Mannheimia haemolytica Leukotoxin Induces Rapid Redistribution of LFA-1 on Bovine Lymphoma Cells.

By

Bryan Keith Taylor

Bachelor of Science

Langston University

Langston, Oklahoma

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CHAPTER I

ADVANCEMENTS IN *MANNHEIMIA* (FORMERLY *PASTEURELLA*) *HAEMOLYTICA* RESEARCH

Since the classification of *M. haemolytica* from *Bacillus bovisepticus* to *Pasteurella haemolytica* (Newsom and Cross, 1932), there has been significant scientific advancement on the role *M. haemolytica* plays in the onset of pneumonic pasteurellosis.

In 1978, Benson and colleagues demonstrated that bovine alveolar macrophages challenged with *M. haemolytica* exhibited severe cytolytic and morphological changes caused by the action of a toxic factor being produced by *M. haemolytica*. In 1982, Shewen and Wilkie demonstrated that the cytotoxin was specific for bovine leukocytic cells, and was termed "leukotoxin" (LKT). Shewen and Wilkie (1985) also demonstrated that LKT production was a result of actively growing bacteria.

Genetic analysis of the genes encoding LKT lead to genetic manipulations and mutant gene constructs. Lo and colleagues (1985) cloned and expressed the LKT gene cluster and demonstrated that homology exists between *Escherichia coli* α-hemolysin and *M. haemolytica* LKT (Lo et al., 1987). This group of cytolysins, along with others, was subsequently termed the Repeats in Toxin (RTX) family (Forestier and Welch, 1991) and characterized as pore formers (Bhakdi et al., 1986). Genetic analysis also resulted in a new classification for the organism from *Pasteurella haemolytica* to *Mannheimia haemolytica* using 16s RNA sequencing and DNA-DNA hybridizations to achieve information about the phylogenetic structure and taxonomic distances within the *P. haemolytica* complex (Angen et al., 1999).

Slocombe and colleagues (1985) demonstrated the importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis. When experimentally exposed to *M. haemolytica*

calves with normal numbers of neutrophils developed lung lesions, that consisted of necrosis of the alveolar walls, intra-alveolar hemorrhage, a severe exudative and necrotizing bronchopneumonia with accumulation of proteinaceaous fluid in alveoli and lymphatics. Cattle depleted of neutrophils had lungs that appeared grossly normal. Watson and colleagues (1995) demonstrated that neutrophil mediated injury involved three major mechanisms: release of reactive oxygen species, secretion or release of enzymes form cytoplasmic granules, and production of arachidonic acid metabolites, which are all factors in lesion formation after the onset of shipping fever.

Ortiz-Carranza and Czuprynski (1992) showed that activation of bovine neutrophils by *M. haemolytica* was Ca^{2+} dependent. Stevens and Czuprynski (1996) later demonstrated that at sublytic concentrations, LKT can activate ruminant leukocytes leading to apoptosis, whereas lytic concentrations of LKT inhibited leukocyte function and is cytolytic (Clinkenbeard et al., 1989b).

Perhaps the most beneficial contributions to research on *M. haemolytica* were made on its mechanisms of binding. After binding its target cell, LKT is hypothesized to insert into target cells membranes forming transmembrane pores (Moayeri and Welch, 1997; Bhakdi et al., 1986). Brown and colleagues (1997) suggested that specific binding sites for *M. haemolytica* LKT exist on bovine leukocytes. Lally and colleagues (1999) confirmed these finding by demonstrating that the RTX toxins recognize a β_2-intergrin on the surface of human target cells. Li and colleagues (1999) defined the receptor as bovine CD18 and Jeyaseelan and colleagues (2000) further characterized the receptor as LFA-1. Leite and collegues (2000) demonstrated that recombinant Interlukin-1β accelerated the effects of LKT on neutrophils in a β_2-integrin dependent manner, thus increasing β_2-integrin affinity for LKT, resulting in a more avid biological response from host.

The studies above represent only a few of the significant findings that have paved the way for current research on *Mannheimia haemolytica*.

Chapter II

Literature Review

Overview: *Mannheimia (*formerly *Pasteurella) haemolytica*

Bovine pneumonic pasteurellosis, or "shipping fever", was coined in North America by Kinsley in 1915 (Gibbs et al., 1984). Kinsley considered it to be the pulmonary form of hemorrhagic septicemia. Hepburn (1925) later described it as a respiratory disease of cattle that usually arose shortly after transport of either Irish or Orkney cattle to Aberdeenshire. Transit fever, as defined by Anderson in 1939 (Gibbs et al., 1984), was a disease which usually affects recently weaned single-suckled calves shortly after they were housed in autumn. The causative agent was later found to be *Mannheimia (*formerly *Pasteurella) haemolytica* biotype A serotype 1. The disease was called "shipping fever" rather than "transit fever", because it occurred chiefly among young cattle recently introduced to feedlot conditions (Gibbs et al., 1984).

Pneumonic pasteurellosis currently causes upwards of one billion dollars in annual losses to the beef cattle industry in North America, which is greater than all other bacterial diseases combined (Babiuk and Lawman, 1987). Based on capsular antigens or the lipopolysaccharide complex, there are 15 serotypes, within two biotype groups (A and T), and numerous untypable strains of *M. haemolytica* (Carter, 1967; Tsai et al., 1988). In clinically healthy cattle, *M. haemolytica* is present in low numbers in the nasal passages and those that are isolated are predominantly biotype A serotype 2 (A2) which are rarely associated with shipping fever (Morck et al., 1988; Morck et al., 1989). Exposure of healthy cattle to stressful agents such as viral infection, change in management practices (marketing, transportation, and processing), and change in environmental (heat/cold) conditions, leads to an explosive growth and selective colonization by *M.*

haemolytica in the upper respiratory tract (Frank, 1979; Frank, 1988; Jones, 1987). In theory, most healthy cattle should not succumb to pneumonic pasteurellosis or become clinically ill when infected because protective mechanisms should stop the progress of a *M. haemolytica* infection. However, the ability of the protective mechanisms to cope with the infection is diminished by stress or viral-induced respiratory disease. In stressed cattle, *M. haemolytica* can multiply rapidly in the nasopharynx to form a large population (Frank and Smith, 1983) that travels from the nasopharynx to the lungs in aerososolized droplets (Grey and Thomson, 1971). When large numbers of *M. haemolytica* enter the lung they produce a devastating anterior ventral pneumonia, characterized by extensive fibrin exudation, neutrophil and macrophage influx, capillary thrombosis, and foci of coagulation necrosis surrounded by a zone of bacteria and degenerating swirling inflammatory cells (Whiteley et al., 1992). In natural cases, the most frequently involved portion of the lung is the anterioventral area. Gross lesions often include acute fibrinous or serofibrinous pleuritis; interlobular septa are distended with serum and fibrin (Rehmtulla and Thomson et al., 1981). The air passages are infiltrated with fibrinous exudates from the blood. The pneumonic areas are clearly distinguished from the nonpneumonic areas.

Virulence Factors

Virulence factors enhance the ability of bacteria to evade host defense mechanisms by enhancing bacterial colonization and infiltration of host tissues. The four major virulence factors associated with *M. haemolytica* are fimbriae, polysaccharide capsule, endotoxin *(lipopolysaccharide),* and LKT (Confer et al., 1990); others include protease, sialidase and outer-membrane components (Weekley, 1998).

FIMBRIAE (PILI)

Surface proteins (adhesions) or bacterial capsules allow bacteria to adhere to eukaryotic cell surfaces. Information on upper respiratory tract (URT) colonization of *M. haemolytica* is very limited. Two major alterations that lead to colonization of the URT by gram negative bacteria are alterations in the mucocilliary apparatus (Pavia, 1987) and loss of fibronectin, an adhesive glycoprotein (Woods, 1987), from epithelial cell surfaces.

Loss of fibronectin, which exposes receptors on cells and permits binding of gram-negative bacteria, appear to be a key event in favoring colonization of the human URT (Woods, 1987; Proctor, 1987). Fimbriae of some gram-negative bacteria are composed of proteins that can act as lectins in recognizing specific cell surface receptors. Specific receptors for fimbriae are present on eukaryotic cells, and the fimbriae-receptor interaction overcomes normal repulsive forces exerted between bacteria and eukaryotic cells. *M. haemolytica* is reported to produce two types of fimbriae, a large rigid nonflexible structure of 12 nm diameter, and a small, thin flexible structure of 5 nm diameter (Morck et al., 1987; Morck et al., 1988). Potter and colleagues (1988) reported that the large, rigid fimbriae of *M. haemolytica* A1 were readily purified by mechanical shearing and centrifugation and is comprised of a 35kDa subunit. Structures resembling fimbriae were seen by transmission electron microscopy on *M. haemolytica* adherent to tracheal epithelium in a naturally infected calf (Morck et al., 1989).

Electron microscopic studies by others have failed to demonstrate fimbriae on the surface of *M. haemolytica*. Morck and colleagues (1987) noted that mechanical factors such as agitating the culture could prevent demonstration of fimbriae. Clarke and colleagues (2000) examined adherence of *M. haemolytica* to mammalian tissue culture cells and found that *M. haemolytica* did adhere to Madin-Darby bovine kidney cells and bovine turbinate cells, suggesting an important role

of adherence in the initial pathogenesis of shipping fever pneumonia. Through electron microscopic studies, Clarke and colleagues (2001) also found that epithelial cell cilia and surface mucus played a role in *M. haemolytica* adherence. *M. haemolytica* adherence maybe brought forth by a variety of virulence factors, but whether these factors are specific or nonspecific in their host interactions are unknown. The difference in percent adherence detected between a single strain of *M. haemolytica* A1 and A2 may be related to serovar-specific expression of bacterial adhesions, but this has yet to be determined (Clarke, 2000).

CAPSULAR POLYSACCHARIDE

M. haemolytica produces a polysaccharide capsule (glycocalyx) during logarithmic growth phase (Corstvet et al., 1982). *M. haemolytica* from infected calves had greater quantities of capsular material than organisms grown in culture (Morck et al., 1988). Capsular material was also seen ultrastucturally on *M. haemolytica* within the alveoli of experimentally and naturally-infected cattle. (Morck et al., 1988; Morck et al., 1989; Whiteley et al., 1992). Examination of purified capsules from five serotypes of *M. haemolytica* showed that these bacterial capsules were complex polysaccharides. The capsule of each serotype has a different sugar composition (Adlam et al., 1986) and is attached to alveolar epithelium via the capsule (Morck et al., 1988). Also, microcolonies of the organism were seen encased in amorphous capsular material within alveoli (Morck et al., 1989).

Studies by Czuprynski and colleagues (1998) revealed that *M. haemolytica* capsular polysaccharide might be an important virulence mechanism. Using bovine neutrophils, they demonstrated that *M. haemolytica* capsular polysaccharide increased directed migration and diminished phagocytosis and killing of *M. haemolytica*, but not *Escherichia coli.* The results of

these studies indicate that *M. haemolytica* capsular material may be important for adherence of the organism to alveolar and bronchiolar surfaces and attraction of neutrophils to the sites of colonization (Confer et al., 1990). Capsular material inhibits neutrophil functions by decreasing their ability to phagocytize and protect the host from bacterial invasion. Capsular material may also inhibit complement-mediated serum killing.

Clarke and colleagues (2000) explored the role of the capsule in adherence of *M. haemolytica*. Mean % adherence to bovine nasopharyngeal tissue of capsulated *M. haemolytica* was significantly higher than that of de-capsulated *M. haemolytica*, further demonstrating the potential role of the capsule in adherence and initial pathogenesis of bovine respiratory disease.

