	7.47
	8	1/30	235	7.06
	13	1/30	228	6.85
	27	1/30	229	6.87
Mare Colostrum	1	1/60	577	34.66
	3	1/30	70	2.11
	8	1/30	10	0.30
	13	1/30	5	0.17
	27	1/30	5	0.15
Foal Serum	1	1/30	246	7.39
	3	1/30	246	7.39
	8	1/30	261	7.85
	13	1/30	179	5.37
	27	1/30	142	4.27

Table 13: 108 Yanders Begone

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
4/12/00	3/18/01	3/25/01 4AM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 3/25/01	(3) 3/28/01	(8) 4/2/01	(15) 4/9/01	(28) 4/22/01

Notes

Mare died 3/27/01. No serum/colostrum samples obtained post-partum. Foal grafted onto Hanks Carli Tari (110). Serum and colostrum samples from 110 represent sample 2.

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	292	8.78
Mare Colostrum	1	1/60	660	39.62
Foal Serum	1	1/30	290	8.72
	3	1/30	241	7.23
	8	1/30	203	6.09
	15	1/30	184	5.53
	28	1/30	148	4.46

Table 14: 130 Cotton Candy

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
4/18/00	3/24/01	4/5/01 1AM

Sampling Information

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 4/5/01	(3) 4/7/01	(7) 4/12/01	(14) 4/19/01	(29) 5/4/01

Notes

Dystosia. Foal physically removed 45-60 minutes after foaling began. Foal aspirated myconium in the womb and immediately placed on antibiotics: Penicillin and Anthicasin. Mare retained placenta and was given Oxytocin and Banamine after more than four hours. Placenta passed by morning. Foal- blowout diarrhea and possible endotoxemia. All symptoms in mare and foal ceased with time and antibiotic time coarse. Foal healthy.

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	433	12.99
	3	1/30	318	9.55
	7	1/30	322	9.66
	14	1/30	216	6.50
	29	1/30	162	4.86
Mare Colostrum	1	1/60	504	30.24
	3	1/30	111	3.34
	7	1/30	50	1.50
	14	1/30	44	1.33
	29	1/30	39	1.17
Foal Serum	1	1/30	250	7.52
	3	1/30	231	6.93
	7	1/30	193	5.80
	14	1/30	156	4.70
	29	1/30	131	3.95

Table 15: 140 Aces It

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
4/28/00	4/3/01	4/2/01 10PM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 4/2/01	(3) 4/5/01	(7) 4/9/01	(15) 4/17/01	(28) 4/30/01

Notes

Retained small pieces of placenta which led to metritus and foundering of front legs.
Levaged 4 days after foaling and treated with antibiotics: Penicillin

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	265	7.96
	3	1/30	311	9.33
	7	1/30	257	7.71
	15	1/30	205	6.15
	28	1/30	251	7.55
Mare Colostrum	1	1/60	405	24.31
	3	1/30	207	6.23
	7	1/30	59	1.79
	15	1/30	58	1.76
	28	1/30	70	2.10
Foal Serum	1	1/30	108	3.26
	3	1/30	212	6.36
	7	1/30	262	7.88
	15	1/30	171	5.15
	28	1/30	226	6.79

Table 16: 109 Ima Tiffany Jewel

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
5/6/00	4/11/01	4/19/01 1AM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 4/19/01	(3) 4/22/01	(7) 4/26/01	(15) 5/4/01	(27) 5/16/01

Notes

Day 27-29 Foal sample was not collected because we were unable to catch the foal.

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	383	11.51
	3	1/30	334	10.04
	7	1/30	270	8.10
	15	1/30	240	7.20
	27	1/30	288	8.64
Mare Colostrum	1	1/60	484	29.04
	3	1/30	123	3.71
	7	1/30	68	2.05
	15	1/30	61	1.84
	27	1/30	40	1.21
Foal Serum	1	1/30	210	6.31
	3	1/30	266	7.99
	7	1/30	300	9.00
	15	1/30	271	8.15

Table 17: 102 Kat

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
5/14/00	4/19/01	4/19/01 8AM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 4/19/01	(3) 4/22/01	(7) 4/26/01	(15) 5/4/01	(27) 5/16/01

