MOLECULAR INTERACTION OF *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN WITH BOVINE LYMPHOCYTES

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What you decide on will be done,
and light will shine on your ways.
—*Job* 22:28

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LIST OF ABBREVIATIONS

AC-Hly	adenylate cyclase-hemolysin
AIDS	acquired immune deficiency syndrome
BCA assay	bicinchroninic acid assay
BL3 cells	bovine lymphoma cell line
BHI	brain-heart infusion
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
CED	cell death defective
CS	culture supernatant
DMSO	dimethylsulfoxide
DR	death receptor
DT	diphtheria toxin
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FHA	filamentous hemagglutinin
HBSS	Hank's balanced salt solution
HL60 cells	human promyelocytic leukaemia cell line
ICE	interleukin - 1β converting enzyme

LFA-1	lymphocyte function-associated antigen 1
LDH	lactate dehydrogenase
LKT	leukotoxin from *Pasteurella haemolytica*
LPS	lipopolysaccharide
Ltx	leukotoxin from *Actinobacillus actinomycetemcomitans*
MAb	monoclonal antibody
MAC	membrane attack complex
MOPS	3-(N-morpholino)propanesulfonate
OD	optical density
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PBST	phosphate buffered saline-0.05% polyoxyethylene-20-sorbitan monolaurate
PHA	phytohemagglutinin
PTX	pertussis toxin
Raji cells	human lymphoma cell line
RTX	repeats-in-toxin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TIF	tagged image file
TU	toxic unit
Stx	Shiga toxin

TNF	tissue necrosis factor
VT	verocytotoxin;
YT	yeast-tryptone

CHAPTER I

INTRODUCTION

Bovine pneumonic pasteurellosis, commonly termed shipping fever, is a severe respiratory disease of feedlot and stocker cattle that is responsible for considerable economic losses to the cattle industry in North America (Weekly, 1998). Although bovine pneumonic pasteurellosis is a multi-factorial disease, *Pasteurella haemolytica* biotype A serotype 1 is the most frequently isolated microorganism in pure culture from pneumonic lungs (Shewen, 1986). *P. haemolytica*, a Gram-negative bacterium, produces several factors including leukotoxin (LKT), endotoxin, capsular polysaccharide, protease, neuraminidase, outer membrane proteins, and fimbriae (Confer, et al, 1988), which enable pathogenic bacteria to colonize the host, multiply and persist in the respiratory tract of cattle. LKT, a member of the repeats-in-toxin (RTX) family of Gram-negative bacterial pore-forming toxins, is produced during logarithmic-growth phase of *P. haemolytica* and is considered to be the primary virulence factor in the pathogenesis of bovine pneumonic pasteurellosis (Welch, 1991; Shewen, 1986; Tatum, et al., 1998). High concentrations of LKT are responsible for the most severe lung lesions during pneumonic pasteurellosis. However, the functions of low concentrations of LKT related to the pathogenesis of this disease are not well defined.

Apoptosis is a particular mechanism of cell death, which is morphologically and biochemically distinct from oncotic cell lysis (Wyllie, et al., 1980). Apoptosis is a fundamental biological process that impacts on the early development, maturation, and homeostasis of multicellular organisms, but apoptosis is also involved in acquisition of diseases (Wyllie, 1980). *In vitro* studies indicated that various cellular insults, including bacterial toxins, that are cytolytic at high concentrations may induce apoptosis at lower concentrations (Lieberthal and Levine, 1996). Recently, it has been reported that several

RTX toxins that cause oncotic cell lysis at high concentrations are capable of inducing apoptosis in their target cells when the toxin concentrations are decreased. Stevens and Czuprynski (1996) reported that low concentrations of *P. haemolytica* LKT-induced morphologic changes in bovine leukocytes viewed at the light microscopic level are consistent with apoptosis *in vitro*. However, DNA fragmentation, a hallmark of apoptosis, was not observed. Furthermore, the low resolution associated with light microscopy made it difficult to interpret the morphologic changes. During initial infection, a small number of *P. haemolytica* are present in the lower airway of cattle; therefore, the concentration of LKT is presumably low. Whether this low concentration of LKT is involved in induction of apoptosis in bovine leukocytes to promote the bacterial colonization has not been investigated.

The overall goals of this research were to 1) optimize culture conditions for *P. haemolytica* LKT production; 2) examine the initial interaction of LKT with lymphoid target cells; and 3) investigate whether *P. haemolytica* LKT can induce morphological and biochemical changes of apoptosis in isolated bovine lymphocytes. Elucidation of the initial interaction of LKT with its target cells and the consequence of this interaction may provide essential information for better understanding the pathogenesis of bovine pneumonic pasteurellosis and for development of comprehensive preventive approaches and formulation of novel therapeutic strategies for this disease.

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CHAPTER II

LITERATURE REVIEW

1. Overview of *Pasteurella haemolytica*, leukotoxin (LKT) and bovine pneumonic pasteurellosis

1.1. General features of *P. haemolytica*

Pasteurella haemolytica, the causative agent of bovine pneumonic pasteurellosis, is classified in the family *Pasteurellaceae*, which comprises three genera: *Pasteurella*, *Actinobacillus*, and *Haemophilus* (Biberstein, 1990). Species of *Pasteurella* are small Gram-negative rods or coccobacilli, which are generally facultative anaerobic, non-motile, oxidase-positive, and non-spore-forming. Almost all species of *Pasteurella* reduce nitrates and utilize carbohydrates fermentatively (Carter, et al., 1995). *P. haemolytica* is an important veterinary microorganism both as a primary and opportunistic pathogen of cattle and sheep. *P. haemolytica* may occur as a commensal in the upper respiratory and digestive tracts and is confined almost exclusively to ruminants, with most adequately characterized strains originating from cattle, sheep, and goats (Biberstein, 1981). A distinct variety of *P. haemolytica* can cause infrequent low-grade infections in poultry (Timoney et al. 1988) and swine. However, the strains of *P. haemolytica* isolated from poultry, or swine differ in certain biochemical characteristics from the conventional ruminant strains, and in addition, the former strains can not be serotyped. The taxonomic position of these "atypical" strains is not clear. Frequently, they are referred to as *P. haemolytica*-like organisms (Carter, et al. 1995).

P. haemolytica was first recognized during studies of bovine hemorrhagic septicemia and named *Bacillus bovisepticus* (Jones, 1921). Because the hemolytic colonies were readily distinguishable from those of *P. multocida*, the newly recognized organism was renamed *P. haemolytica* by Newsom and Cross in 1932, and this has been

widely accepted (Carter, 1968). Two different biotypes (biovars) of *P. haemolytica*, designated A and T, have been recognized based upon the fermentative activity with arabinose or trehalose, respectively (Table 1). Biotype A strains produce acid from arabinose or xylose, but no acid from trehalose or salicin. In contrast, biotype T strains produce acid from trehalose or salicin, but not from arabinose or xylose. These biotypes are each further divided into serotypes. According to the bacterial capsular and somatic antigens, *P. haemolytica* can be divided into at least 16 serotypes, determined by a passive hemagglutination or by a rapid plate agglutination test. Serotyping can be done only on the strains that are encapsulated (Biberstein et al., 1960). Biotype A comprises serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14 and 16. Serotypes 3, 4, 10, and 15 are found only among biotype T strains. It has been proposed that *P. haemolytica*, biotype T could be reclassified as *P. trehalosi* (Mutters, et al., 1985), but this proposal has not been widely accepted (Carter, et al., 1995). The classification for biotypes has biologic significance since these two biotypes differ with regard of reservoir, pathogenicity, antigenic nature, biochemical activity, antimicrobial susceptibility, cultural and serologic traits, and genetic relatedness (Table 1). The distribution of serotypes and biotypes by host species, disease produced, and frequency of occurrence of the serotypes and biotypes within a given host species is presented in Table 2. The most frequent isolate from bovine pneumonic pasteurellosis in pure culture is *P. haemolytica* biotype A serotype 1 (Shewen, 1986).

1.2. Bovine pneumonic pasteurellosis and its etiologic agents

Bovine pneumonic pasteurellosis has been termed as 'shipping fever', 'transit fever', 'stockyard's pneumonia', 'exposure disease', and 'transport fever' (for more

information, see review: Rehmtulla and Thomson, 1981). The various terms used to denote pneumonic pasteurellosis may have delayed a more comprehensive understanding of this disease, and the term pneumonic pasteurellosis did not attained wide recognition until 1968 (Carter, 1968). Although many believe pasteurellosis to be a secondary complication of virus infection, neither has this relationship been demonstrated consistently in field studies, nor has vaccination of cattle against common respiratory viruses had a significant impact on the incidence of pneumonic pasteurellosis (Shewen, 1986). Experimental infection of cattle with *P. haemolytica* biotype A serotype 1 has successfully reproduced the fibrinous bronchopneumonia and pleuritis typical of pneumonic pasteurellosis (Panciera, et al., 1984; Shewen, 1986). This disease is predominantly developed in weaned and yearling cattle that have been recently transported. Outbreak of the disease usually occurs within two weeks after cattle arrive in the feedlot and commonly lasts for about 2 - 4 weeks thereafter (Yates, 1982; Ribble, et al., 1995a, b, c). The clinical signs of bovine pneumonic pasteurellosis may vary from inapparent to severe signs, but the cardinal signs include depression, anorexia, dyspnea, fever, nasal discharge, coughing, and abnormal respiratory sounds (Ribble, et al., 1995a). The gross lesions of bovine pneumonic pasteurellosis are also varied, dependent on the duration of the disease, but usually characterized by severe, fibrinous pleuropneumonia (Ose, 1973; Whiteley, et al. 1990). The histological lesions in the lungs of cattle with pneumonic pasteurellosis are characteristically those of acute fibrinous pneumonia and pleuritis. Depending on the stage when examined, features commonly observed are predominantly fibrinocellular exudate comprising polymorphonuclear and mononuclear

cells, edema, and bronchiolitis. In terminal stages, multifocal areas of coagulation necrosis are present in the parenchyma of affected lobes (Rehmtulla and Thomson, 1981).

It is difficult to accurately estimate the economic impact of bovine pneumonic pasteurellosis, excluding the cost of treatment or preventive measures. It has been estimated that the annual losses attributed to this disease in the United States were 25 million dollars in 1954 (Shaw, 1954) and approximately 800 million dollars in 1980's (Drummond et al., 1981; Weekly et al., 1998).

The studies on bovine pneumonic pasteurellosis indicate that in addition to *P. haemolytica*, many other etiologic agents, including bacterial, mycoplasmal (Schiefer, et al., 1978), and viral (Jericho and Langford, 1978; Jericho and Darcel, 1978) infections also contribute to the pathology of this disease (Table 3). Inadequate management including long distance of shipment, over-crowding, dehydration, insufficient ventilation, and other stress conditions all decrease host defenses (Frank, et al., 1986). It is speculated that more submucosal receptors for *P. haemolytica* colonization are exposed after the development of extensive lesions of mycoplasmal and viral infections. Marked increases in adrenocortical hormone levels during stressful conditions have anti-inflammatory and immunosuppressive effects (Carter, 1973). It has been hypothesized that *P. haemolytica* resides in the upper respiratory tract of cattle as a commensal, and overgrows in the upper respiratory tract upon mycoplasmal, viral infections, or mismanagement-induced stress (Woldehiwet, et al., 1990). The heavily colonized nasal pharynx and tonsilar regions then serve as a source of *P. haemolytica* aerosolation to the lower airways, where *P. haemolytica* produces numerous virulence factors, which breach the host immune defense system and establishes infection (Fig. 1) (Frank, 1986; Whiteley, 1992).

1.3. Optimization of culture conditions for promoting expression of certain virulence factors

P. haemolytica grows well in brain-heart infusion broth or agar plates. Media containing serum or blood promote its growth (Carter, et al., 1995). Different culture conditions may stimulate *P. haemolytica* to express different concentrations of virulence factors (Gentry, et al., 1986; Confer and Durham, 1992; Confer, et al., 1992; Mosier, et al., 1994; Gatewood, et al., 1994; Waurzyniak, et al., 1994; Highlander, 1997). Several potential virulence factors from *P. haemolytica* have been identified (Confer, et al, 1988), including leukotoxin, endotoxin or lipopolysaccharide (LPS), protease, neuraminidase (Frank and Tabatabai, 1981; Straus, et al., 1993a and 1993b; Straus and Purdy, 1994), capsular polysaccharide (Morck, et al., 1987), outer membrane proteins, fimbriae (Potter, et al., 1988) and pilli (Confer, et al, 1988; Whiteley, et al., 1992; Breider and Yang, 1994; Brogden, et al., 1995). Each of these virulence factors may play a role in the pathogenesis of bovine pneumonic pasteurellosis, either promoting bacterial growth by acquisition of nutrients, or colonization by interacting with host cells or damaging the host defense system. Among these virulence factors, LKT is considered to be the primary virulence factor in the pathogenesis of bovine pneumonic pasteurellosis (Petras, et al., 1995; Murphy, et al., 1995; Fedorova and Highlander, 1997; Tatum, et al., 1998).

To study the role of *P. haemolytica* LKT in the pathogenesis of bovine pneumonic pasteurellosis, a significant amount of LKT needs to be produced and purified from the culture supernatants. Supplementation of culture media with serum proteins (10 to 100 mg/ml) enhances the *P. haemolytica* growth rate (3-fold increase) and LKT activity (3- to 25-fold increase) in culture supernatants (Confer and Durham, 1992; Gatewood, et al.,

1994; Highlander, 1997). Although supplementation with serum protein increases LKT production, this supplementation causes a practical problem for LKT purification. Separation of LKT from protein-supplemented media has not been satisfactory (Gentry and Srikumaran, 1994). Therefore, to obtain a large quantity of LKT for purification, it is necessary to develop a serum protein-free medium for optimizing *P. haemolytica* LKT production.

1.4. Biological and genetic characteristics of LKT

P. haemolytica LKT is cytotoxic to ruminant leukocytes and platelets *in vitro* (Shewen and Wilkie, 1982; Clinkenbeard and Upton, 1991). More severe lung lesions are formed in cattle or goats challenged with wild-type *P. haemolytica* than those challenged with LKT-negative mutant strains (Petras, et al., 1995; Tatum et al., 1998). The resistance of calves to experimental challenge with wild-type *P. haemolytica* is closely correlated with higher titers of serum neutralizing antibody against LKT (Gentry et al., 1985; Shewen and Wilkie, 1988; Sreevatsan et al., 1996). These implicate LKT as an important virulence factor in the pathogenesis of pneumonic pasteurellosis and a protective antigen.

All known serotypes and several untypable strains of *P. haemolytica* isolated from ruminants produce LKT during logarithmic growth phase (Shewen and Wilkie, 1985). In contrast, another study indicated that all of four untypable strains of *P. haemolytica* failed to produce LKT (Chang, et al., 1987a). LKT is specific for alveolar macrophages and circulating blood lymphocytes, neutrophils, mast cells, platelets, and cultured leukocyte cell lines from cattle, sheep, and goats but has little or no effect on cells from swine, horses, or humans (Kaeher et al., 1980; Shewen and Wilkie, 1982; Sutherland, 1985; Clinkenbeard and Upton, 1991; Adusu, et al., 1994). In addition, alveolar macrophages

from adult cows are significantly more resistant to intoxication than cells from calves, and neutrophils are more sensitive than either alveolar or mammary macrophages (O'Brien and Duffus, 1987). The mechanism of target cell specificity has not been determined.

The culture supernatants from rapid growing *P. haemolytica* at logarithmic growth phase contain native LKT, which is highly aggregated (≈8,000 kDa) and has low biological activity (Waurzyniak, et al., 1994). The native LKT multimers can be partially disaggregated to 250 – 800 kDa by addition of 0.5% bovine serum albumin resulting in enhanced LKT activity (Waurzyniak, et al., 1994). Resuspension of native LKT in phosphate buffer containing various chaotropic agents also results in partial disaggregation of this native LKT and enhances LKT activity. Guanidine at 3 M disaggregates LKT to a multimer mass of approximately 800 kDa and enhances LKT activity 3 to 20-fold. In 6 M urea or 1 M sodium thiocyanate, LKT multimers are observed ranging in mass from 8000 kDa to 400 kDa, and activity enhancement is less than that for LKT in 3 M guanidine. Several detergents were tested for enhancement of LKT activity, but only 1% Tween 20 enhances LKT activity (4-fold), whereas 1.25% octylglucoside, 10 mM CHAPS, and 5 mM deoxycholate diminished and 1% Triton X-100 abolishes LKT activity (Clinkenbeard, et al., 1995). The LKT activity in the culture supernatants is rapidly lost when the bacterial growth reaches from the late logarithmic to stationary phases (Shewen and Wilkie, 1985), probably due to proteolysis of 102 kDa LKT monomer to one of 95 kDa molecular mass (Chang, et al., 1987b).

The genes encoding LKT (*lktA*), and proteins required for activation (*lktC*), and secretion (*lktBD*) have been cloned and sequenced (Chang, et al., 1987b; Lo, et al., 1987;

Highlander, et al., 1990; Fedorova and Highlander, 1997). The four-gene cluster, with *lktC* first, then *lktA*, and followed by *lktBD*, is controlled by a common operon. The leukotoxin produced from *lktA* is inactive and termed pro-LKT (Fedorova and Highlander, 1997; Stanley, et al., 1998). The pro-LKT is converted in the cytosol to the active form by LKTC-directed fatty acid acylation. After activation, the hydrophobicity of LKT is increased, but this is not required for secretion. LKT is secreted across both membranes by the type I secretion system employing an uncleaved C-terminal recognition signal (Finlay and Falkow, 1997). The LKT secretory apparatus comprises LKTB (an inner membrane traffic ATPase), and LKTD (an inner membrane protein that is suggested, but not shown, to bridge to the outer membrane). Another outer membrane protein TolC, encoded by a gene separated from *hlyCABD* locus, has been identified to be involved in *E. coli* α-hemolysin secretion TolC (Stanley, et al., 1998). However, whether TolC is responsible for LKT secretion is unknown. The paradigm for RTX production, activation, and secretion is presented in Fig. 2.

From the genetic and biochemical point of view, LKT belongs to the repeat-in-toxin (RTX) family (Welch, 1991), which are produced by a variety of Gram-negative bacteria. In addition to *P. haemolytica* LKT, the members in this family also include the hemolysin of *E. coli*, the hemolysins and leukotoxins of *Actinobacillus spp.*, the bifunctional adenylate cyclase/hemolysin of *Bordetella pertussis*, and the hemolysins of *Proteus vulgaris*, *Morganella morganii*, and *Moraxella bovis* (Stanley, et al., 1998) (Table 4). RTX toxins share many common features, such as a glycine-rich, tandem repeated nonapeptide GGXGXDX(L/I/V/W/Y/F)X in their sequence and Ca^{2+}-dependent, target cell-directed pore-forming activity. The number of nonapeptide repeats in RTX

toxins varies from 7 to 43. The tandem repeat regions are thought to be involved in Ca^{2+} binding of the toxin and are vital for toxic activity (Boehm, et al., 1990a and 1990b; Bhakdi and Tranum-Jensen; 1986). The molecular model of LKT structure and its interaction with target cell membranes are shown in Fig. 3.

The pore formation by LKT is thought to be caused by insertion of the toxin into the cytoplasmic membranes of target cells, analogous to the mechanism used by the C5b6789 complement membrane attack complex (MAC) (Clinkenbeard, et al., 1992; Reiter, et al., 1995). By addition of osmotic stabilizing or protection agents such as a series of carbohydrates with different molecular weights to media containing LKT-exposed bovine lymphoma BL3 cells, it is estimated that the transmembrane pore size formed by LKT is approximately 1 nm (Clinkenbeard, et al., 1989a). These small pores allow only dissipation of transmembrane electrochemical gradients of K^+ and Na^+ molecules vital to the osmotic stability of the cells. Because intracellular proteins and other macromolecules are too large (>5 nm) to cross the plasma membrane through the toxin-formed pores, dissipation of the K^+ and Na^+ electrochemical gradient results in a colloid-osmotic imbalance, with the cytoplasm being hypertonic relative to the extracellular space. Water, which diffuses freely across the cytoplasmic membrane, moves into the cell to correct the colloid-osmotic imbalance, and the cell swells (Clinkenbeard, et al., 1992) (Fig. 4). Subsequent to cell swelling, large cytoplasmic membrane defects form (approximately 100 nm), which is a Ca^{2+} dependent process (Clinkenbeard, et al., 1989a). Calcium entering the cytosol may be partially through voltage-sensitive Ca^{2+} channels, because Ca^{2+} channel blockers decreased LKT-induced Ca^{2+} influx (Ortiz-Carranza and Czuprynski, 1992). The function of Ca^{2+} in the cytolytic

activity has not been well defined. Calcium may be required for toxin-induced phospholipase metabolism of cytoplasmic membrane phospholipids (Wang, et al., 1998), or the toxin-induced influx of Ca^{2+} may cause the disruption of the cytoskeleton, resulting in the loss of cytoplasmic membrane strength (Clinkenbeard, et al., 1989). Another possibility for Ca^{2+}-induced cytolytic activity is proteolysis of integral proteins in the cytoplasmic membranes by Ca^{2+}-activated proteases in the cytosol (Fig. 4).

1.5. Initial interaction of leukotoxin with target cells

Generally speaking, RTX toxins can be divided into two classes, hemolysins and leukotoxins, based on their target cell specificity. For example, *E. coli* α-hemolysin intoxicates a wide range of target cells, while *P. haemolytica* LKT and *A. actinomycetemcomitans* leukotoxin (Ltx) only intoxicate subsets of leukocytes from ruminants and primates, respectively (Kaeher, et al., 1980; Tsai, et al., 1984; Taichman, et al., 1991). The basic biologic mechanism that allows certain bacterial toxins to selectively intoxicate certain types of target cells, while not injuring other host cells is partially solved. The target cell selectivity by RTX toxins appears to be related to the presence of specific receptors on target cell surfaces. Lally *et al* (1997) have identified a cell surface receptor for *A. actinomycetemcomitans* Ltx and *E. coli* α-hemolysin, which includes two polypeptide chains of approximate molecular masses of 100 and 170 kDa. Microsequencing of tryptic peptides from the two proteins showed complete homology with CD11a and CD18, the two subunits of the β_2-integrin lymphocyte function-associated antigen 1 (LFA-1). However, several questions still remain to be answered. If LFA-1 is only the molecule responsible for RTX toxin-induced cytolysis, why are red blood cells, and other non-hematopoetic origin cells (i.e. endothelial cells), which do not

have LFA-1 present on cell surface, susceptible to hemolysins? Whether RTX toxins only bind to susceptible cells and then lead to lysis is still contradictory. One study indicated that the biotinylated *P. haemolytica* LKT only binds to susceptible bovine leukocytes, but not non-susceptible porcine leukocytes, as assessed by flow cytometry (Brown, et al., 1997). Therefore, only susceptible cells are lysed. Others have reported that *P. haemolytica* LKT binds to susceptible BL3 cells, as well as non-susceptible porcine alveolar macrophages, but not non-susceptible bovine endothelial cells (Kannan, et al., 1997). It has been shown that *A. actinomycetemcomitans* Ltx binds to human erythroleukemic cells and mouse SP2 myeloma cells, but neither cell types are susceptible to this toxin (Taichman et al, 1991; Sato et al, 1993).

2. Apoptosis

2.1. Apoptosis and oncosis: two morphologically distinct forms of cell death

The term apoptosis, from the Greek word for "falling off" of leaves from a tree, was originally described by Kerr, et al. (1972). Apoptosis is sometimes referred to as "cell suicide" or "programmed cell death" and is an active cellular process of gene-directed self-destruction to remove unwanted cells and is employed to cause specific cell death during embryogenesis, maturation, and homeostasis. In addition, aberrant apoptosis can occur during pathogenesis of both infectious and non-infectious diseases (Wyllie, 1980). Cell death through apoptosis is distinct from the passive, catabolic, and degenerative oncotic cell lysis, which is sometimes referred to as "accidental cell death" or "death by murder". Oncotic cell lysis involves swelling of mitochondria and disruption of membrane integrity and subsequent cellular swelling and lysis, due to the colloid-

osmotic imbalance of the cell (Clinkenbeard, et al., 1989a; Majno and Joris, 1995), while apoptosis is characterized by membrane blebbing, chromatin condensation, and DNA fragmentation into oligonucleosomal fragments (Wyllie, et al., 1980). In the final stages, the apoptotic cells become fragmented into membrane-bound apoptotic bodies which are rapidly eliminated by phagocytic cells without eliciting significant inflammatory damage to surrounding cells (Wyllie, et al., 1980) (Table 5). The apparent benefit of apoptosis over oncotic cell lysis is that with the former, cells die without lysis and the associated inflammatory response that typically occurs with oncosis.

2.2. Biochemical and molecular mechanisms regulating apoptosis

Although the morphologic features of apoptosis have been appreciated for several decades (Kerr, et al., 1972), the biochemical pathways responsible for apoptotic cell death are only beginning to be elucidated. From studies on apoptosis in *in vitro* systems and genetic analysis of cell death during development of the nematode *Caenorhabditis elegans*, a number of genes have been identified to be involved in the apoptotic process. In *C. elegans*, *ced-3*, *ced-4*, and *ced-9* (ced stands for cell death defective) have been identified as genetic cornerstones of the death program that are required for promotion (*ced-3* and *ced-4*) or prevention (*ced-9*) of apoptosis. Remarkably, mammalian homologs of a protein encoded by *ced-3* are a family of at least ten cysteine proteases with aspartate specificity, formerly known as the ICE (interleukin - 1β converting enzyme) family, now called caspases (Table 6). A protein encoded by *ced-9* corresponds in mammals to a family with many members, and the prototype of which is Bcl-2. The mammalian equivalents of the CED-4 molecule have not been identified (Golstein, 1997).

Apoptosis can be triggered by a variety of extrinsic and intrinsic signals, including physiologic activators, bacterial toxins, cytotoxic chemicals, and pharmacological agents (Table 7) (Thompson, 1995). Operationally, apoptosis is induced by death receptors (TNF, Fas, DR3, DR4, and DR5), by p53 dependent and independent cellular stress pathway (mitochondrial pathway), and by the secretion of perforin and granzymes from cytotoxic T cells. Sequentially, in the initiation phase of apoptosis, heterogenic stimuli trigger the apoptotic machinery through signal transduction to stimulate expression of apoptotic genes. During the effector phase, apoptotic gene products interact with cellular components and cells pass a point of no return, thus becoming irreversibly committed to apoptotic cell death due to oxidative damage, altered calcium homeostasis, and abnormal mitochondrial functions. Alterations in mitochondrial permeability transitionally linked to membrane potential disruption precede nuclear and plasma membrane changes. It has been demonstrated that *in vitro* induction of permeability transition in isolated mitochondria provokes the release of a protein factor capable of inducing nuclear chromatin condensation and fragmentation (Petit, et al., 1996). It appears that oxidative damage, disruption of calcium homeostasis, and abnormal mitochondrial functions are three key factors triggering apoptosis pathway to express apoptotic gene products leading to apoptosis. The last stage of apoptosis is the degradation phase, during which vital structures and functions are destroyed and typical apoptotic morphology of cells can be detected (Petit, et al., 1995).

It seems that the apoptosis pathway is similar to the blood coagulation cascade, both of which are involved in activation of various proenzymes. The central component of this machinery is a proteolytic system involving activation of a family of caspases

(Froelich, et al., 1998), which are responsible for DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-bound apoptotic bodies (Thornberry and Lazebnik, 1998). Caspases cleave several proteins involved in cytoskeleton regulation specifically at aspartic residues. During apoptosis, caspases also specifically cleave a nuclease inhibitor that is present as a complex with endonucleases and subsequently activates endonucleases, which cleave nuclear DNA at the sites of internucleosomes to yield about 200 bp fragments (Thornberry and Lazebnik, 1998).

2.3. Detection of Apoptosis

A variety of methods have been developed for detection of apoptosis including morphological, biochemical, and molecular biological techniques. Some of these methods are species specific and others are universal. The most reliable method is electron microscopy, which provides unequivocal information about apoptosis (Wyllie, et al., 1980). At the ultrastructural level, apoptotic cells are readily distinguished from normal and oncotic cells. However, this method is time-consuming and requires specific equipment and skills. Detection of fragmented DNA in apoptotic cells by various assays is another useful and reliable technique. These methods include agarose gel electrophoresis, colorimetric diphenylamine assay, ELISA systems, and *in vitro* or *in situ* methods involving various DNA end-labeling, such as fluorescence and enzyme (i.e. Klenow enzyme). Recently, assay systems have been developed for detection of the caspase family of protease enzymes that are activated during apoptosis.

2.4. Clinical implications of apoptosis

In addition to involvement of apoptosis in early development and maintenance of homeostasis, it has been demonstrated that many diseases are associated with inappropriate induction or inhibition of apoptosis, including neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, or cerebellar degeneration (Solary, et al., 1996; Kitamura, et al., 1998), transplant rejection (Lin, et al., 1998), autoimmune disorders (Thompson, 1995), hemolytic-uremic syndrome (Karpman, et al., 1997), neoplasia (Thompson, 1995), and acquired immune deficiency syndrome (AIDS) (Wang and Klimpel, et, al., 1998). Apoptosis is a pathological feature of polycystic kidney disease and toxin-induced liver diseases (Thompson, 1995). Ischemic injury, such as myocardial infarction or stroke, induces rapid cell death within the central area of ischemia by necrosis. Outside the central ischemic zone, cells die over a more prolonged time period by apoptosis (Solary, et al., 1996). During the course of aging, tissue homeostatic control is altered and equilibrium is shifted toward cell death. The true nature of this age-related cell deletion phenomenon could be apoptosis (Lockshin and Zackeri, 1990) as a consequence of diminished synthesis of various growth factors, transmembrane signaling defects, inability to cope with oxidative stress, or abnormal cell cycle regulation. Some of these diseases are due to the failure of cells to undergo apoptosis, others are associated with excessive apoptotic cell death. Elucidation of apoptosis pathways associated with different diseases may provide valuable information for the formulation of novel strategies in prevention and treatment of diseases.

3. Role of bacterial toxins in induction of apoptosis

3.1. Apoptosis in bacterial pathogeneses

Bacteria have evolved many strategies to survive inside the host, overcoming host immune defenses and resulting in diseases. Some bacteria use their virulence factors to adhere and colonize in the host, to acquire nutrients from the host, or to kill host immune system cells. Other bacterial pathogens alter host immune mechanisms to escape host surveillance. Recently, it has been determined that a number of bacterial pathogens including Gram-positive and Gram-negative bacteria, as well as *Mycobacteria*, are able to induce apoptosis in host cells (Zychlinsky and Sansonetti, 1997; Chen and Zychlinsky, 1994; Li, et al., 1998; Schesser, et al., 1998; Fernandez-Prada, et al., 1998). The bacterial toxins appear to be significant contributors to induction of apoptosis in a variety of host cells.