LIPOPOLYSACCHARIDE (ENDOTOXIN)

Lipopolysaccharide (LPS) is a major component of gram-negative bacterial outer membranes. *M. haemolytica* LPS is similar to LPS produced by other gram-negative bacteria having amphiphilic properties and a hydrophobic fatty-acyl-containing lipid A; a highly charged and hydrophilic core containing 2-keto-3-deoxyoctosnic acid (KDO) substituted with phosphate and ethanolamine; and a polar, noncharged, hydrophilic repeating polysaccharide containing an O-specific chain (Luderitz et al., 1982).

LPS is apparent in most LKT preparations and its separation from LKT is rigorous (Yoo et al., 1995). The quantity of endotoxin in dried *M. haemolytica* cell walls ranges from 12 to 25 % (Keiss et al., 1964). *M. haemolytica* LPS introduced into sheep's lung caused influx of neutrophils and accelerated lesion development (Brogden et al., 1984). Its been proposed that LPS may be necessary for maximal production of some RTX toxins (Czuprynski and Welch, 1995). LPS pre-treatment of rabbit lungs caused an increase in thromboxane release after addition of *E. coli*

hemolysin (Walmrath et al., 1994). Also, mutations in genes involved with LPS synthesis were shown to reduce production and activity of *E. coli* hemolysin (Bauer and Welch, 1997; Stanley et al, 1993).

LPS enters cells by interacting with specific receptors via the core polysaccharide or by insertion into the cell membranes via the lipid A portion of the molecule (Bradley 1985; Morrison and Rudbach, 1981; Haeffner-Cavaillon et al., 1985). LPS binds with a circulating blood protein (lipopolysaccharide binding protein [LPB]), which binds to CD14 molecules. At low doses the LPS complex can directly activate a cascade of cytokines and other mediators (TNF, IL-1, IL-6, IL-8, NO, and PAF). At moderate doses LPS induces fever and increases synthesis of acute phase reactants. Finally, higher doses of LPS result in endotoxic shock, which is characterized by systemic vasodilation (hypotension), diminished myocardial contractility, widespread endothelial injury, and activation of the coagulation cascade (Parrillo, 1993; Ognibene, 1997; Glauser, 1996). Sublethal intravenous and intraarterial injections of *M. haemolytica* LPS in sheep caused an initial increase in pulmonary arterial pressure followed by a decrease in cardiac output with reduction in left arterial, pulmonary venous, and systemic blood pressure (Keiss et al., 1964).

Whiteley and colleagues (1990) found that when endotoxin was released into the inflammatory exudate of the alveolus it was localized in alveolar macrophages, neutrophils in the alveolus, and neutrophils in the bronchial lymph nodes of calf lungs experimentally infected with *M. haemolytica* A1. Intravenous infusion of *M. haemolytica* LPS in calves induced release of thromboxane A_2, prostaglandins, serotonin, cAMP, and cGMP, which may mediate endotoxic effects (Emau et al., 1984; Emau et al., 1987). *M. haemolytica* LPS also caused direct cell membrane damage to bovine pulmonary endothelial cells along with enhanced neutrophil adherence to and arachidonic-acid release from endothelium, indicating endothelial cell activation (Confer et al., 1990).

LEUKOTOXIN

Bacterial cytolysins disrupt target cells by decreasing their plasma membrane integrity. Bacterial cytolysins may be distinguished as pore-forming cytolysins (that disrupt osmotic integrity), enzymatically active cytolysins (which degrade membrane lipids), or surfactant cytolysins (which solubilized cell membranes in a detergent-like action) based upon their mechanisms of action against host target cells.

M. haemolytica produces a leukotoxin (LKT) that belongs to the Repeats in Toxin (RTX) family (Forestier and Welch, 1991). The RTX toxins represent the largest family of gram-negative pore forming cytolysins and can be distinguished by a number of common traits (Ludwig and Goebel, 1999). The C-terminal half of RTX toxin proteins includes a tandem array of glycine and aspartate rich nonameric repeats that contain a consensus sequence (UXGGXG[N/D]DX). RTX toxins are post-transcriptionally activated and their secretion proceeds via a type 1 secretion pathway, allowing translocation across both the inner and outer membrane of bacteria in one step. RTX toxin activity is Ca^{2+} dependent. The genes specifically required for synthesis, activation and secretion are clustered on bacterial chromosomes or plasmids as four contiguous genes (C-A-B-D). Gene C encodes the activator protein, A is the structural gene, and both B and D encode the ABC (ATP binding cassette) protein and the MFP (membrane fusion protein) component of the ABC exporter. Some members of the RTX family include *Escherichia coli* α-hemolysin, *Actinobacillus pleuropneumoniae* hemolysins and cytolysins, *Actinobacillus actinomycetemcomitans* leukotoxin, *Vibrio cholerae* leukotoxin, *Bordetella pertussis* adenylate cyclase toxins, and many more.

The LKT determinant from *M. haemolytica* has been cloned (Lo et al., 1985; Strathdee and LO, 1989a). Four open reading frames encoding polypeptides of molecular sizes 19.8, 101.9, 79.7, and 54.7 kDa can be deduced form the nucleotide sequence (Lo et al., 1985; Strathdee and Lo, 1989a). Thus four open reading frames designated *lktC, lktA, lktB*, and *lktD* respectively, in the order of their genetic organization. *LktA* encodes the structural polypeptide of the toxin that is activated by an intracellular component encoded by *lktC*, whereas *lktB* and *lktD* encode a secretion function (Strathdee and Lo, 1989b). *LktA* consist of 953 amino acids with a molecular size of 101.9 kDa. Two large hydrophobic domains, which are involved with interactions between the toxin and its target cell membrane, can be found on the molecule (Lo, 1990). The chromosomal *lktCABD* operon resembles the *E. coli* α-hemolysin determinant with repect to gene organization and transcriptional polarity (Lo et al., 1987; Highlander et al., 1989, 1990; Strathdee and Lo, 1989a,b). The proteins encoded by the *lkt* genes are structural and functional homologs to the *hly* determinants respectively. The amino acid sequence for *lkt A* is 36.4% identical to that of *E. coli hlyA*. The most pronounced structural difference between these two toxins is that *lktA* has a shorter repeat domain, consisting of only eight instead of 13 consecutive glycine-rich nonapeptide repeats (Strathdee and Lo, 1987).

The cytotoxicity of *M. haemolytica* was first reported in 1978 (Benson et al., 1978). *M. haemolytica* LKT is unique among the RTX toxin family. It is cytolytic for ruminant leukocytes only (Shewen and Wilkie, 1982) and does not affect leukocytes from other species. Forestier and Welch (1991) took advantage of *lkt* and *Hly* gene sequence similarities to examine the domains responsible for their different target cell specificities. They proposed of model for interaction of *hlyA* and *lktA* with host cells. The model assumed that the amino terminal portion of *lktA* was responsible for its interactions with ruminant leukocytes while positions 563 and 739 contained the

structure responsible for *HlyA* erythrocyte lysis. It was later demonstrated that a unique interaction between RTX toxins and β_2 intergrins exsisted, whereby RTX toxins utilized these receptors to destroy host immune cells (Lally et al., 1997). Binding of *M. haemolytica* to its target cell was defined more specifically as bovine CD18, the β_2-chain of the β_2-integrin heterodimer (Li et al., 1999).

LKT is a heat-liable protein exotoxin that is oxygen stable, non-dialyzable, water soluble, and is produced during logarithmic growth phase (Baluyut et al., 1981; Chang et al., 1986; Sutherland and Redmond, 1986; Shewen and Wilkie, 1985). Binding of LKT (lytic concentrations) to its target cell, via bovine CD18, causes formation of a non-functional transmembrane pore (approximately the diameter of sucrose 0.001 to 0.002 μm) (Clinkenbeard et al., 1989b). Pore formation allows the dissipation of the transmembrane electrochemical gradients of K^+ and Na^+ causing an osmotic imbalance. Thus the cytoplasm is hypertonic as compared to the outside of the cell. In order to correct this imbalance, H_20 diffuses into the cell, resulting in a swollen cell (Clinkenbeard et al., 1989c). Cell swelling is followed by the subsequent formation of large cytoplasmic defects, which is a Ca^{2+} dependent process (Clinkenbeard et al., 1989a). Target cells exposed to LKT in media lacking Ca^{2+} are protected against toxin induced cytolysis (Clinkenbeard et al., 1989a; Clinkenbeard et al., 1989b). Ca^{2+} also mediates LKT induced cytolysis by activating membrane phospholipases (Wang et al., 1999) or by causing disruption of the cytoskeleton, thereby resulting in cytolysis.

Although the ability of LKT to selectively destroy the leukocytes of their infected host is an obvious advantage for the bacteria, the activation of leukocytes by low concentrations of LKT may be of even greater importance. Low concentrations of LKT activate bovine neutrophils resulting in stimulated oxidative burst, release of secondary granules, cytoskeletal alterations (Czuprynski et

al., 1991; Maheswaran et al., 1992), and the secretion of inflammatory mediators (5-hydroxyeicosatatraenoic acid and leukotriene B_4) in a dose dependent manner (Clinkenbeard et al., 1994; Hendricks et al., 1992). Stevens and Czuprynski (1996) also reported that at low concentrations of LKT, bovine leukocytes were induced to undergo changes consistent with apoptosis in vitro; observing zeiosis, chromatin condensation, and nuclear fragmentation, which are accepted criteria for apoptotic determination.

Likewise, the effect of LKT on platelets may be important in the pathogenesis of pneumonic pasteurellosis. Leukotoxin-damaged platelets could release fibrinogen and vasoactive compounds contributing to the formation of thrombi and fibrin leakage into alveolar spaces, typically associated with pneumonic pasteurellosis (Clinkenbeard and Upton, 1991). Although *M. haemolytica* LKT is species-specific for ruminant leukocytes, LKT exhibits low level, non species-specific hemolytic activity (Murphy et al, 1995), which is less efficient than its leukolytic activity. Since LKT causes hemolysis it has been suggested that it can bind erythrocytes nonspecifically, independent of its species specific receptor, CD18 (Li et al., 1999; Sun et al., 1999). LKT binding to erythrocytes may not be mediated by a protein receptor (Li et al, 1999). Instead, LKT binding to erythrocytes may involve direct interaction of LKT with membrane phospholipids. Sun and colleagues (1999) proposed two types of LKT binding to lymphoid cells. A high-affinity LKT binding, that leads to efficient leukolysis, and in some lymphoid cells from reputed LKT-nonsusceptible species, a low affinity LKT binding with low efficiency increase in intracellular Ca^{2+} concentration without leading to leukolysis. Non-specific binding of RTX toxins to non-susceptible target cells has been observed (Sato et al., 1993; Sun et al., 1997). Non-specific binding of RTX toxins to erythrocytes most likely involves a domain of RTX toxins that differs from the domain used with binding of species specific bovine CD18.

Summary: Bovine Respiratory Disease Pathogenesis

[1]***Stress*** plays a critical role in shipping fever pathogenesis. Exposure to viral infections (Bovine Herpes Virus or Paralnflunenza), change in management practices (weaning and transport), and change in environmental conditions (fluctuation in temperatures) may lead to growth and colonization of *M. haemolytica* in the upper respiratory tract of cattle (Frank et al., 1983; Confer et al., 1990; Whiteley et al., 1990).

[2]***Bacterial colonization*** is facilitated by fimbriae and/or capsular polysaccharide on the surface of *M. haemolytica* which aid in adhere to eukaryotic cell surfaces. The loss of fibronectin on epithelial cell surfaces also allow bacterial colonization because it exposes receptors on cells and permits binding of gram-negative bacteria (Woods, 1987; Proctor, 1987). Alterations in mucocilliary apparatus also play a role in *M. haemolytica* colonization (Pavia, 1987).