Notes

None

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	359	10.79
	3	1/30	331	9.93
	7	1/30	284	8.53
	15	1/30	347	10.43
	27	1/30	339	10.18
Mare Colostrum	1	1/60	329	19.76
	3	1/30	138	4.14
	7	1/30	74	2.24
	15	1/30	45	1.35
	27	1/30	38	1.14
Foal Serum	1	1/30	228	6.85
	3	1/30	281	8.45
	7	1/30	268	8.04
	15	1/30	270	8.10
	27	1/30	259	7.77

Table 18: Freckles By Doc

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
5/8/00	4/13/01	4/23/01 10:30PM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 4/23/01	(3) 4/26/01	(8) 5/1/01	(15) 5/8/01	(29) 5/29/01

Notes

Mare experienced a retained placenta and was given 40 i.u. of Oxytocin every hour on the hour from 2AM-4AM. Placenta was passed around 5 AM

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	452	13.56
	3	1/30	384	11.54
	8	1/30	299	8.97
	15	1/30	232	6.96
	29	1/30	266	7.99
Mare Colostrum	1	1/60	333	20.03
	3	1/30	105	3.16
	8	1/30	97	2.92
	15	1/30	72	2.16
	29	1/30	73	2.21
Foal Serum	1	1/30	244	7.33
	3	1/30	336	10.10
	8	1/30	313	9.41
	15	1/30	320	9.60
	29	1/30	226	6.79

Table 19: 121 Miss Nita Brook

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
5/11/00	4/16/01	5/01/01 10PM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 5/1/01	(3) 5/3/01	(7) 5/8/01	(15) 5/16/01	(27) 5/28/01

Notes

None

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	375	11.26
	3	1/30	272	8.18
	7	1/30	270	8.10
	15	1/30	250	7.50
	27	1/30	241	7.25
Mare Colostrum	1	1/60	720	43.23
	3	1/30	115	3.45
	7	1/30	93	2.79
	15	1/30	60	1.82
	27	1/30	72	2.18
Foal Serum	1	1/30	167	5.03
	3	1/30	182	5.48
	7	1/30	243	7.31
	15	1/30	218	6.55
	27	1/30	214	6.42

Table 20: Tiger

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
Unknown	Unknown	5/10/01 8:30PM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 5/10/01	(2) 5/12/01	(6) 5/16/01	(13) 5/23/01	(27) 6/06/01

Notes

Tiger was sampled from the residence of Teresa Morrison. Tiger is property of Mrs. Morrison and not the University of Georgia.

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	276	8.29
	2	1/30	338	10.15
	6	1/30	280	8.41
	13	1/30	230	6.90
	27	1/30	205	6.15
Mare Colostrum	1	1/60	171	10.26
	2	1/30	87	2.63
	6	1/30	113	3.40
	13	1/30	78	2.34
	27	1/30	38	1.16
Foal Serum	1	1/30	197	5.93
	2	1/30	212	6.36
	6	1/30	238	7.14
	13	1/30	244	7.33
	27	1/30	232	6.96

Table 21: 127 Wheeling Polly

Pregnancy Information

Last Breeding Date	Due Date	Foaling Date
Unknown	5/1/01	5/21/01 12:15 AM

Sampling Information

Dating (exact date post-partum):

Day 1-Pre-suckle Birth	Day 2-3	Day 6-8	Day 13-15	Day 27-29
(1) 5/21/01	(2) 5/22/01	(8) 5/28/01	(15) 6/4/01	(29) 6/18/01

Notes

Bradford Protein Determination

Sample	Day	Dilution	Protein (μg/mL)	Total Protein (μg/μL)
Mare Serum	1	1/30	335	10.07
	2	1/30	354	10.63
	8	1/30	282	8.48
	15	1/30	280	8.42
	29	1/30	272	8.18
Mare Colostrum	1	1/60	372	22.36
	2	1/30	136	4.09
	8	1/30	103	3.11
	15	1/30	62	1.87
	29	1/30	60	1.82
Foal Serum	1	1/30	270	8.12
	2	1/30	300	9.00
	8	1/30	302	9.08
	15	1/30	279	8.37
	29	1/30	290	8.72