Generally, the bacterial protein toxins can be divided into three groups according to their sites of action. (i) Toxins acting at the cytoplasmic membrane, where they interfere with transmembrane signaling pathways. This group includes the *E. coli* heat-stable enterotoxin ST, which acts directly on the transmembrane guanylate cyclase of intestinal cells. (ii) Toxins that alter membrane permeability, such as pore-forming toxins of the streptolysin O/listeriolysin O family, *Staphylococcus* alpha toxin, and the RTX toxins. (iii) Toxins that act inside cells, where they enzymatically modify a specific cytosolic target. These toxins are often involved in changes to the host cell cytoskeleton or signaling pathways. A growing body of evidence indicates that the toxins that alter cell membrane permeability or act inside cells as an enzyme are potent inducers of apoptosis

in a variety of target cells. For example, the pore-forming alpha-toxin from Gram-positive *S. aureus* induced apoptosis in human peripheral-blood T lymphocytes and rabbit corneal epithelial cells (Jonas, et al., 1994; Moreau, et al., 1997). Blockade of host protein synthesis by a number of toxins, such as Shiga toxin (Stx) 1 and Stx2, verocytotoxin (VT) from *E. coli* (Obrig, 1997) and diphtheria toxin (DT) (Hall, et al., 1998) induce cell death by apoptosis in normal human renal tubular epithelial cells, mesangial cells, and a renal carcinoma cell line ACHN cells (Van Setten, et al., 1997; Kiyokawa, et al., 1998; Taguchi, et al., 1998). Studies *in vitro* have demonstrated that listeriolysin O initiates apoptosis in cultured dendritic cells, a type of antigen presenting cell (Guzman, et al., 1996). In this review I will focus on RTX toxin-induced apoptosis and hypothesize a mechanism of apoptosis pathways.

3.2. Role of RTX toxins in induction of apoptosis

As mentioned above, RTX toxins are calcium-dependent pore-forming toxin proteins which are not only important in the pathogeneses of many Gram-negative bacterial infections, but also could be models to study other porins such as ionophores and perforin of cytotoxic T lymphocytes in cell biology (Reiter, et al., 1995). Since the early 1990's, the increasing numbers of RTX toxins have been recognized as inducers of apoptosis in many different types of cells *in vitro*, which may play a role in the pathogeneses of diseases.

E. coli hemolysin - *E. coli* α-hemolysin is the protype of RTX toxins proven to be influential not only in urinary tract infections but also hemorrhagic intestinal diseases. At high concentrations, *E. coli* α-hemolysin is highly cytotoxic to different cells such as erythrocytes, granulocytes, monocytes, lymphocytes, endothelial cells, and renal tubular

epithelial cells (Jonas, et al., 1993). Exposure of low concentrations of *E. coli* α-hemolysin to human T lymphocytes which have been prestimulated with phytohemagglutinin (PHA) induces DNA fragmentation similar to that observed in cells undergoing apoptosis (Jonas, et al., 1993). However, the authors conclude that DNA fragmentation induced by α-hemolysin in PHA-prestimulated T lymphocytes may represent only an epiphenomenon, because exposure of freshly isolated resting T cells to any ranges of concentrations of α-hemolysin did not exhibit DNA fragmentation (Jonas, et al., 1993). The difference observed between activated and resting T cells might reflect the T cell capacity to enter an apoptotic program (Chen and Zychlinsky, 1994). The ability of *E. coli* α-hemolysin to induce apoptosis was confirmed by a recent report, in which several strains of α-hemolysin-positive enteroaggregative and cytodetaching *E. coli*, but not α-hemolysin-deficient mutants, induce apoptosis in murine macrophage cell line J744 cells (Fernandez-Prada, et al., 1998), while infection of human monocyte-derived macrophages with α-hemolysin-positive *E. coli* result in damage of plasma membrane integrity without changes in nuclear morphology. The morphological changes in J774 cells include compacted and condensed nuclei at light microscopy level, striking condensation and margination of chromatin material at electron microscopy level, and a ladder pattern of DNA fragmentation was also observed on agarose gel electrophoresis (Fernandez-Prada, et al., 1998). Whether a toxin induces oncosis or apoptosis appears to be related to the rapidity of cell membrane damage and to the extent of such damage (Mangan, et al., 1991; Jonas, et al., 1994). This implies that J774 cells are less susceptible to α-hemolysin-induced oncosis than human monocyte-derived macrophages.

B. pertussis adenylate cyclase-hemolysin - *B. pertussis*, the etiologic agent of whooping cough in humans, synthesizes several virulence factors involved either in the infection, such as filamentous hemagglutinin (FHA), agglutinogens, and pertactin, or in the disease, such as pertussis toxin (PTX), adenylate cyclase-hemolysin (AC-Hly), tracheal cytotoxin, and dermonecrotic toxin (Khelef, et al., 1993). AC-Hly is one of the virulence factors necessary to induce lethality in the murine model by inactivation of various G proteins and elevation of an intracellular 3'-5' cyclic AMP (cAMP). The wild-type of *B. pertussis* and a mutant stain deficient in pertussis toxin expression are able to induce apoptosis in J774A.1 cells, a monocyte-macrophage cell line, and murine alveolar macrophages, whereas avirulent mutant or AC-Hly-deficient mutants do not induce apoptosis. These results implicate that induction of apoptosis is dependent on the expression of AC-Hly (Khelef, et al., 1993 and 1995). In vivo study further confirmed this finding (Gueirard, et al., 1998). In a murine respiratory model, intranasal infection of wild-type of *B. pertussis*, not AC-Hly deficient mutant stain, induces extensive apoptosis in neutrophils and macrophages, indicating that apoptosis induced by *B. pertussis* AC-Hly is involved in the pathogenesis of whooping cough in humans (Gueirard, et al., 1998).

A. actinomycetemcomitans Ltx – *A. actinomycetemcomitans* is an etiologic agent of numerous human diseases, such as endocarditis, meningitis, osteomyelitis, and localized juvenile periodontitis (Korostoff, 1998). A majority of clinical isolates of *A. actinomycetemcomitans* synthesize a heat-labile exotoxin, termed leukotoxin (Ltx). Similar to *P. haemolytica* LKT, *A. actinomycetemcomitans* Ltx is a potent virulence factor and has a narrow target cell specificity, being toxic only to polymorphonuclear

neutrophils and mononuclear cells from human and several primates. In contrast, human platelets, fibroblasts, endothelial cells, and epithelial cells are relatively resistant to the cytotoxic effects of Ltx (Mangan, et al., 1991). Exposure of freshly isolated human peripheral blood lymphocytes to purified Ltx for 1.5 hours did not significantly decrease their viability. However, mitogen- and antigen-induced proliferation and immunoglobulin production were inhibited (Rabie, et al., 1988). Prolonged incubation of freshly isolated human lymphocytes to purified Ltx induces cell death by both apoptosis and necrosis, evidenced by uptake of propidium iodide, release of ^{51}Cr from the cytoplasmic membranes, and increases in cleavage of chromosomal DNA into nucleosome-sized fragments (Mangan, et al., 1991). Electron microscopy examination indicated that 60% of lymphocytes died by necrosis, 10% of lymphocytes exhibited cell shrinkage and nuclear chromatin condensation characteristic of apoptosis, and 30% of cells remained unchanged. Quantification of low-molecular-weight DNA in Ltx-treated lymphocytes indicated that Ltx-induced DNA fragmentation was concentration-dependent, with maximal fragmentation occurring in cells treated with Ltx at 100 ng/ml. Agarose gel electrophoresis confirmed DNA fragmentation induced by sublytic dose of Ltx (Mangan, et al., 1991). Recently, it has been reported that incubation of human promyelocytic HL-60 cells with 0.2 Ltx units of recombinant Ltx resulted in the four populations of the cells: viable (43.3%), early apoptotic (10.4%), late apoptotic and/or secondarily necrotic (29.9%) cells, and cellular debris (16.4%) (Korostoff, et al., 1998).

P. haemolytica LKT – Similar to other RTX toxins, *P. haemolytica* LKT at high concentrations causes oncotic cell lysis in bovine leukocytes and platelets by induction of intracellular colloid-osmotic imbalance (Clinkenbeard, et al., 1989a). However, whether

P. haemolytica LKT is able to induce apoptosis in its target cells at lower concentrations remained unclear until 1996 (Stevens and Czuprynski, 1996). In their study, the criteria for judging apoptotic cells were cytoplasmic membrane blebbing (i.e. zeiosis) and nuclear condensation. The maximal induction of apoptosis in bovine leukocytes by *P. haemolytica* LKT was 0.5 U/ml (Stevens and Czuprynski, 1996). However, DNA fragmentation, a hallmark of apoptosis (Wyllie, et al., 1980), was not detected. Furthermore, the morphological changes in their study was examined by light microscopy. The limited resolution associated with light microscopy makes it difficult to interpret the morphological results. Since apoptosis represents an important mode of cell death and has proved to be involved in the pathogeneses of some infectious diseases, it is necessary to clarify whether exposure of sensitive target cells to *P. haemolytica* induces apoptosis as determined by both biochemical and ultrastructural methods. Elucidation of mechanisms involved in *P. haemolytica* LKT-induced apoptosis in bovine leukocytes, especially lymphocytes, will provide essential information for formulation of novel strategy for prevention and treatment of bovine pneumonic pasteurellosis.

3.3. The hypothesis of the mechanism for *P. haemolytica* LKT-induced apoptosis

Studies of other stimuli that induce apoptosis indicated that whether injured cells undergo cell death via apoptosis or oncosis may depend on the severity of cellular insult (Cohen, 1996; Lieberthal and Levine, 1996). Cells that undergo oncosis when exposed to severe ischemic or toxic insults may undergo apoptotic cell death when the insult is lessened (Lieberthal and Levine, 1996). A variety of pore-forming agents, including pore-forming bacterial toxins (RTX toxins and staphylococcal alpha-toxin) (Jonas, et al., 1993 and 1994; Mangan, et al., 1991; Khelef, et al., 1993; Stevens and Czuprynski, 1996),

lymphokine perforin from cytotoxic T lymphocytes (Jans, et al., 1998), and ionophore A23187 (Vrana, et al., 1998), which cause oxidative damage, disruption of intracellular calcium homeostasis and abnormal mitochondrial functions, could induce oncotic cell lysis at high concentration, while at low concentrations, these effectors may trigger apoptosis. It is our hypothesis that after formation of extensive pores on cytoplasmic membranes by high concentrations of *P. haemolytica* LKT, rapid disruption of homeostasis of the target cells induces oncotic cytolysis (Clinkenbeard, et al., 1989a). However, lower concentrations of LKT induce sublethal injuries to target cells, including activation of oxidative burst, alterations of cytoskeleton, release of secondary granules, and inhibition of mitogen-induced cell proliferation (Czuprynski, et al., 1991; Czuprynski and Ortiz-Carranza, 1992; Ortiz-Carranza and Czuprynski, 1992; Majury and Shewen, 1991a and 1991b; Stevens and Czuprynski, 1996). The biochemical events which lead to sublethal effects may trigger the apoptosis pathway and lead to apototic cell death (Cohen, 1996) (Fig. 5).

It is well known that *P. haemolytica* LKT is able to form transmembrane pores on target cells (Clinkenbeard, et al, 1989a), to cause influx of calcium (Ortiz-Carranza and Czuprynski, 1992), increase activity of phospholipase A_2 (Wang and Clarke, et al., 1998), stimulate oxidative burst (Czuprynski, et al., 1991), and lyse target cells (Clinkenbeard, 1989b). Consequences of pore-formation, calcium-influx, oxidative burst, and activation of Ca^{2+}-independent phospholipase A_2 but not cytosolic phospholipase A_2 activity (Atsumi, et al., 1998) lead to apoptosis. However, different types of cells, for example, fibroblasts versus lymphoid cells, may have different responses to a variety of cellular insults (Fig. 5).

Several studies (Carter, 1973; Minton, 1994; Hopster, et al., 1998) have indicated that great increases in the production of adrenocortical hormones and subsequent reduction in peripheral blood lymphocyte numbers in cattle are associated with stress conditions. Adrenocortical hormone-induced apoptosis in various species and types of cells have been extensively studied (Wyllie, 1980). Therefore, it is rational to speculate that *P. haemolytica* LKT, as well as stressful conditions, could be an inducer of apoptosis in sensitive target cells and apoptosis could be a process involved in the pathogenesis of bovine pneumonic pasteurellosis. The study of apoptosis induced by *P. haemolytica* LKT may provide valuable information for the formulation of novel therapeutic strategies to prevent and treat bovine pneumonic pasteurellosis.

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Table 1. Differential characteristics of biotypes of *P. haemolytica* (Carter, et al. 1995)

	Biotype A	Biotype T (P. trehalosi)
Fermentation		
arabinose	+	-
trehalose	-	+
salacin	-	+
xylose	+	-
lactose	+	-
Susceptibility to penicillin	High (except serotype 2)	Low
Serotypes	1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16	3, 4, 10, 15
Principal location in normal host	Nasopharynx	Tonsils

Table 2. Host distribution and frequency of occurrence of biotypes and serotypes of *P. haemolytica* (Timoney, et al. 1988).

Host	Biotype and serotype	Disease	Frequency
Cattle	A1	Bronchopneumonia	Most frequent
	A2	Bronchopneumonia	Frequent
	A6, T3, T4	Bronchopneumonia, mastitis	Sporadic
	A7, A9, A11	Septicemia, meningitis	Sporadic
	Untypable	Bronchopneumonia	Sporadic
Sheep	A2	Bronchopneumonia, mastitis	Most frequent
	T3, T4, T10, T15	Septicemia, arthritis	Sporadic
	A1, A6	Bronchopneumonia, mastitis	Sporadic
	A5, A7, A9, A11, A13, A14	Bronchopneumonia	Infrequent
	Untypable	Bronchopneumonia	Infrequent

Table 3. Etiologic and possible contributory factors in bovine pasteurellosis (Carter, 1973; Biberstein and Zee, 1990).

Bacteria	Mycoplasmas	Viruses	Environmental stress
P. haemolytica	*M. bovis*	IBR[a]	exhaustion, starvation,
P. multocida	*M. dispar*	PI-3[b]	dehydration,weaning,
H. somnus	*Ureaplasma sp.*	BRSV[c]	ration changes,
Salmonella spp.	*M. bovirhinitis*	BVD[d]	over-crowding,chilling, over-
Streptococcus spp.	*M. laidlawii*	Adeno-	heating, excess or irregular,
S. aureus	*M. mycoides*	Rhino-	high energy feed, poor
E. coli		Reo-	ventilation, excess humidity,
C. pyogenes		Herpes-	non-respiratory diseases,
Bacteroides spp.		Entero-	castration, dehorning,
Chamydia psittaci		Calici-	maladjustment
Chlamydia		Corona-	

[a] Infectious bovine rhinotracheitis.

[b] Parainfluenza-3.

[c] Bovine respiratory syncytial virus.

[d] Bovine viral diarrhea.

Table 4. Toxins of the RTX family*#

Bacterium	Toxin	Activity	Target species	Target cells	Size (kDa)	Total no. AA[c]	HlyA identity (%)
Escherichia coli	HlyA	Hemolysin	Human, monkey, pig, rat, mouse, horse, sheep, cow	Erythrocytes, leukocytes, epithelia, endothelia	110	1024	
	EhxA	EH-Hyl[b]	Human, sheep, cow	Erythrocytes, leukocytes	107	998	61
	EaggA[a]				120	ND[d]	?
Proteus vulgaris	PvxA	Hemolysin			110	ND	73
Morganella morganii	MmxA	Hemolysin			110	ND	60
Actinobacillus pleuropneumoniae	ApxIA	Hemolysin	Pig, cow, rabbit	Erythrocytes, leukocytes, endothelia	110	1022	59
	ApxIIA	Hemolysin	Pig, cow, rabbit	Erythrocytes, leukocytes, endothelia	102	956	49
	ApxIIIA	Leukotoxin	Pig	Leukocytes, macrophages	113	1049	56
A. actinomycetemcomitans	AaltA	Leukotoxin	Human, ape, monkey	Leukocytes	114	1050	54
A. suis	AshA	Hemolysin	Pig, horse, sheep, cow	Erythrocytes	102	956	49
P. haemolytica	LktA	Leukotoxin	Cow, sheep, goat	Leukocytes, macrophages, platelets	102	953	49
P. haemolytica-like	PllktA	Leukotoxin	Pig, cow	Leukocytes	102	947	48
B. pertussis	CyaA	AC-Hly	Human, sheep	Erythrocytes, leukocytes, macrophages	178	1706	31
Moraxella bovis	MbxAa	Hemolysin	Sheep, cow	Erythrocytes, lymphocytes, epithelia	110	ND	?

*Stanley, et al., 1998. Microbiology and molecular biology reviews, 62:309-333.

#Welch, 1991. Molecular Microbiology, 5:521-528.

[a]No gene yet identified.

[b]Enterohemorrhagic hemolysin.

[c]AA=amino acid.

[d]Not determined.

Table 5. Comparison of the morphologic and biochemical features of apoptosis and oncosis.

Characteristics	Apoptosis	Oncosis
Causes	Suicide (regulated)	Murder (unregulated)
Cell size	Shrinkage	Swelling
Cell membrane permeability	Normal (trypan blue excluded)	Abnormal (trypan blue included)
Cell membrane budding	An early characteristic feature	Absent
Cytoplasmic vacuolation	Present	Absent
Mitochondrial morphology	Normal	Swollen, with flattened cristae
Nuclear chromatin	Condensed and fragmented	No condensation or fragmentation
Formation of apoptotic bodies	Characteristic	Absent
Ultimate fate of cell	Phagocytosis	Lysis
Inflammation	Absent	Present
Appearance in tissue sections	Inconspicuous, usually underestimated	Injury of cells and surrounding tissue obvious and easily quantified

Table 6. Nomenclature for interleukin-1β-converting enzyme (ICE)/Ced-3-like human cysteine proteases (caspases)*

	Caspase designation	Alternative names
ICE subfamily	Caspase 1	ICE
	Caspase 4	$ICE_{(rel)}$II; TX; ICH-2
	Caspase 5	$ICE_{(rel)}$III; TY
Ced-3 subfamily	Caspase 2	ICH-1
	Caspase 3	Apopain; CPP32; Yama
	Caspase 6	Mch2
	Caspase 7	Mch3; ICE-LAP3; CMH-1
	Caspase 8	FLICE 1; MACH; Mch5
	Caspase 9	ICE-LAP6
	Caspase 10	FLICE 2; Mch 4

* Froelich, et al., 1998. Immunology Today, 19:30-36.

Table 7. Activators or inducers of apoptosis*

Physiologic activators	Damage-related inducers	Therapy-associated agents	Toxins
TNF family	Heat shock	Chemotherapeutic drugs	Ethanol
Fas ligand	Viral infections	Cisplatin	β-amyloid
TNF	Oncogenes	Doxorubicin	peptide
TGFβ[a]	*Myc*	Bleomycin	
Neurotransmitters	*Rel*	Cytosine	
Glutamate	*E1A*	Arabinoside	
Dopamine	Tumor suppressors	Nitrogen mustard	
N-methyl-D-aspartate	P_{53}	Methotrexate	
Growth factor withdrawal	Nutrient deprivation	Vincristine	
Loss of matrix attachment	Antimetabolites	Gamma radiation	
Glucocorticoids	Bacterial Toxins	UV radiation	
Calcium	Cytotoxic T cells		
	Free radicals		
	Oxidants		

* Thompson, 1995.
[a]Transforming growth factor β.

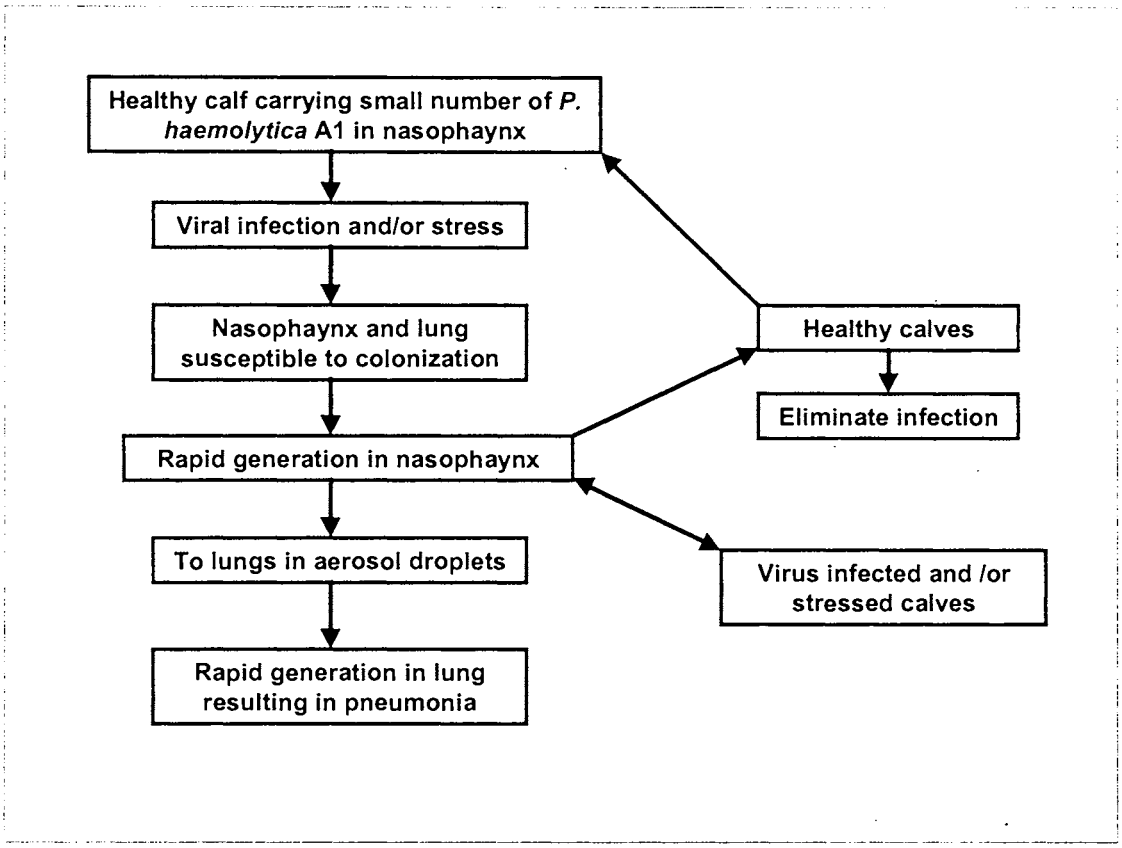

Fig. 1. Epidemiology and pathogenesis of *P. haemolytica* A1 (From Frank, 1986).

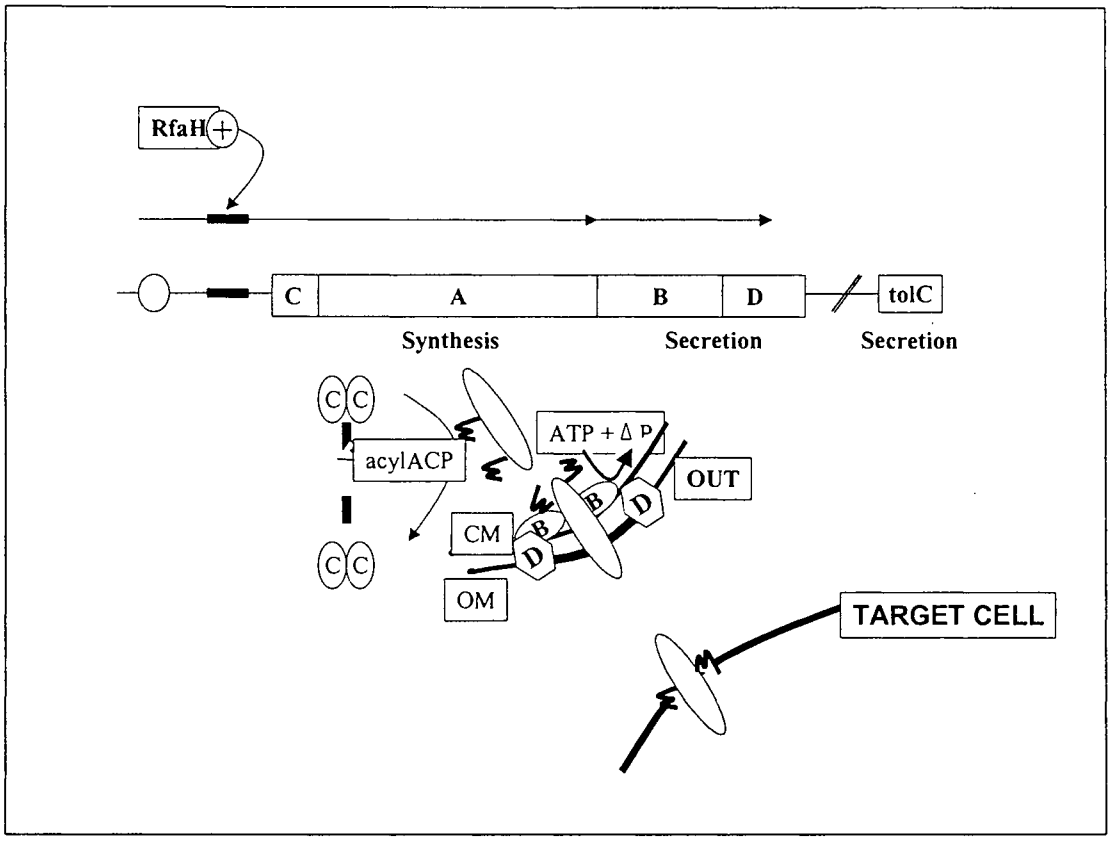

Fig. 2. The molecular model for RTX toxin synthesis, maturation, and export. Adapted from Stanley, et al. (1998). CM, cytoplasmic membrane; OM, outer membrane; ΔP, total proton motive force.

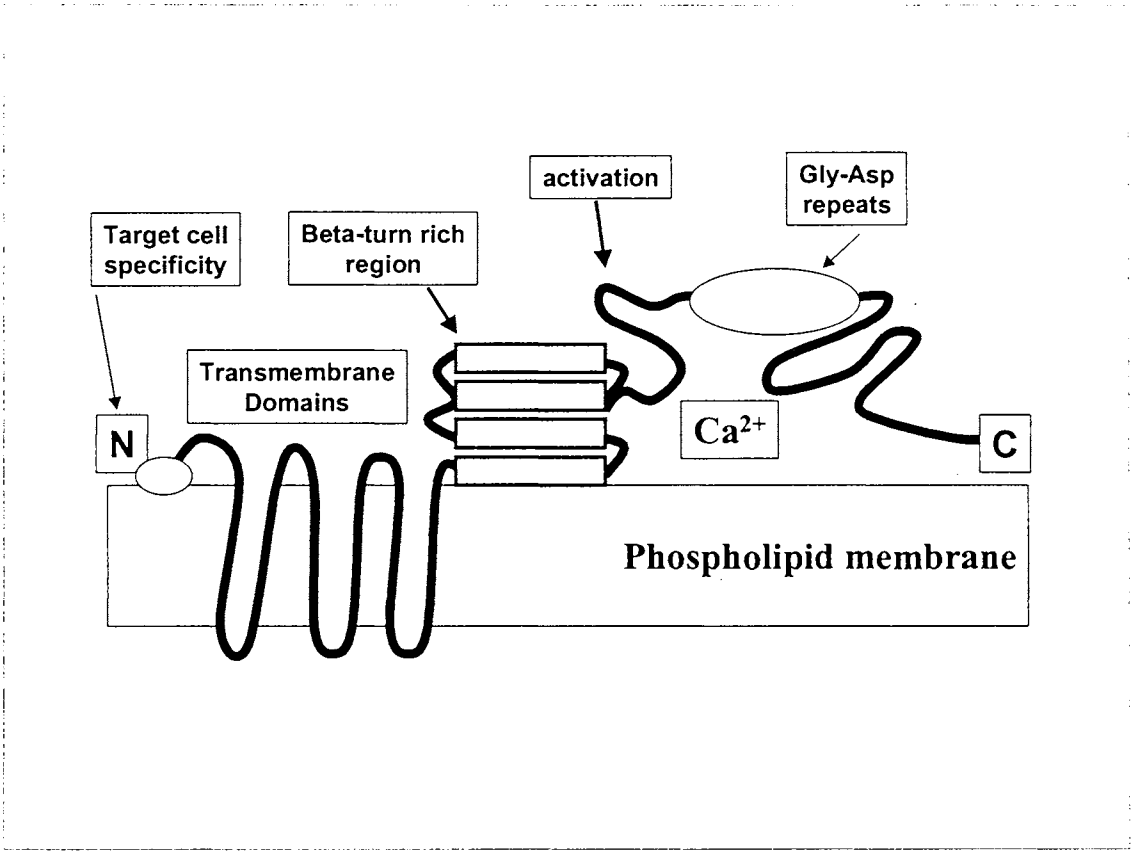

Fig. 3. Molecular model for interaction of LKT with target cell membrane. Adapted from Welch (1991). The model proposes that the transmembrane domains of LKT, either singly or in cooperation with other LKT molecules, form transmembrane pores that allow passage of monovalent and possibly divalent cations.

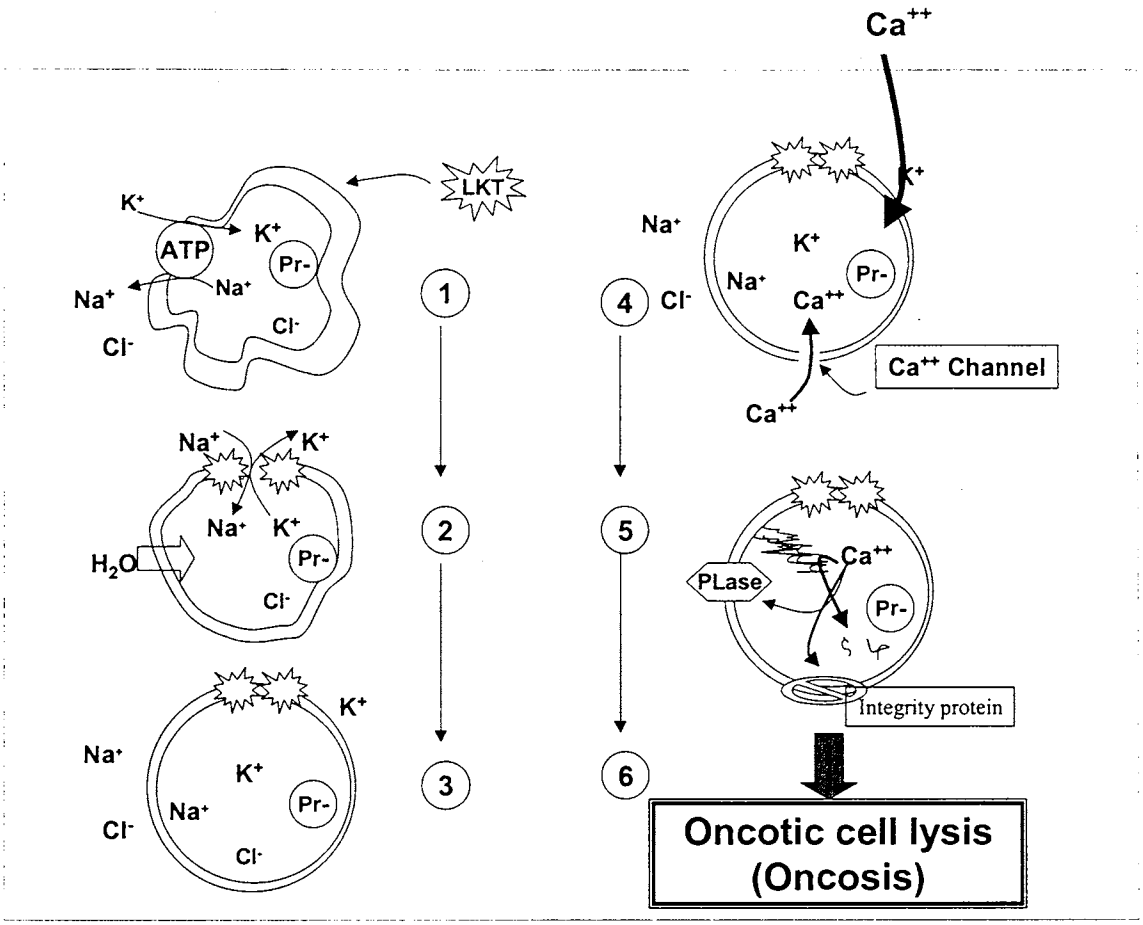

Fig. 4. Mechanism of high concentrations of LKT action on target cells. Adapted from Clinkenbeard, et al. (1992). In this figure, Pr- stands for intracellular proteins and Plase stands for phospholipase. This model illustrates that diffusion of monovalent ions across the transmembrane pores induced by LKT results in cell swelling. Calcium influx into target cells either through calcium channels or LKT-induced large defects on cytoplasmic membrane causes cell lysis.