Once *M. haemolytica* colonizes the lower airways, [3]***endotoxin***, or lipopolysaccharide activates Hageman factor XII, thus initiating the coagulation cascade, kinin system and plasminogen activators (Morrison and Rudbach, 1981). LPS also initiates the onset of both the classical and alternative pathways of complement activation (Morrison and Ulevitch, 1978; Morrison and Rudbach, 1981). LPS activates alveolar macrophages to produce cytokines IL-1 and TNF-alpha, and the lipid mediaters PAF, LTB_4, LTC_4, and LTD_4 both directly (Bradley, 1985) and indirectly via complement activation (Morrison and Ulevitch, 1978; Morrison and Rudbach, 1981). IL-1, TNFα, PAF, and LTB_4 are chemotactic for neutrophils and other leukocytes, they increase the expression of adhesion molecules and stimulate monocytes to produce IL-8. IL-8 is important in

neutrophil migration, and has an autocrine effect on alveolar macrophage activation. LPS is also cytolytic for alveolar macrophages, causing cytolysis and release of nitric oxide, reactive oxygen intermediates, and other proteases (Whiteley et al., 1990; Rimsay et al., 1981).

β_2-integrin's role in LKT- target cell interactions

The β_2-integrins are a family of heterodimeric transmembrane surface glycoproteins that mediate cell to cell interactions during an inflammatory response (Larson and Springer, 1990). The β_2-integrins are composed of a cytoskeletally attached β_2-chain (CD18) that is linked to one of three α-chains. These glycoproteins are commonly referred to as LFA-1 (αL, β_2 or CD11a/CD18), Mac-1 (αM, β_2 or CD11b/CD18) or p150, 95 (αX, β_2 or CD11c/CD18). The β_2-integrins interact with intracellular adhesion molecules (ICAMS) on endothelium were their primary role is extravasation and migration toward chemoattractants. Leukocyte adhesion deficiency (LAD) is an immunodeficiency marked by the absence of one of the three α chains and/or the common β_2-chain on the surface of leukocytes. This disorder is associated with severe and recurrent bacterial infections, impaired extravascular targeting and accumulation of myeloid leukocytes, altered wound healing, and significant morbidity is caused by absent or greatly diminished surface expression of integrins of the β_2 class (Harris et al., 2001). A number of distinct β-chain mutations have been characterized, which either affect the amount of the β-chain precursor synthesized or affect the ability of the β-chain to associate with the α chain in the endoplasmic reticulum after synthesis, including undetectable β-chain mRNA and protein precursor and low levels of β-chain mRNA and protein precursor (Kishimoto et al., 1987).

LFA-1 is distributed highly among cells of the lymphocytic, granulocytic, and monocytic lineages (Springer et al., 1987). LFA-1 surface distribution ranges from 15,000 to 40,000 sites per peripheral lymphocyte, and is expressed mostly on T lymphocytes. LFA-1 is present on 50% of bone marrow cells and is first detected during the pre-B cell and late myeloblast stages. LFA-1 is not seen on myeloid and erythroid cells and is absent on macrophages, unless stimulated by LPS or interferon γ (IFN γ) (Shevach, 1993).

Mac-1 and p150,95 show similarities with respect to expression and function. Mac-1 is present on granulocytes, monocytes, and differentiated myeloid cell lines, Mac-1 is not present on myeloid precursor cells. The p150,95 is expressed on monocytes and granuloclytes and is absent form the majority of peripheral lymphocytes. The p150,95 is present on tissue macrophages in much higher numbers than that of Mac-1 suggesting that after monocytes enter tissues and differetiate into macrophages the expression shifts from Mac-1 to p150,95 (Shevach, 1993).

During log phase growth *M. haemolytica* [4]***secretes LKT*** (Shewen and Wilkie, 1985), LKT binds to leukocytes via bovine CD18 (Li et al., 1999) causing a functional but uncharacterized pore. This pore causes the dissipation of the electrochemical gradients of Na^+ and K^+ (Clinkenbeard et al., 1989c). The inside of the cell becomes hypertonic as compared to that of the outside. In order to correct this osmotic imbalance, extracellular water moves into the cell resulting in a swollen cell. The dissipation of Na^+ and K^+ is followed by the subsequent influx of extracellular Ca^{2+} causing the dissociation and or polymerization of actin filaments resulting in membrane damage, ultimately leading to total cytolysis of the cell (Clinkenbeard et al., 1989c). Membrane damage may also be mediated by Ca^{+2} dependent activation of cystolyic phospholipase A_2, leading to elaboration of lysophospholipids, which are known to have detergent like effects on membrane phospholipids (Wang et al., 1999).

As the onset of acute inflammation ensues, the neutrophils are the first to the site of inflammation. [5]***Neutrophil migration*** is mediated by soluble inflammatory mediators (TNFα, IL-1, IL-8, PAF, and LTB_4). These cytokines increase the expression of intracellular adhesions molecules on the surface of endothelium (I-CAM 1) and the surface of neutrophils (LFA-1 and Mac-1). Neutrophils are slowed in the blood stream through interactions between E and P selectins and the Siayl-Lewis X receptor located on the surface of neutrophils (McEver and Cummings, 1997). Once they are slowed in circulation, they adhere to endothelium through the interaction of the I-CAMs and LFA-I. Neutrophils are then allowed to squeeze through the endothelium through interaction with (PECAMS) CD31, in a process known as diapedesis, after which they migrate toward the inflammatory stimulus through a chemical gradient formed by cytokines (Butcher, 1991).

[6]***Acute Pulmonary Damage*** is initiated by the onset of acute inflammation characterized by neutrophil accumulation. LKT activation results in release of neutrophilic enzymes such as collagenase, elastase, and reactive oxygen intermediates (Watson et al., 1995). The release of enzymes from neutrophils cause generation of LTB_4, and PGE_2, which both mediate tissue damage that occur during neutrophil activation. The end result is a pnuemonic fibropurulent lesion characterized by severe congestion, massive fibrin exudation in air spaces, hemorrhage, and red discoloration. (Rehmtulla and Thomson, 1981; Slocombe et al., 1995).

The final stage of shipping fever pneumonia is the onset of [7]***chronic inflammation*** (Rehmtulla and Thomson, 1981; Whiteley et al., 1990).

β_2-integrin Capping

Membrane dynamics play a crucial role in β_2-integrin's interaction with their ligand. In order to control cell adhesion and communication after ligand binding, transmembrane proteins undergo redistribution and clustering in the area of intracellular contact (Singer, 1992). This phenomema is known as capping.

Capping was first characterized by Roger Taylor and Philip Duffus (1971) through studies with fluorescently conjugated Immunoglobulin antibodies. These researchers demonstrated that when spleen cells were tréated at 0° C, surface fluorescence was entirely ring-like, however when treatment of cells with a fluorescent tagged antibody was carried out at 37°C, the percentage of fluorescence remained the same but seen at only one pole of the cell. Taylor and Duffus showed that cap formation was a metabolically dependent active process which could be induced or inhibited and was completely reversible.

The actin cytoskeleton plays a pivatol role in β_2-integrin membrane distribution. The actin cytoskeleton acts as a bridge that brings membrane components together to activate signaling events. The β cytoplasmic domain has been demonstrated to be associated with the cytoskeletal components of α-actin, vinculin, filamin, and or talin (Pavolko and LaRoche, 1993; Pardi et al., 1995; Sharma et al., 1995; Burn et al., 1988). Treatment of cells with cytocalasin D, which disrupts the cytoskeleton, has been shown to result in activation of LFA-1, which is directly associated with receptor capping. Thus, cytoskeletal constraints keep LFA-1 immobile.

The release of LFA-1 from the cytoskeleton is also thought to be regulated by calpain, a cyteine protease that is activated by local Ca^{2+} fluxes (Stewart et al., 1998). Proteins identified as

potential calpain targets include talin, filamin, and α-actinin, all of which are involved in restraint of LFA-1 to the cytoskeletal network.

β_2-Integrins are signaling receptors, as well as targets of intracellular signals. β_2-Integrins are capable of activating non-receptor tyrosine kinases (Dib and Anderson, 2000). These, along with their downstream effectors, mediate a signaling cascade that causes cytoskeletal rearrangements, resulting in increased adhesion, increased cell motility, and changes in cell shape

Sampath and colleagues proposed this possible mechanism of capping: LFA-1 is released from the cytoskeleton as a result of proteolysis of talin by calpain. These events lead to mobilization of the integrin. Alpha actinin then binds the cytoplasmic domain of the β_2 subunit, stabilizing the interaction between the cyoskeleton and the integrin, which leads to strong adhesion at the site of receptor aggregation after receptor mobilization (Sampath et al., 1998).

CHAPTER III

SUMMARY

Mannheimia haemolytica is the primary pathogen involved in the bovine respiratory disease complex. This bacterium produces an exotoxin, known as leukotoxin (LKT), whose target cell specificity is mediated by recognition of a β_2-integrin. LKT specifically intoxicates leukocytes from ruminants with no affect on leukocytes from other species. It has been hypothesized that LKT inserts into target cell membranes forming transmembrane pores followed by cell swelling, membrane degeneration, and cytolysis. To further understand the mechanisms by which LKT intoxicates target cells, we used immunogold electron microscopy (immunoEM) to localize LKT, CD11a, and CD18 on the surface of bovine lymphoma cells (BL3) before and after LKT exposure. Cells (BL3) were incubated with monoclonal antibodies (mAb) specific for LKT, CD11a, or CD18 and labeled with goat anti-mouse IgG conjugated to 15nm gold particles. Expression of both CD11a and CD18 increased in response to LKT stimulation as seen by flow cytometry. Prior to LKT exposure immunoEM revealed that CD11a and CD18 were dispersed evenly throughout BL3 cell plasma membranes. After 1 min LKT exposure, immunoEM revealed that LKT, CD11a, and CD18 were associated with degenerative areas of BL3 cell plasma membranes, with CD18 localized to both intact and degenerative areas after LKT exposure. ImmunoEM did not detect LKT or CD11a in association with intact areas after LKT exposure, they were only seen in association with degenerative areas of BL3 cell plasma membranes. Rapid re-distribution and increased expression of LFA-1 are key steps in the cascade of events that occur between LKT binding and transmembrane pore formation.

CHAPTER IV

INTRODUCTION

Central to the understanding of shipping fever pneumonia are the mechanisms involved in the interaction of *Mannheimia haemolytica* leukotoxin (LKT), the most important virulence factor associated with shipping fever pneumonia, with its target cell. LKT belongs to the repeats in toxin family (RTX) (Forestier and Welch, 1991) and its species specificity for ruminant leukocytes (Shewen and Wilkie, 1982) is mediated by recognition of a β_2-integrin (Li et al., 1999; Jeyaseelan et al., 2000). The β_2-integrins consist of one of three α-chains paired with one β-chain and are commonly referred to as LFA-1 (αL, β_2 or CD11a/CD18), Mac-1 (αM, β_2 or CD11b/CD18) or p150, 95 (αX, β_2 or CD11c/CD18) (Larson and Springer, 1990). This group of heterodimeric transmembrane receptors interacts with intracellular adhesion molecules (ICAMS) on endothelium where their primary role is extravasation and migration toward chemoattractants during an inflammatory response.

The dynamics of membrane receptors is crucial to β_2-integrin interactions with ligand. In order to control cell adhesion and communication after ligand binding, transmembrane proteins undergo redistribution in the area of intracellular contact (Singer, 1992). The redistribution of membrane receptors, also known as capping, is a temperature dependent, cytoskeletally driven, metabolically dependent active process that may be induced (e.g.colchicine) or inhibited (e.g. cytochalasin B) (Bourguignon and Bourguignon, 1984; Taylor et al., 1971). This phenomenon was first described by Taylor and colleagues (1971) who demonstrated that immunoglobulin's (Ig) distribution on resting lymphocytes is diffuse, while polar distribution may be induced by the

interaction of the anti-Ig antibodies with the Ig molecules of the cell membrane, suggesting a possible mechanism for lymphocyte triggering by antigen.

