

To the Graduate Council:

I am submitting herewith a thesis written by Donald G. Gerbig, Jr. entitled "A Role for Divalent Cations in the Cytotoxicity of Pasteurella haemolytica leukotoxin." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Microbiology.

Robert N. Moore

Robert N. Moore, Major Professor

We have read this thesis
and recommend its acceptance:

Mike A. Breiden

David A. Bemis

Accepted for the Council:

C. W. Mink

Vice Provost
and Dean of The Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Donald J. Schubert

Date 7/31/87

A ROLE FOR DIVALENT CATIONS IN THE CYTOTOXICITY
OF PASTEURELLA HAEMOLYTICA LEUKOTOXIN

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Donald G. Gerbig, Jr.

August 1987

DEDICATION

This work is dedicated to my wife, Frances, for her love and patience throughout this ordeal. I would also like to dedicate this to my parents, Mr. and Mrs. Donald G. Gerbig, Sr., whose love and support through the years have made this possible.

ACKNOWLEDGMENTS

I appreciate the support, interest and patience shown by the members of my thesis committee, Dr. David A. Bemis, Dr. Michael A. Breider and especially Dr. Robert N. Moore. I also appreciate the efforts of Steve Foster for his technical assistance and focus on reality. In general, I would like to express my gratitude for the assistance given to me by the faculty, staff and students in the Department of Microbiology.

ABSTRACT

Pasteurella haemolytica (Biotype A, serotype 1), the primary causative agent of bovine pneumonic pasteurellosis, produces a potent ruminant specific leukotoxin that is believed to be a primary virulence determinant. The nature and mode of action of this toxin is poorly understood. Properties of Pasteurella haemolytica leukotoxin have been further elucidated using leukotoxin culture filtrates generated in RPMI medium 1640 (rpm/1640) with 1% horse serum. The BL-3 cell line was used as the leukotoxin target in an assay for cytotoxicity. Exposure of the target cells to the leukotoxin for as little as 5 min was sufficient to elicit cytotoxicity as assessed by neutral red cytotoxicity assays and thymidine incorporation assays. The leukotoxin killed target cells at 4°C and at 37°C and was not stabilized or destabilized in the presence or absence of serum. Estimated molecular weight of the leukotoxin, as determined by gel filtration chromatography, was approximately 75,000 daltons (d); however, some activity was detected in fractions with estimated molecular weights of 46,000 and 16,000 d.

Leukotoxin-target cell interactions were also investigated. The leukotoxin appeared to have a specific requirement for divalent cations. Using one hr neutral red cytotoxicity assays, leukotoxic activity in concentrated filtrates was reduced following dialysis against divalent-cation free Hanks balanced salt solution (HBSS). Cytotoxicity was recovered by the addition of physiological concentrations of Ca^{2+} , Ba^{2+} , Co^{2+} , and Sr^{2+} , but not by Mg^{2+} or Mn^{2+} . Furthermore, two Ca^{2+} chelators, Ethylenediamine tetraacetic acid

(EDTA) and Ethyleneglycol-bis-(B aminoethyl ether) N,N'-tetraacetic acid (EGTA), abrogated the activity of leukotoxic filtrates. In addition, Verapamil and the ionophore A23187, both known to influence Ca^{2+} channels, reduced cytotoxicity in one hr assays when used with leukotoxin diluted in HBSS containing Ca^{2+} and Mg^{2+} . The protective effect of Verapamil was overcome by increasing the Ca^{2+} concentration.

Results from additional experiments, however, indicated that divalent cations were not absolutely essential for cytotoxicity. Cytotoxicity was determined over time in RPMI 1640 with and without EGTA using neutral red cytotoxicity, trypan blue exclusion and chromium⁵¹ release assays. Cell death occurred in two phases: a rapid death requiring divalent cations and a prolonged death that was independent of divalent cations (specifically Ca^{2+}). The cytotoxic effect of P. haemolytica leukotoxin, therefore, was influenced by the presence of divalent cations (specifically Ca^{2+}). The requirement for Ca^{2+} , however, was not absolute.

TABLE OF CONTENTS

CHAPTER	PAGE
1. INTRODUCTION	1
Statement of Purpose	10
2. MATERIALS AND METHODS	11
Materials	11
<u>Pasteurella haemolytica</u> Lipopolysaccharide	12
Cell Culture	13
Pulmonary Lavage Cells	13
Bacterium	14
Leukotoxin Production	14
Assays	15
Neutral red cytotoxicity assay	15
Trypan blue exclusion assay	16
Chromium release assay	16
Thymidine incorporation assay	17
Gel filtration column chromatography	18
Statistical Analysis	18
3. RESULTS	19
Sensitivity of the BL-3 Cell Line to Leukotoxic Filtrates	19
Optimal Conditions for Generation of Leukotoxic Filtrates	22
Characteristics of Leukotoxic Activity in Culture Filtrates	29
Target Cell-Toxin Interaction	35
Calcium as a Requirement for Leukotoxicity	41

CHAPTER	PAGE
3. (continued)	
Effects of Verapamil and the Ionophore A23187 on Leukotoxicity	47
4. DISCUSSION	54
LIST OF REFERENCES	62
VITA	70

LIST OF TABLES

TABLE	PAGE
1. Influence of Serum on Leukotoxin Production	26
2. Leukotoxin Production as a Function of Incubation Time .	28
3. Stability of Leukotoxic Filtrates when Incubated for Three Hours in the Presence of Increasing Serum Concentrations	31
4. Effect of Incubation Temperature on the Sensitivity of BL-3 Cells to <u>P. haemolytica</u> Leukotoxin	34
5. Cytotoxicity of One Unit of Leukotoxin in Buffer Containing Chelating Agents	40

LIST OF FIGURES

FIGURE	PAGE
1. Cytotoxicity of <u>P. haemolytica</u> Culture Filtrate for Bovine Pulmonary Lavage Cells and Cells of the BL-3 Cell Line	20
2. Cytotoxicity of <u>P. haemolytica</u> Culture Filtrate for Cells of the Murine Cell Line P388-D1 and the Bovine BL-3 Cell Line	21
3. Assessment of the Cytotoxic Potential of <u>P. haemolytica</u> LPS for BL-3 Cells	23
4. Leukotoxin Production by <u>P. haemolytica</u> as a Function of the Initial Concentration of Bacteria	24
5. Growth Kinetics of <u>P. haemolytica</u>	27
6. Sensitivity of <u>P. haemolytica</u> Leukotoxin to Heating at 56°C for 30 min	30
7. Sephacryl S-200 Elution Profile of Leukotoxic Activity in <u>P. haemolytica</u> Filtrates	32
8. Leukotoxin Exposure Time Required for Induction of Cytotoxicity for BL-3 Cells	36
9. Lack of Effect of Dexamethasone Pretreatment on Sensitivity of BL-3 Cells to <u>P. haemolytica</u> Leukotoxin	38
10. Influence of Ca^{2+} and Mg^{2+} on Sensitivity of BL-3 Cells to <u>P. haemolytica</u> Leukotoxin	39
11. Restoration of Leukotoxin Sensitivity of BL-3 Cells by Addition of Ca^{2+} but not Mg^{2+}	42
12. Restoration of Leukotoxin Sensitivity of BL-3 Cells by Addition of Ca^{2+} , Ba^{2+} , Co^{2+} and Sr^{2+}	43
13. Leukotoxin Cytotoxicity Time Course for BL-3 Cells in the Presence or Absence of EGTA Assessed by the Release of ^{51}Cr	44
14. Leukotoxin Cytotoxicity Time Course for BL-3 Cells in the Presence or Absence of EGTA Assessed by Exclusion of Trypan Blue	45

15.	Leukotoxin Cytotoxicity Time Course for BL-3 Cells in the Presence or Absence of EGTA Assessed by the Uptake of Neutral Red	46
16.	Leukotoxin Cytotoxicity Time Course for BL-3 Cells in the Presence or Absence of EGTA Assessed by Incorporation of ³ HTdR	48
17.	Protective Effect of Verapamil for BL-3 Cells Exposed to <u>P. haemolytica</u> Leukotoxin	49
18.	Reversal of Verapamil Protection of Leukotoxin-Treated BL-3 Cells by Exogenously Added Ca ²⁺	50
19.	Protective Effect of A23187 Ionophore for BL-3 Cells Exposed to <u>P. haemolytica</u> Leukotoxin	51
20.	Failure of Phorbol Myristate Acetate to Protect BL-3 Cells from <u>P. haemolytica</u> Leukotoxin	53

LIST OF ABBREVIATIONS

CFU	Colony Forming Unit
cpm	Radioactive counts per minute
dH ₂ O	Distilled water
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(B aminoethyl ether) n,n'- tetraacetic acid
hr	Hour
HBSS	Hank's balanced salt solution
LPS	Lipopolysaccharide
min	Minute
RPMI 1640	RPMI 1640 growth medium
U	Unit
μm	Micron

CHAPTER 1

INTRODUCTION

Bovine respiratory disease (BRD) complex is a vague term describing many respiratory diseases of cattle. Bovine shipping fever pneumonia is a major component of the BRD complex and a major problem affecting the cattle industry in North America (71). The disease occurs when cattle are transported from the range to the stockyards, hence, the colloquial name shipping fever (6). The disease is characterized by fever, anorexia, nasal discharge, weakness, inflammation of the airways, fibrous exudate, lymphatic clots, thromboses and necrotic foci (21). The economic losses plaguing the cattle industry result not only from death, but also weight loss, delayed marketings and a smaller supply of beef (20). Compounding these problems are the expenses of medical treatment and extra labor. This disease has affected the cattle industry for many years, and an in-depth understanding has been the focus of numerous investigations (6,9,21).