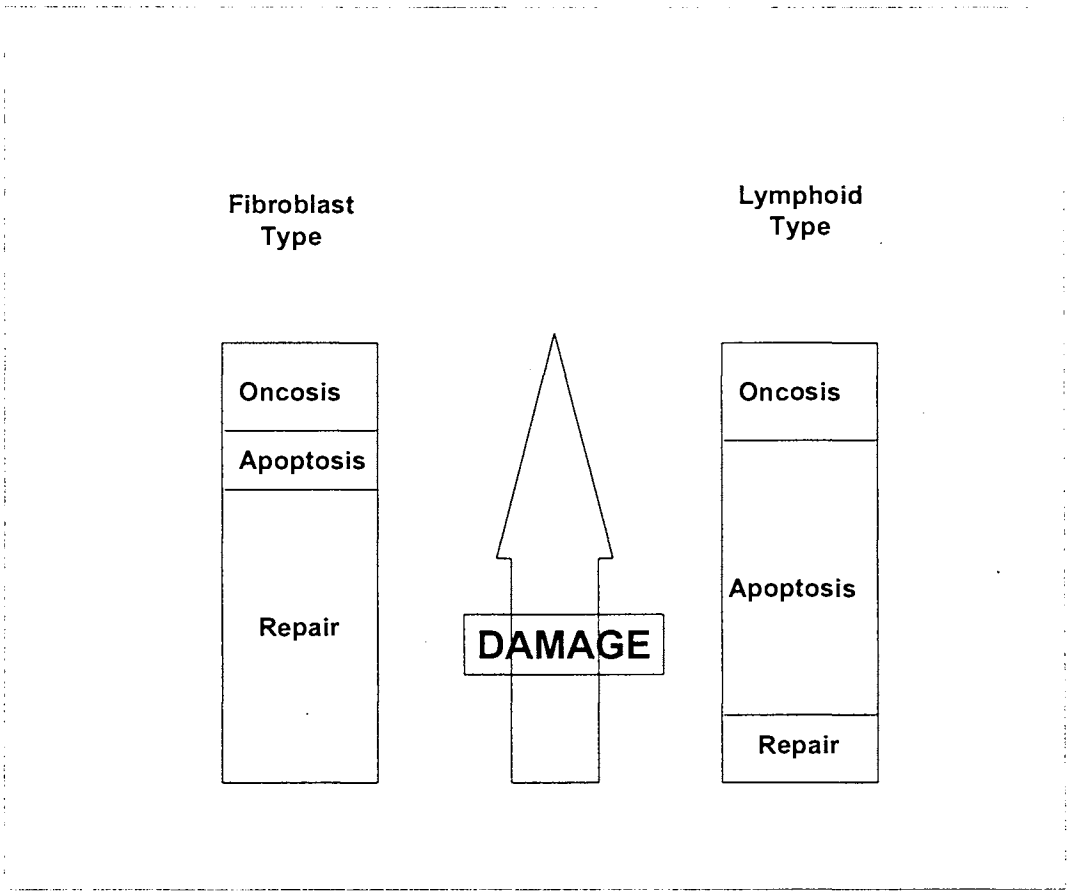

Fig. 5. Relationships between cell death by apoptosis *versus* oncosis and the severity of cellular insults versus cell types. Adapted from Cohen (1996) and Lieberthal and Levine (1996). Different cells have different capabilities of tolerating cellular insults, but generally, moderate insults induce cell death by apoptosis.

CHAPTER III

HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

Based on the observation that agents that are cytolytic at high concentrations may induce apoptosis at low concentrations, I hypothesized that *P. haemolytica* LKT, a cytolytic toxin at high concentrations, may induce apoptotic cell death in bovine lymphocytes at low concentrations.

This hypothesis was tested by characterization of apoptosis in isolated peripheral blood bovine lymphocytes incubated with different concentrations of LKT for various designated times. The criteria for assessment of apoptosis included:

1). Quantification of DNA fragmentation in bovine lymphocytes incubated with LKT using the diphenylamine colorimetric assay;

2). Verification and visualization of a DNA ladder pattern extracted from bovine lymphocytes incubated with LKT by agarose gel electrophoresis and cyto- or histochemical methods;

3). Demonstration of ultrastructural changes by transmission electron microscopy of bovine lymphocytes incubated with various concentration of LKT.

The specific objectives of this study were:

1). To optimize culture conditions for *P. haemolytica* LKT production as a source of LKT;

2). To examine the initial interaction of *P. haemolytica* LKT with target cells using the whole cell and isolated cytoplasmic membrane LKT binding assays.

3). To investigate whether *P. haemolytica* LKT is able to induce apoptosis in isolated bovine lymphocytes;

4). To determine whether DNA fragmentation is involved in LKT-induced apoptosis in bovine lymphocytes; and

5). To demonstrate the ultrastructural changes of bovine lymphocytes incubated with different concentrations of LKT and to determine whether LKT induces cell death by apoptosis or oncosis.

CHAPTER IV

SERUM-FREE CULTURE OF *PASTEURELLA HAEMOLYTICA* OPTIMIZED FOR LEUKOTOXIN PRODUCTION

This chapter was written in the format of and for publication in

American Journal of Veterinary Research

Structured abstract

Objectives - To screen supernatants of *Pasteurella haemolytica* cultures grown in 4 serum-free culture media for maximal leukotoxin (LKT) production and minimal protein concentration as an optimal source of LKT for purification.

Sample population - One *P haemolytica*, biotype A, serotype 1 strain originally isolated from the pneumonic lung of a calf.

Procedure - *P haemolytica* was grown in brain-heart infusion broth, yeast-tryptone broth, RPMI-1640 medium, and McCoy's modified 5A medium. Culture biomass and the protein concentration, LKT activity, and LKT concentration in culture supernatants were measured. Effects of media pH and supplementation with metal cations and glucose on growth rate of *P haemolytica* and culture supernatant parameters were evaluated.

Results - *P haemolytica* cultivated in brain-heart infusion broth or RPMI-1640 medium containing 0.1 M phosphate (pH6.8) produced the highest concentrations of LKT. Supplementation of RPMI-1640 medium with 0.36 mM $FeCl_3$ or 1.0 mM $MgSO_4$ further increased LKT specific activity in culture supernatant, but addition of 1 % glucose did not enhance LKT production. LKT production in $MgSO_4$-supplemented RPMI-1640 was comparable to that in serum protein-supplemented medium.

Conclusions - Although BHI was superior to RPMI-1640 for *P haemolytica* growth and LKT production, the higher protein concentration and lower LKT specific activity made BHI a less desirable medium compared to RPMI-1640. Growth rate and LKT production with minimal protein content was optimal in pH 6.8 phosphate-buffered $MgSO_4$-supplemented RPMI-1640 medium.

Clinical relevance -This medium can serve as a source of culture supernatant for purification of LKT.

Introduction

Pasteurella haemolytica, a facultative anaerobic Gram-negative coccobacillus, is a commensal of the oropharynx and upper respiratory tract of ruminants. [1] During conditions which can produce stress in cattle, such as shipment, viral infection, weaning, or overcrowding, *P haemolytica* proliferates in the upper respiratory tract, subsequently colonizes the lower airway, produces various virulence factors, and induces a profound inflammatory reaction, resulting in pneumonic pasteurellosis. [2] A ruminant leukocyte-specific cytolytic **leukotoxin (LKT)** produced by *P haemolytica* is considered to be a primary virulence factor. *Pasteurella haemolytica* LKT kills alveolar macrophages and neutrophils, thereby diminishing the general immune response against bacterial infection in the lung.

Pasteurella haemolytica LKT is produced at high concentrations only during late logarithmic growth phase.[3,4] The amount of LKT activity produced appears proportional to the rate of bacterial growth.[5,6] Numerous factors may affect the growth rate of *P haemolytica*, and thereby the concentration of LKT produced. Production of LKT, as described by Benson and coworkers[7] involves growth of *P haemolytica* in **brain-heart infusion (BHI)** broth for 4.5 hours followed by centrifugal collection and resuspension of the bacterial pellet in Hank's Basic Salts Solution-based Media 199 containing 10 to 20% fetal bovine serum. A common protocol currently in use was developed by Shewen and Wilkie[4] and is a modification of the original protocol, in which BHI broth culture of *P haemolytica* is used to inoculate RPMI-1640 tissue culture medium containing 7% FBS (replaces step involving resuspension in Media 199). This bacterial culture is then grown

in RMPI-1640 medium for 1 to 2 hours to logarithmic growth phase, and LKT in the culture supernatant separated by centrifugation.

Supplementation of culture media with serum proteins yields the highest growth rate (3-fold increase) and highest concentration of LKT activity (3- to 25-fold increase) in culture supernatants.[5,6,8] Use of selective supplementation of culture media with various serum proteins allowed identification of lactoferrin and **bovine serum albumin (BSA)** as serum components that enhance bacterial growth and LKT production.[5,9,10]

Although supplementation with serum protein enhances LKT production, this supplementation causes a practical problem for LKT purification. **Culture supernatants (CS)** from media supplemented with serum or BSA typically contain approximately 10-fold higher protein concentration than in CS from nonsupplemented media. Separation of LKT from protein-supplemented media has not been satisfactory.[11] The present study was undertaken to optimize culture of *P haemolytica* for LKT production in a serum-free medium to serve as a source of LKT for purification.

Materials and Methods

Culture protocol – A strain of *P haemolytica* biotype A serotype 1, originally isolated from the pneumonic lung of a calf and stored frozen as 15% glyceral-BHI stock culture at –135 C, was grown at 37 C with 5% CO_2 for 18 hours on BHI,[a] 5% bovine blood agar. Single colonies were used to inoculate 10 ml of BHI, **yeast-tryptone (YT)** broth containing 1.2% tryptone and 0.5% yeast extract, RPMI-1640,[b] or McCoy's modified 5A[c] liquid media. Inoculated cultures were incubated at 37 C with 5% CO_2 for

8 hours, after which 10 ml were used to inoculate 100 ml of the corresponding type of liquid medium (test medium) in 500 ml Erlenmeyer flasks. These cultures were incubated at 37 C with aerobic conditions and rotatory shaking at 120 oscillations/min. Five milliliters aliquots of cultures were collected at 0, 1, 2, 3, 4, and 5 hours for measurement of growth (absorbance at 600 nm [OD_{600nm}]) and pH, using a pH meter.[d] Culture supernatants were prepared from 5 ml aliquots by centrifugation at 12,000 x *g* for 30 minutes at 4 C followed by filtration through a 0.22-μm disposable filter[e] and storage at -135 C for assay of protein concentration, LKT activity, and LKT concentration. Protein concentration was measured with a **bicinchroninic acid (BCA)** assay.[f] The CS parameters were measured at 0, 1, 2, 3, 4, and 5 hours of culture.

Effect of media pH on growth and LKT production - The described culture protocol was used except that 0.1 M sodium phosphate or 0.1 M citric acid-phosphate was added to each of the test media and pH was adjusted by addition of HCl or NaOH to pH 6.0, 6.4, 6.8, 7.2, 7.4, 7.6, or 8.0 (sodium phosphate buffer) or to pH 5.2 or 5.6 (citric acid-phosphate buffer) prior to sterilization. Media pH, culture growth, and CS parameters were measured at 0, 1, 2, 3, and 4 hours.

Effect of metal cation or glucose supplementation on *P haemolytica* growth and LKT production - The described culture protocol was used with RPMI-1640 buffered with 0.1 M sodium phosphate, pH 6.8 as a basal medium to which supplements of 361.4 μM $FeSO_4$,[12] 361.4 μM $FeCl_3$,[6,9] 9.0 mg/100 ml $CaCl_2$,[13] 1 mM $MgSO_4$,[14] 1.2 μM $CuCl_2$,[15] 0.1μM $ZnSO_4$,[16] 10 μM $MnCl_2$,[16,17] 0.5g/100ml BSA,[10] or 1g/100 ml glucose[b,5] were added. Culture growth and CS parameters were measured at 0, 1, 2, 3, and 4 hours.

LKT activity - Activity of LKT was assayed by measuring leakage of the intracellular enzyme **lactate dehydrogenase (LDH)** from bovine lymphoma cells (BL3 cells).[g,18] Bovine lymphoma cells were enumerated by a hemocytometer, and collected by centrifugation at 700 x *g* for 15 minutes. Cell pellet was resuspended in RPMI-1640 medium, and the final concentration of BL3 cells was adjusted to 4 x 10^6 cells/ml. Aliquots of 100 µl of BL3 cells were added to 100 µl of serially diluted CS in a 96-well round-bottom microtiter plate. Leakage of LDH was maximized by addition of 0.1% t-octylphenoxypolyethoxyethanol[h] (v/v) and background leakage was determined by exposure of cells to RPMI-1640 only. Plates were incubated at 37 C for 2 hours, and exposure terminated by centrifugation at 700 x *g* for 10 minutes. One hundred-µl aliquots of supernatant were transferred to 96-well flat-bottom microtiter plates, and plates were warmed to 37 C. Activity of LDH was assessed kinetically by adding 100 µl of assay reagent LDH-L 50,[b] held at 37 C, to all wells and measuring the absorbance at 340 nm (OD_{340nm}) per minute in a thermally controlled, kinetic microtiter plate reader[i] for 2 minutes at 37 C. Specific leakage of LDH was calculated as a percentage as follows:

$$\% \text{ specific LDH leakage} = [(A-B)/(C-B)] \times 100$$

in which A = LKT-induced LDH leakage, B = RPMI-1640 negative control, and C = t-octylphenoxypolyethoxyethanol positive control. The LKT activity was enumerated as **toxic units (TU)** that were determined by graphing specific LDH leakage versus the CS dilution factor. One TU was defined as the dilution factor at which the specific LDH leakage under the assay conditions described is 50%.[18]

LKT concentration - Concentrations of LKT were assayed by a sandwich ELISA.[j] Plates[k] were coated at 37 C for 2 hours with rabbit polyclonal antisera produced

against *P haemolytica* LKT diluted 1:500 in 15 mM carbonate, 35 mM bicarbonate buffer (pH 9.6). After washing the plate 3 times with **phosphate buffered saline (PBS)-0.05% polyoxyethylene-20-sorbitan monolaurate[l] (PBST)** to remove unbound antibody, wells were blocked with 200 µl of 0.3% nonfat dried milk in PBS for 2 hours at 37 C. One hundred-µl aliquots of serial dilutions of CS, partially purified LKT, or control media in PBST-0.3% nonfat dried milk were added to wells and incubated for 2 hours at 37 C. After washing the plate 3 times with PBST to remove unbound LKT, 100 µl of 1:500 diluted anti-LKT **monoclonal antibody (MAb)** C6 [19] was added to wells and incubated for 2 hours at 37 C. Removal of unbound MAb was followed by addition of 100 µl of 1:500 diluted anti-mouse IgG (γ-chain specific) biotin conjugate[b] (in PBST-0.3% nonfat dried milk) and incubation for 2 hours. After washing the plate with PBST, 100 µl of 1:1,000 avidin-horseradish peroxidase[m] in PBST-0.3% nonfat dried milk was added and incubated at 37 C for 2 hours. Finally, plates were washed 3 times with PBST followed by the addition of substrate (0.8 mM 2, 2'-azino-bis ethybenzthiazoline-6-sulfonic acid,[b] 0.001% H_2O_2 in 0.1 M citrate buffer, pH 4.8). Plates were incubated for at least 45 minutes at room temperature (approximately 25 C) and absorbance was measured colorimetrically at 405 nm (OD_{405}) with a microtiter plate reader.[n] Partially purified *P haemolytica* LKT was used as the toxin standard.[20] This LKT was judged to be >90% homogenous by silver stained band density on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The major contaminant of this LKT was nonbicinchroninic acid-reactive lipopolysaccharide. The amount of LKT in CS was determined, using a microplate manager reader program.[o] The correlation coefficient for the standard curve was 0.95. Controls in this assay included: 1) partially purified LKT as standard; 2) diluent without LKT, anti-

LKT MAb C6 without LKT, and LKT-negative mouse ascitic fluid as negative controls. This assay was validated by deletion of each major component (ie, capture antibody rabbit polyclonal antiserum, LKT, detector antibody MAb C6, anti-mouse IgG biotin conjugate, or avidin-horseradish peroxidase), which did not increase background absorbance of this sandwich ELISA.

Statistical analysis - Experimental assays were conducted in triplicate. Data analyses were performed by ANOVA, one way comparison.[p] Differences were considered at $P < 0.05$, unless stated otherwise.

Results

Initial OD_{600nm} of the cultures at 0 hours was approximately 0.05. After a 1-hour lag period during which growth was initiated, test cultures entered logarithmic growth. Late-logarithmic growth phase was reached at approximately 4 hours and stationary phase at approximately 5 hours (data not shown). Production of LKT paralleled growth rate, and was maximal during late-logarithmic growth phase and declined during stationary phase. For culture media with an initial pH of 7.2, the 4-hour cultures were slightly acidified (< 0.1 pH units) for BHI and YT (without added buffer), but moderately alkalinized for 26 mM $NaHCO_3$ buffered RPMI-1640 (0.7 pH units) and McCoy's modified 5A (0.3 pH units). Replacement of bicarbonate buffer with 0.1M phosphate decreased alkalization of the cultures in RPMI-1640 and McCoy's modified 5A to < 0.1 pH units.

By use of 0.1 M sodium phosphate- or citrate-phosphate-buffered test media, the shortest culture biomass doubling times (data not shown) and maximal LKT activity (Fig 1, data not shown for YT or McCoy's modified 5A media) occurred in all test media at pH 6.8. Of the 4 test media, use of BHI and RPMI-1640 yielded the shortest doubling times and highest CS LKT concentration and LKT activity in CS (Table 1).

Protein concentration of uninoculated media was 3.3 and 1.6 mg/ml for BHI and YT media, respectively, but uninoculated RPMI-1640 and McCoy's modified 5A did not contain protein. Growth of *P haemolytica* in BHI or YT resulted in a decreased protein concentration of CS, whereas growth in RPMI-1640 or McCoy's modified 5A media resulted in increased CS protein concentration (Table 1).

For protein purification, specific activity (TU/mg CS protein) related the amount of LKT to the amount of protein from which it must be separated during purification. The highest LKT specific activity was obtained by use of RPMI-1640 medium. Although more LKT (activity and ng toxin) was produced in BHI medium, the 10-fold higher protein concentration in CS by use of BHI made this medium inferior to RPMI-1640 medium, which yielded a similar LKT concentration, but had much lower protein concentration in CS.

Phosphate buffered RPMI-1640 medium, pH 6.8 was chosen as a basal medium in which to test effects of supplementation with Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, glucose or combinations of these supplements. This medium, supplemented with 0.5% BSA, was used as a positive control for maximization of LKT activity. The shortest doubling times, highest culture OD_{600nm} and CS LKT activities were obtained for RPMI-1640 supplemented with Fe^{3+}, Mg^{2+}, and BSA (Table 2). The positive effect of BSA

supplementation in RPMI-1640, pH 6.8 was much less than that previously reported[10] for bicarbonate-buffered RPMI, pH 7.2. Combining Fe^{3+} with BSA supplementation resulted in a longer doubling time and lower LKT activity and concentration. Addition of Mg^{2+} to this combined supplementation resulted in a further diminution of effect. Supplementation of RPMI-1640 with glucose did not have a significant positive effect on doubling times or LKT activity and concentration in CS, but resulted in < 0.1 pH unit acidification of the 0.1 M sodium phosphate-buffered RPMI-1640 medium.

Supplementation of RPMI-1640 with Mg^{2+} resulted in the highest LKT specific activity. The optimum level of Mg^{2+} supplementation was tested in 0.1 M sodium phosphate-buffered RPMI-1640 medium by supplementation with (0.1, 1.0 and 10.0 mM) or without $MgSO_4$. The LKT activity and concentration was enhanced for 1.0 mM, but was similar to non-supplemented RPMI-1640 for 0.1 and 10.0 mM $MgCl_2$-supplemented RPMI-1640.

Discussion

The LKT produced by *P haemolytica* is difficult to purify.[11] Leukotoxin is produced only during late-logarithmic phase,[4] and then, as determined in this study, at low concentrations (100 to 500 ng LKT/ml). The requirement for 10 to 100 mg/ml of serum protein in culture medium to obtain maximal concentrations of LKT makes subsequent removal of these serum proteins an additional barrier to purification.[5,11]

Using the Shewen-Wilkie culture protocol[4], supplementation of RPMI-1640 with 0.5% BSA results in approximately 4-fold increase in LKT concentration,[8] which may be

caused by increased growth rate of the bacteria during late logarithmic phase.[10] The role of bovine serum albumin as a nutrient is supported by the decrease in CS protein concentration from 5.0 to 1.4 mg/ml during 4-hour growth in 0.5% BSA supplemented RPMI-1640 observed in the present study (Table 2). However, BSA may have undergone catabolism without being incorporated into cells or LKT. The amount of LKT produced appears proportional to the growth rate of the culture. Therefore, if catabolism of BSA increased LKT concentration by increasing the growth rate of *P haemolytica*, then optimization of media pH and metal cation supplementation to increase the growth rate of *P haemolytica* would likely result in increased LKT production.

A variety of culture media have been used for LKT production, but serum-supplemented BHI and RPMI-1640 are the most common. Of the serum-free media screened for *P haemolytica* growth and LKT production in our study, BHI and RPMI-1640 proved superior. Brain-heart-infusion medium supported superior growth and LKT production compared to that in YT, a similar complex medium, which may be related to the medium's protein concentration. Brain-heart-infusion medium has a higher protein concentration than YT, and higher catabolism of media protein hypothetically supports more rapid growth of *P haemolytica* and higher LKT production.[5,6] Likewise, RPMI-1640 and McCoy's modified 5A media have similar formulations. However, substances responsible for superior growth in RPMI-1640 compared with McCoy's modified 5A medium were not apparent. Recently, Highlander[5] identified several substances in a chemically defined medium that inhibited *P haemolytica* growth; however, RPMI-1640 and McCoy's modified 5A media contain only 2 of these substances, L-serine and L-methionine, and in similar concentrations.

Acidification of media to a pH < 6.8 is one factor that limits *P haemolytica* growth in culture.[21] The buffer for RPMI-1640 medium in the Shewen-Wilkie protocol was not specified, but 25 mM bicarbonate-buffered RPMI-1640 medium is the most common form supplied. Medium pH typically used for LKT production is 7.2 to 7.3. As indicated by results of our study, bicarbonate-buffered RPMI-1640 was insufficient to maintain a stable culture pH. Paradoxically, we observed that bicarbonate-buffered tissue culture media was alkalinized rather than acidified. Alkalinization may develop by a combination of neutralization of the bicarbonate by fermentation acid and subsequent loss of CO_2 from the medium and by catabolism of amino acids in the medium to ammonium ions.

Replacement of bicarbonate buffer with 0.1 M sodium phosphate buffer was sufficient to maintain a stable pH throughout the growth period. Concentration of phosphate buffer > 0.05 M inhibits *P haemolytica* growth in a casein hydrolysate medium[21], but addition of 0.025 M potassium phosphate to the medium is not sufficient to maintain a stable pH. Medium with a pH of 7.5 is acidified to approximately pH 6.5 during fermentation of 1.0% galactose.[21] In our study, 0.1 M sodium phosphate was not inhibitory if added to RPMI-1640 medium, and 0.1 M phosphate-buffered RPMI-1640 was sufficient to maintain a stable pH even during fermentation of 1.0% glucose. Optimal pH for all 4 test media for culture of *P haemolytica* reported herein was pH 6.8. In RPMI-1640, changing the medium pH from 7.2 to 6.8 resulted in a 25-minute shorter doubling time and a 6-fold increase in LKT concentration.

Supplementation with magnesium had previous been reported to promote an optimal rate and total amount of growth of *P haemolytica*.[21] Likewise, in our study,

$MgSO_4$ supplementation optimized growth, and that was associated with increased LKT production. Nonsupplemented RPMI-1640 medium contains 0.4 mM $MgSO_4$, however, the concentration of free Mg^{2+} in 0.1 M phosphate-buffered RPMI-1640 is approximately 10 μM. Supplementation of 0.1 M phosphate-buffered RPMI-1640 with 1.0 mM $MgSO_4$ increases the concentration of free Mg^{2+} to approximately 50 μM. It is possible that, in addition to the effect of Mg^{2+} on increased growth rate, Mg^{2+} supplementation may have some direct effects on LKT activity. The LKT activity-to-concentration ratio was 1.7 times greater in 1 mM $MgSO_4$-supplemented versus nonsupplemented RPMI-1640 medium.

Iron-containing compounds enhance LKT production by *P haemolytica*,[9] but non-supplemented RPMI-1640 does not contain iron. Ferric or ferrous salt supplementation of RPMI-1640 in the absence of suitable organic or protein siderophores has been reported to be ineffective for increasing LKT production.[9] Our results differed. Although Fe^{3+} supplementation did increase LKT concentration in CS, it also increased protein concentration such that specific activity of LKT was not optimized as compared with that obtained $MgSO_4$ supplementation.

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[1] DIFCO Laboratories, Detroit, Mich.

[b]Sigma Chemical Co, St Louis, Mo.

[c]KC Biological, Lenexa, Kan.

[d]Corning Inc., Corning, NY.

[e]Nalgene Filtunit TYP S CN, Nalge, Rochester, NY.

[f]Pierce, Rockford, Ill.

[g]BL3 cells, CRL 8037, American Type Culture Collection, Rockville, Md

[h]Triton X-100, Sigma Chemical Co., St. Louis, Mo.

[i]Thermomax, Molecular Devices Palo Alto, Calif.

[j]Sun YD, Nagaraja TG, Chengappa MM, et al. 75th annual meeting of the conference of research workers in animal diseases. 20. 1994.

[k]Immulon 1, Dynatech Laboratories Inc., Virg.

[l]Tween® 20, Fisher Scientific, Fair Lawn, NJ.

[m]Bio-Rad Laboratories, Hercules, Calif.

[n]Molecular Devices, Menlo Park, Calif.

[o]BioRad/Microsoft-Window, Bio-Rad Laboratories, Hercules, Calif.

[p]SigmaStat, Jandel Scientific Software, San Rafael, Calif.

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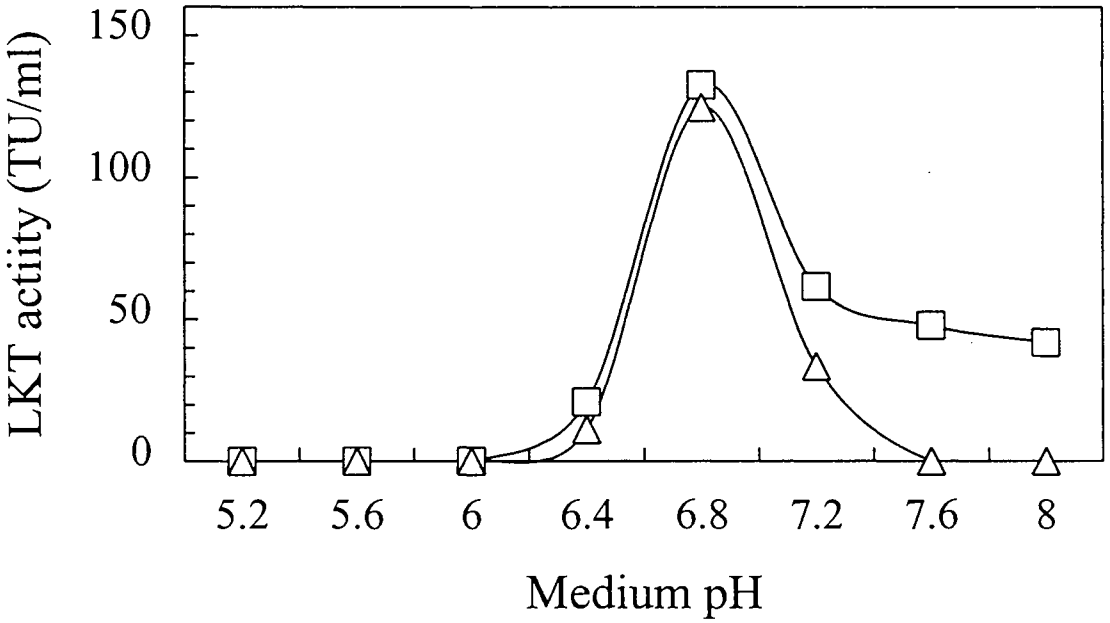

Fig. 1. Activity of leukotoxin (LKT) against bovine lymphoma cells of culture supernatants from the phosphate buffered RPMI (Δ) or BHI (□) with different pH values. LKT activity units were defined as the highest LKT dilution that caused 50% LDH leakage from target cells.

Table 1. Growth characteristics of *Pasteurella haemolytica* and leukotoxin (LKT) production in phosphate-buffered test media at pH 6.8

Medium	Biomass*	Doubling time (min)	Protein concentration in CS (mg/ml)	LKT concentration† (μg/ml)	LKT activity‡ (TU/ml)	LKT specific activity (TU/mg CS protein)
Brain-heart-infusion	0.594[a]	40	2.51[a]	62.5[a]	174[a]	69
Yeast-tryptone	0.485[b]	90	1.33[b]	19.6[b]	22[b]	16
RPMI	0.621[a]	75	0.24[c]	44.2[c]	165[c]	688
McCoy's	0.492[b]	100	0.20[c]	5.5[d]	44[d]	222

*Absorbance at 600nm after 4 hour incubation.