It is hypothesized that initial binding of LKT via its receptor involves a change in LKT conformation (Moayeri and Welch, 1997) followed by its insertion into target cell membranes causing the formation of a transmembrane pore (Bhakdi et al., 1986). Pore formation results in a cascade of cytolytic events. These include, the dissipation of the electrochemical gradients of K^+ and Na^+, the diffusion of H_20 resulting in a swollen cell, the rapid influx of extracellular Ca^{2+} (Clinkenbeard et al., 1989a), membrane degeneration, and cytolysis (Clinkenbeard et al., 1989b). While the mechanisms that occur after pore formation have been well defined, the mechanisms that occur between LKT receptor recognition and pore formation have yet to be elucidated.

The β_2-integrins are signaling receptors, however, they are also targets of and are functionally affected by intracellular signals (Dib and Andersson, 2000). The β_2-integrins are composed of a cytoskeletally attached β_2-chains (CD18). Tyrosine phosphorylation of CD18 regulates cytoskeletal rearrangement (Dib and Andersson, 2000), which regulates cell motility and effects β_2-integrin membrane distributions (Southwick and Stossel, 1983). A recent report shows that *Mannheimia haemolytica* LKT induces tyrosine phosphorylation of the CD18 tail of LFA-1 in bovine leukocytes, thus inducing their biological effects (Jeyaseelan et al., 2001). In this study, we used immunoEM to test the supposition that LKT intoxication of BL3 cells involves the rapid redistribution of LFA-1 molecules on BL3 cell plasma membranes prior to pore formation.

CHAPTER V

MATERIALS AND METHODS

Cultivation of Tissue Culture Cells

Tissue culture cells (BL3) were obtained from and cultured as indicated by the American Type Culture Collection, Rockville, MD, USA. Briefly, bovine lymphoma cells (BL3) were grown in suspension culture in 50% Leibovitz L-15[e]-50% Eagle minimal essential medium (GIBCO Laboratories, Grand Island, NY) with 20% fetal bovine serum to which l-glutamine (2mmol/L), gentamicin (50 mg/L), and $NaHCO_3$ (2.2 g/L) was added at 37°C with 5% $C0_2$ (GIBCO Laboratories, Grand Island, NY).

Preparation of Mannheimia haemolytica LKT

LKT preparations were made form wild type *M. haemolytica* strain SH1217 and mutant strain SH1562 that contains a non-polar insertion in the lktC gene that produces inactive pro-LKT (Fedorova and Highlander, 1997; Sun and Clinkenbeard, 1998). LKT preparations were late-logarithmic phase 60% ammonium sulfate concentrated culture supernatants produced in RPMI 1640 medium (Sigma Chemical Co.). The concentrated culture supernatants (CCS) were dialyzed against phosphate buffered saline (PBS), pH 7.2.

LKT activity assay

Quantification of LKT activity was determined by leakage of the large cytoplasmic enzyme, lactate dehydrogenase (LDH), from BL3 cells as described previously (Clinkenbeard et al., 1989a). LKT activity was quantified as toxic units (TU), which were determined by graphing specific LDH leakage versus the culture supernatant dilution factor. One TU was defined as the dilution factor of LKT which caused specific LDH leakage of 50% for 4 X 10^6 BL3 cells. Concentrated culture supernatant (CCS) preparations of LKT (50μl) were serially diluted in a round bottom 96-well microtiter plate containing 100μl of RPMI 1640 (pH 7.2) (Sigma Chemical Co.). A BL3 cell suspension (100μl; 4 X 10^6 cells/ml) was added to each well and the plate incubated at 37°C for 120 minutes. Exposure ended by centrifugation at 2,000 x *g* for 5 min and 100μl of supernatant was placed in a flat bottom 96-well microtiter plate. Flat bottom plate was warmed to 37°C and 100μl of LDL-50 (LDH assay reagent, re-hydrated by addition of 25ml H_20; Sigma Chemical Company) was placed in each well. LDH activity was measured in a thermally controlled kinetic microtiter plate reader (thermomax) at 340nm for 2 minutes at 37°C. Data was reported as 10^{-3} OD/min. Triton X-100 (1%) was used to assay maximal LDH leakage, and buffer or cells only were used in place of LKT sample to measure background LDH leakage.

LKT Exposure

Pre- and post-fixed BL3 cells (2 X 10^6 cells/ml) were exposed to *M. haemolytica* strain SH1217 CCS LKT or SH1562 mutant strain CCS pro-LKT (1:10 dilution, 347 TU) for 1 minute at 37°C and immediately centrifuged at 10,000 x *g* for 3 min. Unbound CCS LKT was discarded and

cell pellet gently washed by re-suspension in 1 ml of phosphate buffered saline + 1% bovine serum albumin (PBSA) (pH 7.2). Cells were centrifuged at 10,000 x *g* for 3 minutes and cell pellet was re-suspended in 4% paraformaldehyde and 0.2% gluteraldehyde in 0.05% cacodylate buffer (pH 7.4) (fixative) for 20 minutes and washed 3x, as indicated above, centrifuging at 10,000 x *g* for 3 min between each wash.

Immunohistochemistry

Fixation and immunogold staining techniques were modified based on a previously described method (Jiaviriyaboonya et al., 1991). Pre-fixed BL3 cells were incubated in 10ml fixative for 20 minutes and gently washed 3x, by re-suspension in PBSA, centrifuging at 2,000 x *g* for 5 minutes after each wash. BL3 cells ($2x10^6$cell/ml), pre- and post-fixed, were blocked for 15 minutes in PBSA and subsequently incubated with a 1:10 anti-CD18 mAb (BAQ30A) or a 1:10 anti-CD11a mAb (F10-150) (VMRD; Pullman, WA, USA.) for 90 min, and washed as indicated above 3x in 1ml of PBSA, centrifuging at 10,000 x *g* for 3 minutes after each wash. Cells were then incubated with a 1:10 goat anti-mouse IgG $F'(ab)_2$ gold conjugated (15nm particle size) (Electron Microscopy Sciences; Fort Washington, PA, USA) for 90 minutes at 37°C and washed 3x, as indicated above.

BL3 cells exposed to LKT were incubated with 1:10 murine anti-LKT C6 (Murphy et al., 1995), 1:10 anti-CD11a mAb, or 1:10 anti-CD18 mAb for 90 min at 37°C and washed 3x as indicated above. BL3 cells were then incubated with 1:10 goat anti-mouse IgG $F'(ab)_2$ gold conjugated (15nm particles size) for 90 min and wash 3x, as indicated above, in PBSA.

Fixation and Embedding

Gold-labeled cells were fixed in 4% paraformaldehyde and 0.2% gluteraldehyde in 0.05% cacodylate (pH 7.4) for 2 hours at 20°C. Cells were washed 3x in PBSA as indicated (centrifuging at 10,000 x *g* for 3 minutes for the first two washes, and at 10,000 x *g* 10 minutes for the last wash). Cells were post fixed in osmium tetroxide in 0.2M cacodylate buffer for 3 hours and washed (without re-suspending cell pellet) 3x10 minutes in 0.1M cacodylate buffer. Cells were dehydrated in ethanol series (50, 70, 90, 95, and 100%[3x]) for fifteen minutes and infiltrated with 1:1 LR white resin and 100% ethanol for 24 hours at 4°C. Cells were further infiltrated by replacing 1:1 LR White resin and 100% ethanol with 100% LR White for 24 hours at 4°C. Cells were embedded with 100% LR White resin by placing in oven at 65°C for 48 hours. Thin sections (70-90nm thickness) were cut from blocks using a Diatome and placed on nickel coated grids. Sections were stained for 15 minutes in uranyl acetate and for 10 minutes in lead citrate. Grids were examined on the JEOL 100 CXII transmission electron microscope.

Flow Cytometry

BL3 cells (2 X 10^6 cells/ml RPMI 1640), both LKT unexposed and LKT exposed (as indicated above), were incubated in the dark for 1 hour at 4°C 1:500 (0.2μg) with anti-CD11a (F10-150), anti-CD11b (MM12A), anti-CD11c (BAQ153A), or anti-CD18 (BAQ30A) (VMRD; Pullman, WA. USA). Cells were washed 3x as indicated above in PBS + 0.1% Na azide, and incubated for 1 hour, in the dark, with 1:500 (0.2μg) Alexa-Fluor 488 goat anti-mouse IgG polyclonal antibody (A-11001). Cells were washed 3x as indicated above and analyzed for presence of CD11a, CD11b,

CD11c, and CD18 using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), 10,000 events per sample.

Statistical Analyses and ImmunoEM Quantification

Gold labeling was quantified by a single individual (n=15 BL3 cells / treatment group). The use of a light microscope was employed to quantify gold label on low magnification negatives. The amount of degenerative and intact membrane was measured on BL3 cells exposed to LKT by a single individual using a circular rotary measuring tool. Gold label data colleted were analyzed by t-test using GraphPad Prism®, (GraphPad Software, San Diego, California, USA, www.graphpad.com). In all cases, $p < 0.05$ was considered statistically significant.

CHAPTER VI

RESULTS

Expression of β_2-integrins on BL3 cells increased after exposure to LKT for 1 minute at 37°C as indicated by the increase in fluorescent intensities when LKT exposed BL3 cells were subsequently fixed and incubated with anti-CD11a, CD11b, CD11c, and CD18 followed by addition of Alexa-Fluor 488 goat anti-mouse IgG polyclonal antibody. After LKT exposure LFA-1 had the largest increase in expression and made up the highest percentage of BL3 cell's β_2-integrin expression. Expression of all three α-chains and the common β-chain increased as a result of LKT stimulation when compared to their constitutive expression prior to LKT stimulation (Table 1).

Exposure of BL3 cells (2 X 10^6cells/ml) to LKT (1:10 dilution, 347 TU) for 1 min at 37°C resulted in a loss of normal membrane morphology for 18.9 ± 0.5% (n=15) of the membrane's circumference. Loss of membrane integrity was typically restricted to one area of the cell and was marked by loss of intact plasma membrane. Fixation in 4% paraformaldehyde and 0.2% gluteraldehyde in 0.05% cacodylate buffer prior to LKT exposure inhibited LKT-induced plasma membrane damage (Figure 1).

The percent of total gold label specific for anti-LKT that localized to degenerative areas of BL3 cell plasma membranes was statistically greater ($p < 0.05$) than gold label specific for anti-LKT associated with intact areas (Figure 2). Pre-fixed BL3 cells exposed to LKT exhibited almost undetectable amounts of gold label specific for anti-LKT (data not shown). However, when un-fixed BL3 cells were exposed to LKT, immunoEM revealed that gold label specific for anti-LKT was associated with degenerative areas of BL3 cell plasma membranes (Figure 3), no gold label specific for anti-LKT was associated with intact areas (Figure 3). Use of pro LKT resulted in no

LKT-induced cytolysis and gold labeling was not seen with either intact or degenerative regions of BL3 cell plasma membranes when the primary antibody was omitted (data not shown).

Prior to exposure of BL3 cells to LKT, immunoEM revealed that gold label specific for anti CD11a and anti-CD18 surround the entire circumference of BL3 cell plasma membranes (Figures 4 and 5). Following exposure of BL3 cells to LKT, the number of gold particles specific anti-CD11a were significantly increased and the percent of label on degenerative areas was increased compared to the percent of total label associated with intact areas of BL3 cell plasma membranes ($p < 0.05$) (Figure 2). After addition of LKT for 1 minute, ImmunoEM revealed gold label specific for anti-CD11a was associated with only degenerative areas of BL3 cell plasma membranes (Figure 6). Gold particles (15nm) specific for anti-CD11a were not seen in association with intact areas (Figure 6). In contrast, LKT exposed BL3 cells labeled with gold specific for anti-CD18 did not differ statistically in the percent of total gold label associated with degenerative areas of BL3 cell plasma membranes when compared with intact areas ($p > 0.05$) (Figure 2). After addition of LKT for 1 minute ImmunoEM revealed that gold label specific for anti-CD18 was found associated with both degenerative and intact areas of BL3 cell plasma membranes (Figure 7) with $47.5 \pm 7.0\%$ of gold label concentrated in degenerative areas.