In general, a number of factors are assumed to contribute to the pathogenesis of shipping fever. These influences include stressor factors in combination with infectious agents such as viruses and bacteria. Stressor factors include prolonged transport involving anxiety and fatigue, temperature extremes, inadequate feeding and watering, changes in diet, increased humidity, crowding, inhalation of dust, and drafts (6). There is little doubt that these factors are involved, but the extent is uncertain. Viruses associated with the

disease include parainfluenza-3 virus (PI-3) and bovine herpes virus-1 (BHV-1) (22,24,30,57). Bacteria involved include Mycoplasma spp., Pasteurella multocida and Pasteurella haemolytica (65). Although Mycoplasma spp. have been isolated from pneumonic lungs, by far the most important etiological agents of the disease are Pasteurella multocida serotype 3 and Pasteurella haemolytica serotype 1 (9,21). Of these two organisms, Pasteurella haemolytica serotype 1 is the most frequently isolated bacterium from pneumonic lungs (6). Thus, pneumonic pasteurellosis is a predominant form of shipping fever.

Experimentally the disease can be induced with a combination of viruses and bacteria or bacteria alone. Virus infection is assumed to act as a predisposing factor (18,57,58). In conjunction with Pasteurella spp., both PI-3 and BHV-1 induce pneumonic pasteurellosis (22,23,24). Interestingly, aerosol vaccination of cattle with BHV-1 induces protective immunity to subsequent challenge with BHV-1 and P. haemolytica (24). These results imply that viral infections may play a major role in the pathogenesis of pneumonic pasteurellosis, perhaps by hindering lung clearance of the bacteria (22,24). Studies have shown that low numbers of P. haemolytica can be adequately cleared from the lung in healthy cattle, whereas, larger inocula of the bacterium can induce fibrinous pneumonia (71). Alterations of normal clearance function by viral infection may enhance susceptibility to the bacterium.

Although the pathogenesis of pneumonic pasteurellosis has not been elucidated, preventative measures have been implemented, albeit with mixed results (12,71). The most obvious solution would be better

management of cattle from the farm to the stockyards, taking care to avoid stressor factors. However, vaccination regimes have become the focus of attention to combat the problem of shipping fever (13).

Since P. haemolytica serotype-1 is the most frequently isolated organism, vaccinations have concentrated upon this organism.

Vaccinations have been attempted with killed bacterins, live organisms, and products of the bacterium. Unfortunately, killed bacterins have been used with limited success against experimental challenge with P. haemolytica. In one study, animals vaccinated parenterally with a killed bacterin fared far worse when experimentally challenged than control animals vaccinated with phosphate buffered saline (67). In another study, vaccination with live organisms injected subcutaneously protected animals against experimental challenge compared to animals vaccinated with killed organisms (12). Vaccination with live organisms, therefore, appears to protect animals from experimental challenge, whereas killed bacterins appear either to be ineffective or to elicit inappropriate responses. Live organisms produce factors or maintain antigenic structures that may aid in conferring immunity that killed bacterins do not. However, vaccination with live organisms injected intramuscularly or subcutaneously sometimes affords little protection. The live organisms may be rapidly inactivated in healthy animals (13). Since the bacterium is a respiratory pathogen and a mucosal immune response is essential in protection, the most successful route of vaccination may be through aerosol exposure (65). Unfortunately, aerosol exposure is not the most practical route of vaccination.

Immunity elicited by this route is short lived and requires multiple exposure to antigens (65). Therefore, vaccinating a large number of animals by this route would not be practical for field use (13).

The age of the culture used to immunize influences the effectiveness of vaccination for pneumonic challenge with P. haemolytica. Aerosol immunization with log phase cultures protected better than stationary phase cultures (11), indicating, perhaps, that something is produced by log phase cultures that is not present or is inadequate in stationary phase cultures. The growth phase factor produced by live organism could be capsular material or some other antigen associated with a live organism. Aerosol immunization with young encapsulated cultures provides better protection against experimental challenge than aerosol immunization with older, nonencapsulated cultures (11). Additional factors such as neuraminidase (54), proteases (42), or cytotoxin (4,47) released by younger cultures could also be involved with protection associated with cells from logarithmic phase cultures.

Pasteurella haemolytica produces a number of possible virulence factors that might be involved in the disease as well as confer protection observed with immunization. Among these are endotoxin, neuraminidase, proteases and a potent cytotoxin. Endotoxin in the lung activates the alternative complement pathway, increases vascular permeability, and can cause thromboses in veins, capillaries, and lymphatics that result in necrosis (43,56,59). In addition, endotoxin as well as antigen-antibody complexes can cause disseminated intravascular coagulation (56). Much of the damage seen in pneumonic

pasteurellosis may be attributed to the effects of endotoxin produced by P. haemolytica (10,28,56).

Neuraminidase may be a potential virulence factor in pneumonic pasteurellosis. Vibrio cholera produces a neuraminidase that unmasks a cell receptor for cholera toxin (61). A similar role for the neuraminidase of P. haemolytica has been postulated (54). It is possible that the potent cytotoxin produced by the organism (47) requires neuraminidase to unmask a receptor for its action on target cells. However, the role for neuraminidase has not been identified.

A neutral, metal dependent protease produced by P. haemolytica that is specific for sialoglycoprotein has been implicated also as a potential virulence factor in pneumonic pasteurellosis (42). As with the neuraminidase, the role this factor plays in disease is unknown. Other bacterial proteases have been implicated as potential virulence factors (reviewed in 8), indicating that further work on the protease may be necessary to establish if it has a role in disease.

Pasteurella haemolytica produces a potent cytotoxin that has received much attention as a potential virulence factor. This cytotoxin is also known as a leukotoxin since it is specifically toxic for leukocytes (46). Such a leukotoxin, produced in vivo, could be a major factor acting to enhance survival of the organism in the lower respiratory tract (46,51, reviewed in 8). The primary leukocyte in the healthy bovine lung is the alveolar macrophage (62,66). However, inhalation of P. haemolytica elicits a neutrophilic response in the bovine lung (48,66). The damage seen in the lung of an animal with pneumonic pasteurellosis includes necrosis of the alveolar walls,

intra-alveolar hemorrhage with the accumulation of proteinaceous fluid in the alveoli and lymphatics (28). Much of this lung injury is attributed to neutrophil degranulation, most likely damaged by the leukotoxin (32,48,62). Moreover, in vitro log phase P. haemolytica is not phagocytosed by pulmonary macrophages, while supernatant medium from log phase cultures, in sublethal doses, depresses the oxidative burst of neutrophils (3,8). Thus, the leukotoxin may act at two different levels in the disease by enhancing bacterial survival as well as promoting leukocyte death and degranulation.

Data from recent investigations indicate the leukotoxin may be an important component in protective vaccination (31,38). Protection from the disease is associated with the presence of leukotoxin neutralizing antibodies in bovine lung secretions (37,38). Since leukotoxin production is apparent only in log phase cultures of the bacteria (47), successful vaccination observed with log phase P. haemolytica may involve exposing the animal to leukotoxin.

The mode of action of the leukotoxin is poorly understood. Death of leukocytes exposed to P. haemolytica in the log phase of growth or log phase culture filtrates is rapid with the loss of organization in the cytoplasm, rounding of the cell, nucleolysis and rupture (2,4,65). The toxin may act at the membrane level or traverse the membrane to an intracellular target.

A number of characteristics of the leukotoxin are known. Leukotoxic activity is seen in lag and log phase culture filtrates, but only declining amounts are detected in stationary phase filtrates (2,8,47). Thus, the leukotoxin appears to be product of actively

growing bacteria. In addition, the activity is very labile and does not appear to be cell associated (25,47). There is also a ruminant specificity associated with the leukotoxin. The leukotoxin affects only lymphoreticular cells of cattle, sheep, and goats, but not swine, horses or humans (26). This host specificity is not unique to P. haemolytica. Both Staphylococcus aureus and Actinobacillus actinomycetemcomitans produce leukotoxins with a narrow host range specificity (60,68). The leukotoxin also appears to be a protein. Toxin containing culture medium heated at 56°C for 30 min (47, Moore, et al., unpublished), or trypsinized (2,8) loses its leukotoxic activity. However, the leukotoxin may be a glycoprotein as amylase treatment of culture filtrates has been reported to abrogate leukotoxic activity (8).