†Measured using a sandwich ELISA with rabbit anti-LKT polyclonal antibody as capture antibody and C6 monoclonal antibody against LKT as detector antibody. Partially purified LKT was used as standard.

‡TU = toxic units; defined as the highest LKT dilution at which leakage of lactate dehydrogenase from target cells was 50%.

Values in a column with different superscripts are significantly different from one another at $P < 0.05$.

CS = culture supernatant.

Table 2. Effects of supplementation of phosphate-buffered RPMI-1640 medium on growth characteristics of and leukotoxin (LKT) production by *Pasteurella haemolytica*

Supplement	Biomass*	Doubling time (min)	Protein concentration in CS (mg/ml)	LKT concentration† (μg/ml)	LKT activity‡ (TU/ml)	LKT specific activity (TU/mg CS protein)
None	0.719[c]	75	0.24[d,e]	41.7[c]	147[c]	613
Fe^{3+}	0.870[b]	60	0.37[d]	47.4[a]	234[b]	632
Fe^{2+}	0.570[e]	90	0.11[f]	36.1[d]	111[e]	1000
Ca^{2+}	0.430[g]	100	0.26[d,e]	17.3[e]	84[f]	323
Mg^{2+}	0.972[a]	50	0.31[d]	63.7[b]	378[a]	1220
Cu^{2+}	0.870[b]	70	0.31[d]	44.3[a,c]	223[b]	719
Zn^{2+}	0.813[d]	70	0.33[d]	42.2[c]	194[d]	583
Mn^{2+}	0.522[f]	100	0.45[c]	33.0[d]	128[c,e]	284
BSA	0.863[b]	50	1.43[b]	47.7[a]	239[b]	167
BSA+Fe^{3+}	0.692[c]	80	1.43[b]	41.9[c]	169[c]	118
BSA+Fe^{3+}+Mg^{2+}	0.445[g]	120	2.09[a]	6.0[f]	24[g]	15
Mg^{2+} + glucose	0.965[a]	50	0.32[d]	59.6[b]	351[a]	1097

Key see Table 1. BSA = bovine serum albumin. Additional details concerning formulation of media appears in Materials and Methods.

Table 3. Effect of concentration of Mg^{2+} in phosphate-buffered RPMI-1640 on leukotoxin (LKT) production

Supplement	Biomass*	Doubling time (min)	Protein concentration in CS (mg/ml)	LKT concentration† (μg/ml)	LKT activity‡ (TU/ml)	LKT specific activity (TU/mg CS protein)
None	0.750[a]	75	0.26[a]	42.1a	159[a]	612
0.5% BSA	0.901[b]	50	1.62[b]	49.2[b]	279[b]	172
0.1 mM Mg^{2+}	0.876[c]	60	0.30[a]	43.9[a]	172[a]	573
1 mM Mg^{2+}	0.960[d]	50	0.32[c]	64.2[c]	386[c]	1206
10 mM Mg^{2+}	0.882[c]	60	0.28[a]	44.1[a]	187[a]	668

See Table 1 for key.

CHAPTER V

BINDING OF *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN TO TARGET CELLS FROM DIFFERENT SPECIES

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Abstract

Pasteurella haemolytica, the causative agent of bovine pneumonic pasteurellosis, produces a leukotoxin (LKT), which is specific for intoxication of ruminant leukocytes and platelets. In this study, we examined the interaction of LKT with three cultured cell lines (BL3, Raji, and HL60 cells) and isolated peripheral blood lymphocytes from four species of animals (bovine, equine, porcine and canine) by measurement of LKT binding, intracellular Ca^{2+} concentration, and cytoplasmic enzyme lactate dehydrogenase leakage (LDH). LKT bound to all target cells, except HL60 cells, in a concentration-dependent manner at room temperature, however, only bovine origin cell line BL3 cells and isolated bovine lymphocytes were susceptible to LKT-induced increases in intracellular Ca^{2+} concentration and cytoplasmic enzyme LDH leakage. Exposure of HL60 cells to LKT at low temperature (0 °C), or in the presence of protease inhibitor cocktail at room temperature enhanced LKT binding, implying that at room temperature, no detectable LKT binding was due to proteolysis of LKT by HL60 cells. Treatment of BL3 cells with 80 to 200 μg ml^{-1} protease K prior to LKT exposure decreased LKT binding and LDH leakage, but decrease in LDH leakage was not proportional to the reduction in LKT binding. We conclude that *P. haemolytica* LKT binds to both susceptible and non-susceptible target cells, but simply binding is not enough to induce Ca^{2+} influx and LDH leakage from target cells.

Key words: Dot blot; Leukotoxin; *Pasteurella haemolytica*; Receptor; Western blot

1. Introduction

Pasteurella haemolytica, the causative agent of bovine pneumonic pasteurellosis, produces several virulence factors, including leukotoxin (LKT), endotoxin, protease, neurominidase and outer membrane proteins (Confer, et al, 1988). Among these virulence factors, LKT is considered to be the primary virulence factor (Petras, et al. 1995; Murphy, et al., 1995; Fedorova and Highlander, 1997; Tatum, et al., 1998). *P. haemolytica* LKT is a member of the "repeats-in-toxins" (RTX) family of gram-negative bacterial pore-forming toxins (Welch, 1991). Generally, RTX toxins are divided into two classes, hemolysins and leukotoxins, based on their target cell specificity. For example, *Escherichia coli* α-hemolysin is able to intoxicate a broad range of target cells, but *P. haemolytica* LKT and *Actinobacillus actinomycetemcomitans* leukotoxin (Ltx) only intoxicate a narrow range of target cells limited primarily to leukocytes from ruminant and primates, respectively (Shewen and Wilkie, 1982; Tsai, et al., 1984). The molecular basis of this cell-type and species specificity to RTX toxin-mediated intoxication is partially solved. A receptor for Ltx and *E. coli* α-hemolysin mediated cell lysis has been identified as lymphocyte function-associated antigen 1 (LFA-1) on susceptible cells (Lally, et al., 1997). However, several questions still remain to be answered. For example, LFA-1 is only present on leukocytes but not on red blood cells, nor on cells of non-hematopoietic origin, both of which are susceptible to hemolysin-mediated lysis. Furthermore, whether RTX toxins only bind to susceptible cells and lead to cell lysis is

still controversial (Taichman, et al., 1991; Sato, et al., 1993; Iwaki, et al., 1995; Brown, et al. 1997).

Studies on *A. actinomycetemcomitans* Ltx binding indicated that Ltx not only binds to susceptible cells, such as human neutrophils and mononuclear leukocytes but also binds to non-susceptible cells K562 human erythroleukemic cells and mouse SP2 myeloma cells (Taichman, et al., 1991; Sato, et al., 1993). Adenylate cyclase-hemolysin (AC-Hly) from *Bordetella pertussis* unsaturably binds to sheep erythrocytes, rather than through high-affinity surface receptors for AC-Hly binding (Iwaki, et al., 1995). An indirect evidence also suggests that *E. coli* α-hemolysin unsaturably binds to the artificial liposomal membranes which are made with phospholipids. In the presence of Ca^{2+}, it is able to form pores in protein-free liposomes (Ostolaza and Goñi, 1995; Menestrina, 1988), which implicates that *E. coli* α-hemolysin does not need specific receptors to execute its cytolytic function. For *P. haemolytica* LKT, it has been reported that the biotinylated *P. haemolytica* LKT only binds to susceptible bovine leukocytes rather than non-susceptible porcine leukocytes, as assessed by flow cytometry (Brown, et al., 1997), therefore, only susceptible cells are intoxicated. Others reported that *P. haemolytica* LKT binds to susceptible BL3 cells, as well as some non-susceptible porcine alveolar macrophages, but not non-susceptible bovine endothelial cells (Kannan, et al., 1997). These contradictory reports have impeded our better understanding of LKT-target cell interaction and molecular pathogenesis of bovine pneumonic pasteurellosis.

Elucidation of the initial interaction of LKT with its target cells may provide information beneficial for formulation of novel therapeutic strategies. Therefore, the purpose of this study was to investigate whether *P. haemolytica* LKT binding is specific

to susceptible target cells. In the present study, two binding assays, the whole cell LKT binding and cytoplasmic membrane binding, were employed to determine LKT binding. LKT-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cytoplasmic enzyme lactate dehydrogenase (LDH) leakage were used to determine the susceptibility of target cells to LKT-mediated intoxication.

2. Materials and methods

2.1. Preparation of LKT and LKT-minus mutant concentrated culture supernatants

The LKT and LKT-minus mutant concentrated culture supernatants were obtained from a wild type strain of *P. haemolytica* and its isogenic strain with an allelic *lktCA*$^-$ replacement mutation (Murphy, et al., 1995). The concentrated culture supernatants were prepared by inoculating 1 liter of RPMI medium containing 2.2 g l^{-1} $NaHCO_3$ to an OD_{600nm} = 0.25 with wild type or mutant *P. haemolytica* prepared by growth overnight on 5% bovine blood agar and then in brain-heart infusion media to late logarithmic phase. The cultures in RPMI medium were grown at 37°C with 120 oscillations/minute for approximately 2.5 hours to an OD_{600nm} = 0.9 to 1.0 (Sun and Clinkenbeard, 1998). All subsequent steps were conducted at 4°C. The bacteria were removed by centrifugation, and culture supernatant was concentrated and partially purified by fractional precipitation (0-60% saturation by addition of 361 g l^{-1} solid ammonium sulfate). The precipitates were resuspended at 0.5 mg protein ml^{-1} in 20 ml of 50 mM sodium phosphate, 100 mM sodium chloride buffer, pH 7.0 (phosphate-NaCl), dialyzed against the same buffer, and

stored at -135°C. The protein content of the LKT preparations was ≈ 50% greater than the LKT-minus mutant preparations (Murphy, et al., 1995).

2.2. Cell culture

Bovine lymphoma BL3 (CRL8037), human promyelocytic leukemia HL60 (CCL240), and human Burkitt lymphoma Raji (CCL86) cells were obtained from and cultured as described by the American Type Culture Collection (Rockville, MD).

2.3. Isolation of bovine peripheral blood lymphocytes

Two healthy beef calves (approximately 200 Kg) served as blood donors for isolation of lymphocytes. Lymphocytes were isolated as previously described (Reeves and Renshaw, 1978) with modifications. Briefly, the blood sample was collected from healthy cattle by venipuncture of the jugular vein, and anticoagulant (sodium heparin, 15 U ml^{-1}) was added. Four-five ml of heparinized blood in a 50-ml conical centrifuge tube was centrifuged at 600 x *g* for 30 minutes at 22°C. The plasma was aspirated down to about 10 -15 mm above the interface between the erythrocytes and the plasma. The cells at the interface were carefully pipetted into another centrifuge tube with as few erythrocytes as possible and were diluted (1:3, v/v) with Hank's balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free). Thirty to thirty-five ml of diluted cells were carefully layered onto 15 ml of Ficoll-Paque (Sigma Chemical Co, St. Louis, MO) and centrifuged at 500 x *g* for 20 minutes with the brake in the off-position. After centrifugation,

lymphocytes and monocytes in the interface between plasma and the Ficoll-Paque were pipetted into another 50 ml conical centrifuge tube, washed twice with 30 ml of HBSS and resuspended in RPMI medium containing 10% fetal bovine serum (FBS) at 5 x 10^6 cells ml^{-1}. This final cell suspension was free of erythrocytes and contained approximately 90% lymphocytes (mean 89.9, range 72 - 99%) and 5% monocytes (mean 4.5, range 0 - 9%), based on differential counting. To remove the monocytes, 5 ml of cell suspension were placed into a 50 mm tissue culture-treated Nunclon petri dish (Nalge Nunc International, Milwaukee, WI) and incubated at 37°C for 2 hours. Following the incubation, monocytes adhered onto the plate surface, and 99% of the non-adherent cells were lymphocytes. Greater than 95% of the cell population was viable based trypan blue exclusion.

The procedures for isolation of equine, porcine, or canine peripheral blood lymphocytes from healthy animals were basically the same as that for bovine lymphocytes.

2.4. Isolation of cytoplasmic membranes from cultured cell lines

Cytoplasmic membranes were prepared from 1 x 10^9 PBS-washed cultured cells (BL3, Raji, or HL60 cells) in 50 ml of 50 mM 3-(N-morpholino)propanesulfonate (MOPS), 100 mM NaCl buffer, pH 7.0 (MOPS-NaCl) by gently breaking cells by 5 passes of a Potter-Elvenhjem tissue grinder with 0.1 mm clearance. Unbroken cells and nuclei were removed by centrifugation at 700 x *g* for 15 minutes, the plasma membranes collected by centrifugation at 25,000 x *g* for 45 minutes, washed twice with distilled

water, and resuspended in MOPS-NaCl buffer at a concentration of 2 mg protein/ml (BCA microprotein assay, Pierce Chemical Co., Rockford, IL) and stored at -135°C. The isolated membranes had similar phospholipid to protein ratios (0.76 , 0.60 , and 0.70 mg phospholipid mg^{-1} protein for BL3, HL60 and Raji cells, respectively) (Zhou and Arthur, 1992).

2.5. Assay of intracellular Ca^{2+} concentration using Flou3

PBS-washed tissue cultured cell lines (BL3, Raji, or HL60 cells) protected from light were loaded with Fluo3 (Molecular Probes Inc., Eugene, OR) by incubating cells in 5 μM acetoxymethyl ester of Fluo3 (in DMSO containing 0.14% pluronic acid) for 30 minutes at 25°C with constantly mixing on a cell rotator (Angenics, Cambridge, MA). The Fluo3 loaded cells were collected by centrifugation at 200 x *g* for 15 minutes at 4°C, washed with 10 ml PBS, resuspended in 3 ml HBSS without phenol red, Ca^{2+}, or Mg^{2+} (HBSS-Ca-Mg), then enumerated by hemocytometer. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured on Fluo3 loaded BL3, HL60, or Raji cells (1.0 x 10^7 cells ml^{-1}) incubated at 37°C in HBSS-Ca-Mg containing 1 mM $CaCl_2$ and 1:250 anti-fluorescein antibody and exposed in triplicate to 1:50 diluted LKT or LKT-minus mutant, 4 μM 4-bromo A23187, or PBS. Fluorescence intensity (490 nm excitation, 523 nm emission) was measured in a fluorescence plate reader (Cytofluor 2300 Fluorescence Measurement System, Millipore Corporation, Bedford, MA).

2.6. Assay of cell lysis

Following measurement of Fluo3 fluorescence intensity, the cells were spun down at 700 x *g* for 5 minutes, and cell lysis was determined by measuring intracellular enzyme lactate dehydrogenase (LDH) leakage. LDH was assayed by transfer of 100 µl incubation supernatant to wells of a 96-well flat bottom microtiter plate. The plate was warmed to 37 °C, and then 100 µl of LDH assay reagent (LDH-L 50) (Sigma Chemical Co., St. Louis, MO) at 37°C was added to each well. LDH activity was measured in a thermally-controlled kinetic microtiter plate reader (ThermoMax, Molecular Devices, Palo Alto, CA) at 340 nm for 2 minutes at 37°C. Data were reported as mOD $minute^{-1}$. Maximal LDH leakage was determined by exposing cells to 0.1% Triton X100, and a set of PBS control cells containing no Fluo3 was used as the background LDH leakage.

2.7. Whole cell LKT binding detected with immunological dot blot

The cultured cell lines (BL3, Raji, and HL60 cells) and isolated blood lymphocytes from bovine, equine, porcine and canine were exposed to various concentrations of LKT, or LKT-minus mutant, or PBS at room temperature for 0.5, 1, 3, 5, 10 or 20 minutes. After incubation, the cells were washed two times with PBS, and resuspended at 3 x 10^6 cells ml^{-1} in PBS. One hundred µl of cells were placed in each well of the Millipore MilliBlot-D system (Millipore Corporation, Bedford, MA). After

vacuum, the cells in each well were blotted onto the nitrocellulose membrane. The membrane was washed with PBS for 5 minutes at room temperature and blocked with 3% gelatin-PBS for 1 hour, followed by two washes (5 minutes each time) with PBS-0.15% Tween 20. The series of controls were set up as follows: 1) cell control; 2) negative mouse ascites control; 3) unrelated monoclonal antibody (MAb) control; and 4) LKT positive control. The nitrocellulose membrane was incubated with 1:500 diluted anti-LKT MAb C6, followed by goat anti-mouse IgG, and streptavidin-alkaline phosphatase (with appropriate incubation and wash steps). The dot blot membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (fast BCIP/NBT, Sigma Chemical Co., St. Louis, MO). The blots were scanned into tagged image files (TIF) using a flat bed scanner (HP ScanJet IIcx, Hewlett-Packard Co., Boise, ID) and the density vs distance from origin measured (SigmaScan, Jandal Scietific, San Rafael, CA). The values of dot spots were expressed as relative scanning density (scale is white = 0, black = 255).

2.8. Cytoplasmic membrane LKT binding detected with western blot

LKT binding was also assessed to further validate the whole cell LKT binding by incubating isolated membranes (50 μg protein) with 1 μg LKT or LKT-minus mutant or the equivalent volume of phosphate-NaCl buffer (no LKT) in 250 μl of RPMI, pH 7.2 at 25°C for 2 minutes. Incubation was terminated by dilution in ice-cold 2.5 ml MOPS-NaCl. Unbound LKT was removed from the membranes by 2 washes with 2.5 ml MOPS-NaCl at 4°C. Washed membranes were dissolved in 50 μl SDS-PAGE sample buffer,

boiled for 1.5 minutes, 15 μl subjected to 10% SDS-PAGE, and blotted onto nitrocellulose membranes. A no membrane control was run in which the assay setup was identical to that containing membranes, but following the 2-minute incubation, instead of the termination and washing steps, a 25 μl aliquot of the assay mixture was removed and mixed with 25 μl of SDS-PAGE sample buffer, the sample processed as above, and 15 μl subjected to electrophoresis and blotting. Following blocking, the nitrocellulose membranes were incubated with 1:500 mouse ascites for anti-LKT MAb C6 (Sun, et al., 1997), followed by biotinylated goat anti-mouse IgG, and streptavidin-alkaline phosphatase (with appropriate incubation and wash steps). Bands were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Fast BCIP/NBT, Sigma Chemical Co., St. Louis, MO) color development.

2.9. Inhibition of LKT binding and LDH leakage

BL3 or Raji cells (3×10^6 cells ml^{-1}) were incubated with 0, 20, 40, 80, 120, 150, or 200 μg ml^{-1} protease K (Sigma Chemical Co., St. Louis, MO) at room temperature for 10 minutes, then washed twice with PBS. After removal of unreacted enzymes, the cell pellets were resuspended in RPMI (3×10^6 cells ml^{-1}) for LKT-induced LDH leakage assay or in PBS (3×10^6 cells ml^{-1}) for the whole cell LKT binding assay.

2.10. Assay of LKT binding to HL60 cells under different conditions

The primary experiments indicated that LKT did not bind to HL60 cells at room temperature. The reason for this is possibly involved in proteolysis of LKT by HL60 cell secreted enzymes. In a separate experiment, the entire procedure for LKT binding to HL60 cells was performed at 0°C or at room temperature in the presence of a protease inhibitor cocktail containing antipapain-HCl, bestatin, chymostatin, E-64, leupeptin, pepstain, phosphoranmidon, Pefabloc® SC, EDTA and aprotinin, which is known to inhibit the following proteases: pankreas extrext, pronase, chymotrypsin, thermolysin, trypsin and papain (Boehringer Mannheim, Indianapolis, IN).

2.11. Experimental reproducibility and statistical analysis

Binding assays including the whole cell and isolated cytoplasmic membrane LKT binding experiments were repeated with essentially the same results. Experiments measuring $[Ca^{2+}]_i$ and LDH leakage were conducted in triplicate and were repeated with essentially the same results. A statistical analysis program (SigmaStat, Jandel Scientific Software, San Rafael, CA) was used to calculate standard deviations and assess statistical differences and pair-wise comparisons. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Determination of target cell susceptibility to LKT-mediated intoxication

Cultured cell lines (BL3, Raji, and HL60 cells) and isolated blood lymphocytes (bovine, equine, porcine, and canine) were tested for susceptibility to LKT-mediated intoxication. Increases in intracellular Ca^{2+} concentration and cytoplasmic enzyme LDH leakage were used to determine the susceptibility of target cells to LKT-mediated intoxication. For the Ca^{2+} influx assay, the data were calculated using a formula in which the fluorescence intensity induced by Ca^{2+} ionophore A23187 was defined as 100% and PBS exposure was defined as 0%. The data in Table 1 indicated that LKT, not LKT-minus mutant induced a significant Ca^{2+} influx in bovine-origin BL3 cells and isolated bovine lymphocytes. Although Ca^{2+} influx in other tested cells was also observed after exposed to LKT, the amount of influx was usually below 10% (Table 1). Similar to LKT-mediated Ca^{2+} influx, exposure of cultured cell lines (BL3, Raji, and HL60 cells) and isolated blood lymphocytes (bovine, equine, porcine, and canine) to LKT resulted in significant LDH leakage from bovine-origin BL3 cells and isolated bovine lymphocytes, not any other types of cells. Increase in LDH leakage was also observed from isolated bovine lymphocytes exposed to LKT-minus mutant, but the level was much lower than that induced by LKT (Table 1). Therefore, we conclude that only bovine-origin leukocytic cells are susceptible to LKT-mediated intoxication.

3.2. Binding of LKT to intact target cells

To investigate whether LKT bound to target cell surface, an immunological whole cell binding assay was developed. In this assay, the cultured cell lines (BL3, Raji, or HL60 cells) or isolated lymphocytes (3×10^6 cells ml^{-1}) of bovine, equine, porcine, or

canine origin were incubated with 40 μg LKT at room temperature for 3 minutes. After removal of unbound LKT, the amount of LKT binding to target cells was detected using immunological dot blot with anti-LKT MAb C6 as primary antibody. This assay was validated by deletion of each major component (primary antibody, biotinylated secondary antibody, streptavidin alkaline phosphatase) or in place of anti-LKT MAb C6 with negative control mouse ascites. The amount of LKT binding was expressed as a binding index, which was defined as the ratio of the relative scanning density of LKT binding to target cells vs that to BL3 cells. The binding indexes of LKT to cultured cell lines and isolated lymphocytes were shown in Table 1. The results indicated that LKT was essentially bound to all types of cells tested (binding indexes ranged from 0.88 to 1.54), except HL60 cells (binding index = 0.02).

3.3. Binding of LKT to isolated cytoplasmic membranes

To further validate the whole cell LKT binding assay, a cytoplasmic membrane LKT binding assay was developed, in which isolated cytoplasmic membranes from BL3, Raji, and HL60 cells were incubated with LKT or LKT-minus mutant, then unbound LKT was removed by twice washes. After fractionalization in SDS-PAGE and transfer onto the nitrocellulose membrane, the bound LKT was detected using a MAb C6 against LKT. The results were shown in Fig. 1. The significant and similar amounts of 102 KDa LKT band were recovered from both BL3 cells and Raji cells, however, negligible amounts of LKT bound to HL60 cells. Deletion of membranes or LKT from the assay or deletion of any major components (anti-LKT MAb C6, biotinylated goat anti-mouse IgG,

or streptavidin-alkaline phosphatase) or substituting irrelevant MAb for MAb C6 resulted in no detectable 102 KDa band for BL3 and Raji cells. The lack of significant LKT binding to the HL60 membranes did not appear to be a problem of selective lower recovery of HL60 membranes compared to BL3 or Raji membranes. Judging from recovery of the major constitutive membrane phosphatase activities at ≈70 KDa, recovery of membranes was similar for all 3 cell types (Fig. 1). In some repeats of this experiment, a faint LKT band was detected with HL60 membranes, but this band was < 5% the density of the LKT band density for LKT binding to BL3 cells. No 102 KDa LKT band was observed when the isolated BL3, Raji, or HL60 cytoplasmic membranes were incubated with LKT-minus mutant supernatant. These results were consistent with those of the whole cell LKT binding assay.

3.4. Concentration and time dependence of LKT binding

The binding of LKT to target cell surface was also evaluated by incubation of BL3 or Raji cells with various concentrations of LKT for different time periods. The binding of LKT to BL3 cells was concentration-dependent (Fig. 2). The binding curve was near linear from 0.07 to 40 $\mu g\ ml^{-1}$ LKT, and this binding was not saturable. The time course of LKT binding to BL3 and Raji cells was shown in Fig. 3. Exposure of BL3 or Raji cells to 40 $\mu g\ ml^{-1}$ LKT at room temperature for different times resulted in rapid binding with maximal binding between 2 to 3 minutes. Although the amount of LKT binding to Raji cells was higher than that to BL3 cells, the pattern of LKT binding was essentially similar (Fig. 3).

3.5. Inhibition of LKT binding by protease K

Pre-treatment of BL3 cells with protease K resulted in decrease in LKT binding and LDH leakage, but decrease in LDH leakage was not proportional to LKT binding reduction (Fig. 4). Further experiments indicated that effect of protease K on LKT binding Raji cells was similar to BL3 cells (data not shown).

3.6. Detection of LKT binding to HL60 cells under different conditions

To test whether failure of LKT binding to HL60 cells was due to proteolysis, the whole cell LKT binding assay was conducted at 0°C or at room temperature in the presence of protease inhibitor cocktail. At 0°C, a significant amount of LKT binding to HL60 cells was observed, but was peaked at 1 minute exposure, then reduced to basal level (Fig. 5). The protease inhibitor cocktail also enhanced LKT binding to HL60 cell with no significant decline after 3 minute exposure (data not shown).

4. Discussion

Our study indicated that target cell susceptibility to LKT-mediated intoxication such as increases in $[Ca^{2+}]_i$ and cytoplasmic enzyme LDH leakage was limited to bovine leukocytes, which was same as those reported previously (Shewen and Wilkie, 1982; Confer, et al., 1988). The causes for slight increases (below 10%) in $[Ca^{2+}]_i$ and LDH

leakage from non-bovine-origin cells exposed to LKT or LKT-minus mutant supernatant were unknown. It is possible that a small amount of lipopolysaccharide (LPS) presented in LKT preparation was responsible for the action.

In the present study, two new binding assays for detecting the initial interaction of LKT with target cells were described. The detection is specific for LKT, because we used anti-LKT MAb C6 as a primary antibody and bound LKT to cytoplasmic membrane was recovered from a western blot. The results of LKT binding to intact cell surfaces from the immunological dot blot were consistent with those from the cytoplasmic membrane binding assay. The reproducible results indicated that these two binding assays are suitable for detection of LKT binding. Compared to cell membrane binding, the whole cell LKT binding detected with immunological dot blot is simpler and time-saving, although the cytoplasmic membrane LKT binding assay detected with western blot remains valuable as an alternative method for measurement of toxin binding.

Our data indicated that *P. haemolytica* LKT is basically binding to all target cells, except HL60 cells, although the relative amount of binding is variable among the cells that we tested. The molecular mechanism of LKT binding to target cell membranes is not clear. However, it appears that the initial interaction is involved in acyl-groups or certain amino acids of mature LKT and phospholipids in the cytoplasmic membranes. LKT protein produced by the *lktA* gene, termed pro-LKT, lacks biological activity, unless it is acylated by LKTC protein, which functions as acyltransferase (Stanley, et al., 1998). Acylation of LKT not only activates its biological activity (cytolysis), but also increases its intrinsic hydrophobicity (Stanley, et al., 1998). The hypothesis for the interaction of RTX toxin proteins with cytoplasmic phospholipids of target cells has been partially

confirmed by Sato, et al. (1993). In their study, the purified *A. actinomycetemcomitans* Ltx was essentially bound to both Ltx susceptible cells HL60 and Ltx non-susceptible cells human erythroleukemia K562, as determined by ELISA (Sato, et al., 1993). Importantly, the percentage of Ltx binding decreased in the presence of phosphatidylcholine (PC), which suggests that externally added PC competes with phospholipids in cytoplasmic membranes for the binding to Ltx. *E. coli* α-hemolysin unsaturably binds to the artificial liposomal membranes which were made with egg PC and egg phosphatidylethanolamine (PE). In the presence of Ca^{2+}, bound α-hemolysin induces lysis of the liposomal membranes (Ostolaza and Goñi, 1995), which apparently lack proteins. Interestingly, as the amount of PC in liposomal membranes was increased, the percentage of bound α-hemolysin was enhanced (Ostolaza and Goñi, 1995). This implicates that RTX toxins preferentially bind to PC, other than PE.

The hallmarks of receptor-mediated molecular binding include high affinity (dissociation constant range from pM to nM) and saturable binding (10^2 to 10^5 receptors per cell). In the present study, the concentration and time dependence of LKT binding indicated that the binding of LKT to BL3 or Raji cells was not saturable. After initial maximal binding between 2 to 3 minutes, a significant amount of LKT was dissociated from the cell surfaces, then reached a plateau. These findings further suggested that LKT binding to target cell surface was not protein receptor-mediated, although LKT executes its biological function (cytolysis) through a receptor (Lally, et al., 1997).

One may argue that treatment of target cells with protease K resulted in reduction of LKT binding is due to damage of protease K-sensitive protein receptors for LKT binding (Brown, et al., 1997). But apparently it is not appropriate to draw this conclusion,

because proteinase K is not normally a highly specific enzyme, which cleaves many proteins in the C-terminal region, including phospholipoproteins (Morris, et al., 1985). It is possible to speculate that proteolysis of phospholipoproteins by protease K releases phospholipids, which in turn compete for binding to LKT with target cell membranes and consequently, result in decrease in LKT binding to target cells.

The question may arise about why LKT did not non-specifically bind to HL60 cells. To answer this question, further binding assays for HL60 cells were conducted at 0°C or in the presence of protease inhibitor cocktail, which resulted in significant LKT binding. These findings suggest that HL60 cells may secrete certain proteolytic enzymes. HL60 cells are derived from human promyelocytic leukemia, a malignant tumor (American Type Culture Collection, Rockville, MD). It is well known that one of typical characteristics of malignant tumor cells is invasion and destruction of surrounding tissues (Cotran, et al, 1994). To do so, some tumor cells secrete proteolytic enzymes, while others induce host cells to elaborate proteases (Steeg, 1992). HL60 cells could be in the former group, whereas BL3 and Raji cells may belong to another group. Further experiments are need to confirm this speculation.

It has been demonstrated that *A. actinomycetemcomitans* Ltx- and *E. coli* α-hemolysin-mediated cell lysis is through the interaction of toxins with CD11a and CD18, the two subunits of the β2 integrin, LFA-1. Anti-CD11a or CD18 blocks toxin-mediated cell lysis in susceptible HL60 cells. Transfection of CD11a and CD18 integrin genes into a cell line (K562) that is not susceptible to either RTX toxin resulted in LFA-1 expressing cells, KL/4, that are susceptible to both toxins (Lally, et al., 1997). The question is why some cells, such as red blood cells, do not bear LFA-1 on their surface, but are

susceptible to RTX toxins, like *E. coli* α-hemolysin. Even *P. haemolytica* LKT possesses a weak hemolytic activity (Murphy, et al., 1995; Fedorova and Highlander, 1997). The question about non-LFA-1 borne target cells susceptible to RTX toxin-mediated intoxication indicates that other component(s) may be involved in the interaction of RTX toxins with target cells.