Redistribution of gold label specific for anti-CD11a and anti-CD18 from their native distribution (around the entire circumference of BL3 cell plasma membranes) to their distribution after LKT exposure (degenerative areas of BL3 cell plasma membranes) was characterized by the decreased amounts of gold particles seen in association with intact areas of BL3 cell plasma membranes after LKT exposure. The amount of gold label surrounding BL3 cell plasma membranes decreased significantly ($p < 0.05$) after LKT exposure (Figure 8).

After LKT exposure CD18 is localized on both intact and degenerative areas of BL3 cell plasma membranes. The amount of gold label specific for anti-CD18 associated with degenerative areas was comparable to that seen associated with degenerative areas of BL3 cells exposed to LKT and labeled with anti-CD11a. There was not a statistical difference in the amount of gold label associated with degenerative areas of BL3 cells labeled with either anti-CD18 or anti- CD11a ($p > 0.05$) (Figure 9).

Table 1. β_2-Integrin expression on LKT exposed and unexposed BL3 cells.*

	β_2-Integrin Expression	
β_2-Integrin	– LKT	+ LKT
CD11a	8.6%	95.1%
CD11b	7.1%	67.7%
CD11c	53.3%	93.1%
CD18	80.1%	98.7%

* BL3 cells (2 x 10^6 cells/ml) were exposed to LKT for 1 minute at 37°C, rinsed, and fixed. Fixation was followed by incubation with antibody specific for CD11a, b, c, or CD18 for 30 minutes at 0°C and rinsed. Cells were incubated with Alexa-Fluor 488 goat anti-mouse IgG polyclonal antibody (A-11001) for 30 minutes at 0°C, rinsed, and analyzed on FACSCalibur (10,000 events/sample). Values represent percent fluorescent intensity before (-LKT) and after (+LKT) exposure.

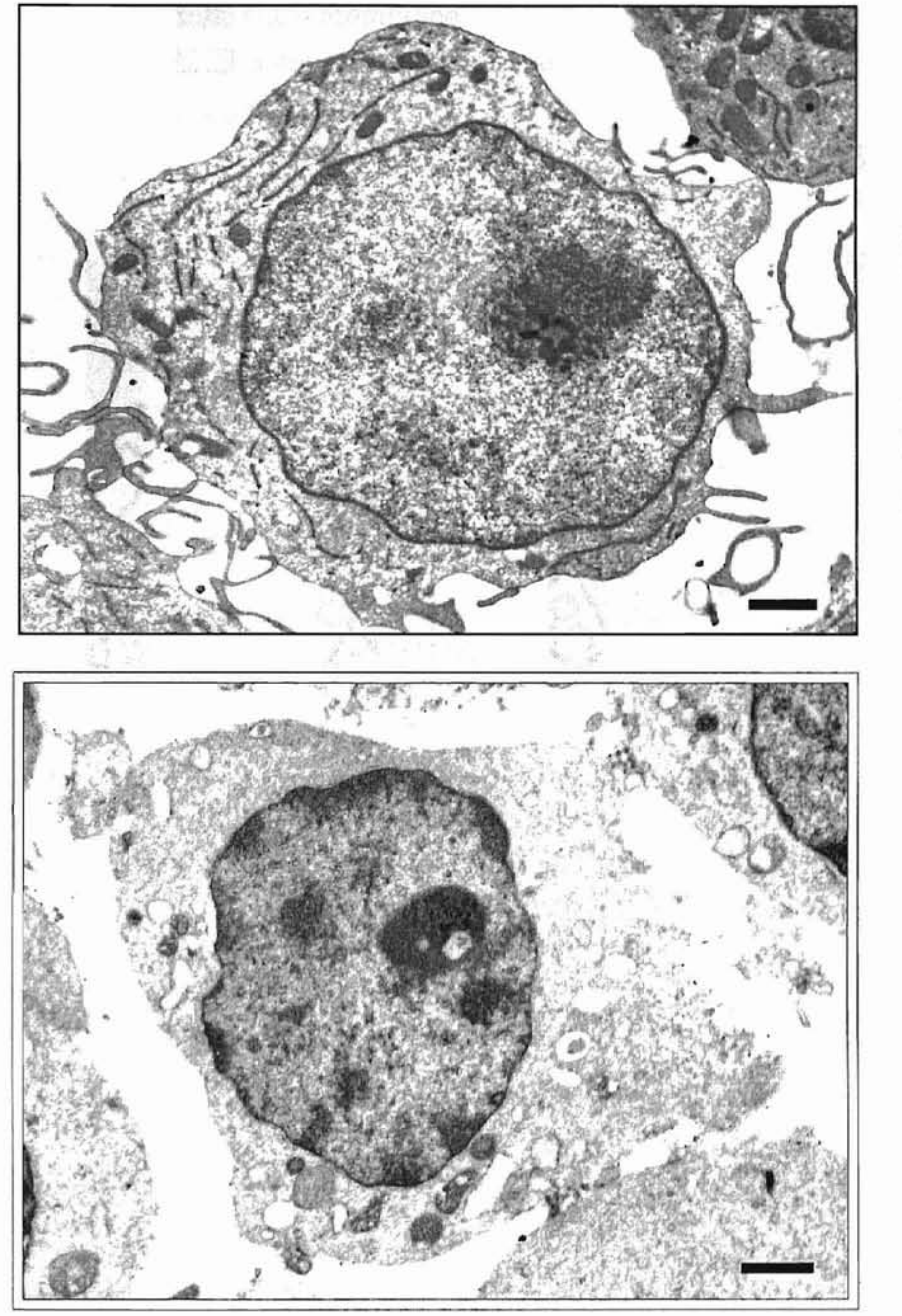

Figure 1. Electron micrographs of BL3 cells exposed to LKT for 1 minute at 37°C. Fixation in 4% paraformaldehyde and 0.2% gluteraldehyde in 0.05% cacodylate buffer inhibited LKT induced plasma membrane damage (top; 9,000x), while no fixation resulted in an 18.89 ± 0.5% loss of normal plasma membrane morphology (n=15) (bottom; 10,285x). Bar = 1μm.

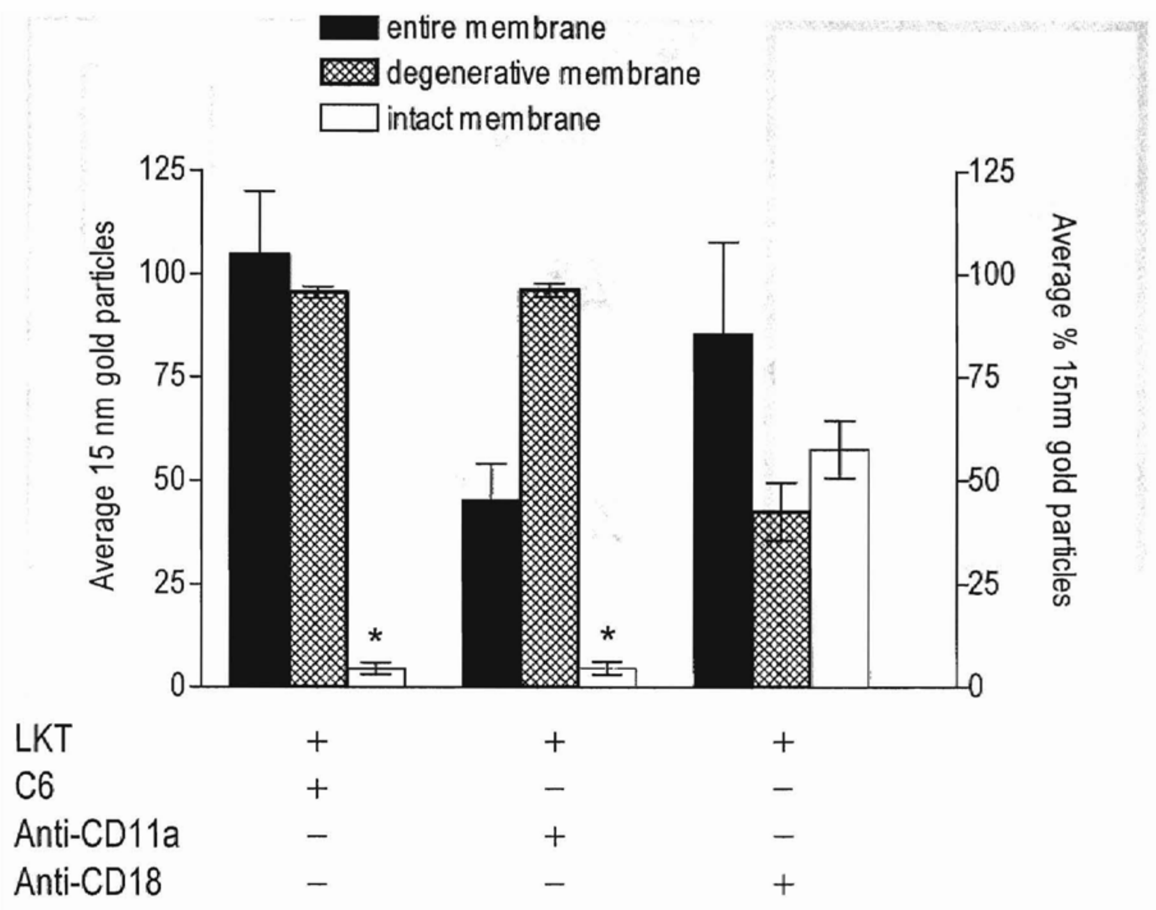

Figure 2. Distribution of LKT, CD11a, and CD18 on BL3 cells after 1 minute LKT exposure. The percent of total gold label (15nm) that localized to degenerative regions after LKT exposure was significantly higher than that found on intact membrane for both LKT and CD11a. There was not a significant difference in the percent of gold label specific for anti-CD18 that localized to either degenerative or intact areas of BL3 cells ($p > 0.05$). Left y-axis is average number of gold particles per cell (black; n=15). Right y-axis is percent gold label detected in association with either degenerative (shaded) or intact (white) membrane. Asterisks represent values significantly different from the percent of total gold label seen in association with intact plasma membrane before LKT exposure ($p < 0.05$).