Controversy has surrounded reports of the apparent molecular weight of the leukotoxin. Molecular weights of 300,000 (2), 160,000 (39), 150,000 (14), 100,000 (29), and 75,000 (Moore et al., unpublished) daltons (d) have been reported. Much of this discrepancy may be explained by different methods of determining molecular weight and various culture conditions used to produce leukotoxin. Baluyut et al. (2) generated leukotoxin from log phase P. haemolytica cultures in RPMI 1640 medium without a serum source and based the molecular weight estimate of the toxin, 300,000 d, on retention by ultrafiltration membranes. Mosier et al. (39) generated leukotoxin from log phase cultures in RPMI 1640 with 7% fetal calf serum and estimated the molecular weight of the toxin to be 160,000 d by gel filtration chromatography. Himmel et al. (14) generated leukotoxin in dialysis

culture in brain heart infusion medium with 10% calf serum from stationary phase cultures of P. haemolytica. Molecular weight estimation using ultrafiltration, size exclusion chromatography, and rabbit anti-P. haemolytica antisera identified the leukotoxin to be 150,000 d. Lo et al. (29) using recombinant DNA technology and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reported a molecular weight of 100,000 d for the leukotoxin. Moore et al. (unpublished) generated leukotoxin from log phase P. haemolytica cultures in RPMI 1640 with 5% fetal calf serum and estimated the molecular weight to be 75,000 d by gel filtration. Until the molecule(s) responsible for the leukotoxin activity are purified, an accurate molecular weight will not be determined.

Purification of the molecule responsible for leukotoxicity is of paramount importance. All studies done to date have used culture filtrates or a derivative to study leukotoxin. Unfortunately, there are many other bacterial products produced by P. haemolytica (i.e. neuraminidase, proteases, endotoxin) in crude or partially pure filtrates that could be involved in the cytolysis seen with the leukotoxin.

Much of the work on the leukotoxin, especially purification, has a goal of producing a suitable antigen for vaccination (29,31,reviewed in 38,39). However, little work has been undertaken on understanding the action of the toxin. Many other toxins have been purified and their mode of action understood. Diphtheria toxin from Corynebacterium diphtheriae (35), exotoxin A from Pseudomonas aeruginosa (35), and shiga toxin from Shigella dysenteriae (44) are

three examples of toxins that have been purified and their modes of action defined.

All three toxins are secreted as inactive proenzymes that require proteolytic cleavage and reduction or denaturation to express full biological activity (35). Additionally, all three toxins have similar biological activities, that is, inhibition of protein synthesis. Diphtheria toxin and exotoxin A catalyze NAD-dependent ADP ribosylation of EF-2 (35) and shiga toxin inactivates the 60S subunit of the eukaryotic ribosome (35,44).

Leukotoxins from Staphylococcus aureus and Actinobacillus actinomycetemcomitans have been studied and to some extent characterized. S. aureus leukotoxin, also called the Pantan-Valentine toxin, consists of two components, an F component with a molecular weight of 32,000 d, and an S component with a molecular weight of 38,000 d. Some controversies exist as to the exact mode of action of the Pantan-Valentine leukotoxin (40), however, it is generally agreed that the toxin acts to produce a cation permeable channel in the target cell membrane (40,68). The leukotoxin from A. actinomycetemcomitans has a molecular weight of approximately 180,000 d (60) and is thought to be a membrane acting toxin (55), although the exact mode of action has not been elucidated. Both leukotoxins have similarities to the P. haemolytica leukotoxin. The leukotoxins from A. actinomycetemcomitans and S. aureus have a narrow host range specificity. A. actinomycetemcomitans leukotoxin affects neutrophils and monocytes from humans, but not rabbit, rat, mouse or guinea pig (60). S. aureus leukotoxin affects only the leukocytes of humans and

rabbits (69). P. haemolytica leukotoxin exhibits a narrow host range specificity, affecting only the leukocytes of ruminants (26). Although the mode of action of the leukotoxins from A. actinomycetemcomitans and S. aureus are not fully understood, they, along with P. haemolytica leukotoxin, induce similar rapid morphological changes in target cells. These changes include cessation of normal membrane movement and degranulation (2,55,70). In addition, all three leukotoxins share the characteristics of susceptibility to heat (47,44,68), proteolytic enzymes (8,60,68), and neutralization with antisera (37,69,72).

Statement of Purpose

Factors involved in the cytotoxicity of Pasteurella haemolytica leukotoxin are not well characterized. The time of exposure necessary to induce cytotoxicity and parameters involved in the production and stability of the molecule are unknown. Moreover, divalent cations, specifically calcium, are involved in toxin-cell interactions involving diphtheria toxin (45), shiga toxin (44), and staphylococcal leucocidin (70). No cation involvement has been reported for P. haemolytica leukotoxin. The purposes of this study were to further characterize the leukotoxin. Parameters investigated were conditions for leukotoxin production and stability, physical properties of the toxin, toxin-cell interactions and the influence of divalent cations in the cytotoxic process.

CHAPTER 2

MATERIALS AND METHODS

Materials

The divalent cations Ba^{2+} , Ca^{2+} , Co^{2+} , Mg^{2+} and Sr^{2+} (Fisher, Norcross, GA) were in the chloride form and used at 1 mM concentration unless otherwise stated.

The chelating agents Ethylenediamine tetraacetic acid (EDTA, Fisher) and Ethyleneglycol-bis-(B aminoethyl ether) N,N'-tetraacetic acid (EGTA, Fisher) were disodium salts and used as either 1 mM or 3 mM concentration.

Verapamil hydrochloride (5-[(3,4-dimethoxyphenethyl)methylamino]-2-(2,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride, Sigma Chemical Co., St. Louis, MO) was dissolved in divalent cation-free HBSS (Grand Island Biological Co., GIBCO, Grand Island, NY) containing 10% dimethylsulfoxide (DMSO, Sigma) to make a stock solution of 1 mM. The stock solution was used to make the appropriate dilutions of Verapamil in HBSS. Ca^{2+} and Mg^{2+} were added to the final Verapamil dilution for the appropriate concentration (1 mM) of divalent cations in the assay.

The ionophore A23187 (Sigma) was prepared as a stock solution in DMSO at a concentration of 1 mM. This stock was diluted in HBSS with 1 mM Ca^{2+} and mM Mg^{2+} to the appropriate concentration of A23187.

Phorbol myristate acetate (PMA, Sigma) was prepared as a stock solution in 95% ethanol (Sigma) at a concentration of 1 mg/ml. Dilutions were made in RPMI medium 1640 (RPMI 1640, GIBCO)

supplemented with 10% fetal bovine serum (Hyclone, Sterile Systems, Logan, UT) for the appropriate concentration of PMA.

Dexamethasone (Sigma) was prepared as a stock solution in DMSO at a concentration of 1 mM. Dilutions were made in RPMI 1640 containing 10% fetal bovine serum for the appropriate concentration.

Pasteurella haemolytica Lipopolysaccharide

Lipopolysaccharide (LPS) was extracted from Pasteurella haemolytica (Biotype A, serotype 1) by the method of McIntyre et al. (34). P. haemolytica was grown in BHI agar (GIBCO). Cells were collected by centrifugation (Sorvall RC2-B centrifuge, DuPont, Doraville, GA) at 10,000g for 20 min at 4°C and washed in HBSS. The following procedure was carried out at 4°C unless otherwise noted. To each 5 kg of cell paste 5 l of 88% phenol (Fisher) and 10 l of dH₂O were added. After 36 hours of stirring, the suspension was allowed to settle for 7-9 days. The cloudy supernatant was decanted, concentrated in vacuo at 35°C to approximately 2 l and allowed to stand overnight. The aqueous (upper) layer was dialyzed at constant volume against several changes of dH₂O for 4 days. The dialyzed solution was stirred and heated at 65°C for 15 min with an equal volume of 88% phenol. After cooling overnight, the aqueous phase was separated and dialyzed 4-5 days against several changes of dH₂O. This solution was made 0.15 M with NaCl (Fisher) and the LPS removed by sedimentation for 4-6 hours at 29,000g, 5°C. The sediment was resuspended in saline and centrifuged again. The LPS gel was

suspended in a minimum of dH₂O, dialyzed, and lyophilized. LPS powder was stored dry at 4°C.

Cell Culture

The BL-3 cell line was provided by Dr. G. H. Theilen, University of California, Davis. This cell line is a bovine leukemia-derived B lymphocyte cell line and was used as the target cell for cytotoxicity assays. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin, streptomycin and L-glutamine. The cells were incubated at 37°C in humidified 5% CO₂ in air atmosphere.

The P388-D1 cell line, a murine macrophage cell line was grown in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin, streptomycin and L-glutamine. The cells were incubated at 37°C in humidified 5% CO₂ in air atmosphere.