The results in our study are obviously contradictory to those previously reported by Brown, et al. (1997). We can not explain this discrepancy at this point. However, several possibilities might be involved in these outcomes. For example, native LKT used in our study rather than the biotinylated LKT, which may have changed conformation after conjugation with biotin. Secondly, the immunological dot blot and western blot were used in the present study instead of flow cytometry, which might have certain cut-off points for measurement of binding.

In conclusion, *P. haemolytica* LKT binds to both susceptible and non-susceptible target cells, but binding alone is not enough to induce Ca^{2+} influx and LDH leakage from target cells. Further work is needed to identify which component(s) on the cell surface is involved in LKT binding and cell lysis of non-LFA-1 expressing cells.

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Figure legend

Fig. 1. The binding of LKT to isolated cytoplasmic membranes detected by western blot. Significant amounts of LKT bound to both the LKT susceptible cell type, BL3 cell cytoplasmic membranes and to the LKT non-susceptible cell type, Raji cell cytoplasmic membranes, but negligible amounts of LKT bound to the LKT non-susceptible cell type, HL60 cell cytoplasmic membranes. Membranes were exposed to LKT (+) or LKT-minus mutant (-) supernatants or to phosphate-NaCl buffer (no LKT, n). Controls were identical exposure mixtures lacking membrane processed without the termination-dilution or washing steps.

Fig. 2. Dose dependence of LKT binding to BL3 cells. The concentration of LKT was 40 $\mu g\ ml^{-1}$ and target cells (3×10^6 cells ml^{-1}) were exposed to LKT for 3 minutes at room temperature. The amount of LKT binding was expressed as relative scanning density.

Fig. 3. The time course of LKT binding to BL3 (■) or Raji (▲) cells at room temperature. The cell concentration was 3×10^6 cells ml^{-1} and LKT was 40 $\mu g\ ml^{-1}$. The amount of LKT binding was expressed as relative scanning density.

Fig. 4. Effects of protease K on LKT binding (□) to and LDH leakage (■) from BL3 cells. The cell concentration was 3×10^6 cells ml^{-1} and LKT was 40 $\mu g\ ml^{-1}$.

Fig. 5. Effect of temperature on LKT binding to HL60 or BL3 cells. BL3 (cycle) and HL60 (square) cells (3×10^6 cells ml^{-1}) were exposed to LKT (40 $\mu g\ ml^{-1}$) at 0°C (solid lines and solid symbols) or at room temperature (broken lines and blank symbols) for 3 minutes. The amount of LKT binding was expressed as relative scanning density.

Fig. 1.

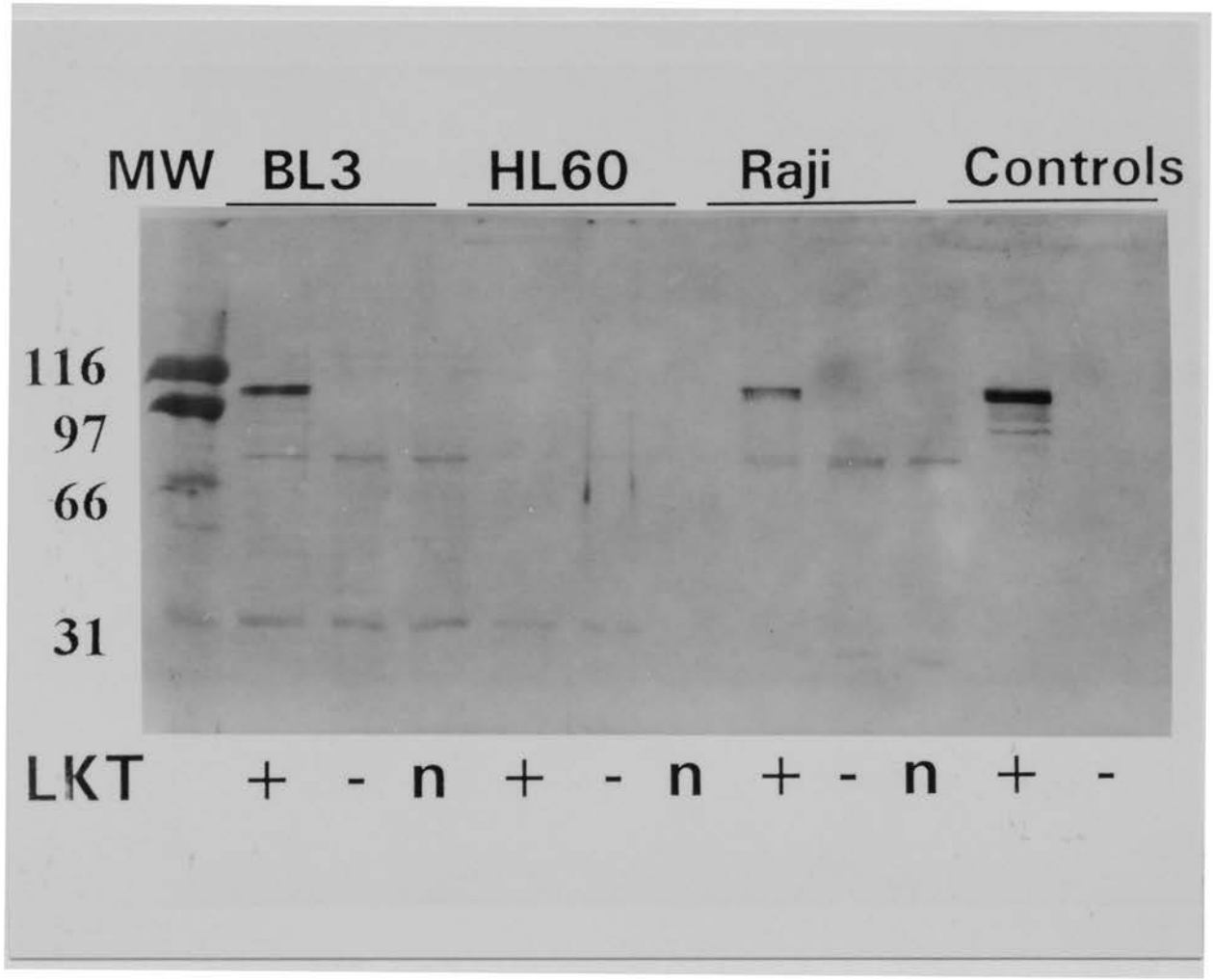
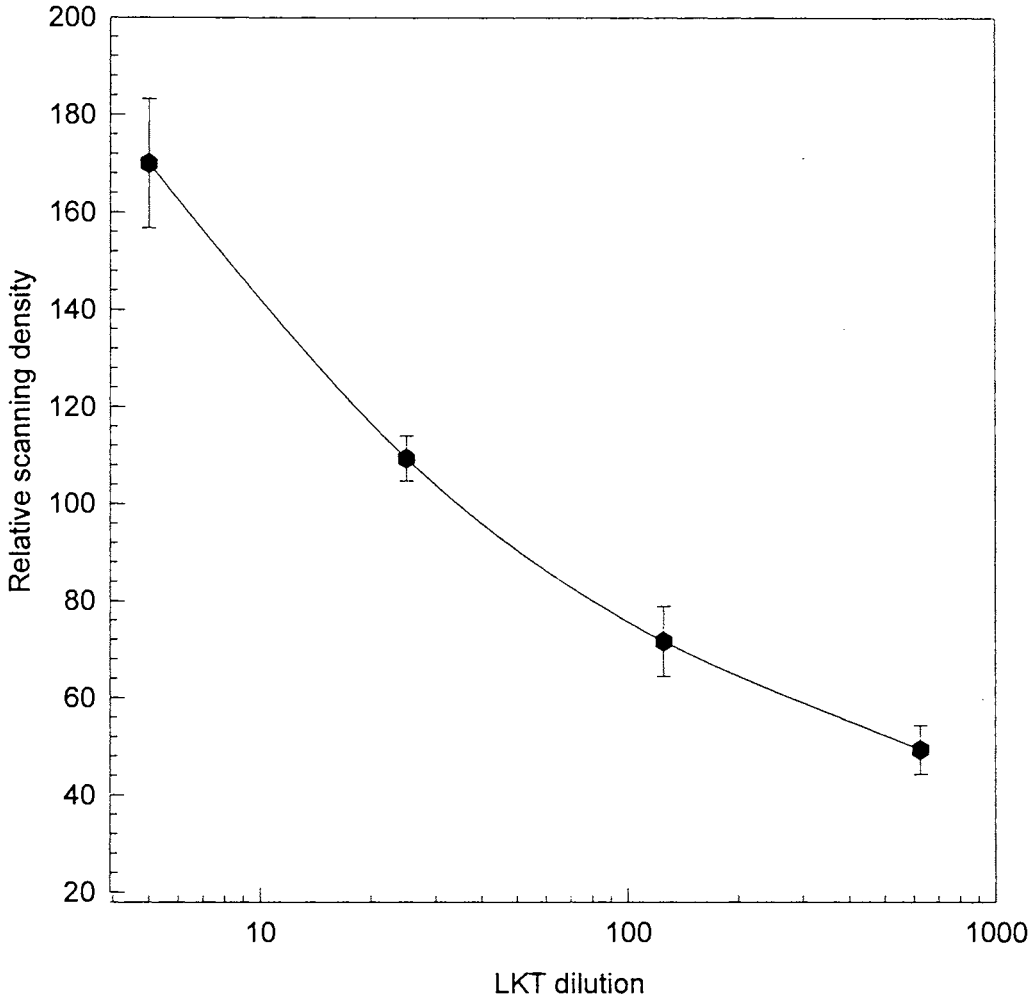


Fig. 2.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180
160
140
120
100
80
60
40
20
Relative scanning density
10
100
1000
LKT dilution

Fig. 3.

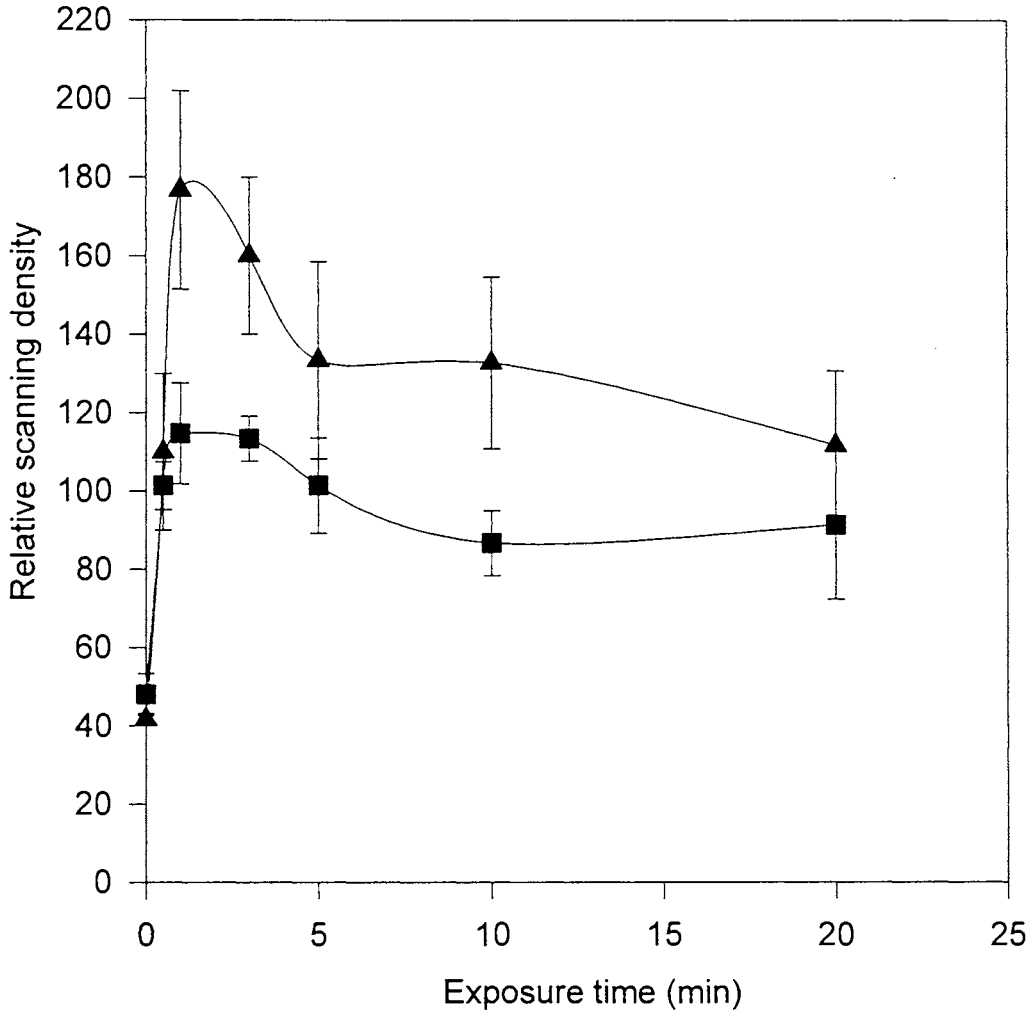
220
200
180
160
140
120
100
80
60
40
20
0
Relative scanning density
0
5
10
15
20
25
Exposure time (min)

Fig. 4.

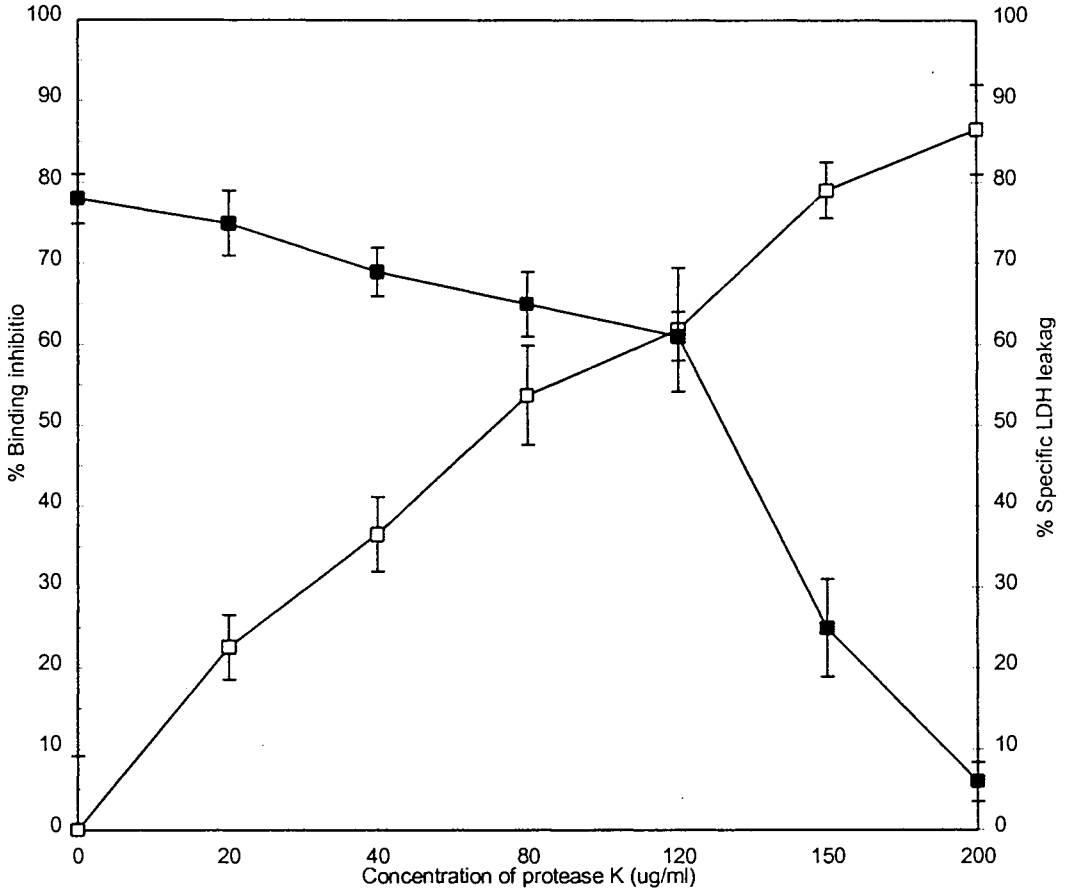
100
90
80
70
60
50
40
30
20
10
0
% Binding inhibitio
% Specific LDH leakag
0
20
40
80
120
150
200
Concentration of protease K (ug/ml)

Fig. 5.

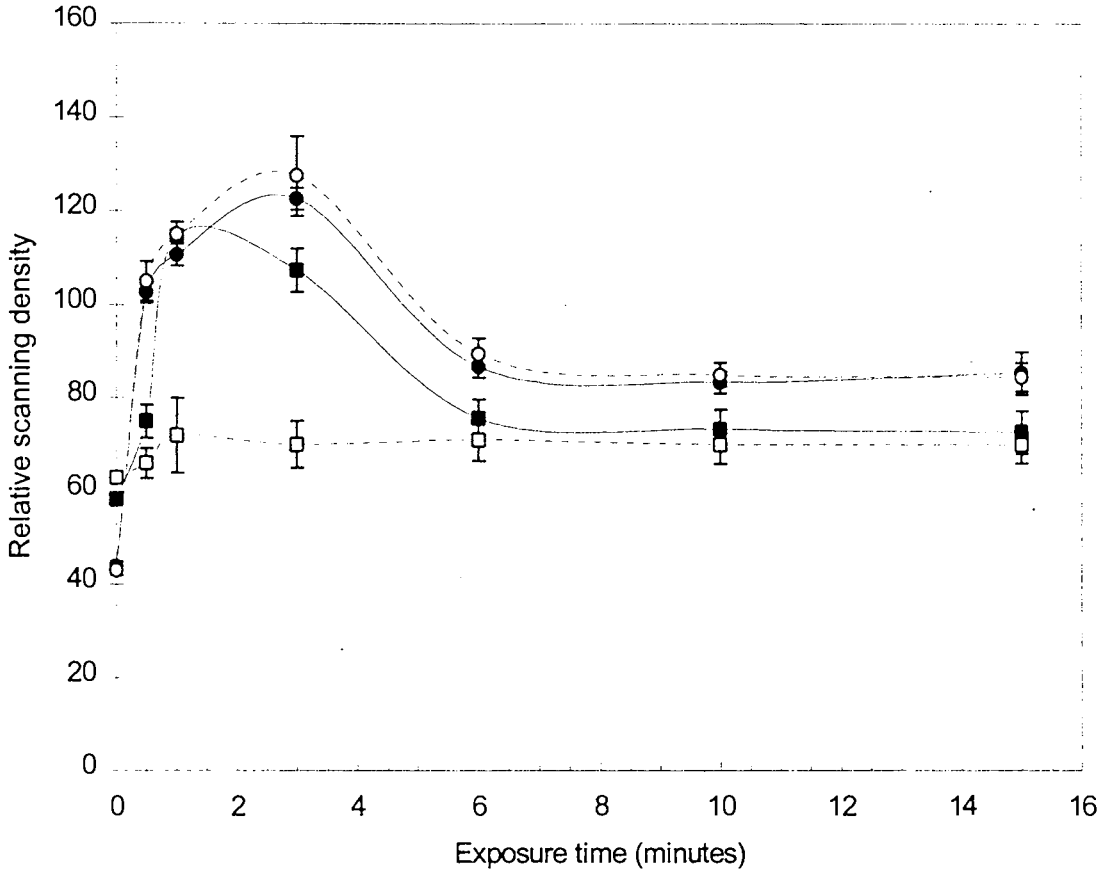
160
140
120
100
80
60
40
20
0
Relative scanning density
0
2
4
6
8
10
12
14
16
Exposure time (minutes)

Table 1

Determination of target cell susceptibility to *P. haemolytica* LKT and binding of LKT to intact cell surface detected with immunological dot blot

Target cells[1]	%A23187 Fluo3 fluorescence intensity[2]		%Specific LDH leakage[3]		LKT binding index[4]	
	LKT[5]	LKT (-)[5]	LKT	LKT(-)	LKT	LKT (-)
BL3	144.9 ± 7.5[a6]	8.0 ± 4.2[a]	64.8 ± 6.2[a]	0.0 ± 0.4[a]	1.00	0.05
HL60	8.1 ± 1.3[b]	5.0 ± 2.1[b]*	1.0 ± 1.5[b]	1.5 ± 0.9[b]	0.02	0.00
Raji	9.0 ± 2.6[b]	5.2 ± 1.8[b]*	0.2 ± 2.0[b]	0.0 ± 0.5[a]	1.54	0.01
BL	72.7 ± 4.8[c]	1.1 ± 0.8[c]	84.1 10.5[a]	8.9 ± 2.3[c]	1.45	0.08
EL	-0.3 ± 1.1[d]	-0.3 ± 1.2[c]	1.0 ± 0.9[b]	0.5 ± 1.2[b]	0.88	-0.01
PL	8.2 ± 1.9[b]	-1.1 ± 1.1[c]*	2.1 ± 1.5[b]	1.7 ± 1.5[b]	1.25	0.00
CL	-0.1± 0.9[d]	-0.1 ± 1.3[c]	0.5 ± 1.2[b]	2.4 ± 1.8[b]	1.19	0.03

[1] BL = bovine lymphocytes; EL = equine lymphocytes; PL = porcine lymphocytes; CL = canine lymphocytes.

[2] % A23187 Fluo3 fluorescence intensity = [(Fluo3 fluorescence intensity of LKT or LKT-minus mutant - Fluo3 fluorescence intensity of PBS) / (Fluo3 fluorescence intensity of A23187) - (Fluo3 fluorescence intensity of PBS)] x 100.

[3] % Specific LDH leakage = [(mOD of LKT or LKT-minus mutant - mOD of PBS) / (mOD of Triton-X 100 - mOD of PBS)] x 100.

[4] LKT binding index = scanning density of LKT binding to target cells / scanning density of LKT binding to BL3 cells.

[5] The concentration of LKT or LKT-minus mutant was 40 μg ml^{-1}.

[6] The mean in a column with different lettered superscripts differ ($P < 0.05$).

* The means between paired data within one type of cells differ ($P < 0.05$).

CHAPTER VI

PASTEURELLA HAEMOLYTICA LEUKOTOXIN INDUCED APOPTOSIS OF BOVINE LYMPHOCYTES INVOLVES DNA FRAGMENTATION

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Abstract

It has been reported that Pasteurella haemolytica leukotoxin (LKT) induces morphologic changes in bovine leukocytes consistent with apoptosis *in vitro*, but DNA fragmentation was not observed. In this study, we investigated whether bovine lymphocytes undergo DNA fragmentation during LKT-induced apoptosis. Bovine peripheral blood lymphocytes were isolated by density gradient centrifugation and exposed to LKT or inactive pro-LKT protein from a lktC⁻ mutant strain. After exposure, DNA fragmentation in lymphocytes was quantified colorimetrically by diphenylamine assay and visualized by agarose gel electrophoresis. At high LKT concentrations, bovine lymphocytes were lysed, but at low concentrations, LKT caused DNA fragmentation characteristic of apoptosis. Maximal DNA fragmentation in bovine lymphocytes was induced by 0.1 TU/ml LKT following 3 hour exposure, but only a background level of DNA fragmentation was observed with the inactive pro-LKT. Equine lymphocytes that are resistant to LKT intoxication did not show DNA fragmentation following exposure to LKT. Preincubation of LKT with a neutralizing anti-LKT monoclonal antibody MM601 inhibited LKT-induced DNA fragmentation. Electrophoresis of DNA from bovine lymphocytes treated with 0.1 TU/ml LKT demonstrated the typical "ladder" pattern of internucleosomal DNA cleavage, the hallmark of apoptosis associated with activation of endonucleases. LKT-induced DNA fragmentation was inhibited by 0.5 mM $ZnCl_2$, an endonuclease inhibitor. The results indicated that LKT at low concentrations induced apoptotic cell death of bovine lymphocytes, which may play a role in initiation and persistence of P. haemolytica infection.

Key words: Pasteurella haemolytica, leukotoxin, lymphocyte, apoptosis, DNA fragmentation

1. Introduction

P. haemolytica, the causative agent of bovine shipping fever pneumonia, produces several virulence factors including LKT, lipopolysaccharide, capsular polysaccharide, fimbriae, glycoprotease, neuraminidase, and outer membrane proteins, which may be important in establishing disease (Confer, et al., 1990). LKT, a repeats-in-toxin (RTX) toxin, is considered to be the primary virulence factor (Petras, et al., 1995; Tatum, et al., 1998). Like other RTX toxins, LKT is thought to act by forming transmembrane pores in the plasma membrane of target cells resulting in colloid - osmotic cell lysis, termed oncosis (Clinkenbeard, et al., 1989b). In addition to oncosis, several RTX toxins have been found to cause apoptosis of target cells exposed to low concentrations of toxins (Mangan, et al., 1991; Khelef, et al., 1993). Recently, Stevens and Czuprynski (1996) reported that P. haemolytica LKT induced morphologic changes in bovine leukocytes consistent with apoptosis *in vitro*. However, DNA fragmentation, a hallmark of apoptosis, was not observed.

Oncosis and apoptosis are two principal pathways of mammalian cell death. These pathways have distinctive morphologic and biochemical characteristics that can be used to distinguish apoptosis from oncosis. Oncosis is typically initiated by changes in plasma membrane permeability resulting in colloid-osmotic cell and organelle swelling, balloon-like degeneration, early rupture of cytoplasmic membrane and mitochondria, but with minimal changes in the nucleus. The biochemical marker of oncosis is dissipation of transmembrane ion gradients and leakage of cytoplasmic enzymes (Clinkenbeard, et al., 1989b). In contrast, apoptosis is a process with early and prominent condensation of

nuclear chromatin, decreased cell size and ultimate formation of membrane-bound apoptotic bodies (Kerr, et al., 1972; Fady, et al., 1995; Lieberthal and Levine, 1996). The biochemical hallmark of apoptosis is endonuclease-mediated cleavage of internucleosomal DNA linker sections which initially generates subchromosomal fragments of 50 to 300 kbp, and subsequently mono- or oligo-nucleosomal DNA fragments that are multiples of approximately 200 bp nucleotides. The DNA fragments produced by this process yield so-called "DNA ladders" after resolution in agarose gels.

Numerous cellular insults cause oncosis including cytotoxic agents, ischemia, heat shock, viral infection, and bacterial toxins. Exposure to ethanol or oxidants are also capable of inducing apoptosis. It appears that agents that are cytolytic at high concentrations may induce apoptosis at lower concentrations. Whether injured cells undergo cell death via apoptosis or oncosis may depend on the severity of insult (Cohen, 1996; Lieberthal and Levine, 1996). Cells that undergo oncosis when exposed to severe ischemic or toxic insults, may undergo apoptotic cell death when the insult is lessened (Lieberthal and Levine, 1996).

In a recent study, it was reported that P. haemolytica culture supernatants caused membrane blebbing and nuclear condensation of bovine leukocytes, but DNA fragmentation was not observed (Stevens and Czuprynski, 1996). In this study, we report that P. haemolytica LKT at high concentrations is cytolytic to bovine lymphocytes, but at low concentrations it induces DNA fragmentation characteristic of apoptosis. The effect of LKT is specific for susceptible cells and is not observed in non-susceptible cells nor can inactive pro-LKT cause apoptosis in susceptible cells. Quantification of DNA

fragmentation and observation of DNA laddering are consistent with LKT-mediated apoptosis in bovine lymphocytes.

2. Materials and methods

2.1. Isolation of bovine peripheral blood lymphocytes

Isolation of lymphocytes was as previously described (Reeves and Renshaw, 1978; Williams, et al., 1986) with some modifications. Briefly, the blood sample was collected from healthy cattle by venipuncture of the jugular vein, and anticoagulant (sodium heparin, 15 U/ml) was added. Forty-five ml of heparinized blood in a 50 ml conical centrifuge tube was centrifuged at 600 x g for 30 minutes at 22 °C, then the plasma was aspirated down to about 10 -15 mm above the interface between the erythrocytes and the plasma. The cells at the interface were carefully pipetted into another centrifuge tube with as few erythrocytes as possible and were diluted (1:3, v/v) with Hank's balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free). Thirty to thirty-five ml of diluted cells were carefully layered on 15 ml of Ficoll-Paque (Sigma Chemical Co, St Louis, MO, USA) and centrifuged at 500 x g for 20 minutes with the brake off. After centrifugation, lymphocytes and monocytes in the white interface between plasma and the Ficoll-Paque were pipetted into another 50 ml conical centrifuge tube, washed twice with 30 ml of HBSS and resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS) at 5 x 10^6 cells/ml. This final cell suspension was free of erythrocytes and contained approximately 90% lymphocytes (mean 89.9, range 72 - 99%) and 5%

monocytes (mean 4.5, range 0 - 9%), based on differential counting. To remove the monocytes, 5 ml of cell suspension was put into a 50 mm tissue culture-treated Nunclon petri dish (Nalge Nunc International., Milwaukee, WI, USA) and incubated at 37 °C for 2 hours. Following the incubation, monocytes adhered onto the plate surface, and 99% nonadherent cells were lymphocytes. Greater than 95% of the cell population was capable of excluding trypan blue in all experiments (Reeves and Renshaw, 1978). Equine lymphocytes were isolated as described above for bovine lymphocyte isolation.

2.2. Preparation of LKT and inactive pro-LKT concentrated culture supernatants

Concentrated culture supernatants from a wild type P. haemolytica strain SH1217 and its isogenic strain SH1562 containing a non-polar insertion in the lktC gene were used as the LKT preparation and as LKTC(-) negative control, respectively (Fedorova and Highlander, 1997). Concentrated culture supernatants were prepared by inoculating 1L RPMI-1640 medium containing 0.1M phosphate buffer (pH 6.8) to an OD_{600nm} = 0.25 with wild type or mutant strain of P. haemolytica prepared by growth overnight on 5% bovine blood agar and then in 100 ml of RPMI-1640 under 5% CO_2 to late logarithmic phase (Sun and Clinkenbeard, 1998). The cultures in RPMI-1640 were grown at 37°C with 120 oscillations/minute for approximately 4 hours to an OD_{600nm} = 0.9 to 1.0. All subsequent steps were conducted at 4°C. The bacteria were removed by centrifugation at 8000 x g for 30 minutes, and culture supernatant concentrated and partially purified by fractional ammonium sulfate precipitation (0-60% saturation by addition of 361 g/L solid ammonium sulfate). The precipitates were resuspended at 0.5 mg protein/ml in 20 ml of

50 mM sodium phosphate, 100 mM sodium chloride buffer, pH 7.0 (phosphate-NaCl), dialyzed against the same buffer, and stored at -135°C. The culture supernatant protein concentrations of the LKT and inactive pro-LKT preparations were not significantly ($P > 0.05$) different (Fedorova and Highlander, 1997).

2.3. LKT activity and LDH leakage assays

LKT activity was quantified as toxic units (TU) by measurement of intracellular lactate dehydrogenase (LDH) leakage caused by serial dilution of the LKT preparation (Clinkenbeard, et al., 1989b). TU was determined by graphing specific LDH leakage versus the culture supernatant dilution factor. One TU was defined as the dilution factor at which the specific LDH leakage was 50% for 4 x 10^6 BL3 cells/ml exposed for 2 hours at 37 °C (Clinkenbeard, et al., 1989b).