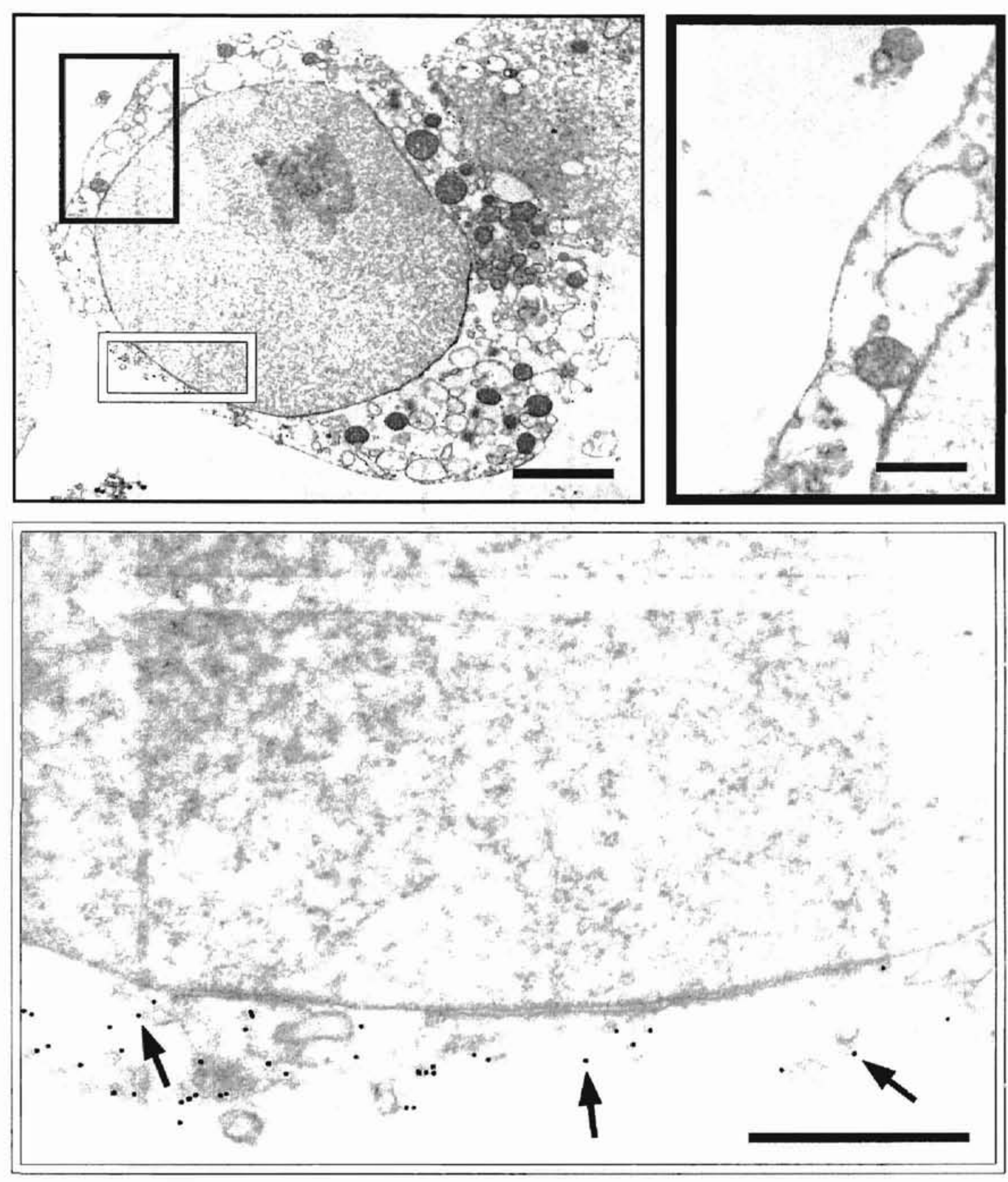

Figure 3. Immunogold localization of LKT on BL3 cells (top left; 7,613x, bar = 2μm). Gold particles (15nm) specific for anti-LKT localized to degenerative regions of BL3 cell plasma membranes (white frame; 65,250x, bar = 0.5μm). Gold label were not detected on intact areas of BL3 cell plasma membranes (black frame; 27,066x, bar = 0.5μm).

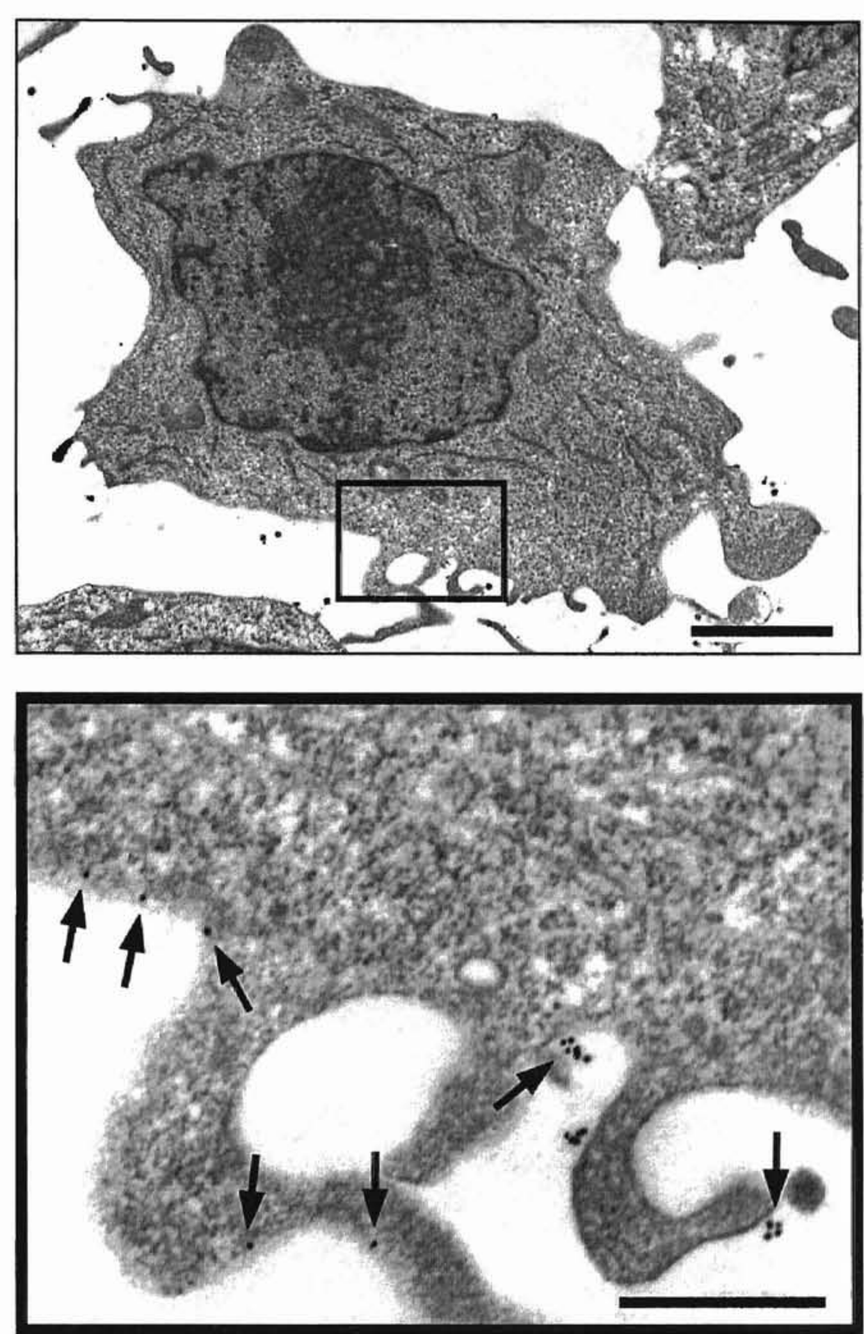

Figure 4. Immunogold localization of CD11a on pre-fixed BL3 cells (top; 9,318x, bar = 2μm). Gold particles (15nm) specific for anti-CD11a were dispersed diffusely around entire BL3 cell plasma membranes (black frame; 54,000x, bar = 0.5μm).

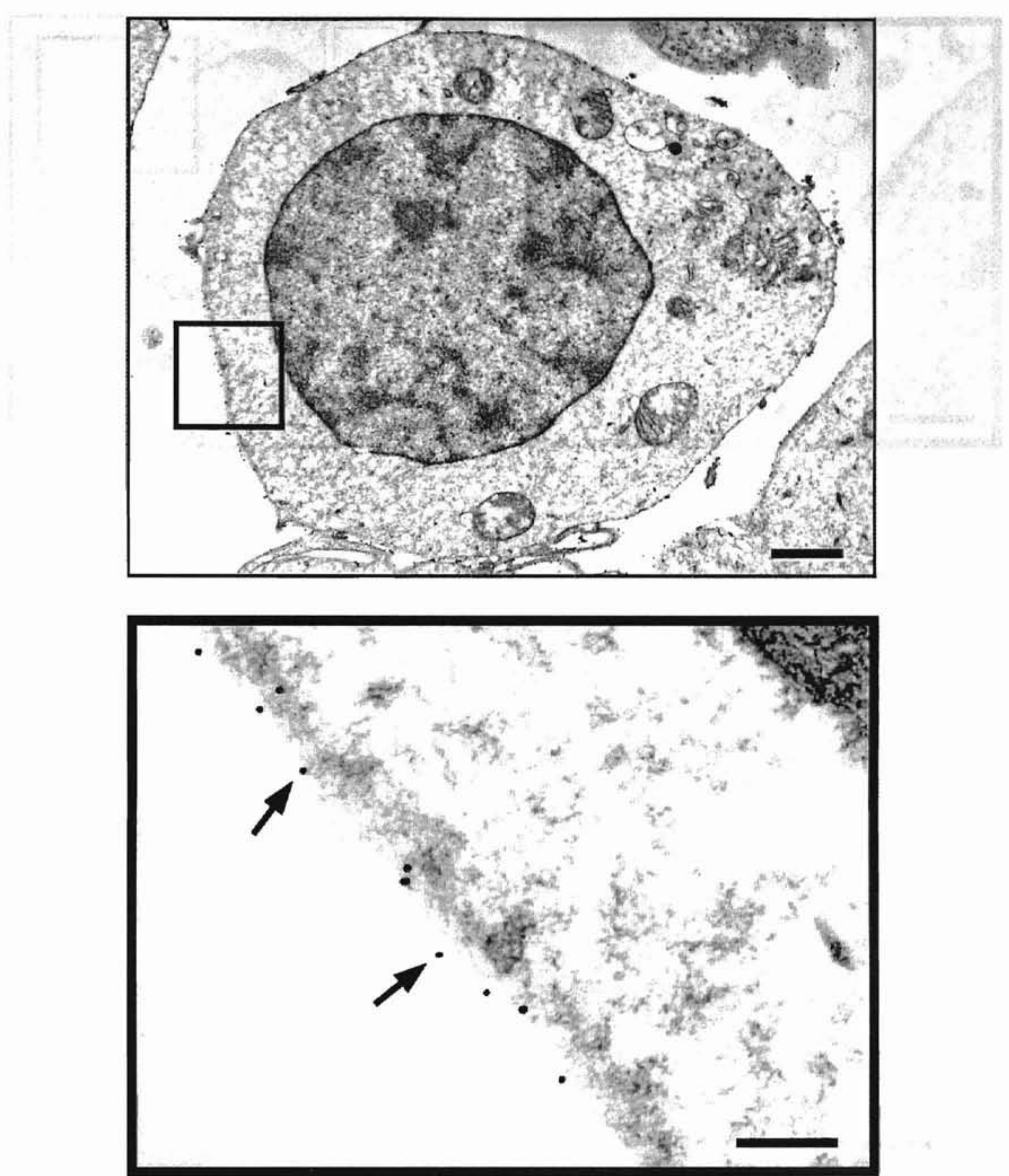

Figure 5. Immunogold localization of CD18 on pre-fixed BL3 cells (top; 10,000x, bar = 1μm). Gold particles (15nm) specific for anti–CD18 were dispersed diffusely around entire BL3 cell plasma membranes (black frame; 75,600, bar = 0.2μm).