Pulmonary Lavage Cells

Bovine pulmonary lavage leukocytes were provided by Dr. T. Bowersock, Dept. of Pathobiology, University of Tennessee, College of Veterinary Medicine, Knoxville, TN. Calves were anesthetized, one lung catheterized and the airway to that lung occluded. Fifty ml of sterile, buffered, glucose-supplemented saline solution (pH 7.2) with 0.2% EDTA was infused into the lung. Aspiration of this solution followed immediately. The infusion-aspiration procedure was repeated until 1000 ml of lavage fluid had been infused into the lung of each calf. The lavage fluid was clarified by centrifugation at 500g for 10 min at room temperature. The resulting cell pellet (Greater than 94%

macrophages) was compared to the BL-3 cell line for sensitivity to leukotoxin.

Bacterium

Pasteurella haemolytica (isolate 853788, Biotype A, serotype 1) was used. The organism was originally isolated from the pneumonic lung of a calf. It was passed twice in calves by intrapulmonic inoculation and the reisolated organism was stored, suspended in skim milk at -70°C . The organism was maintained on blood agar consisting of BHI Agar (GIBCO) supplemented with 1% yeast extract (GIBCO), 1% horse serum (GIBCO) and 5% defibrinated sheep blood (63).

Leukotoxin Production

Leukotoxin filtrates were prepared basically as described by Shewen and Wilkie (47). Several colonies from an overnight blood agar plate were inoculated into BHI Broth and incubated with shaking in a water bath for 5 hr at 37°C , the time the culture was in the logarithmic phase of growth. The cultures were centrifuged (Sorvall RC2-B centrifuge, DuPont) at 10,000g for 20 min at 4°C and supernatants were discarded and the bacterial pellets were suspended in RPMI 1640 with 1% horse serum (GIBCO) at a concentration, determined spectrophotometrically, of 4×10^8 CFU/ml. This bacterial suspension was incubated for an additional 90 min with shaking in a water bath at 37°C . Immediately following this 90 min incubation, the suspension was centrifuged (Sorvall RC2-B centrifuge, DuPont) at 10,000g for 20 min at 4°C and the supernatant was filtrated through a 0.2 μm filter (Gelman, Ann Arbor, MI). Culture filtrates were stored

at -70°C until use. Concentrated culture filtrates were used in some studies. Filtrates were concentrated 100 fold using a Pellicon concentrator equipped with a 30,000 d exclusion membrane (Millipore, Bedford, MA). One unit (U) of leukotoxin activity is defined as the amount of culture filtrate required for 50% cytotoxicity.

Assays

Neutral Red Cytotoxicity Assay

Cytotoxicity was assessed by measuring the uptake of the vital stain neutral red by a modification of the assay reported by Moore et al. (37). Briefly, target cells were placed in a 96 well round bottom microtitre plate (NUNC, Denmark) at a concentration of 2×10^5 cells/well. The cultures were centrifuged (GLC-1 centrifuge, Sorvall, DuPont) at 500g for 10 min at room temperature and the medium discarded by pipet. Leukotoxic culture filtrates were added to the wells at the appropriate concentration in 100 μ l and the cultures were incubated for 1 hr, or as indicated, at 37°C. One hundred μ l of 0.036% neutral red (GIBCO) in physiological saline was added to each well and the cultures incubated at 37°C for an additional 20 min. The cultures were washed three times with physiological saline to remove free neutral red and 100 μ l of lysis buffer (95% ethanol (Fisher) in 0.02 M phosphate buffered saline, pH 6.8) was added to each well. Following a rapid freeze thaw to complete stain release, absorbance of individual wells at 540 nm was determined with an automated microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). Percent cytotoxicity was determined by comparing the mean optical density in

test wells ($n = 4$) containing leukotoxin with the mean optical density in control wells ($n = 4$) containing medium without leukotoxin. The neutral red cytotoxicity assay was performed with cells and leukotoxic culture filtrates diluted in RPMI 1640 or HBSS with various divalent cations as indicated. Results are presented as percentage of cytotoxicity plus or minus 1 standard error.

Trypan Blue Exclusion Assay

Target cells were incubated with undiluted culture filtrates or concentrated culture filtrates diluted in RPMI 1640 or RPMI 1640 with 1mM EGTA in 12 mm x 75 mm polypropylene tubes (Fisher) and incubated at 37°C or 4°C. Samples were taken at indicated time intervals and diluted 1:2 with 0.4% trypan blue (GIBCO). Viability in this assay is considered to equate with the exclusion of this stain. By inspection at 100x magnification, viability of at least 4 x 100 cells per treatment culture group was assessed. Results are presented as percentage of viability plus or minus 1 standard error.

Chromium Release Assay

BL-3 cells were suspended at 1×10^7 cells in 0.5 ml of RPMI 1640 with 10% fetal bovine serum. Five hundred μCi of $\text{Na}_2^{51}\text{CrO}_4$ (ICN, Irvine, CA, specific activity 316 mCi/mg) was added and the cells incubated for 2 hours at 37°C. The cells were washed five times in RPMI 1640, suspended in RPMI 1640 and counted. The cells were diluted to 2×10^5 cells/ml and used in the chromium release assay (47). The labeled BL-3 cells were placed in a 96 well flat bottom microtitre plate (Corning, Corning, NY) in 100 μl , and the appropriate

concentration of leukotoxin added. The plates were centrifuged at 500g for 10 min at room temperature. Spontaneous release wells contained 100 μ l of labeled BL-3 cells and 100 μ l of RPMI 1640. Total release wells contained 100 μ l of labeled BL-3 cells and 100 μ l of 3% Triton X-100 (Sigma). Incubation was at 37°C. At the appropriate times, 50 μ l of supernatant fluid was harvested from each well. Radioactivity for each sample was determined in an LKB-Wallac Riagamma 1274 spectrometer. All tests were performed in quadruplicate. Percent chromium release was calculated using the following formula:

$$\begin{aligned} \% \text{ chromium}^{51} \text{ release} = \\ \frac{\text{avg. test well cpm} - \text{avg. spontaneous release}}{\text{avg. total release cpm} - \text{avg. spontaneous release}} \end{aligned}$$

Results are presented at the mean percentage of chromium⁵¹ release plus or minus 1 standard error.

Thymidine Incorporation Assay

BL-3 cells in RPMI 1640 with 10% fetal bovine serum were placed in 96 well flat bottom microtitre plates (Corning) at a concentration of 2×10^5 cells/well. Leukotoxic culture filtrates were added at the appropriate concentrations. After 1 hr at 37°C with the leukotoxin, 1 μ Ci of tritiated thymidine (ICN, specific activity of 6.7 Ci/mM) was added for an additional 6 hr incubation. In the time course determinations, tritiated thymidine was added at time 0. Plates were frozen at -70°C until harvested. Cells were harvested with an automated harvester (PHD Cell Harvester, Cambridge Technology, Inc.,

Cambridge, MA) onto glass fiber filter strips (Cambridge Technology, Inc.) and the cpm determined in liquid scintillation cocktail (Ecolume, ICN) in a Beckman LS 7000 scintillation counter. Results are the mean of quadruplicate samples plus or minus 1 standard error.

Gel Filtration Column Chromatography

A Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column was used (column length 96 cm, column diameter 3 cm). The buffer was 0.01 M phosphate buffer diluted in 0.15 M NaCl with 0.05% polyethylene glycol (PEG, Sigma). One half ml of concentrated leukotoxic filtrate was mixed with a few drops of glycerol (Fisher) and loaded onto the column. The flow rate was set at 0.5 ml/min, and 2.5 ml fractions were collected and tested for leukotoxicity in the thymidine incorporation assay. All column work was done at 4°C.

Statistical Analysis

Standard error was determined from the formula:

$$\text{Standard deviation} / \sqrt{n},$$

where n equals the number of samples.

Best fit lines, where applicable, were determined by linear regression analysis.