The LDH leakage assay was also used to monitor the relationship between LKT-induced target cell lysis and apoptosis. Briefly, exposed target cells were collected by centrifugation at 700 x g for 10 minutes and 100 µl of supernatant from each treatment (see below) was transferred into a 96-well flat bottom microtiter plate which was then warmed to 37 °C. LDH release was performed by addition of 100 µl of 37 °C assay reagent LDH-L 50 (Sigma Chemical Co., St. Louis, MO, USA). LDH activity was measured in a thermally-controlled kinetic microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA, USA) at 340 nm for 2 minutes at 37 °C. Data were reported as mOD/min.

2.4. Preparation of cell treatments

Isolated peripheral blood lymphocytes were placed in complete RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 10% FBS. The positive control for apoptosis was prepared by incubation of lymphocytes (5 ml, 4 x 10^6 cells/ml) with 10 μM dexamethasone at 37 °C for 24 hours with 5% CO_2. For time-dependent induction of apoptosis by P. haemolytica LKT, 0.5, 0.1, 0.01, and 0.001 TU/ml of LKT or same extent dilutions of inactive pro-LKT were incubated with 0.5 ml of isolated bovine or equine peripheral lymphocytes (4 x 10^6 cells/ml) at 37 °C for 0.5, 1.5, 3.0, 6.0, and 24 hours. Since the primary experiments indicated that 3 hour incubation of lymphocytes with LKT induced the maximal DNA fragmentation, LKT concentration-dependent induction of apoptosis assays were performed by incubation of the isolated bovine peripheral lymphocytes (0.5 ml, 4 x 10^6 cells/ml) with 1.0, 0.5, 0.4, 0.3, 0.2, 0.1, 0.01, and 0.001 TU/ml of LKT or same extent dilutions of inactive pro-LKT at 37 °C for 3 hours. For DNA gel electrophoresis, 5.0 ml of the bovine lymphocytes (4 x 10^6 cells/ml) in complete RPMI-1640 with 1.0 or 0.1 TU/ml of LKT or same extent dilution of inactive pro-LKT as 0.1 TU/ml of LKT, or without LKT, or 0.1 TU/ml of LKT in the presence of 0.5 mM of $ZnCl_2$ were incubated for 3 hours, then DNA was extracted (see below). The equine lymphocytes were incubated only with 0.1 TU/ml of LKT for 3 hours.

2.5. Monoclonal antibody neutralizing leukotoxic activity

Leukotoxic activity of LKT was neutralized by preincubation of 250 µl diluted LKT (0.2 TU/ml) with 250 µl of 1:200 murine anti-LKT monoclonal antibody MM601 for 30 minutes at 4 °C, then the mixture of MM601 and LKT was incubated with the isolated bovine peripheral blood lymphocytes for 3 hours to investigate whether MM601 could block LKT-induced DNA fragmentation.

2.6. Quantification of DNA fragmentation

A diphenylamine colorimetric assay (Liles, et al., 1995; Squier and Cohen, 1997) was used to quantify DNA fragmentation. Briefly, 0.5 ml of 4 x 10^6 target cells/ml were centrifuged at 300 x g for 10 minutes to separate supernatant from cell pellets. One hundred µl aliquots of supernatants were used for LDH leakage assay and the remaining supernatant (designated as S) was used for DNA fragmentation assay. The cell pellets were lysed with 0.5 ml of a hypotonic solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 10 minutes at 13,000 x g to separate high molecular weight, intact chromatin (pellet, designated as B) from cleaved, low molecular weight DNA (top solution, designated as T). The supernatant (S), low molecular weight (T) and high molecular (B) fractions were precipitated overnight with 0.5 ml of 25% trichloroacetic acid (TCA) (Sigma Chemical Co, St Louis, MO, USA), sedimented at 13,000 x g for 10 minutes, and hydrolyzed in 80 µl of 5% TCA at 90 °C for 15 minutes. All fractions were incubated with 160 µl of diphenylamine (Fisher Scientific, Pittsburgh, PA, USA) reagent at 37 °C for 4 hours, in which 150 mg of diphenylamine was dissolved in 10 ml of glacial acid, then 150 µl of concentrated sulfuric acid and 50 µl of acetaldehyde solution were added and mixed well. The amount

of DNA in each sample was estimated from its absorbance at 570 nm in a plate spectrophotometer. The results were calculated using the following equation:

$$\% \text{ DNA fragmentation} = [(S + T)/(S + T + B)] \times 100$$

where S is the amount of DNA in the supernatant, T is the amount of low molecular weight, cleaved DNA in the top solution and B is the amount of high molecular weight, intact chromatin DNA.

2.7. Extraction and electrophoresis of DNA

DNA gel electrophoresis was performed in order to verify DNA fragmentation (Hébert, et al., 1996). After treatments, cellular DNA from different groups was isolated using a DNA extraction kit according to the manufacturer's instruction (Stratagene, La Jolla, CA, USA). Briefly, lymphocytes were resuspended in lysis buffer and incubated for 1 hour at 60 °C in a shaking water bath. The nucleic acids were separated from protein by centrifugation at 2,000 x g for 15 minutes at 37 °C and the supernatant collected. RNA was eliminated by incubating with RNase for 15 minutes at 37 °C. The DNA was precipitated by adding 2.5 volumes of cold absolute ethanol, and incubated overnight at – 20 °C. The DNA precipitates were recovered by centrifugation at 10,000 x g for 20 minutes at 4 °C. After drying, DNA was resuspended in TE buffer containing 10 mM of Tris-HCl, pH 7.4, 1 mM EDTA and quantitated spectrophotometrically at OD_{260} nm (Sambzook, et al., 1989). Two μg of DNA per lane were electrophoresed on a 1% agarose gel in Tris-borate buffer (pH 8.0) at 50 V for approximately 7 hours. After

electrophoresis, DNA was stained with ethidium bromide (2.5 μg/ml), visualized with UV illumination and photographed.

3. Results

3.1. Comparison of dexamethasone and LKT-induced DNA fragmentation

Exposure of isolated bovine lymphocytes (4 x 10^6 cells/ml) to 10 μM dexamethasone at 37 °C for 24 hours or to 0.1 TU/ml LKT at 37 °C for 3 hours under 5% CO_2 resulted in increased DNA fragmentation as quantified by diphenylamine assay (Fig. 1). DNA fragmentation induced by LKT or dexamethasone was inhibited by 0.5 mM $ZnCl_2$, an endonuclease inhibitor.

3.2. Gel electrophoresis of LKT-induced DNA fragments

Exposure of isolated bovine lymphocytes to 0.1 TU/ml of LKT resulted in DNA fragmentation into multiples of approximately 200 bp of nucleotides as detected by the characteristic apoptotic "ladder" pattern on gel electrophoresis (Fig. 2, lane 7) and this DNA laddering was similar to that of dexamethasone-induced apoptosis (Fig. 2, lane 6). This LKT-induced fragmentation was specific for bovine lymphocytes and was not observed when equine lymphocytes were exposed to LKT (Fig. 2, lane 1). Likewise, non-acylated, inactive pro-LKT failed to cause DNA fragmentation in bovine lymphocytes (Fig. 2, lane 4), and preincubation of active LKT with neutralizing monoclonal anti-LKT

antibody MM601 also blocked LKT-induced DNA fragmentation in bovine lymphocytes (Fig. 2, lane 8). Inclusion of 0.5 mM $ZnCl_2$ in the LKT (0.1 TU/ml) exposure blocked LKT-induced DNA fragmentation (Fig. 2, lane 5). At a lytic dose of LKT (1.0 TU/ml), no DNA fragmentation was observed (Fig. 2, lane 3).

3.3 Time-dependence of LKT-induced DNA fragmentation

Exposure of isolated bovine lymphocytes to various concentrations of LKT for 0.5 to 24 hours at 37 °C determined that maximal DNA fragmentation occurred at 3 hours of exposure (Fig. 3). LKT-induced cytolysis as measured by leakage of the intracellular enzyme LDH was also maximal at 3 hours of exposure (Fig. 4). Although maximal at 3 hours of exposure, cytolysis appeared to develop more rapidly than DNA fragmentation. A marked difference in LKT concentration dependence for DNA fragmentation versus cytolysis was observed with high LKT dose causing cytolysis with minimal DNA fragmentation, and low LKT dose causing maximal DNA fragmentation and limited cytolysis. Exposure of equine lymphocytes to various concentrations of *P. haemolytica* LKT or inactive pro-LKT for the same period of incubation as bovine lymphocytes, did not increase DNA fragmentation and LDH leakage (data not shown).

3.4. LKT concentration dependence of DNA fragmentation

Exposure of isolated bovine lymphocytes to various LKT concentrations for 3 hours at 37 °C resulted in a biphasic DNA fragmentation curve with increasing

fragmentation from 0.01 to 0.1 TU/ml followed by a decline from 0.1 to 0.4 TU/ml LKT (Fig. 5). In contrast, the concentration dependence of LKT-induced cytolysis was near linear from 0.1 to 1.0 TU/ml LKT (Fig. 6). The inactive pro-LKT at various culture supernatant concentrations as LKT did not increase DNA fragmentation in bovine lymphocytes and did not cause LDH leakage, either (Fig. 5 and 6).

4. Discussion

LKT is a pore-forming, Ca^{2+} dependent cytolysin specific for ruminant leukocytes and platelets at high concentrations (Shewen and Wilkie, 1982; Clinkenbeard, et al., 1989a, b, c; Clinkenbeard and Upton, 1991). At low concentrations, LKT activates bovine neutrophils as demonstrated by stimulation of an oxidative burst, cytoskeletal alterations and release of secondary granules (Czuprynski, et al., 1991). Exposure of bovine or ovine lymphocytes to low concentrations of LKT inhibits mitogen-induced blastogenesis (Majury and Shewen, 1991a, b; Sharma and Woldehiwet, 1991). Recently, it has been reported that sublytic concentrations of LKT induced morphologic changes of bovine leukocytes consistent with apoptosis. In their study, Stevens and Czuprynski (1996) used cytoplasmic membrane blebbing (zeiosis) and nuclear chromatin condensation and margination in bovine leukocytes detected by light and UV microscopy to implicate LKT as an inducer of apoptosis. Several other RTX toxins have been implicated in induction of apoptosis in their target cells (Mangan, et al., 1991; Khelef, et al., 1993; Kato, et al., 1995; Gueirard, et al., 1998). The criteria for assessment of apoptosis generally include morphologic changes examined by light or electron

microscopy and DNA fragmentation detected by colorimetric assay or visualization of fragmented DNA ladder patterns by agarose gel electrophoresis (Mangan, et al., 1993). In our study, we used the diphenylamine assay for quantification of DNA fragmentation and agarose gel electrophoresis for verification of DNA ladder patterns that are multiples of approximately 200 bp nucleotides.

We determined that LKT is the P. haemolytica culture supernatant component that elicits DNA fragmentation in isolated bovine lymphocytes. The DNA fragmentation formed the typical ladder pattern of multiple nucleosomal sized nucleotides, a hallmark of apoptosis (Mangan, et al., 1993). The evidence that the DNA fragmentation observed in bovine lymphocytes exposed to P. haemolytica culture supernatants was caused by LKT was that no DNA ladder was observed in agarose gel after bovine lymphocytes were exposed to culture supernatants from a mutant strain, which carries a nonpolar insertion in the lktC gene (Fedorova and Highlander, 1997). This mutant strain of P. haemolytica synthesizes and secretes LKTA protein, however, it is biologically inactive, since it cannot be activated by LKTC. In addition, preincubation of LKT with a neutralizing monoclonal antibody MM601 (Gentry and Srikumaran, 1994) prior to exposure of bovine lymphocytes inhibited DNA fragmentation. In accordance with the narrow target cell specificity of LKT cytolysis at high concentrations (Kaehler, et al., 1980), low concentrations of LKT induced DNA fragmentation in bovine lymphocytes, but not in equine lymphocytes.

Dexamethasone, a long-acting glucocorticoid, has numerous pharmacological functions in vivo, including anti-inflammatory activity. However, many *in vitro* studies indicate that glucocorticoids activate the apoptosis pathway in both thymocytes and

mature lymphocytes (Wyllie, 1980; Garvy, et al., 1993; Brunetti, et al., 1995). Glucocorticoid-induced apoptosis is associated with activation of endogenous endonucleases as evidenced by DNA laddering (Wyllie, 1980). Based on these studies, we selected 10 μM dexamethasone as a positive control of apoptosis in bovine peripheral blood lymphocytes. Our results indicated that incubation of bovine lymphocytes with dexamethasone increased DNA fragmentation as determined by diphenylamine assay and observed as a typical apoptotic DNA ladder pattern in agarose gel electrophoresis. The LKT-induced and dexamethasone-induced DNA fragmentation were similar and revealed the same nucleosomal sized DNA ladder pattern in bovine lymphocytes. The mechanism of P. haemolytica LKT-induced apoptosis in bovine lymphocytes is unknown. However, the formation of DNA fragmentation after exposure of bovine lymphocytes to LKT clearly implicates activation of endogenous endonucleases. The structural components of nucleosomes include an octamer histone core, which consists of 2 H_2A, 2 H_2B, 2 H_3 and 2 H_4 histones, and 2 coils of double helix DNA wound around the histone core. The length of DNA coils is 165 bp (20 bp less tightly bound) and the linker DNA between 2 nucleosomes is 25 to 90 bp. Brief digestion of nucleosomes by endonuclease yields approximately 200 bp fragments. More extensive digestion yields 165 bp fragments and continued digestion yields 145 bp fragments. Fragments consistent with such cleavage were observed when bovine lymphocytes were treated with low concentration of LKT.

The effects of zinc on the apoptosis pathway have been extensively studied (Garvy, et al., 1993; Fady, et al., 1995; Solary, et al., 1996; Perry, et al., 1997). Zinc is thought to block apoptosis through a direct inhibition of putative endonucleases (Solary, et al., 1996). In the present study, 0.5 mM $ZnCl_2$ exhibited strong inhibition of

spontaneous, dexamethasone or LKT induced apoptosis. It has been known that glucocorticoids activate endogenous endonucleases (Wyllie, 1980; Garvy, et al., 1993; Brunetti, et al., 1995). Therefore, the inhibition of LKT-induced apoptosis in bovine lymphocytes by zinc suggests that low concentrations of LKT activate endogenous endonucleases.

In the present study, the results of the concentration and time dependent induction of apoptosis characterized by DNA fragmentation in target cells are generally in agreement with previous studies (Mangan, et al., 1991; Stevens and Czuprynski, 1996). Low concentrations of P. haemolytica LKT caused cell death by apoptosis, while at high concentrations LKT killed target cells by oncotic cytolysis. However, our study indicated that 0.1 TU/ml of LKT caused the maximal induction of apoptosis in bovine lymphocytes, while Stevens and Czuprynski (1996) defined 0.5 TU/ml of LKT as maximal induction of apoptosis in bovine leukocytes. In addition, they failed to demonstrate a DNA ladder pattern by agarose gel electrophoresis. These discrepancies may be caused by use of different methods for measurement of LKT activity (LDH leakage versus trypan blue exclusion) and differences in the number of target cells and experimental methods used for detection of DNA fragments. The number of target cells used in our experiments was four times higher than that in the study of Stevens and Czuprynski (1996). Therefore, the amount of fragmented DNA in our study was higher. Also, the diphenylamine assay for detection of DNA fragments is more sensitive than that of agarose gel electrophoresis (Squier and Cohen, 1997).

RTX toxins share many common genetic and biochemical features (Welch, 1991). The ability of RTX toxins to induce apoptosis may be another common RTX strategy for

the parent bacteria to escape the surveillance of host immune defense mechanisms. It has been shown that Bordetella pertussis, the causative agent of whooping cough in humans, causes cell death by apoptosis as determined by morphology and DNA fragmentation (Khelef, et al., 1993). Wild-type and pertussis toxin-deficient strains are able to induce apoptosis, while adenylate cyclase-hemolysin-deficient mutant does not. Thus the bifunctional adenylate cyclase-hemolysin is required for apoptotic cell death (Khelef, et al., 1993). LKT from Actinobacillus actinomycetemcomitans induces human lymphocyte death by oncosis or by apoptosis, dependent on the concentration of the toxin (Mangan, et al., 1991). The capacity of P. haemolytica LKT to induce apoptosis in bovine lymphocytes might be important in the initiation and progression of bovine shipping fever pasteurellosis, because the initial host inflammatory reaction may be reduced (Wyllie, et al., 1980). Apoptosis caused by low concentrations of LKT may be the mechanism responsible for LKT-inhibition of mitogen-induced blastogenesis in bovine lymphocytes and immunosuppression observed in shipping fever pneumonia. Further work is needed to examine the potential in vivo roles of low concentrations of P. haemolytica LKT.

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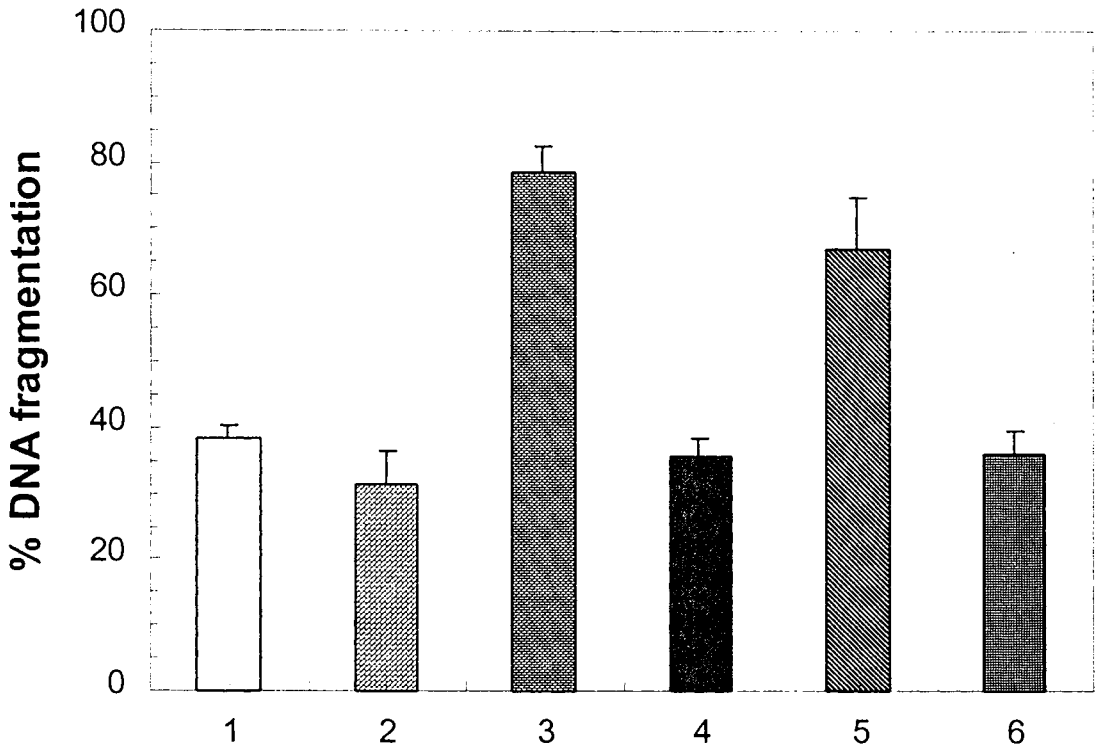

Fig.1. LKT-induced DNA fragmentation as determined by diphenylamine assay. Bovine lymphocytes were: 1, in complete RPMI-1640 medium; 2, in complete RPMI-1640 medium in the presence of 0.5 mM $ZnCl_2$; 3, incubated with 10 μM dexamethasone for 24 hours; 4, incubated with 10 μM dexamethasone for 24 hours in the presence of 0.5 mM $ZnCl_2$; 5, incubated with 0.1 TU/ml of LKT for 3 hours; or 6, incubated with 0.1 TU/ml of LKT in the presence of 0.5 mM $ZnCl_2$ for 3 hours. The data in this figure represents the means of three separate experiments.

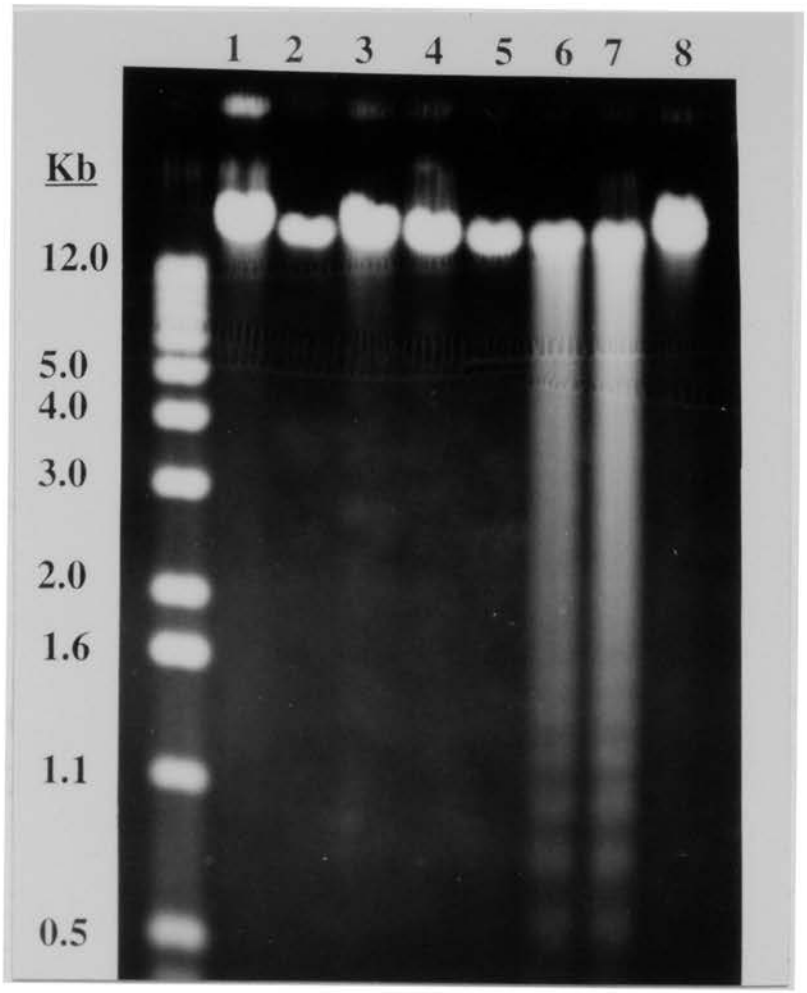

Fig. 2. DNA fragmentation "ladder" pattern is specific for LKT and LKT sensitive target cells. For lane 1, equine lymphocytes were exposed to 0.1 TU/ml of LKT. For lane 2 – 8, bovine lymphocytes were exposed to RPMI-1640 medium(lane 2); to cytolytic dose of LKT (1.0 TU/ml) (lane 3); to the inactive pro-LKT (same extent dilution of 0.1 TU/ml of LKT) (lane 4); to 0.1 TU/ml of LKT in the presence of 0.5 mM $ZnCl_2$ (lane 5); to 10 μM dexamethasone (lane 6); to 0.1 TU/ml of LKT (lane 7);, and to 0.1 TU/ml of LKT preincubated with an anti-LKT neutralizing monoclonal antibody MM601 (lane 8). The results represent four separate experiments with identical results. All treatments were incubated for with 3 hours at 37 °C with 5% CO_2, except dexamethasone which was incubated for 24 hours.

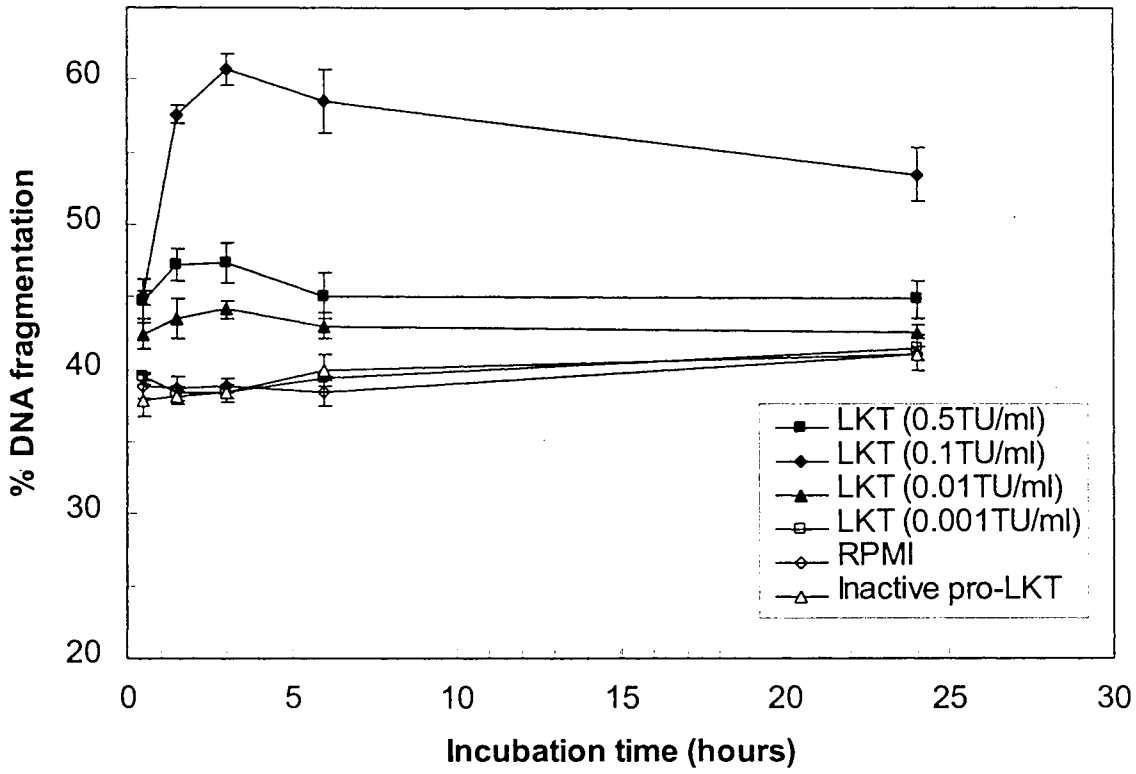

Fig. 3. Time dependence of LKT induced apoptosis as determined by diphenylamine assay. DNA fragmentation was quantitated after exposure of bovine lymphocytes to 0.5 (■), 0.1 (◆), 0.01 (▲), 0.001 (□) TU/ml of LKT, or the inactive pro-LKT with same extent dilution as 0.1 TU/ml of LKT (Δ) or RPMI-1640 only (◇).

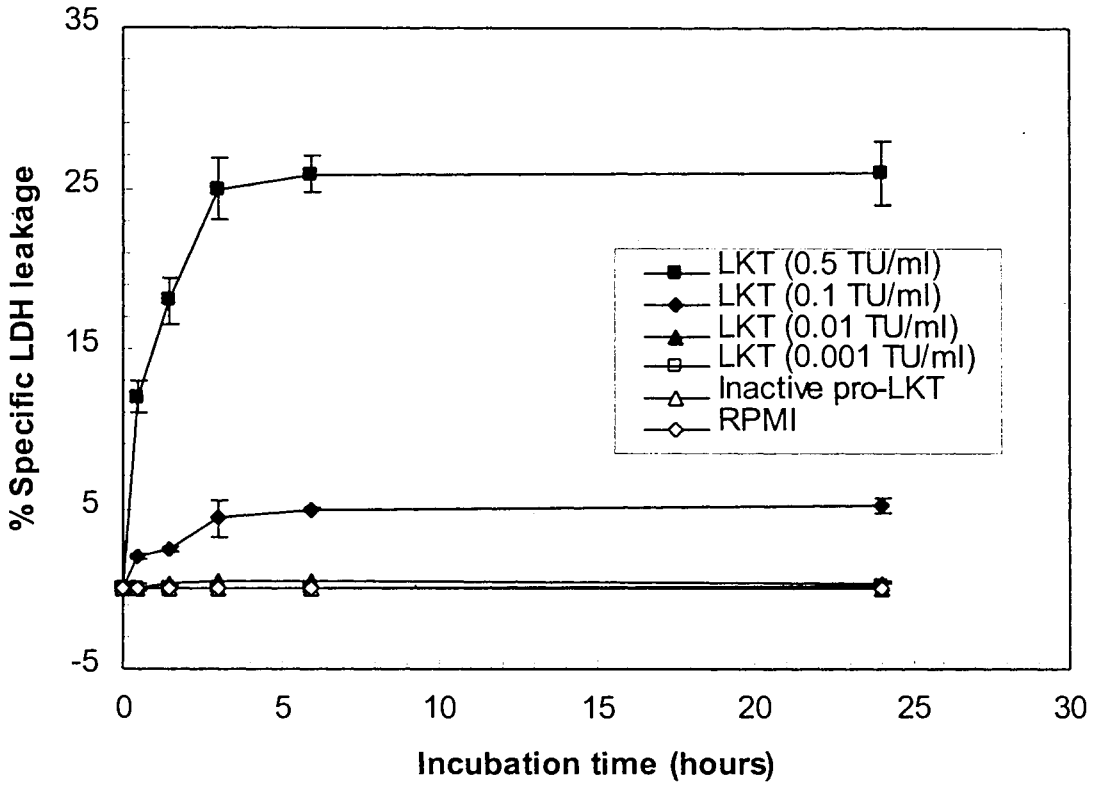

Fig. 4. Time dependence of LKT induced cytolysis as determined by measurement of intracellular enzyme lactate dehydrogenase (LDH) leakage. Percent specific LDH leakage was measured after exposure of bovine lymphocytes to 0.5 (■), 0.1 (◆), 0.01 (▲), 0.001 (□) TU/ml of LKT, or inactive pro-LKT with same extent dilution as 0.1 TU/ml of LKT (Δ) or RPMI-1640 only (✧).