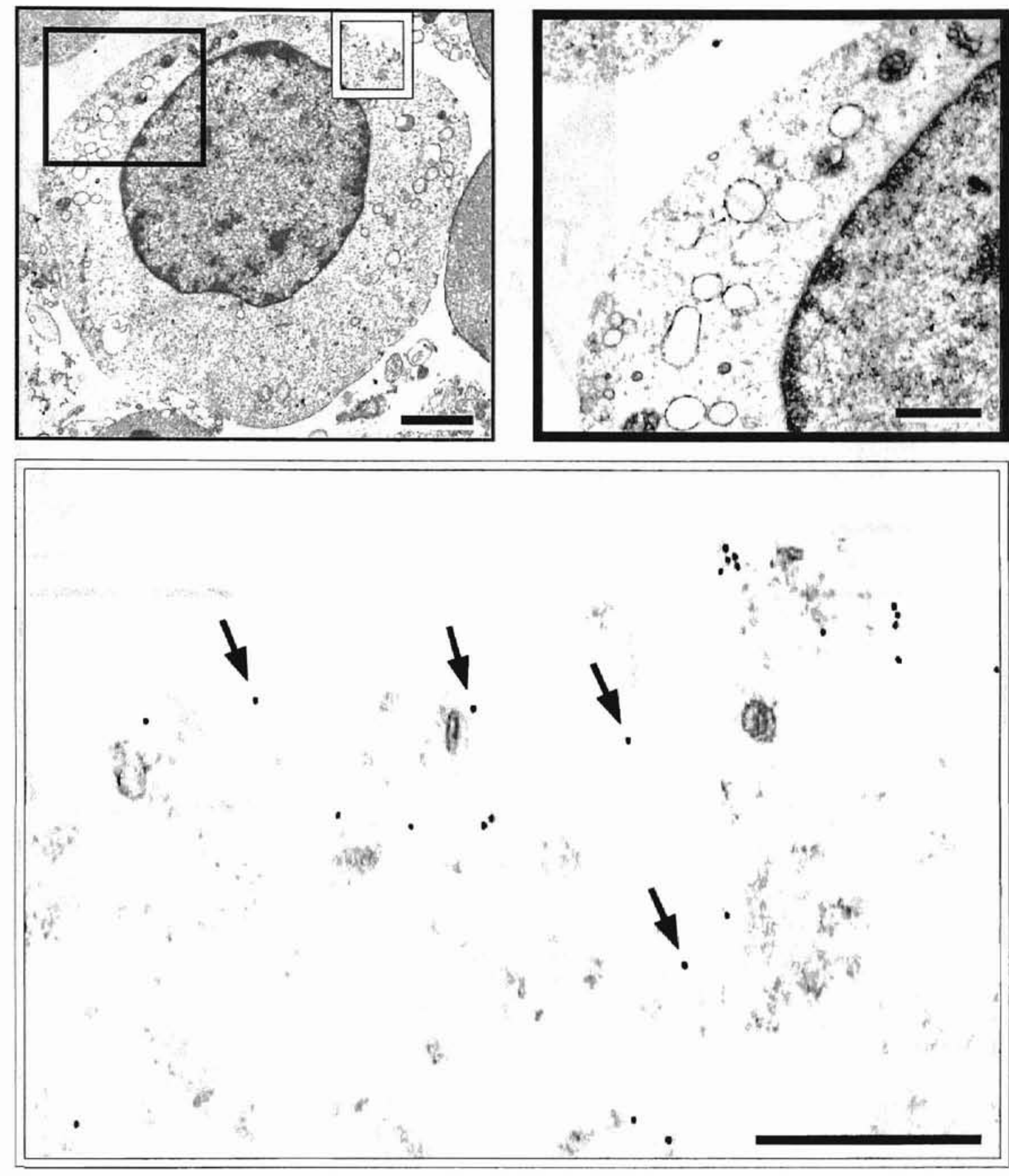

Figure 6. Immunogold localization of CD11a on BL3 cells exposed to LKT for 1 minute at 37°C (top left; 5.324x, bar = 2μm). After LKT exposure, 15nm gold particles specific for anti-CD11a localized to degenerative regions of BL3 cell plasma membranes (white frame; 66,700x, bar = 0.5μm). After LKT exposure gold label was no longer seen in association with intact areas of BL3 cell plasma membranes (black frame; 12,938x, bar = 1μm).

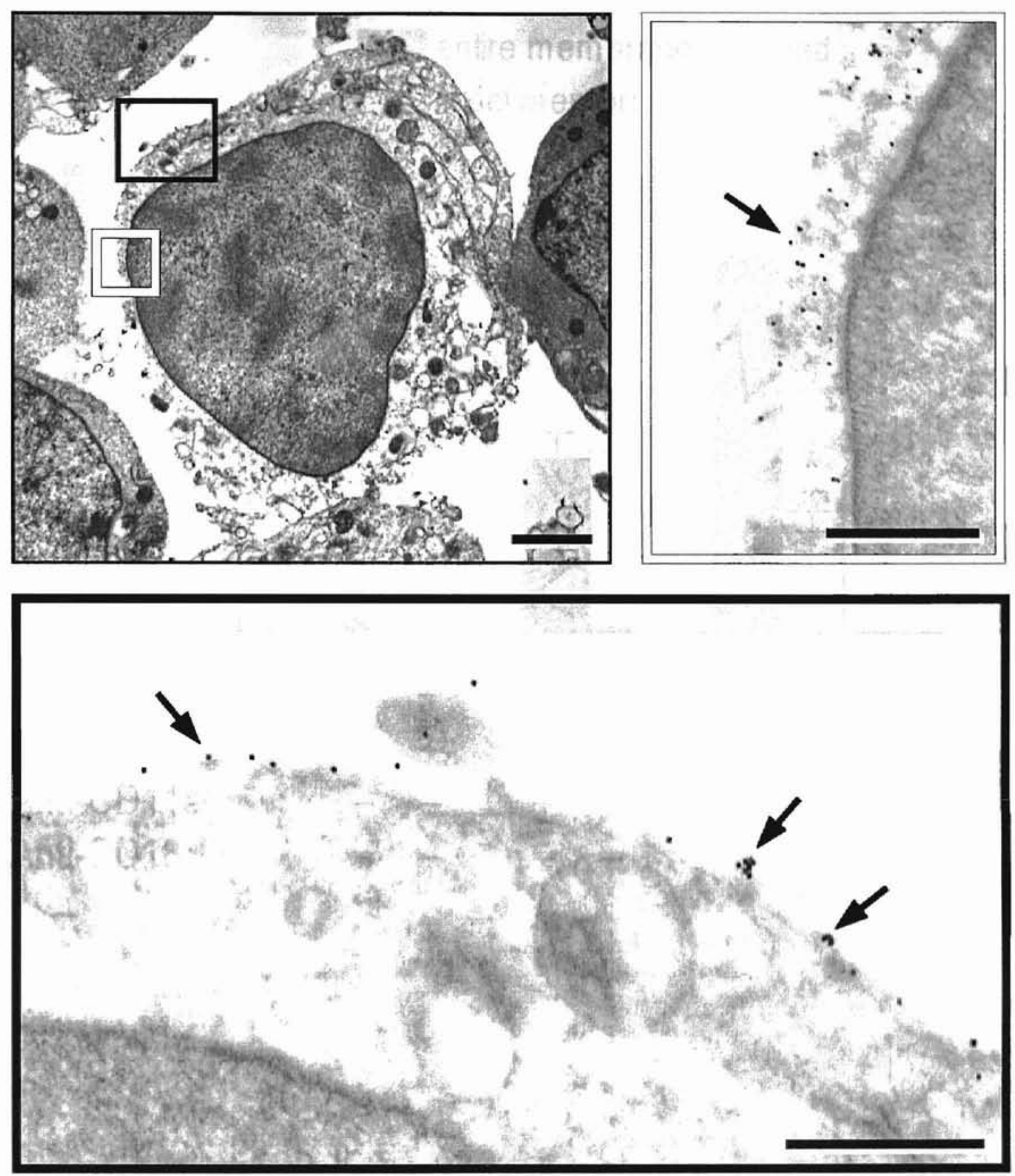

Figure 7. Immunogold localization of CD18 on BL3 cells exposed to LKT for 1 minute at 37°C (top left; 6,023x, bar = 2μm). After LKT exposure 15nm gold particles specific for anti-CD18 localized to both degenerative (white frame; 45,500x, bar = 0.5μm) and intact (black frame; 58,800x, bar = 0.5μm) regions of BL3 cell plasma membranes.

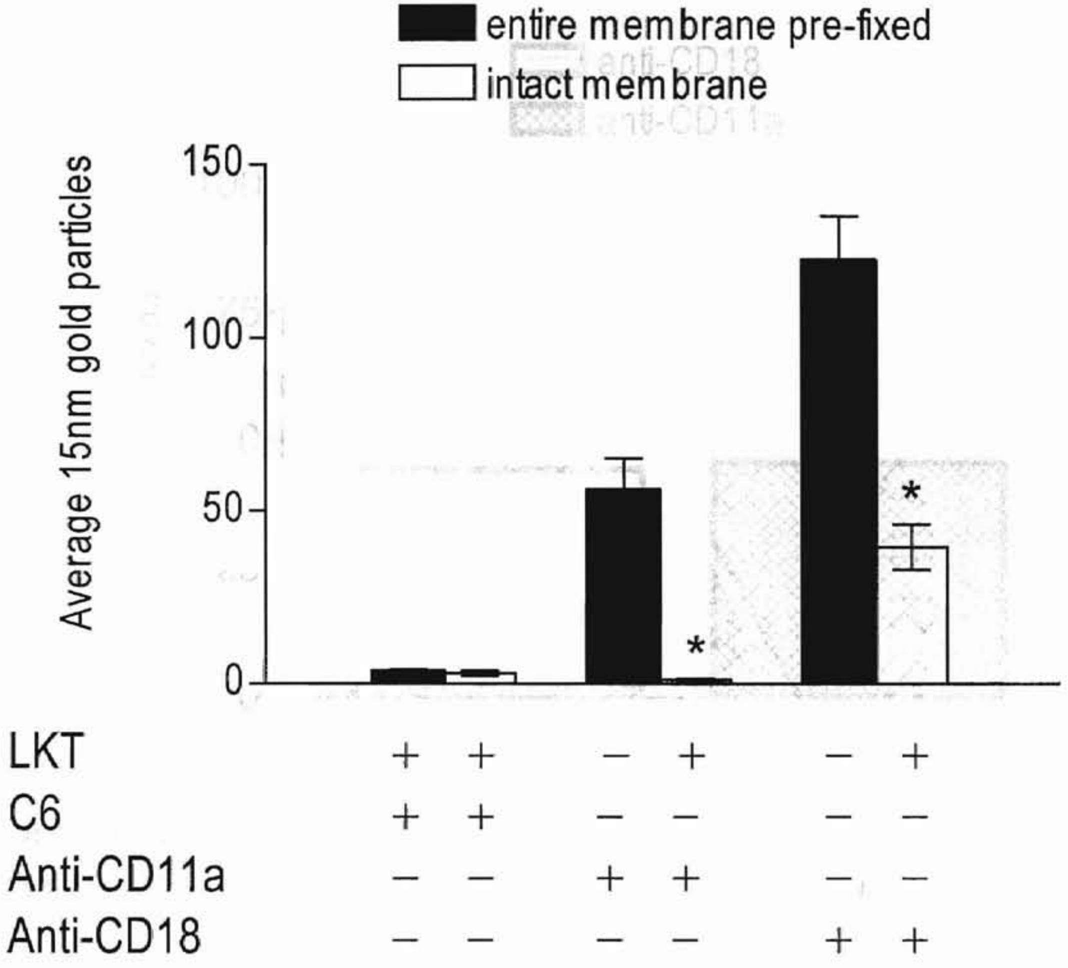

Figure 8. Localization of LKT, CD11a, and CD18 on intact BL3 cell membranes after LKT exposure. The was not a statistical difference in the amounts of gold label specific for anti-LKT in association with intact BL3 cell plasma membranes either before or after LKT exposure ($p > 0.05$). The amount of gold label specific for both anti-CD11a and anti-CD18, in association with intact membrane, decreased as a result of LKT exposure. Black bar represents amount of label on pre-fixed cells; for CD11a and CD18 prior to LKT exposure (n=15). White bar represents the amount of label present on intact membrane after LKT exposure (n=15). Asterisks represent values statistically different from the LKT un-exposed group ($p < 0.05$).