CHAPTER 3

RESULTS

Sensitivity of the BL-3 Cell Line to Leukotoxic Filtrates

The BL-3 cell line, a bovine leukemia-derived B lymphocyte cell line, was used as a target for leukotoxin cytotoxicity assays. To determine if this cell line was sensitive to Pasteurella haemolytica's leukotoxin, a comparison was made with bovine pulmonary lavage cells (Figure 1) using uptake of neutral red to assess cytotoxicity. Results indicated that the cell line was as sensitive, if not more, to the effects of 90 min culture filtrates. As the cell culture filtrates were diluted, the percent cytotoxicity decreased for both the BL-3 cell line and calf pulmonary lavage cells. Fifty percent cytotoxicity was observed for the BL-3 cells at approximately 2% filtrate, whereas 6-7% filtrate concentration was required to achieve the same cytotoxicity with freshly derived bovine lavage cells. Furthermore, the bovine cell line, when compared to the P388-D1 murine macrophage cell line, was more sensitive to leukotoxic filtrates confirming the ruminant specificity associated with the leukotoxin (26). The data given in Figure 2 was derived by assessing incorporation of ^3H -TdR by leukotoxin treated culture wells. As the culture filtrate was diluted, DNA synthesis increased for both cell lines but cytotoxicity for the P388 celline did not decrease at the same rate. The rapid loss of cytotoxicity for the P388 cell line can probably be attributed to limited nutrients remaining in the bacterial culture filtrates. Even at 50% leukotoxic filtrate, cpm of ^3H -TdR

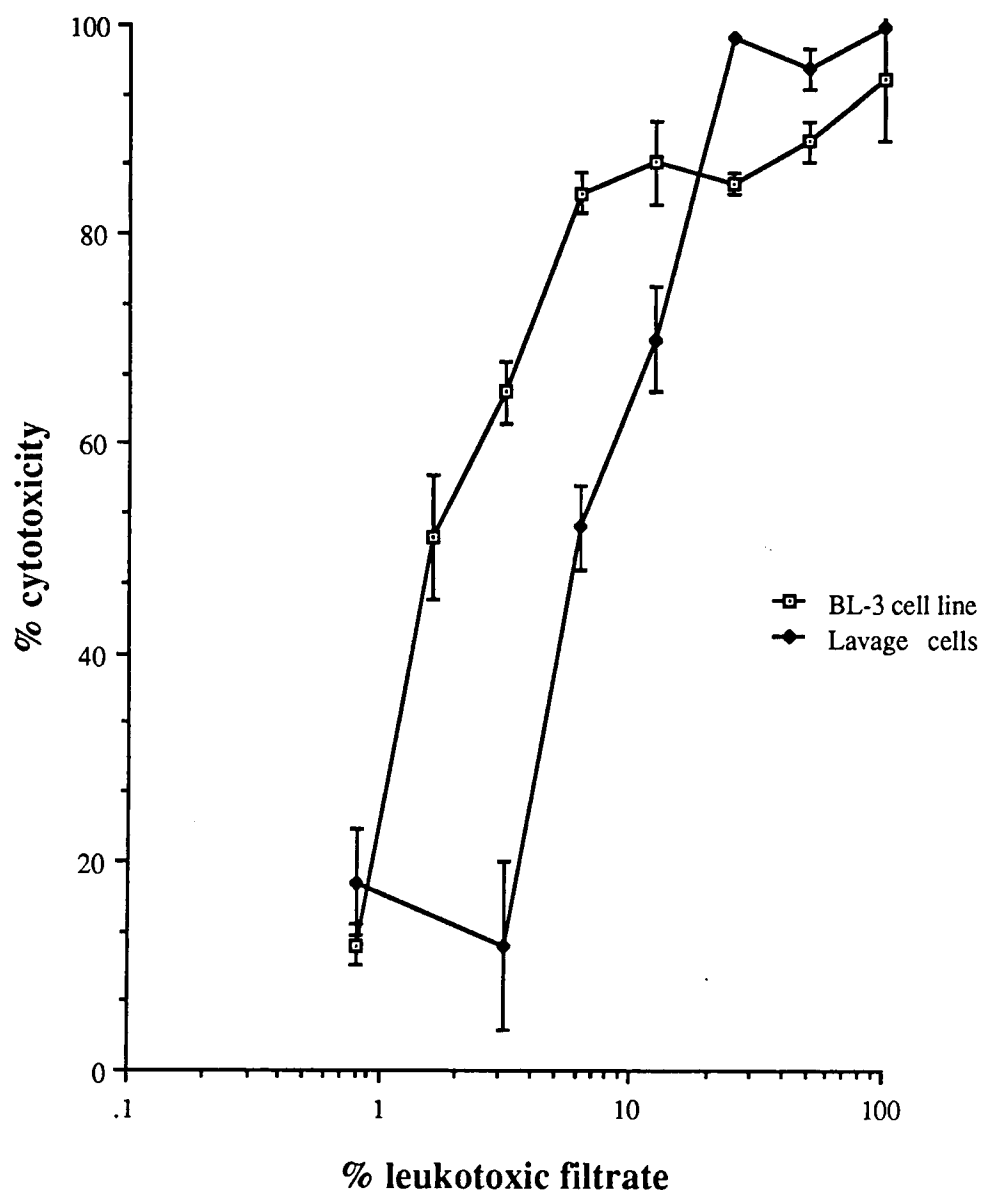


Figure 1. Cytotoxicity of *P. haemolytica* culture filtrate for bovine pulmonary lavage cells and cells of the BL-3 cell line. Cytotoxicity was assessed by diminution of neutral red uptake.

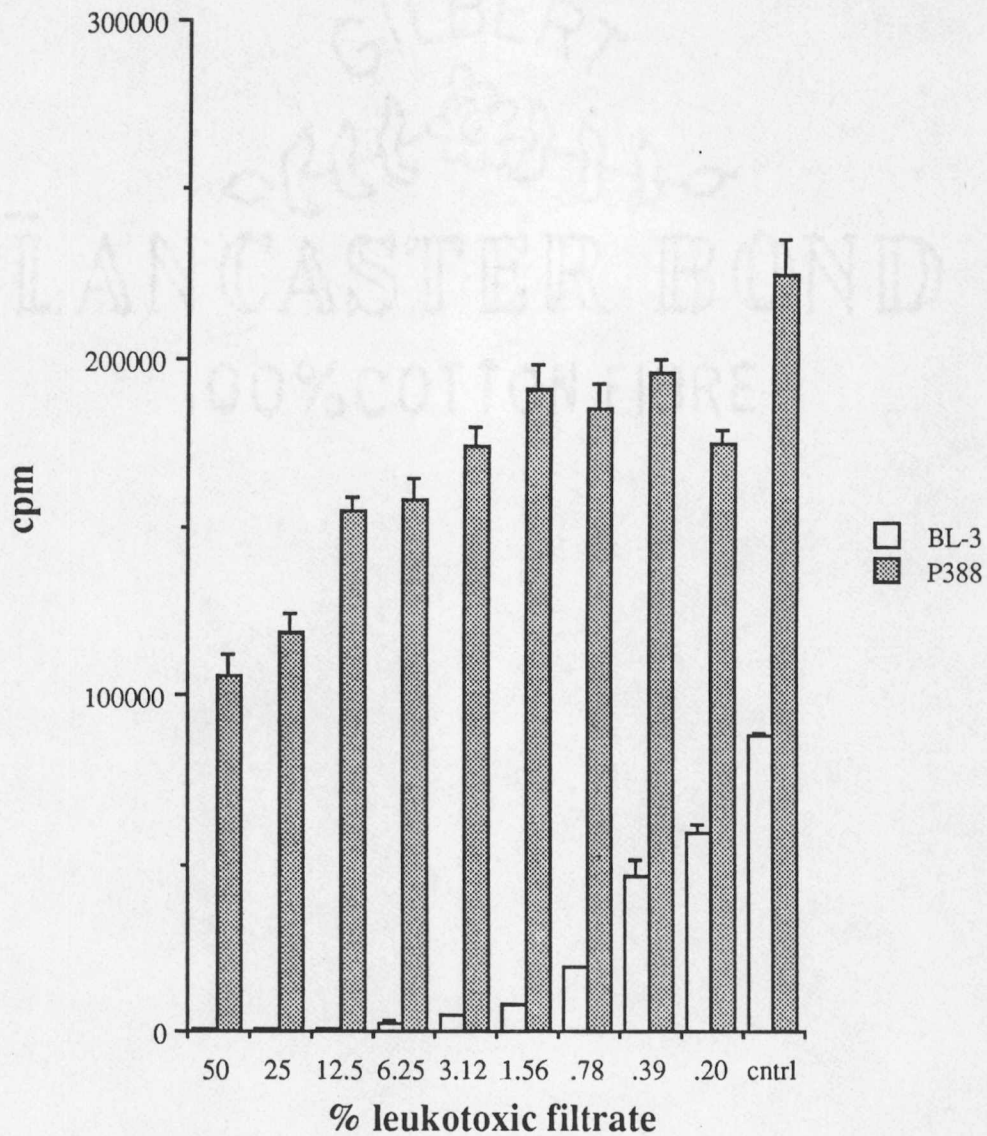


Figure 2. Cytotoxicity of *P. haemolytica* culture filtrate for cells of the murine cell line P388-D1 and the bovine BL-3 cell line. Toxicity was assessed on the basis of ^3H -TdR incorporation by treated cells.

incorporated by the murine cell line was reduced by only 50%, while a similar level of reduction was noted at approximately 0.4% filtrate with the bovine cells.

To test the possibility that endotoxin in the culture filtrates was responsible for the observed cytotoxicity, purified P. haemolytica LPS was assessed for cytotoxicity at dosages up to 33 ug/ml. LPS did not induce measurable cell death, as assessed by neutral red uptake (Figure 3), demonstrating that it was not directly responsible for the cytotoxicity observed in the culture filtrates.