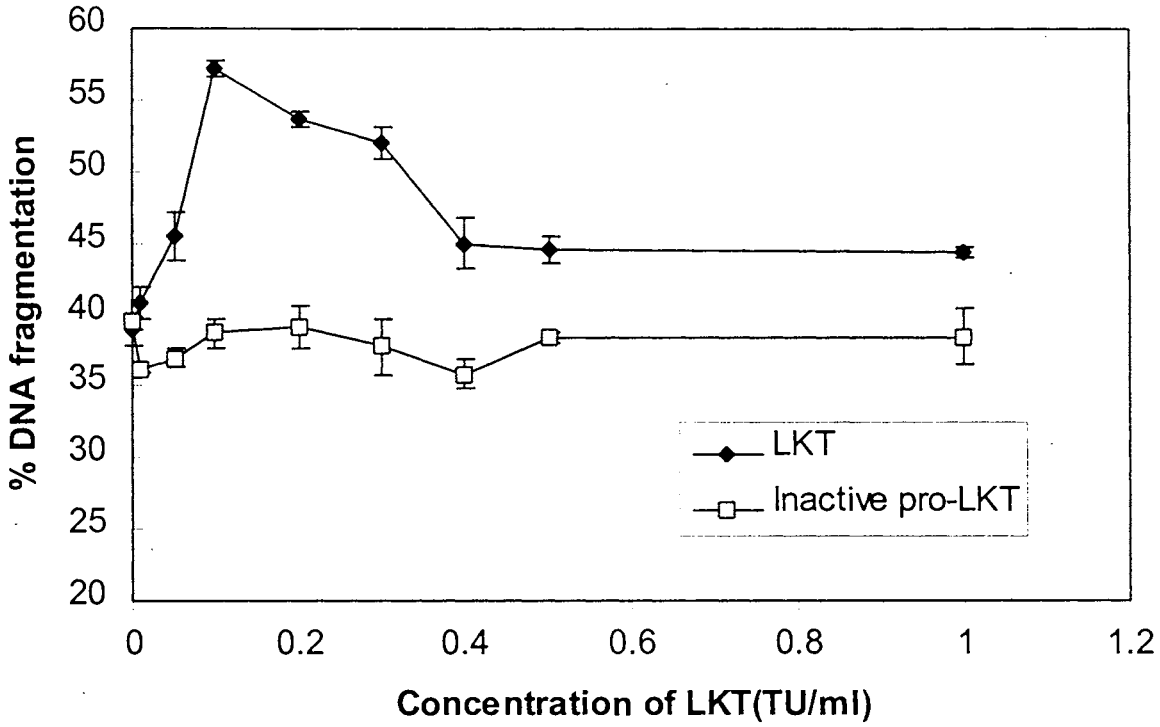

Fig. 5. Concentration dependence of LKT induced DNA fragmentation as determined by diphenylamine assay. DNA fragmentation was quantitated after exposure of bovine lymphocytes to different concentrations of LKT (◆), or inactive pro-LKT (□) for 3 hours.

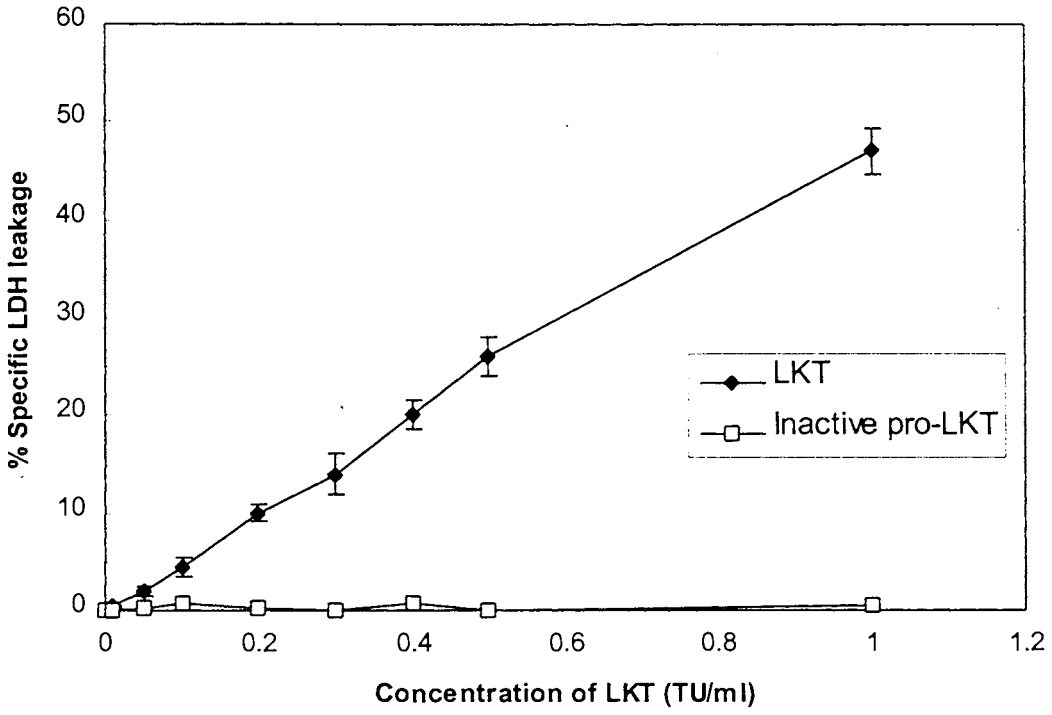

Fig. 6. Concentration dependence of LKT induced cytolysis as determined by measurement of intracellular enzyme LDH leakage. Percent specific LDH leakage was measured after exposure of bovine lymphocytes to different concentrations of LKT(◆), or inactive pro-LKT (□) for 3 hours.

CHAPTER VII

ULTRASTRUCTURAL CHARACTERIZATION OF APOPTOSIS IN BOVINE LYMPHOCYTES INDUCED BY *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN

This chapter was written in the format of and for publication in

American Journal of Veterinary Research

Structured abstract

Objective - To characterize ultrastructural changes of bovine lymphocytes exposed to *P haemolytica* leukotoxin.

Sample population - Partially purified leukotoxin and inactive pro- leukotoxin from one wild type and from its isogenic mutant strain of *P haemolytica* biotype A serotype 1. Isolated bovine lymphocytes were from two healthy calves.

Procedure - Isolated bovine lymphocytes were incubated with various concentrations of leukotoxin and pro- leukotoxin for 3 hours at 37 C. After washing, the cells were prepared for transmission electron microscopy. Thin sections (70 - 90 nm) were examined and photographed with a JEOL JEM-100 CX II transmission electron microscope at an accelerating voltage of 80 kV.

Results – At high concentrations of leukotoxin (0.5 TU/ml), bovine lymphocytes exhibited rupture of cytoplasmic and nuclear membranes and swelling or lysis of mitochondria. At low concentrations of leukotoxin (0.1 TU/ml), bovine lymphocytes exhibited nuclear membrane blebbing, formation of various vacuoles in the cytoplasm, chromatin condensation, nuclear fragmentation, and membrane-bound apoptotic bodies. Exposure of bovine lymphocytes to extremely low concentrations of leukotoxin (0.001 TU/ml) or various concentrations of inactive pro- leukotoxin did not alter the ultrastructure of bovine lymphocytes. Inclusion of 0.5 mM $ZnCl_2$, an endonuclease inhibitor, in exposure medium blocked leukotoxin-induced ultrastructural changes in bovine lymphocytes.

Conclusion – Low concentrations of leukotoxin induce apoptosis, and high concentrations of leukotoxin cause oncotic cell lysis in bovine lymphocytes.

Clinical Relevance - The ability of low leukotoxin concentrations to induce apoptosis in host leukocytes may allow the bacteria to escape host immune surveillance and colonize the lower airways in the host.

Introduction

Bovine shipping fever pneumonia caused by *P haemolytica* is a common and economically important disease in feedlot cattle.[1] During the conditions which can induce stress in cattle, such as shipment, viral infections, weaning, or overcrowding, *P haemolytica* proliferates in the upper respiratory tract, subsequently colonizes the lower airway, and produces various virulence factors, resulting in severe fibrinous pleuropneumonia.[2,3] **Leukotoxin** (LKT), a protein exotoxin produced by *P haemolytica*, is considered to be the primary virulence factor in the pathogenesis of shipping fever.[4,5] As a member of the **repeats-in-toxin** (RTX) family, LKT shares many common genetic and biochemical features with other RTX toxins.[6] Like other members of RTX toxins, LKT at high concentrations is thought to act by forming transmembrane pores in the plasma membrane of target cells resulting in colloid - osmotic cytolysis, termed oncotic cell lysis.[7,8] In addition to oncotic cell lysis, several RTX toxins have been found to cause apoptosis in target cells exposed to low toxin concentrations.[9-11] The ability of bacterial toxins to kill immune and phagocytic cells by apoptosis may allow the bacteria to escape host immune surveillance, to prolong pathogen survival and to establish colonization in the host.[12-14]

Apoptosis is a mode of cell death characterized by two remarkable features. One of these features is a distinct series of morphological changes that include nuclear chromatin condensation, cellular shrinkage, plasma membrane blebbing, and formation of membrane-bound apoptotic bodies. The other hallmark of apoptosis is a specific form of DNA degradation in which the genome of the apoptotic cell is degraded at

internucleosomal sites by endogenous endonucleases.[15,16] In contrast, oncotic cell lysis is typically initiated by plasma membrane permeability changes resulting in colloid-osmotic cell and organelle swelling, early rupture of cytoplasmic membrane, mitochondria, and other organelles.[8] The different features between apoptosis and oncotic cell lysis can be used to identify the specific mode of cell death.

Recently, Stevens and Czuprynski[17] reported that sublytic concentrations of *P haemolytica* LKT induced morphologic changes detected at the light microscopic level in bovine leukocytes consistent with apoptosis. However, DNA fragmentation, a biochemical hallmark of apoptosis, was not observed and the lower resolution associated with light microscopy made it difficult to interpret the morphological results. We subsequently determined that low concentrations of LKT causes increased DNA fragmentation characterized by internucleosomal cleavage[h]. In the present study, isolated bovine peripheral blood lymphocytes were incubated with various concentrations of *P haemolytica* LKT, and the morphological features of bovine lymphocytes were examined by transmission electron microscopy. Since the morphology of dying cells at the ultrastructural level provides reliable information about the mechanism of cell death, the objectives of this study were to characterize the ultrastructural changes of bovine lymphocytes after exposure to *P haemolytica* LKT and to determine whether low LKT concentrations induce ultrastructural changes consistent with apoptosis or oncotic cell lysis.

Materials and Methods

Isolation of bovine peripheral blood lymphocytes - Two healthy beef calves (approximately 200 kg) served as blood donors for isolation of lymphocytes. Lymphocytes were isolated as previously described[18] with certain modifications. Briefly, the blood sample was collected from healthy calves by venipuncture of the jugular vein, and anticoagulant (sodium heparin, 15 U/ml) was added. Forty-five ml of heparinized blood in a 50 ml conical centrifuge tube were centrifuged at 600 x *g* for 30 minutes at 22 C, then the plasma was aspirated down to about 10 -15 mm above the interface between the erythrocytes and the plasma. The cells at the interface were carefully pipetted into another centrifuge tube with as few erythrocytes as possible and were diluted (1:3, v/v) with **Hank's balanced salt solution** (HBSS, Ca^{2+} and Mg^{2+} free). Thirty to thirty-five ml of diluted cells were carefully layered on 15 ml of Ficoll-Paque[a] and centrifuged at 500 x *g* for 20 minutes with brake in the off position. After centrifugation, lymphocytes and monocytes in the white interface between plasma and the Ficoll-Paque were pipetted into another 50 ml conical centrifuge tube, washed twice with 30 ml of HBSS and resuspended in RPMI-1640 containing 10% **fetal bovine serum** (FBS) at 5 x 10^6 cells/ml. This final cell suspension was free of erythrocytes and contained approximately 90% lymphocytes (mean 89.9, range 72 - 99%) and 5% monocytes (mean 4.5, range 0 - 9%), based on differential counting. To remove the monocytes, 5 ml of cell suspension was placed into a 50 mm tissue culture-treated Nunclon petri dish[b] and incubated at 37 C for 2 hours. Following the incubation, monocytes adhered onto the plate surface, and

99% of nonadherent cells were lymphocytes. Greater than 95% of the cell population was capable of excluding trypan blue in all experiments.[18]

Preparation of LKT and inactive pro-LKT concentrated culture supernatants - Concentrated culture supernatants from a wild type *P haemolytica* A1 strain SH1217 and its isogenic strain SH1562 containing a non-polar insertion in the *lktC* gene were used as the LKT preparation and as inactive pro-LKT negative control, respectively.[19] Concentrated culture supernatants were prepared by inoculating 1L RPMI-1640 medium containing 0.1M phosphate buffer (pH 6.8) and 1 mM Mg^{2+} to an OD_{600nm} = 0.25 with wild type or mutant strain of *P haemolytica* prepared by growth overnight on 5% bovine blood agar and then in 100 ml of RPMI-1640 under 5% CO_2 to late logarithmic phase.[20] The cultures in RPMI-1640 were grown at 37 C with 120 oscillations/minute for approximately 4 hours to an OD_{600nm} = 0.9 to 1.0. All subsequent steps were conducted at 4 C. The bacteria were removed by centrifugation at 8,000 x *g* for 30 minutes, and the culture supernatant was concentrated and partially purified by fractional ammonium sulfate precipitation (0-60% saturation by addition of 361 g/L solid ammonium sulfate). The precipitates were resuspended at 0.5 mg protein/ml in 20 ml of 50 mM sodium phosphate, 100 mM sodium chloride buffer, pH 7.0 (phosphate-NaCl), dialyzed against the same buffer, and stored at –135 C. The culture supernatant protein concentrations of the LKT and inactive pro-LKT preparations were not significantly ($P > 0.05$) different.[19]

Determination of LKT activity - Activity of LKT was assayed by measuring leakage of the intracellular enzyme **lactate dehydrogenase** (LDH) from bovine lymphocytes. These cells were enumerated by a hemocytometer, and collected by centrifugation at 700 x *g* for 15 minutes. The cell pellet was resuspended in RPMI-1640 medium, and the final concentration of bovine lymphocytes was adjusted to 4×10^6 cells/ml. Aliquots of 100 µl of lymphocytes were added to 100 µl of serially diluted culture supernatants in a 96-well round-bottom microtiter plate. Leakage of LDH was maximized by addition of 0.1% t-octylphenoxypolyethoxyethanol (v/v) and background leakage was determined by exposure of cells to RPMI-1640 only. Plates were incubated at 37 C for 2 hours, and exposure terminated by centrifugation at 700 x *g* for 10 minutes. One hundred-µl aliquots of cell supernatant were transferred to clean 96-well flat-bottom microtiter plates, and plates were warmed to 37 C. Activity of LDH was assessed kinetically by adding 100 µl of assay reagent LDH-L 50,[a] held at 37 C, to all wells and measuring the absorbance at 340 nm (OD_{340nm}) per minute in a thermally controlled, kinetic microtiter plate reader[c] for 2 minutes at 37 C. Specific leakage of LDH was calculated as a percentage as follows:

$$\% \text{ specific LDH leakage} = [(A-B)/(C-B)] \times 100$$

in which A = LKT-induced LDH leakage, B = RPMI-1640 negative control, and C = t-octylphenoxypolyethoxyethanol positive control. The LKT activity was enumerated as **toxic units** (TU) that were determined by graphing specific LDH leakage versus the culture supernatant dilution factor. One TU was defined as the dilution factor at which the specific LDH leakage under the assay conditions described is 50%.

Preparation of cell treatments - Isolated bovine peripheral blood lymphocytes were placed in complete RPMI-1640 medium containing 10% FBS. The positive control for apoptosis was prepared by incubation of lymphocytes (5 ml, 4 x 10^6 cells/ml) with 10 μM dexamethasone at 37 C for 18 hours with 5% CO_2 or 1.0, 0.1, 0.001 TU/ml LKT or same diluted scales of inactive pro-LKT as LKT for 3 hours. After incubation, the cells were pelleted by centrifugation at 500 x *g* for 10 minutes. The cell pellets were used for transmission electron microscopy preparation and the supernatants were used for LDH leakage assay to monitor the relationship between LKT-induced oncotic cell lysis and apoptosis in target cells. Data were reported as % specific LDH leakage.

Electron microscopy - Sample preparation for electron microscopy was based on a modified protocol from Anderson and Ownby.[21] Briefly, 0.5 ml of control and leukotoxin-treated cells (4 x 10^6 cells/ml) were pelleted by centrifugation at 500 x *g* for 1 minute and washed twice with 0.1 M cacodylate buffer (pH 7.4). Fixation was performed in 1.6% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4). Fixed cells were washed in 0.1M cacodylate buffer and postfixed in 1 % osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer for 2 hours. After washing, cells were dehydrated in ethanol (50, 70, 90, 95, and 100%), transferred to propylene oxide for 1 hour, then embedded in 100% Polybed resin[e]. Thin sections (70 - 90 nm) of selected areas of the plastic embedded blocks were taken with Sorvall MT-6000 ultramicrotome[f] using a Diatome diamond knife[g]. Sections were picked up and mounted on copper grids (200 mesh) and stained with aqueous uranyl acetate and lead citrate. Samples were examined and photographed

with a JEOL JEM-100 CX II transmission electron microscope[h] at an accelerating voltage of 80 kV.

Results

In the present study, the synthetic glucocorticoid hormone dexamethasone was used as the positive control for apoptosis.[16, 22] The typical ultrastructural features in bovine lymphocytes incubated with 10 μM dexamethasone for 18 hours at 37 C included cytoplasmic vacuolation, chromatin condensation and margination, nuclear lobulation, and formation of membrane-bound apoptotic bodies (Fig. 1A). In contrast, the ultrastructure of bovine lymphocytes incubated in RPMI-1640 medium for 18 hours without dexamethasone was not altered (Fig. 1B). These cells were characterized by the scant cytoplasm and large, slightly indented nucleus. The nuclear chromatin was typical with a relatively condensed heterochromatin/euchromatin pattern.

To monitor the relationship between the oncotic cell lysis and apoptosis induced by LKT, the leakage of intracellular enzyme LDH, a marker of cell lysis, was measured after exposure of bovine lymphocytes to different concentrations of LKT or inactive pro-LKT. The results in Fig. 2 indicated that 1.0 TU/ml of LKT induced 55% specific LDH leakage and 0.1 TU/ml of LKT induced only about 5% specific LDH leakage from exposed bovine lymphocytes. Various concentrations of inactive pro-LKT and 0.001 TU/ml of LKT did not induce specific LDH leakage.

Incubation of lymphocytes with low concentration of *P haemolytica* LKT (0.1 TU/ml) for 3 hours induced marked alterations in the cell ultrastructure. These were characterized by irregular cell shapes and by nuclear and cytoplasmic abnormalities (Fig.

3). Nuclear membrane blebbing was commonly observed (Fig. 3A). The nuclei with irregular nuclear shapes were either fragmented, condensed, or polylobated. Condensed chromatin was frequently observed at the periphery of the nucleus in the shape of a horseshoe (Fig. 3B). Cytoplasmic vacuoles with variable size and shape were also observed (Fig. 3C). In addition, apoptotic bodies of various sizes and shapes were frequently observed (Fig. 3D). All of these ultrastructural changes induced by 0.1 TU/ml of *P haemolytica* LKT were similar to apoptosis induced by dexamethasone (Fig. 1A). In contrast, treatment with 0.001 TU/ml of LKT (Fig. 4A), with various concentrations of inactive pro-LKT (Fig. 4B) with RPMI-1640 medium (Fig. 5) did not alter the ultrastructural changes of bovine lymphocytes.

After incubation of bovine lymphocytes with 1.0 TU/ml of *P haemolytica* LKT at 37 C for 3 hours, rupture of nuclear, mitochondrial and plasma membranes was observed (Fig. 6). In some cells, mitochondria were swollen with ruptured cristae, and the nuclear envelope and plasma membrane were ruptured (Fig. 6A). Some cells were completely lysed with only a remnant of membrane present (Fig. 6B), however other cells with ruptured cytoplasmic membranes contained some intact organelles and discernable nuclei within ruptured nuclear membranes (Fig. 6B).

It has been previously shown that certain concentrations of Zn^{2+} inhibit formation of apoptosis by inhibition of endonuclease activity (Solary, et al., 1996).[23] To test the effect of Zn^{2+} on dexamethasone- or LKT-induced apoptosis, bovine lymphocytes were incubated with 10 μM dexamethasone or 0.1 TU/ml of LKT in the presence of 0.5 mM Zn^{2+}. As shown in Fig. 7, exposure of bovine lymphocytes to dexamethasone or the low

concentration of LKT in the presence of 0.5 mM Zn^{2+} blocked apoptotic ultrastructural changes.

Discussion

Apoptosis and oncotic cell lysis are two distinct mechanisms of cell death, each of which has its typical morphologic and biochemical features.[22] The criteria for assessment of apoptosis generally include demonstration of ultrastructural morphology by electron microscopy and DNA ladder pattern of multiple 200 bp fragments by agarose gel electrophoresis.[9,22] In the present study, we have used transmission electron microscopy, a most reliable method for recognizing the morphology of apoptotic cells.[22] Although other methods, such as light or fluorescence microscopy may be used for detection of apoptotic cells, transmission electron microscopy provides unequivocal detailed ultrastructural features of apoptosis.[22]

We found that after exposure of bovine lymphocytes to 0.1 TU/ml of *P haemolytica* LKT, the cells underwent ultrastructural changes, including chromatin condensation and margination, nuclear fragmentation, and formation of cytoplasmic vacuoles and membrane-bound apoptotic bodies, characteristic of apoptosis.[9,10,16,22-28] We observed apoptotic bodies, which were surrounded by an intact membrane with no spillage of intracellular contents. Therefore, conventional methods for determination of cell death, such as ^{51}Cr release, LDH leakage assays and trypan blue exclusion test, may underestimate the proportion of dead cells,[22] because these methods measure cell death

dependent on increased plasma membrane permeability, due to the defects in the plasma membranes.

Several other members of the RTX toxin family, such as *Escherichia coli* α-hemolysin, *Actinobacillus actinomycetemcomitans* leukotoxin, and *Bordetella pertussis* adenylate cyclase/hemolysin, are also capable of inducing apoptosis of target cells at low concentrations.[9-11,28] It seems that a particular threshold of RTX toxin-induced cytoplasmic membrane disruption may be required to cause oncotic cell lysis. Below this threshold, the apoptotic cell death pathway is activated.[11] These RTX toxin-producing bacteria may use this function as a common strategy to escape the host immune surveillance, to prolong their survival, and to establish colonization in the hosts during the initial stage of infection.[12,14]

LKT-induced oncotic cell lysis in ruminant leukocytes has been extensively examined by measuring ^{51}Cr release, trypan blue exclusion, intracellular enzyme leakage, or by scanning electron microscopy.[29] These methods measure cytoplasmic membranes integrity, or demonstrate the surface appearance of target cells. Using these methods, the severity of damage in a population of target cells was estimated, but the exact intracellular ultrastructural changes of individual cells could not be determined. The present study directly demonstrated the intracellular ultrastructural changes in bovine lymphocytes treated with a high concentration of LKT. The electron micrographs documented swollen mitochondria and rupture of nuclear, organelle and plasma membranes. Chromatin condensation, usually used as a criterion for identification of apoptosis, was also observed in oncotic cell lysis. However the chromatin condensation

observed with was easily distinguished at the ultrastructural level from that in apoptosis, because the latter was surrounded by an intact nuclear membrane.

The components of culture supernatants from an isogenic mutant stain of *P haemolytica* were similar to that from the wild parent strain, except that the mutant strain only produced an inactive pro-LKT.[19] Treatment of bovine lymphocytes with various concentrations of inactive pro-LKT did not alter the ultrastructural morphology of bovine lymphocytes, which indicates that the active component in *P haemolytica* culture supernatants is LKT. It also implies that the post-translational modification of an RTX toxin is required for apoptosis or oncotic cell lysis. The results in this study indicated that high concentration of LKT causes oncotic cell lysis and low concentration of LKT induces apoptosis in bovine lymphocytes. At extremely low LKT concentrations, exposed bovine lymphocytes undergo neither oncotic cell lysis nor apoptosis. The pro-LKT had no effect at any concentration tested.

The ability of LKT to induce apoptosis in bovine lymphocytes could be the reason for LKT-mediated inhibition of mitogen-induced blastogenesis of bovine or ovine lymphocytes in vivo.[30-32] Indeed, the induction of apoptosis in cells directly involved in the immune response, as shown in the case of LKT from *P haemolytica*, may enhance initiation and extension of the bacterial infection.[33]

In conclusion, the ultrastructural changes in bovine lymphocytes treated with low concentration of *P haemolytica* LKT were characteristic of those of apoptosis. The capability of LKT to induce apoptosis in bovine lymphocytes may be important in the initiation and progression of *P haemolytica* infection and could explain the immunosuppressive conditions observed in bovine shipping fever. Further work will be

concentrated on LKT-induced apoptosis in vivo and the effects of LKT-induced apoptosis on host immune responses.

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[a] Sigma Chemical Co, St. Louis, Mo.

[b] Nalge Nunc International., Milwaukee, Wisc.

[c] Thermomax, Molecular Devices Palo Alto, Calif.

[d] Polysciences, Warrington, Penn.

[e] Du Pont Company, Diagnostic and BioResearch Systems, Wilmington, Dela.

[f] Diatome U.S., Fort Washington, Penn.

[g] JEOL LTD, Tokyo, Japan.

[h] Sun Y, Clinkenbeard KD, Clarke CR, et al. The proceedings of 79th Conference of Research Workers in Animal Diseases, 1998; 137.

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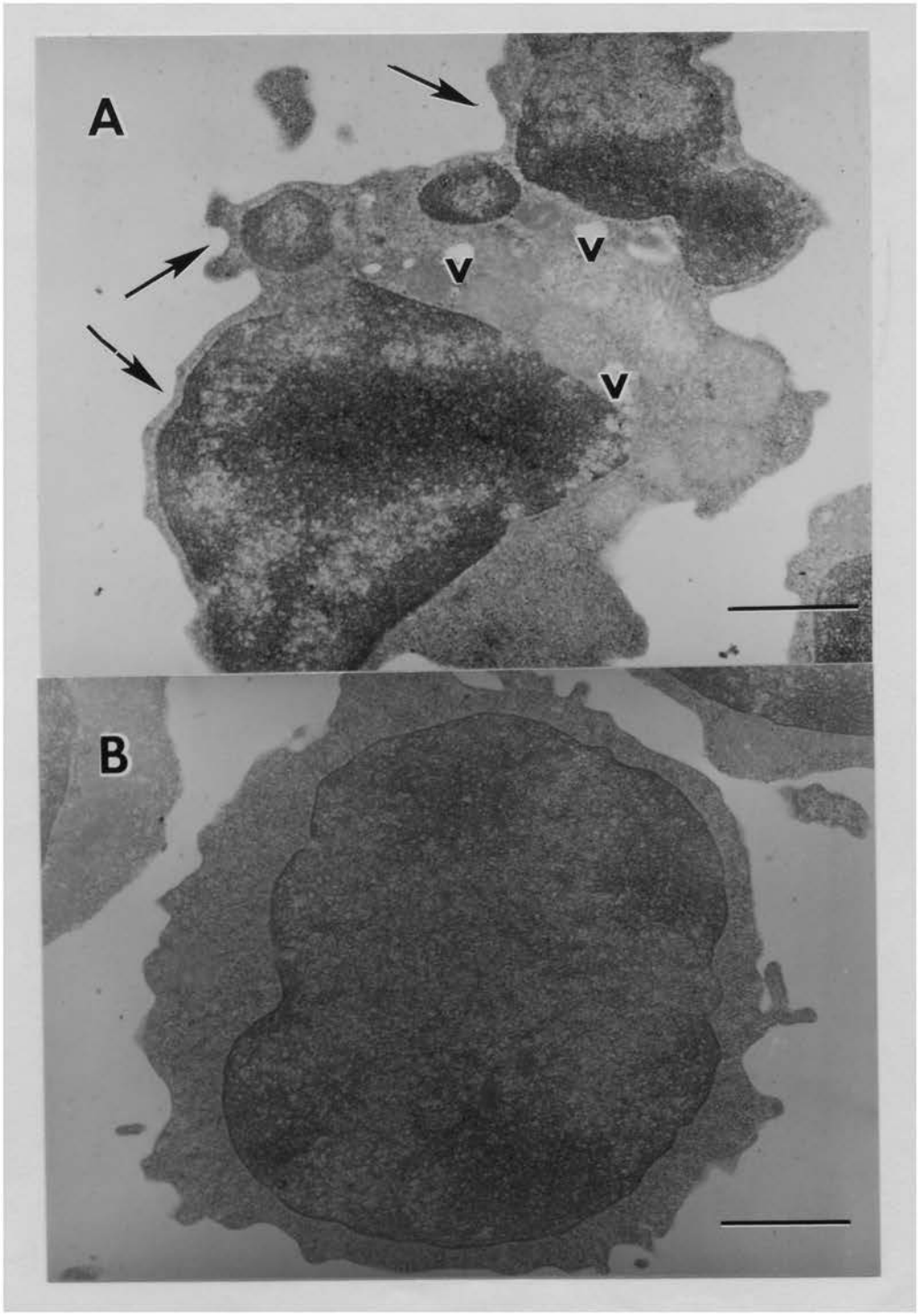
A
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B

Fig. 1. Transmission electron micrographs of bovine lymphocytes. The isolated bovine lymphocytes were incubated with 10 μl dexamethasone (A) or in RPMI-1640 alone (B) for 18 hours and then fixed, processed, and analyzed by transmission electron microscope. Relative to control, dexamethasone-treated cell exhibited cytoplasmic vacuolation (v), chromatin condensation and margination, nuclear lobulation, and formation of membrane-bound apoptotic bodies (arrow). Bar = 1 μm.