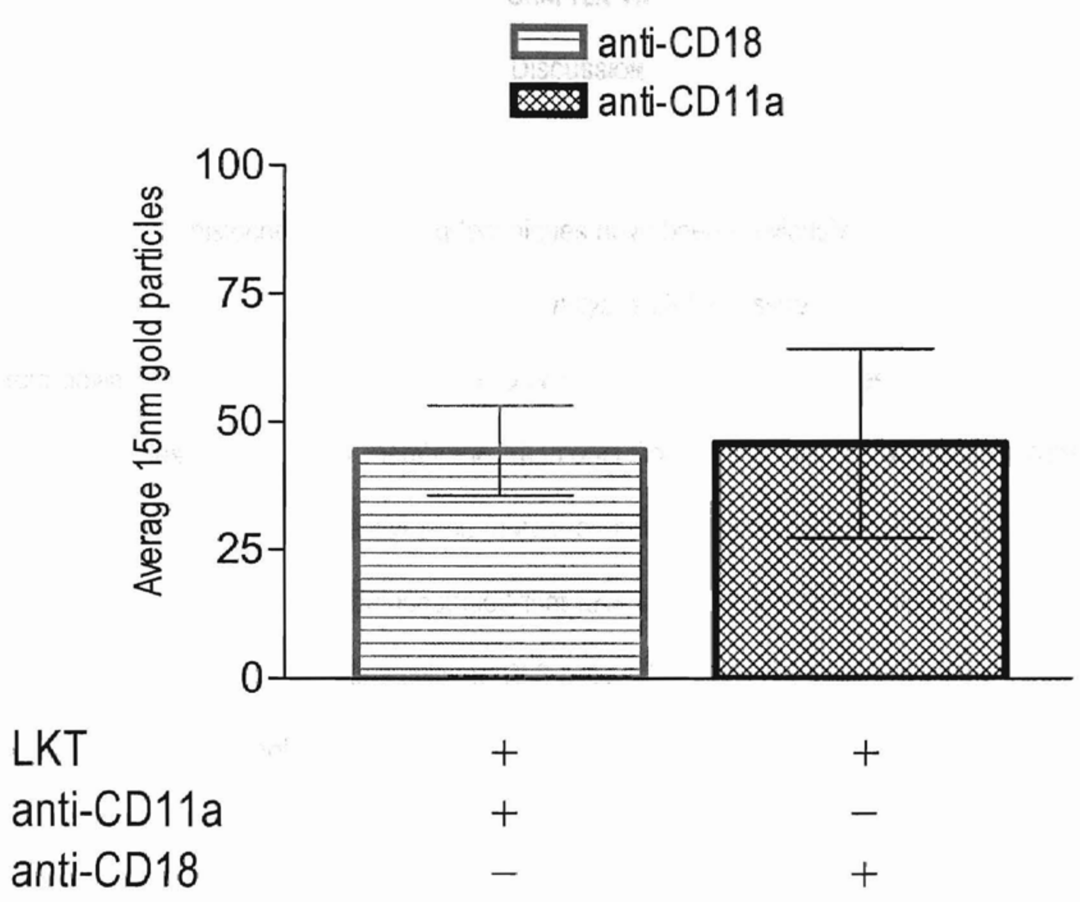

Figure 9. Amounts of CD11a and CD18 localized to degenerative areas of BL3 cell plasma membranes after 1 minute LKT exposure. The amounts of gold label specific for either anti-CD11a or anti-CD18 that localized to degenerative areas of BL3 cell plasma membranes after 1 min LKT exposure were not significantly different ($p > 0.05$; n=15).

CHAPTER VII

DISCUSSION

Immunohistochemical labeling techniques have been previously employed at the light and electron microscopic levels to localize *M. haemolytica* LKT in tissue sections of pneumonic lungs from bovine inoculated with *M. haemolytica* (Whiteley et. al., 1990). These findings suggest that LKT associates primarily with membrane fragments from degenerating inflammatory cells in the alveolus, with no immunoreactivity on areas of lung sections with intact inflammatory cells (Whiteley et. al., 1990). We demonstrated that LKT localized to precise degenerative regions of plasma membranes on individual cultured BL3 cells. Our study differed from that of Whiteley and colleagues in that pathology samples from cattle experimentally infected with *M. haemolytica* were used, whereas our study used isolated cells exposed to LKT. Their method was less definitive in several ways. LPS, a major component of *M. haemolytica* outer membranes, played a major role in damage of lung sections during their studies. They were unable to provide estimations of LKT exposure time because exposure time estimations during experimental infections are less efficient than when using CCS LKT, from which reactions can be initiated and terminated more readily. Finally, they used whole lung sections, which provided a less accurate estimation of exact LKT localization on individual inflammatory cells. In this study, LKT binding was examined at more stringent ultrastructural levels and precise regions of LKT localization on BL3 cells was determined at a specific time point during exposure.

Gold label specific for anti-LKT was readily detected after exposure of BL3 cells to LKT. However, when BL3 cells were pre-fixed very little gold label was detected. Plausible explanations for the extremely low amounts of 15nm gold particles specific for anti-LKT after 1 min LKT

exposure on pre-fixed BL3 cells may be the result of fixation in 4% paraformaldehyde and 0.2% gluteraldehyde in 0.05% cacodylate buffer. Fixation cross-links proteins to fixed cytoskeletal and membranous constituents throughout the cell (Bozzola and Russell, 1992). These constituents of the cell are unified into a single interlocking structure or meshwork held together by a multitude of fixative molecules. Cross-linkage of the β_2-integrins may have masked the epitopes responsible for interaction of LKT with its target cell receptor. Fixation also compromises antigenicity. The type of fixative used, and its concentration determine the extent of antigenic reduction. Gluteraldehyde has the ability to denature proteins and low concentrations of gluteraldehyde have less denaturing effects than higher concentrations. Although fixation of BL3 cells was mild and followed by adequate rinsing, fixative residue may have slightly denatured LKT decreasing its affinity by some degree, thus LKT would not be able to recognize its receptor on pre-fixed BL3 cells.

The α-chain of the β_2-integrin heterodimer, CD11a, was dispersed diffusely throughout BL3 cell membranes prior to LKT exposure. After 1 minute LKT exposure CD11a redistributed to areas of degeneration. CD18 was also dispersed evenly throughout BL3 cell plasma membranes prior to LKT exposure, however, only a portion of CD18 was located in degenerative areas of the membrane after LKT exposure. CD18 is associated with one of three alpha chains, CD11a, b, or c, with that of CD11a expression being the highest of the total β_2-integrin expression on BL3 cells after 1 min LKT exposure. When compared with gold label specific for anti CD18 that localized in degenerative areas of BL3 membranes, the amounts of 15nm gold particles specific for anti-CD11a were not statistically different (Figure 9). Thus, the amount of CD18 represented in areas of degeneration may be that of the CD11a/CD18 heterodimer, further supporting the role of redistribution of LFA-1 after 1 min LKT exposure. We only examined localization of CD11a after

LKT exposure. No experiments were done with either CD11b or CD11c. Whether these subunits redistribute is unknown.

Flow cytometry showed an increase in β_2-integrin expression after BL3 cells were exposed to LKT for 1 minute at 37°C, however, immunoEM revealed a slight decrease in gold label after LKT exposure. Gold labeling is not as efficient as fluorescent labeling, and has the potential to be lost during tissue processing for electron microscopy. Vigorous rinsing steps, sectioning, and grid staining all contribute to the loss of 15nm gold particles during the tissue processing steps. These complications decrease the amounts of gold label that are detected after LKT exposure.

Leukotoxin-induced plasma membrane defects were visible after BL3 cells were exposed to LKT for 1 minute at 37°C. This contrasts with earlier reports that variable-sized membrane defects are not evident until after 20 minutes of exposure to LKT (Clinkenbeard et al., 1989c). Previous reports have shown that LKT stimulates the production of leukotriene B_4 (LTB_4), which involves the subsequent increase of phospholipase A_2 (PLA_2) activity (Wang et al., 1999). Phospholipase A_2 damages plasma membranes by hydrolyzing phospholipids. This leads to the elaboration of lysophospholipids, which have detergent like effects on membranes (Wang et al., 1999). Although LKT-induced cytolysis is inhibited by fixation, no reports show that LKT-induced PLA_2 activity is inhibited by fixation. Thus, the plasma membrane defects that are present after 1 minute LKT exposure may be the result of LKT-induced PLA_2 activity, which continues to hydrolyze membrane phospholipids even after termination of the exposure. In addition, factors may be differences in exposure temperatures, in LKT concentrations, or inadequate reaction termination during experimentation. Increased temperatures may potentiate the lytic effects of LKT on BL3 cells, and likewise increased concentrations of LKT may cause plasma membrane damage to become evident at earlier time periods during LKT exposure. Consideration should also be given

to the centrifugation and washing steps, upon which LKT may have continued to react with BL3 cells. BL3 cells were exposed to LKT for 1 minute, immediately spun down, washed, and fixed. What may have been theoretically a 1 minute exposure could have resulted in a 10-15 minute exposure.

Jeyaseelan and colleagues (2001) found that LKT induced a species-specific nonreceptor tyrosine kinase signaling cascade resulting in tyrosine phosphorylation of the CD18 (β-chain) tail of LFA-1. Their results support our supposition that LKT causes rapid redistribution of LFA-1. Engagement of β_2-Integrins induces activation of tyrosine kinases that lead to phosphorylation of proteins involved with changes in the actin cytoskeleton resulting in cytoskeletal rearrangements that control cell motility and activation (Dib and Anderson, 2000). Since the β-chain of the β_2-Integrins is linked to the cytoskeleton, rearrangement of cytoskeletal components also results in redistribution of the β_2-Integrins. Thus, LKT induced tyrosine phosphorylation of the CD18 tail of LFA-1 is a contributing factor in LFA-1 redistribution after LKT exposure.

Our findings indicate that binding of LKT to BL3 cells results in rapid redistribution and increased expression of the β_2-integrins. ImmunoEM demonstrated that LKT exposed BL3 cells caused rapid redistribution of CD11a from a diffuse pattern on BL3 cells to only areas of degeneration along with LKT. In lieu of these similarities in localization, there arises speculation of some involvement of receptor redistribution in aggregation of LKT to specific sites on BL3 cell membranes were pore formation might be initiated. Amplification of the β_2-integrins by recombinant bovine interleukin-1β was shown to enhance binding and amplify the biological effects of LKT (Leite et al., 2000). The action of LKT on BL3 cells caused increased β_2-integrin expressions (Table 1). Thus, LKT may act in an autocrine manner; increasing β_2-integrin expression and redistribution to enhance LKT binding and induced cytolysis.

CHAPTER VIII

LITERATURE SITED

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VITA

Bryan Keith Taylor

Candidate for the Degree of

Master of Science

Thesis: *MANNHEIMIA HAEMOLYTICA* LEUKOTOXIN INDUCES RAPID REDISTRIBUTION OF LFA-1 ON BOVINE LYMPHOMA CELLS.

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Data: Born in Tulsa Oklahoma, on August 18, 1974, the son of Lennice Marie Taylor. Married with two children.

Education: Graduated from Booker T. Washington High School in Tulsa, Oklahoma in May, 1992. Attended Langston University in Langston, Oklahoma and received a Bachelor of Science in Biology in May, 1997. Completed the requirements for the degree of Master of Science at Oklahoma State University in Stillwater, Oklahoma in December, 2001.

Professional Experience: Employed by the Department of Pathobiology in the College of Veterinary Medicine at Oklahoma State University as a Research Associate from May 1997 to December 2001.

Professional Memberships: American Society for Cell Biology (ASCB), American Society for Microbiology (ASM), Conference of Research Workers in Animal Diseases (CRWAD), Oklahoma Microscopy Society (OMS), and National Institutes of Science (NIS).