Optimal Conditions for Generation of Leukotoxic Filtrates

As described in the previous section, 90 min bacterial culture filtrates were cytotoxic for the BL-3 cell line and the cytotoxicity was not due solely to LPS. The following experiments were performed to determine optimal conditions for leukotoxin production during the 90 min incubation. First, the optimal initial concentration of bacteria was determined. Bacteria were grown in BHI to log phase, centrifuged and resuspended in RPMI 1640 with 1% horse serum. Serial dilutions were made from this original stock into RPMI 1640 with 1% horse serum, and a parallel dilution series in sterile dH₂O was plated to determine the number of bacteria. The RPMI 1640 dilutions were incubated for 90 min, immediately centrifuged and the supernatant medium filtered and frozen for later testing. The optimal initial cell concentration was approximately 4×10^8 CFU/ml (Figure 4). In the experiment shown in the figure, leukotoxic activity increased as the cell concentration increased, up to 80 units at 4×10^8 CFU/ml.

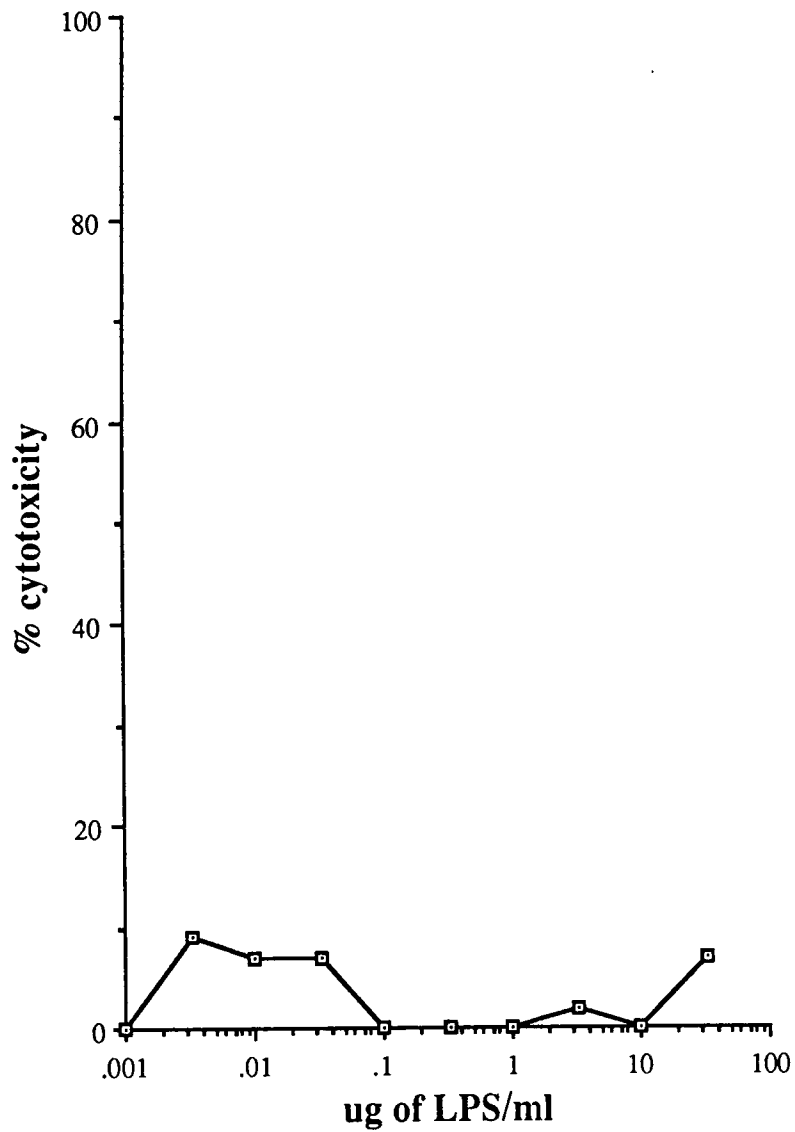


Figure 3. Assessment of the cytotoxic potential of *P. haemolytica* LPS for BL-3 cells. Cytotoxicity was assessed by diminution of neutral red uptake.

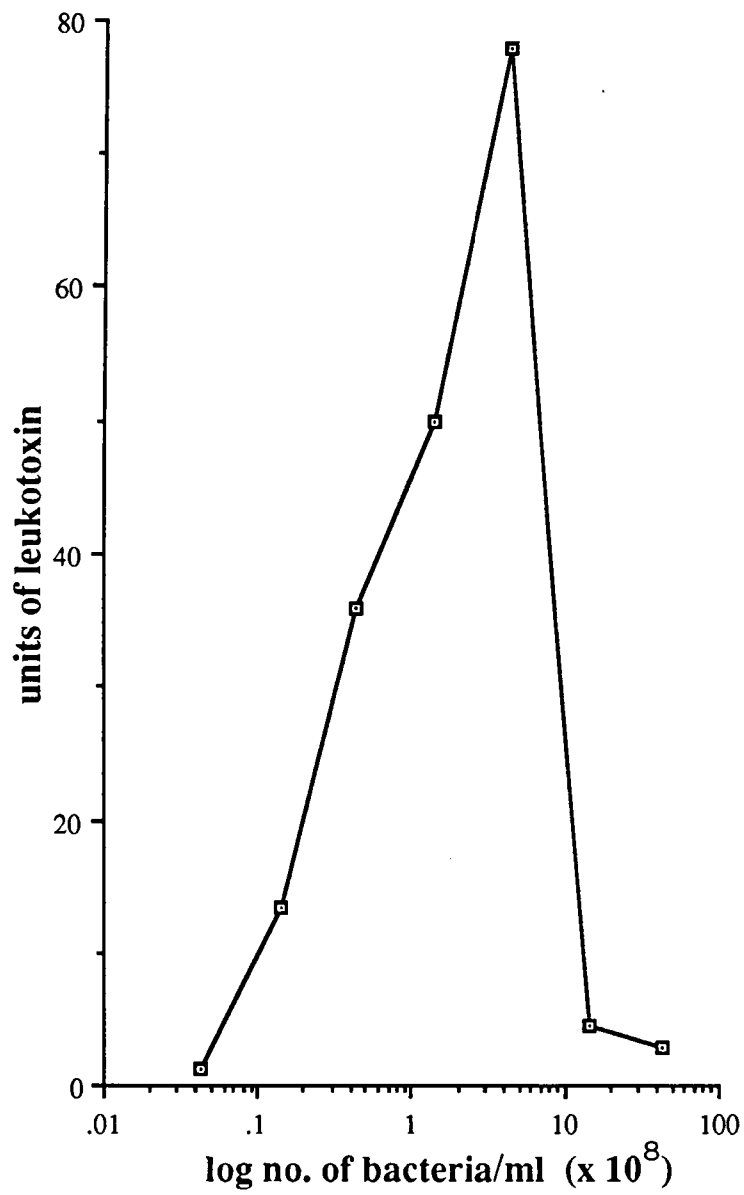


Figure 4. Leukotoxin production by *P. haemolytica* as a function of the initial concentration of bacteria.

Toxic activity decreased precipitously at cell concentrations greater than 4×10^8 CFU/ml. To verify both the time to harvest cell filtrates and the growth phase of the culture, approximately 4×10^8 CFU/ml (determined spectrophotometrically and later determined by plate count to be 2×10^8 CFU/ml) was the initial concentration of bacteria used to generate leukotoxic filtrates. At selected intervals, a 1 ml sample was taken to determine the number of CFU/ml and units of leukotoxic activity. Results indicated that the optimal time to harvest leukotoxic filtrates was 90 min. The units of leukotoxin continued to increase from 2 units at 0 min to 60 units at 90 min and decreased afterward (Table 1). This corresponded to either the end of log phase growth or the beginning of the stationary phase (Figure 5). Moreover, it was noted that a greater concentration of bacteria used to generate leukotoxin actually decreased the amount of leukotoxic activity in the culture filtrates. The peak time to harvest culture filtrates with an initial inoculum of 2×10^9 CFU/ml was 30 min which corresponded to the end of log phase growth (Table 1, Figure 5). However, the units of leukotoxin detected at this time was only 17 compared to 60 at 90 min with an initial inoculum of 2×10^8 CFU/ml.

Serum increased the amount of leukotoxic activity in culture filtrates (Table 2). With no serum in the RPMI 1640 medium the amount of leukotoxin detected in culture filtrates was 14 units. With 1% horse serum the amount of leukotoxin detected increased to 35 units and continued to increase as additional serum was added. At 10% horse serum 76 units were detected. Culture filtrates thereafter were

Table 1. Influence of serum on leukotoxin production

% Horse Serum	Units of Leukotoxin
0.0	14
1.0	35
3.3	46
10.0	76

Pasteurella haemolytica was grown to log phase in BHI, centrifuged and resuspended at 4×10^8 CFU/ml (determined spectrophotometrically) in RPMI 1640 with the appropriate concentration of horse serum. The cultures were incubated for 90 minutes at 37°C in a water bath, centrifuged and the medium filtered and frozen at -70°C until tested for leukotoxicity. Units of leukotoxin were quantified using the BL-3 cell line. Each unit value is the reciprocal of the dilution required to induce 50% cytotoxicity of target cells.