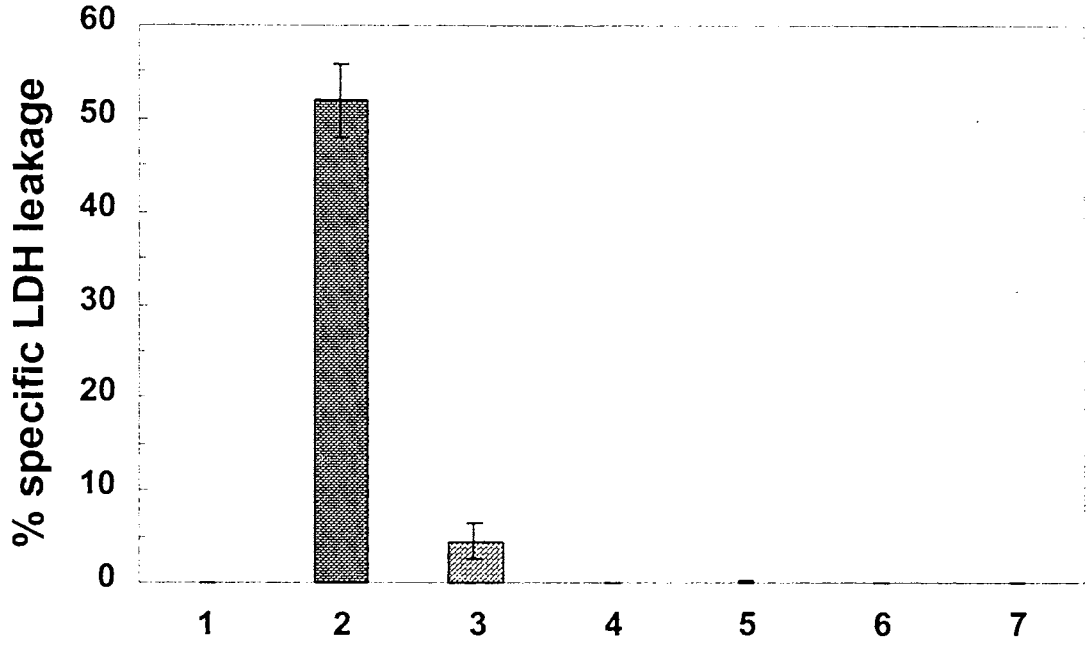
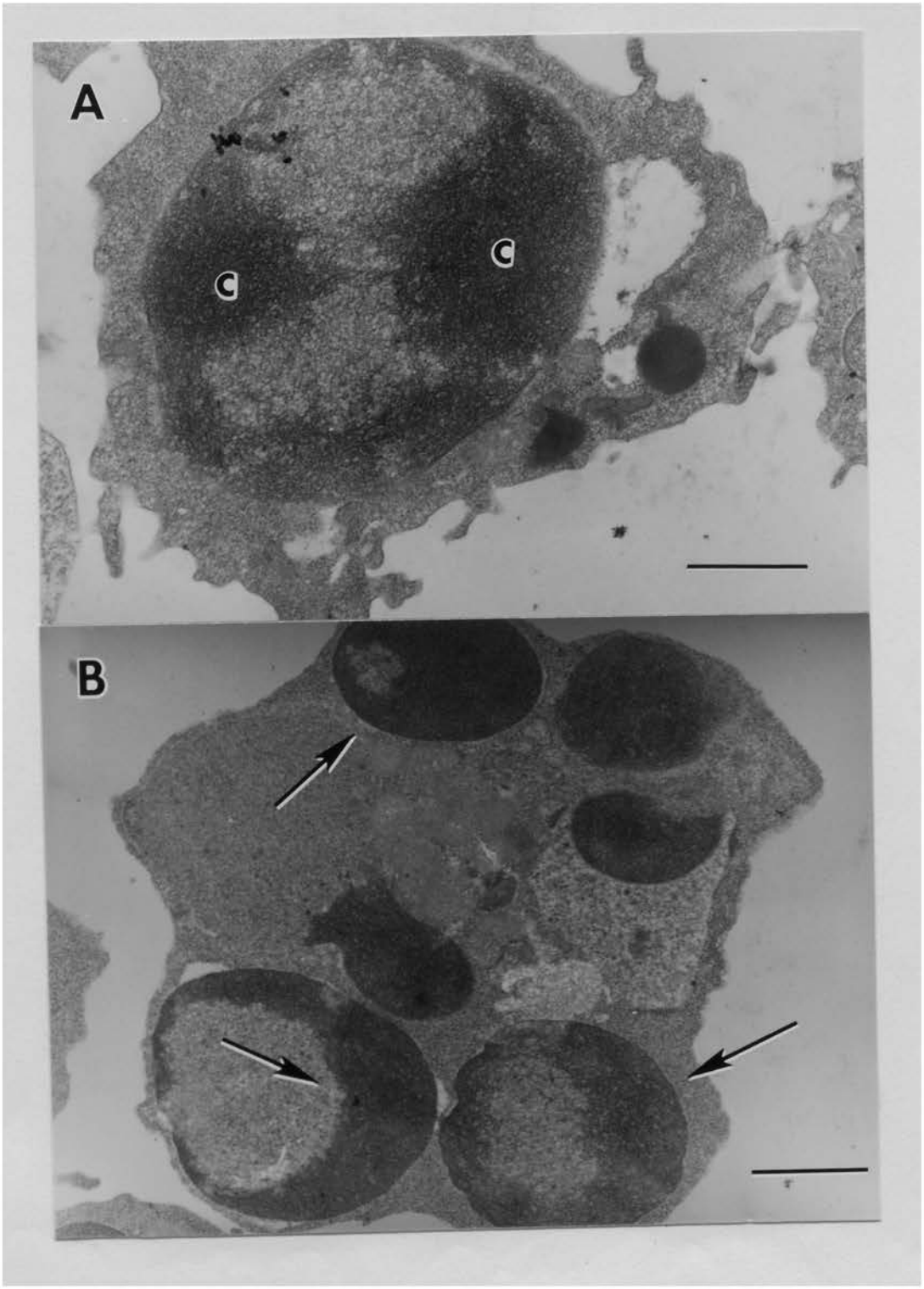


Fig. 2. The effect of LKT on LDH leakage from isolated bovine lymphocytes incubated with *P haemolytica* LKT. The bovine lymphocytes were incubated for 3 hours in RMPI-1640 alone (1), with 1.0 TU/ml (2), 0.1 TU/ml (3), 0.001 TU/ml (4) of LKT, or incubated with same diluted scales of pro-LKT (5, 6, and 7). Per cent specific LDH leakage was defined as compared net LDH leakage from the cells treated with LKT to that from the cells treated with t-octylphenoxypolyethoxyethanol.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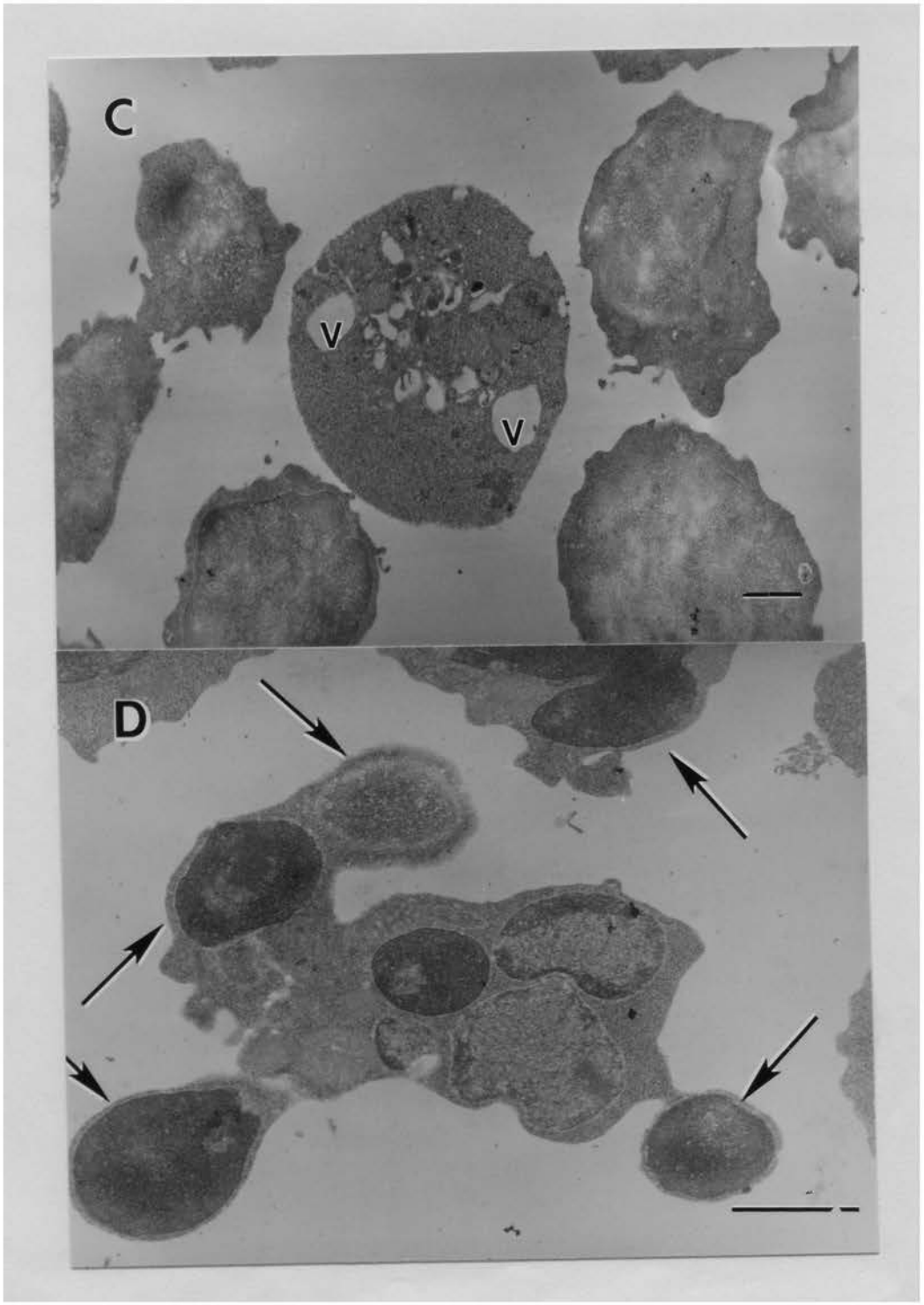
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Fig. 3. Transmission electron micrographs of bovine lymphocytes treated with 0.1 TU/ml LKT. Isolated bovine lymphocytes were incubated with 0.1 TU/ml of LKT for 3 hours and then fixed, processed, and examined with the transmission electron microscope. The ultrastructural changes included (A) chromatin condensation and margination (c), nuclear membrane blebbing (arrow); (B) nuclear fragmentation and chromatin condensation to horseshoe shape (arrow), (C) formation of various vacuoles in the cytoplasma (v), and (D) membrane-bound apoptotic bodies (arrows). Bar = 1 μm.

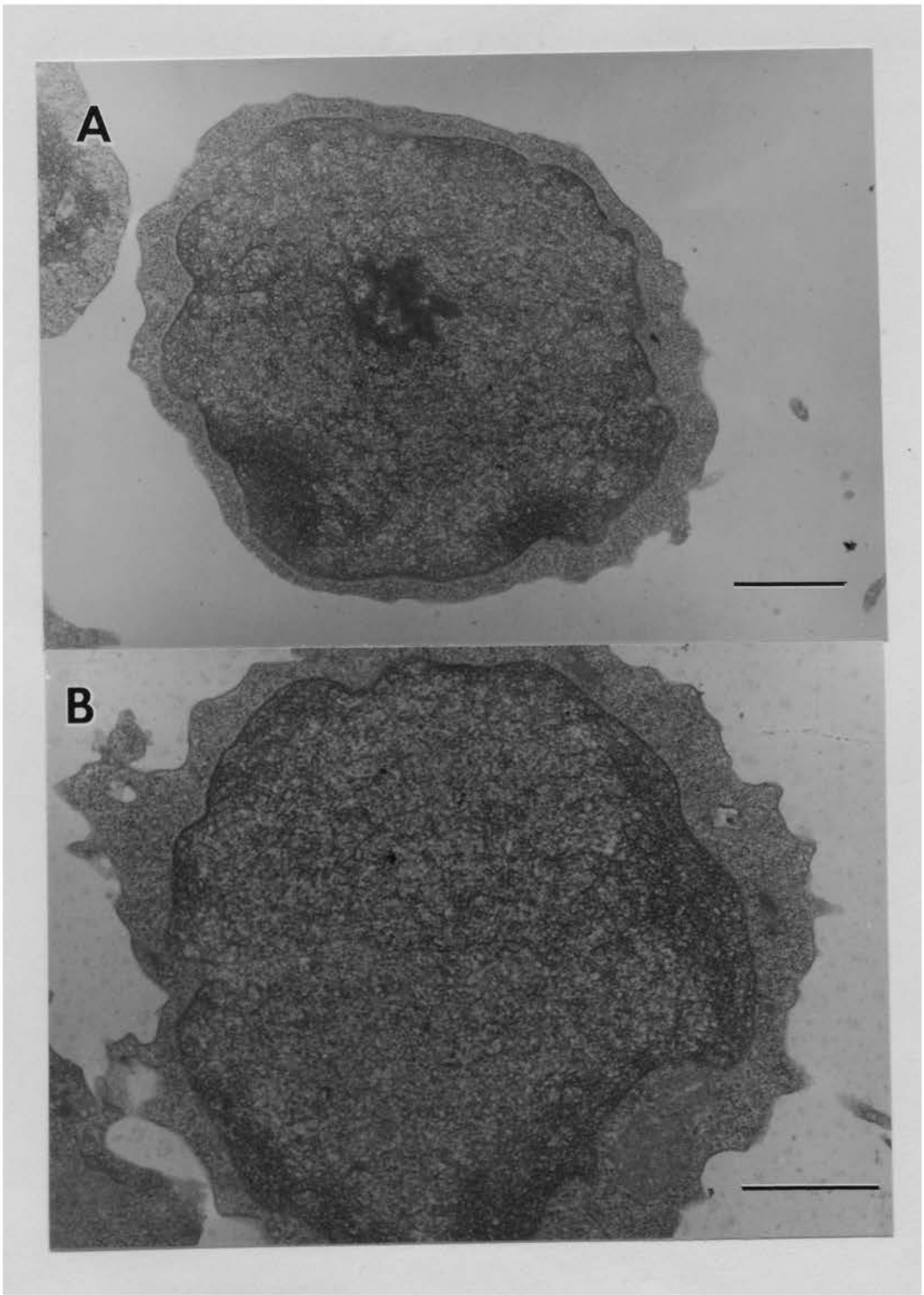
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Fig. 4. Transmission electron micrographs of bovine lymphocytes treated with 0.001 TU/ml LKT or pro-LKT. The isolated bovine lymphocytes were incubated with 0.001 TU/ml of LKT (A, bar = 1 μm.) or pro-LKT (B, bar = 1μm.) for 3 hours and then fixed, processed, and examined by transmission electron microscope. Compared to control in Fig. 5, the ultrastructural morphology of these cells was not altered.

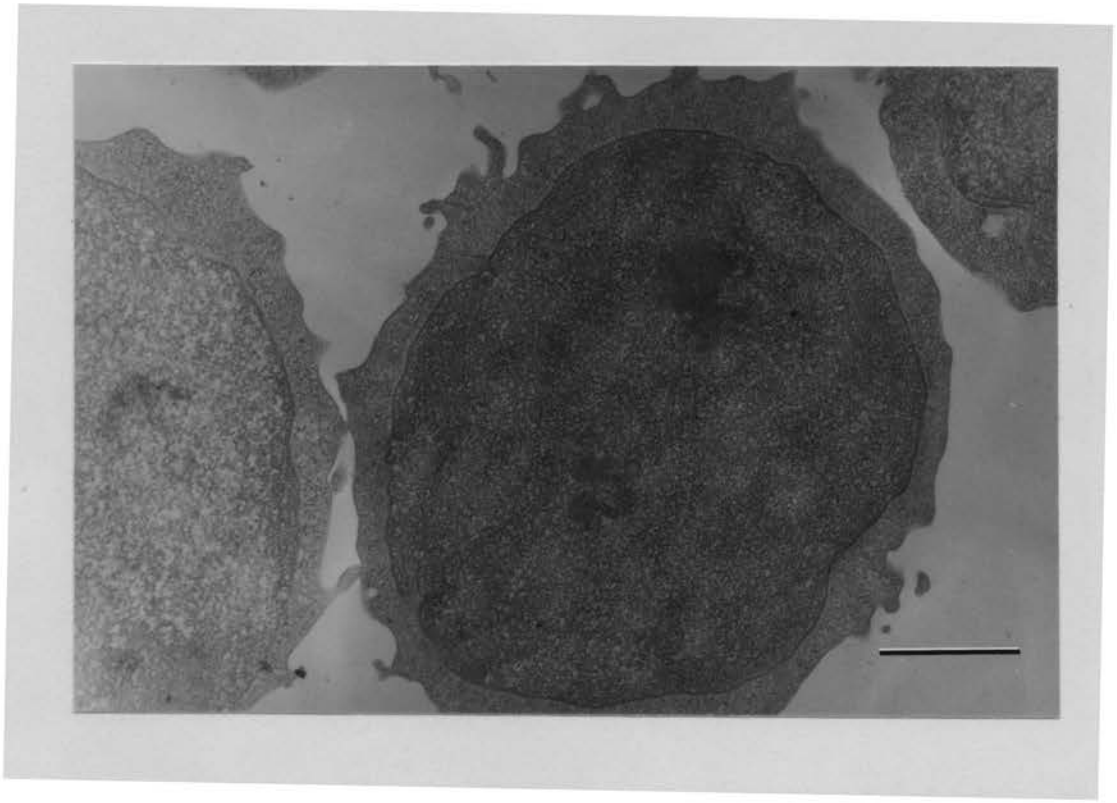

Fig. 5. Transmission electron micrographs of bovine lymphocytes incubated in RPMI-1640 for 3 hours. The isolated bovine lymphocytes were incubated in RPMI-1640 for 3 hours and then fixed, processed, and analyzed by transmission electron microscope. The ultrastructural characteristics of these cells included the scant cytoplasm and large, slightly indented nucleus. The nuclear chromatin with the typical heterochromatin/euchromatin patterns was relatively condensed. Bar = 1 μm.

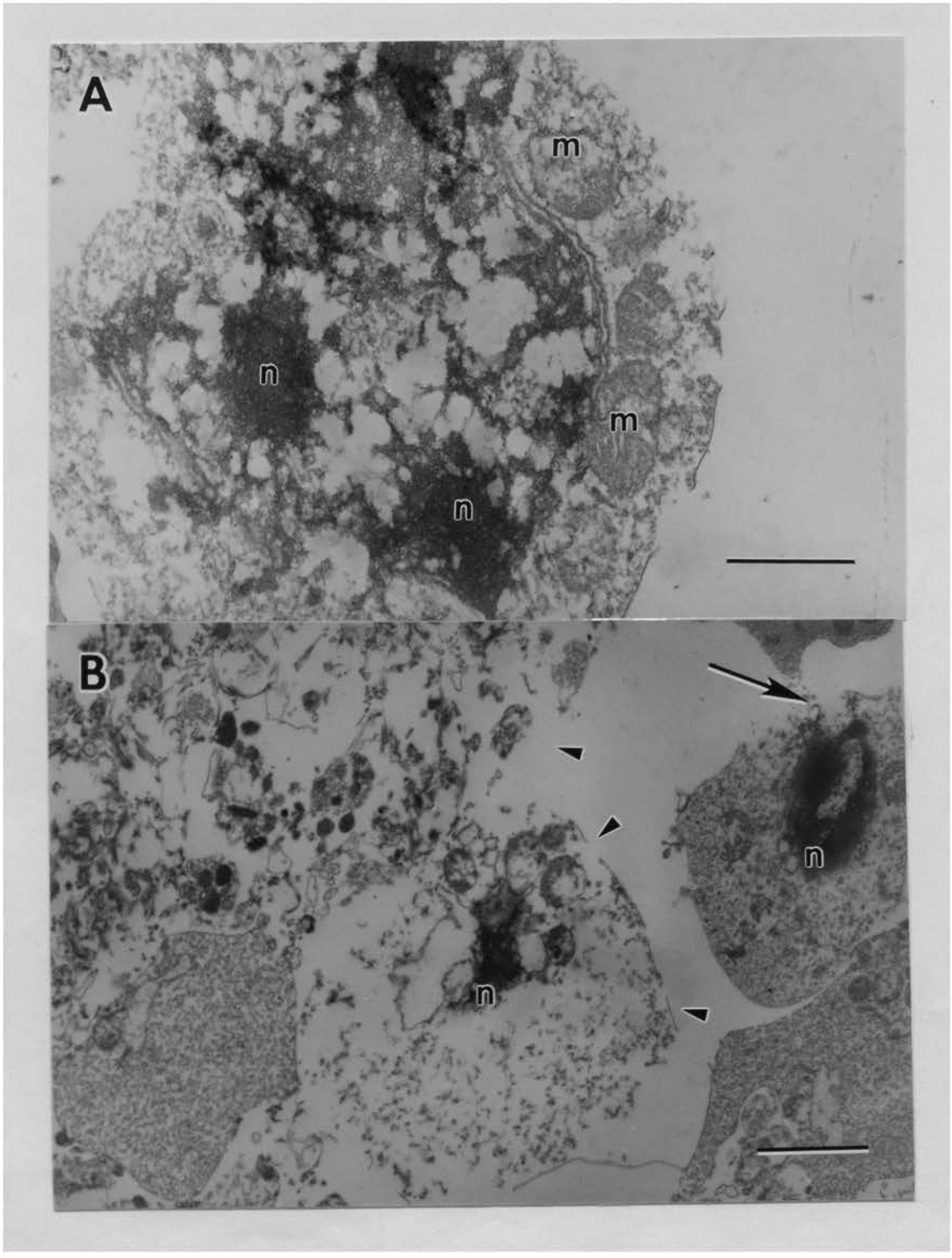
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Fig. 6. Transmission electron micrographs of bovine lymphocytes treated with 1.0 TU/ml LKT. The isolated bovine lymphocytes were incubated with 1.0 TU/ml of LKT for 3 hours and then fixed, processed, and analyzed by transmission electron microscope. (A) Cell with ruptured cytoplasmic membrane (arrowhead) and damaged mitochondria (m), (B) Cell with condensed nuclear chromatin (n), nuclear envelope was ruptured (arrow). Bar = 1 μm.

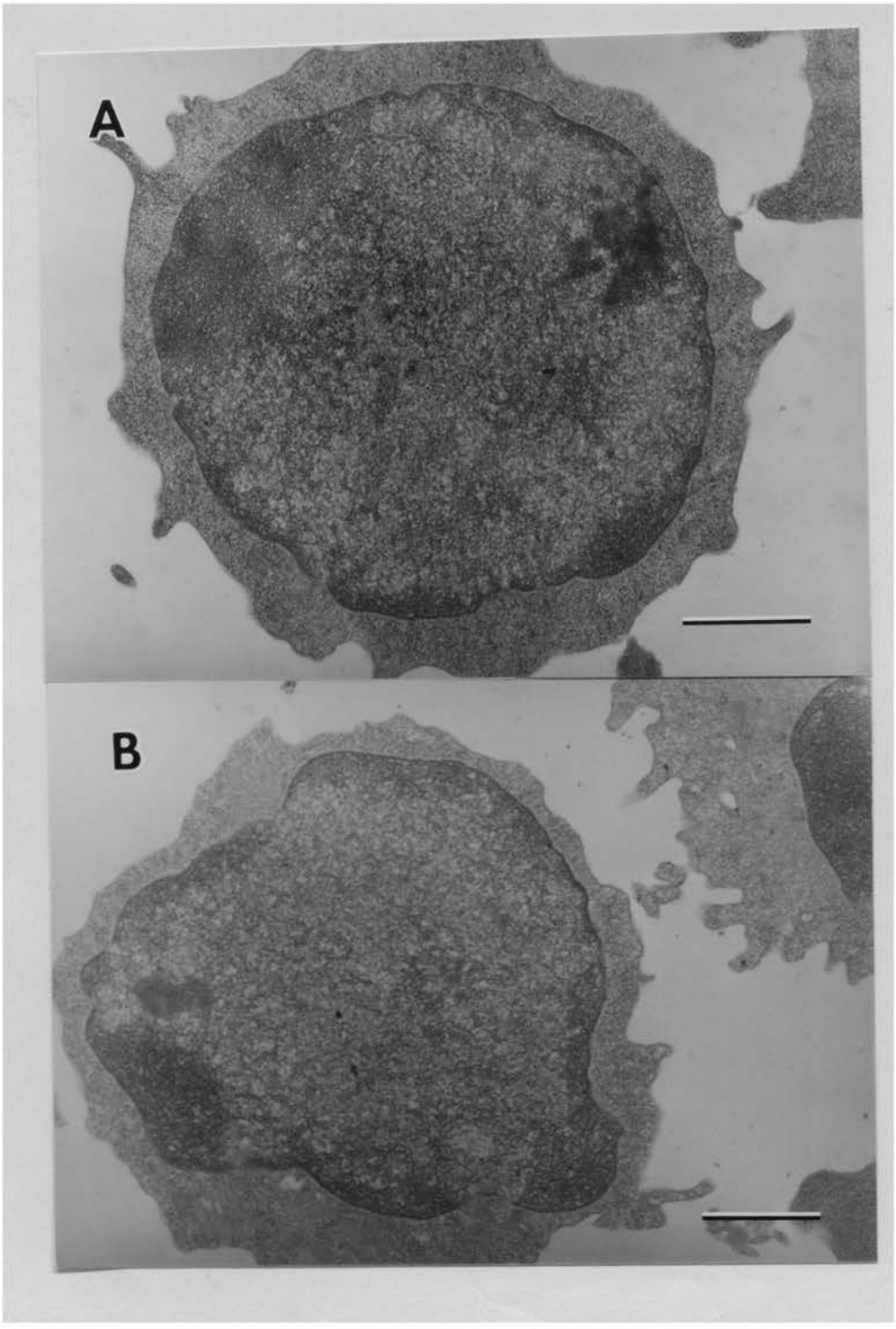
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Fig. 7. Effect of Zn^{2+} on apoptosis. The isolated bovine lymphocytes were incubated with 0.1 TU/ml of LKT in the presence of 0.5 mM Zn^{2+} for 3 hours and then fixed, processed, and analyzed by transmission electron microscope. LKT-induced ultrastructural morphological changes were blocked by 0.5 mM Zn^{2+}. Bar = 1 μm.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Pasteurella haemolytica, a facultative anaerobic gram-negative coccobacillus, is the primary etiological agent of bovine shipping fever pneumonia responsible for great economic losses in the North American cattle industry. *P. haemolytica* produces several virulence factors, including exotoxin, endotoxin, fimbriae, outer membrane proteins, neuraminidase, and capsular polysaccharide, to diminish the host defense systems and establish infection. In the present study, I have focused on the exotoxin, termed as leukotoxin (LKT), the primary virulence factor and an important protective antigen for bovine pneumonic pasteurellosis.

In Chapter IV, a serum-free culture medium for optimization of *P. haemolytica* LKT production was defined, which can be served as a source of LKT for purification. By comparison with brain-heart infusion (BHI) broth, yeast-tryptone broth, RPMI-1640, and McCoy's modified 5A media with different pH values as basal media for cultivation of *P. haemolytica* biotype A serotype 1, pH 6.8 is the optimal pH value for *P. haemolytica* growth and LKT production in all test media. LKT activity and concentration were significantly higher ($P<0.05$) in BHI and RPMI-1640 media. For protein purification, it is very important to measure specific activity, which is defined as a protein interested related to the total protein in the culture supernatant. By comparison of LKT specific activity in BHI and RPMI-1640, it was found that *P. haemolytica* produces higher LKT specific activity in RPMI-1640 medium. After these preliminary experiments, RPMI-1640 was chosen as a basal medium for the following studies. RPMI is often used as tissue culture medium and is buffered with bicarbonate. As tissue culture under 5% CO_2, bicarbonate is able to maintain a stable pH value, but not for bacterial culture under aerobic conditions, because loss of CO_2 from the medium resulted in

alkalization. The results in this study indicated that replacement of bicarbonate buffer with 0.1 M sodium phosphate buffer was sufficient to maintain a stable pH value throughout the bacterial growth period. To enhance LKT production, RPMI-1640 as a basal medium was supplemented with different metal ions or glucose for cultivation of *P. haemolytica*. The results indicated that supplementation of Mg^{2+} or Fe^{3+} to RPMI-1640 could enhance LKT activity and concentration as bovine serum albumin (BSA), but LKT specific activity (LKT activity vs total protein concentration in culture supernatant) was much higher than BSA supplementation. Addition of 1% glucose to phosphate buffered and Mg^{2+}-supplemented RPMI-1640 did not further enhance LKT production.

P. haemolytica LKT is a member of the repeats-in-toxin toxin family of gram-negative bacterial pore-forming toxins. In addition to *P. haemolytica* LKT, other members of RTX toxins include α-hemolysin of *Escherichia coli*, leukotoxin of *Actinobacillus actinomycetemcomitans*, hemolysins and leukotoxins of *A. pleuropneumoniae* and *A. suis*, and adenylated cyclase/hemolysin of *Bordetella pertussis*. Unlike other RTX toxins, *P. haemolytica* LKT and *A. actinomycetemcomitans* leukotoxin (Ltx) intoxicate a narrow range of target cells limited primarily to leukocytes from ruminant and primates, respectively. The molecular basis of this cell-type and species specificity for intoxication is mediated by binding of the toxins to lymphocyte function-associated antigen 1 (LFA-1) on susceptible cells. However, whether RTX toxins are specifically bound to only susceptible target cells, the initial step of the interaction, is still controversial. In Chapter V, the initial interaction of LKT with its target cells was investigated and two LKT binding assays based on the principals of immunological dot blot and western blot were developed. The susceptibility of target cells to LKT-mediated

intoxication was determined by measurement of increases in intracellular Ca^{2+} concentration and leakage of cytoplasmic enzyme lactate dehydrogenase from target cells. LKT is able to bind both susceptible cells, including bovine lymphoma BL3 cells and isolated bovine blood lymphocytes, and non-susceptible cells, including human lymphoma Raji cells and human promyelocytic leukemia HL60 cells, although the relative amount of bound LKT was variable. The binding of LKT was time- and concentration-dependent, and no saturation was observed in all target cells that we tested. These implicated that LKT, like several other members of RTX (repeats-in-toxin), is able to non-specifically bind to both susceptible and non-susceptible cells, although execution of its biological function (cytolysis) is through the interaction of the toxin to LFA-1 on susceptible cells.

Apoptosis is an important mode of cell death, which is distinct from oncosis. Apoptosis is an active cellular process of gene-directed self-destruction and has been shown to be a fundamental biological process that impacts on the early development, maturation, homeostasis, and acquisition of disease states of multicellular organisms. While oncosis is a passive, catabolic, and degenerative cell death. Oncotic cell lysis involves swelling of mitochondria and disruption of membrane integrity and subsequent cellular swelling and lysis, due to the colloid-osmotic imbalance of the cell. While apoptosis is characterized by membrane blebbing, chromatin condensation, DNA fragmentation into oligonucleosomal fragments giving a characteristic ladder pattern on ethidium bromide-stained agarose gels, and formation of membrane bound apoptotic bodies. It has been reported that several members of RTX toxins including *P. haemolytica* LKT are able to induce apoptosis in their target cells, implying that

induction of cell death by apoptosis may be another common strategy of RTX toxins. In Chapters VI and VII, whether LKT was able to induce apoptosis in isolated blood lymphocytes was investigated. The biochemical, histo- and cytochemical, electron microscopical methods were used to characterize LKT-induced cell death. Like higher concentrations of LKT-induced cytolysis, lower concentrations of LKT-mediated apoptosis in isolated lymphocytes was target cell specific. LKT-induced DNA fragmentation, a hall marker of apoptosis, occurred only in bovine lymphocytes, not in equine lymphocytes. Pre-incubation of LKT with its neutralizing monoclonal antibody MM601, or in the presence of 0.5 mM $ZnCl_2$ in LKT exposure blocked LKT-induced apoptosis, implicating that LKT was only component in the culture supernatant of *P. haemolytica* responsible for formation of DNA fragments and LKT-induced DNA fragmentation was through activation of endonucleases. The ultrastructural morphology of LKT-induced apoptosis in bovine lymphocytes included chromatin condensation and margination, vacuolation, formation of membrane-bound apoptotic bodies. Inclusion of 0.5 mM $ZnCl_2$ in LKT exposure also blocked LKT-induced ultrastructural changes. The ability of *P. haemolytica* LKT at low concentrations to induce apoptosis in bovine lymphocytes might be important in the initiation and progression of bovine pneumonic pasteurellosis, because the initial host inflammatory reaction may be reduced. Apoptosis caused by low concentrations of LKT may be the mechanism responsible for LKT-inhibition of mitogen-induced blastogenesis in bovine lymphocytes and immunosuppression observed in shipping fever pneumonia. Further work is needed to examine the potential in vivo roles of low concentrations of *P. haemolytica* LKT.

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