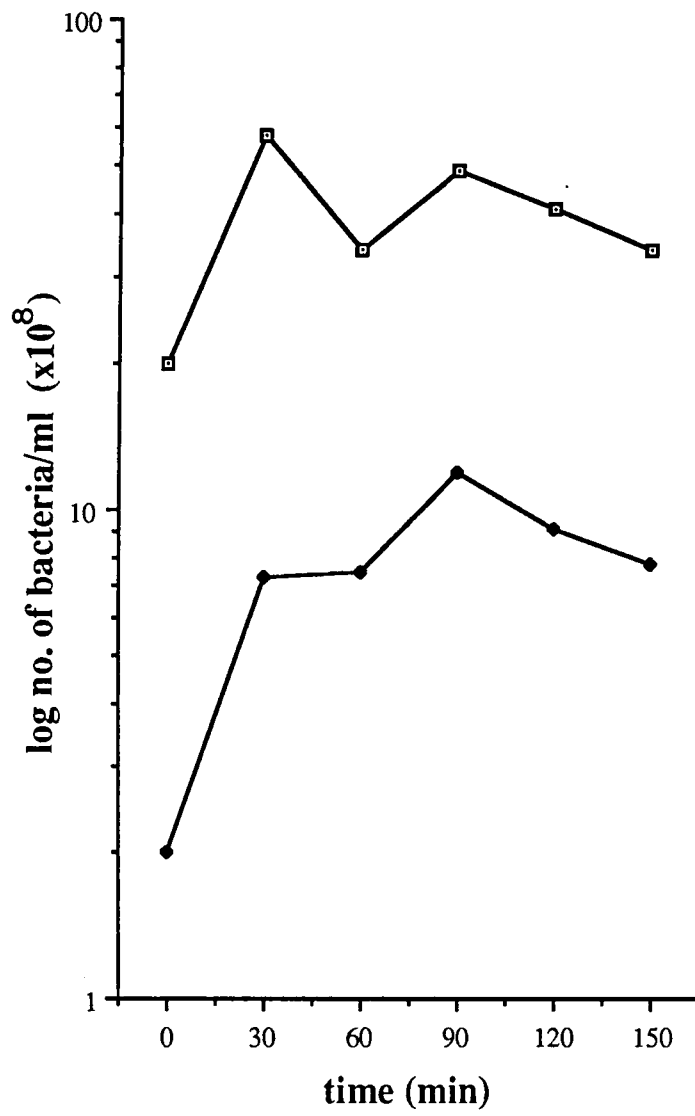


Figure 5. Growth kinetics of *P. haemolytica*.

Table 2. Leukotoxin production as a function of incubation time

Time (min)	<u>Initial Bacterial Inoculum/ml</u>	
	2×10^8	2×10^9
0	2	6
30	26	17
60	33	3
90	60	3
120	43	2
150	13	3

Pasteurella haemolytica was grown to log phase in BHI, centrifuged and resuspended at either 2×10^9 CFU/ml or 2×10^8 CFU/ml (determined spectrophotometrically and later verified by plate counts) in RPMI 1640 with 1% horse serum. At the appropriate times a 1 ml sample was taken, centrifuged, the medium filtered and frozen for later testing for leukotoxicity. BL-3 cells were used as the target cells. Each unit value is the reciprocal of the dilution required for 50% cytotoxicity of target cells.

routinely generated with 1% horse serum to avoid subsequent problems encountered with concentration by ultrafiltration. Although horse serum was used to generate filtrates, it was noted that bovine serum gave higher units of leukotoxin. At 1% horse serum, 35 units of leukotoxic activity were detected in the medium, whereas at 1% bovine serum, 38 units of activity were detected. However, at 10% horse serum, 76 units of leukotoxic activity were detected, whereas at 10% bovine serum, 98 units were detected.

Characteristics of Leukotoxic Activity in Culture Filtrates

Leukotoxic activity in culture filtrates was inactivated by heating at 56°C for 30 min (Figure 6), verifying previous reports of heat lability (47). In addition, leukotoxicity in culture filtrates was reduced 25 fold following incubation at 37°C for 3 hours (Table 3). This lability could not be overcome by the addition or deletion of horse serum (0.1 to 10%) during the three hour incubation (Table 3).

Gel filtration of concentrated filtrates indicated that the leukotoxic activity was found in one major peak, between 70,000 and 85,000 daltons, and in minor peaks at 46,000 and 16,000 daltons (Figure 7).

Culture filtrates were toxic for target cells at 4°C, as well as 37°C, indicating that the leukotoxin may act at lower temperatures (Table 4). However, the rates of cytotoxicity differed at the two temperatures. Viability at 37°C dropped considerably from 72% to 43% in 30 min and by 60 min, viability was only 15%. At 4°C, viability

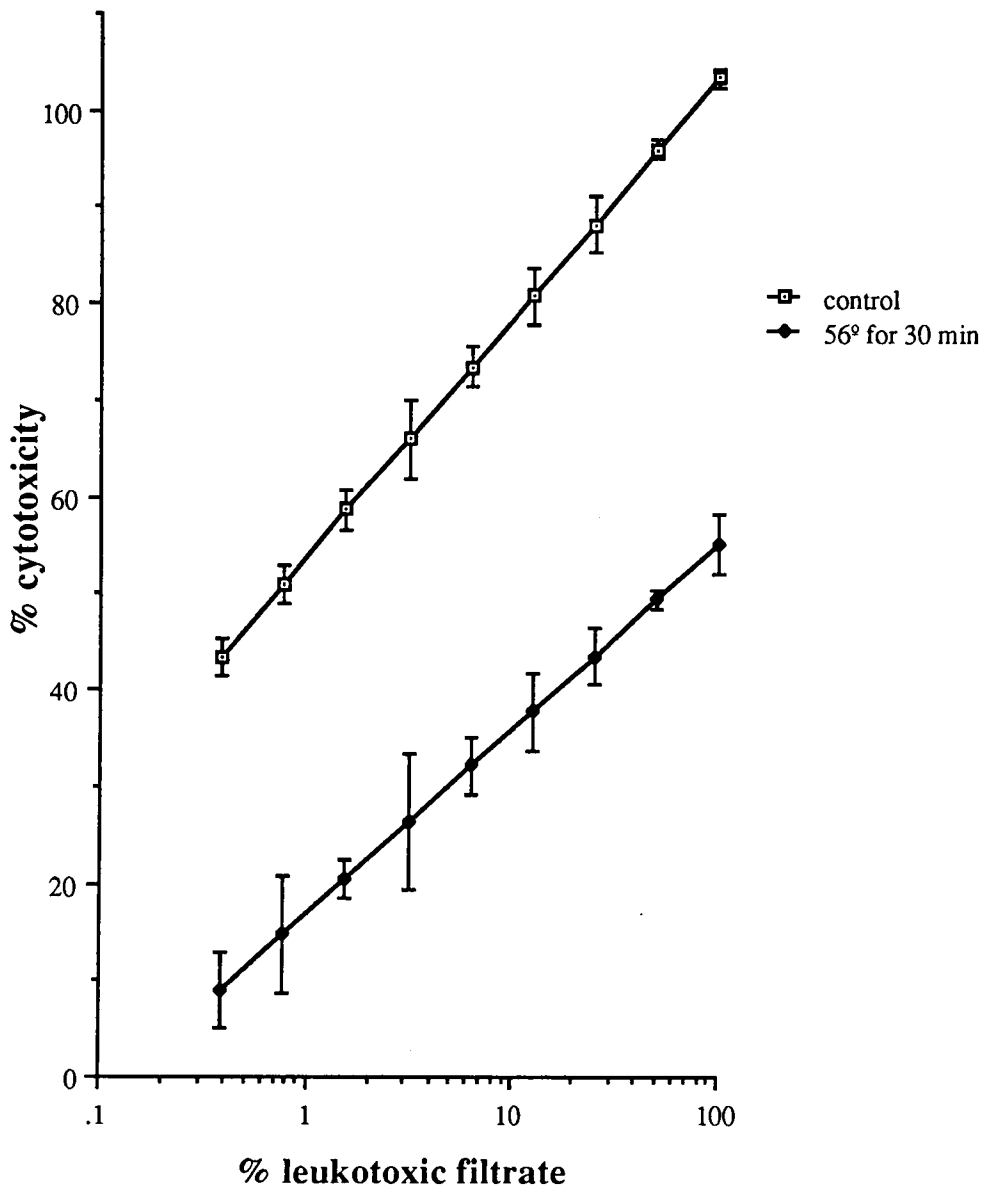


Figure 6. Sensitivity of *P. haemolytica* leukotoxin to heating at 56°C for 30 min. Cytotoxicity was assessed by diminution of neutral red uptake by treated BL-3 cells.

Table 3. Stability of leukotoxic filtrates when incubated for three hours in the presence of increasing serum concentrations

% Serum	Leukotoxic Units
10.00	< 2
3.30	7
1.00	10
0.33	7
0.10	< 2
1.00 ^a	256

^aControl without 3 hr incubation

Ninety minute P. haemolytica culture filtrates were generated in RPMI 1640 with 1% horse serum. Horse serum or RPMI 1640 was added to the leukotoxic filtrates to obtain the appropriate final concentration of serum. The filtrates were then incubated for 3 hr at 37°C and tested for leukotoxicity using the BL-3 cell line as a target. Each unit value is the reciprocal of the dilution required for 50% cytotoxicity of target cells.

Figure 7. Sephacryl S-200 elution profile of leukotoxic activity in P. haemolytica filtrates. Cytotoxicity of eluted fractions was assessed as diminution of neutral red uptake by treated BL-3 cells. Fractions collected were 2.5 ml volume and are numbered consecutively after the blue dextran exclusion peak. Molecular weight standards shown are immunoglobulin G (IgG, 150,000 d), bovine serum albumin (BSA, 67,500 d) and cytochrome c (cyto c, 12,800 d).

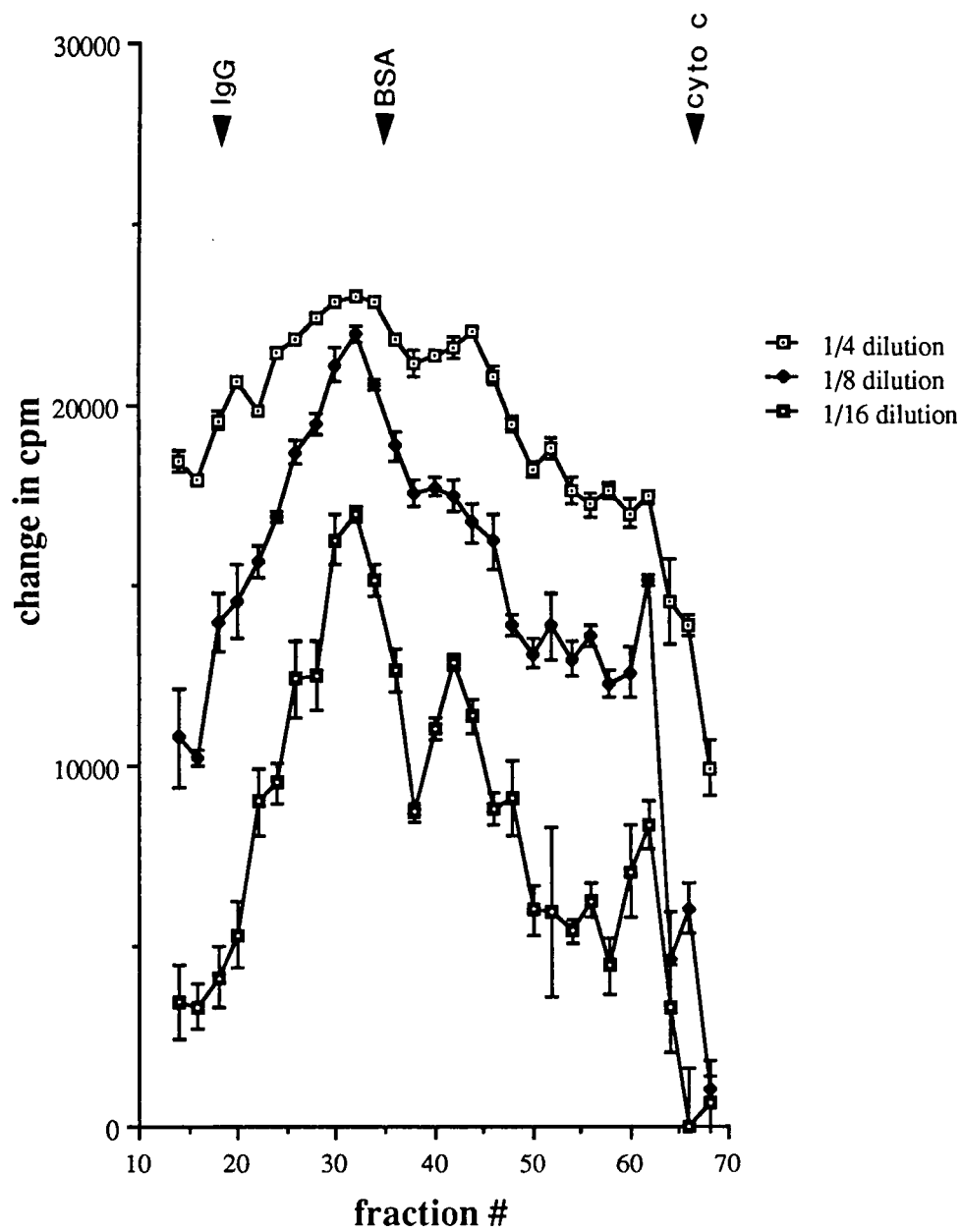


Table 4. Effect of incubation temperature on the sensitivity of BL-3 cells to P. haemolytica leukotoxin

Time	4°C		37°C	
	Control	Leukotoxin	Control	Leukotoxin
0	73 ± 0	72 ± 1	72 ± 1	72 ± 1
30	72 ± 1	63 ± 1	72 ± 1	43 ± 1
60	73 ± 1	45 ± 1	72 ± 3	15 ± 1
90	73 ± 1	44 ± 2	72 ± 2	16 ± 2
120	74 ± 1	38 ± 1	73 ± 1	11 ± 2

BL-3 cells were incubated with 90 min P. haemolytica culture filtrates for the appropriate time. A sample was taken, mixed with trypan blue and the percentage of viability determined. One hundred cells were counted (in quadruplicate) to determine viability. Results are given as % viability plus or minus 1 standard error.

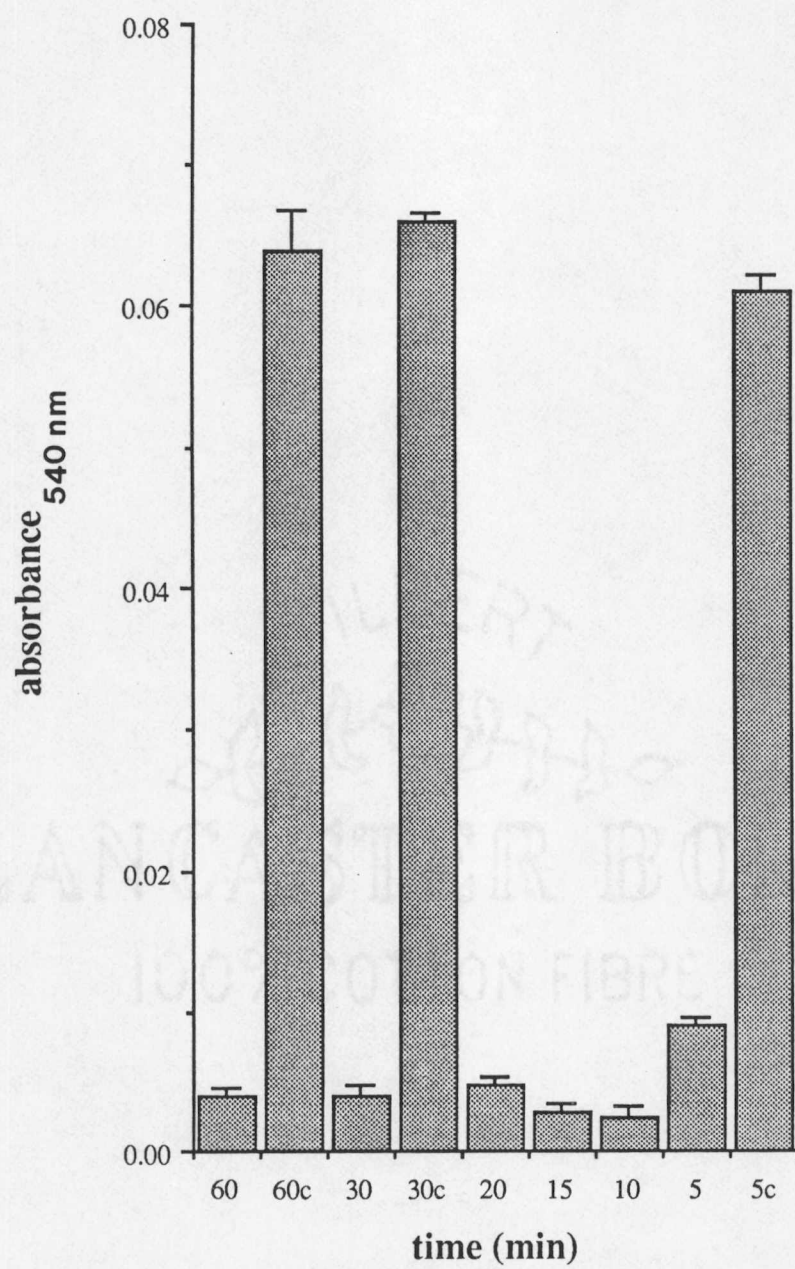
did not drop appreciably until 60 min when it decreased from 72% to 45% and continued to be close to this value for the duration of the experiment. The leukotoxin also acted rapidly. A determination was made as to the minimal time necessary for target cells to be exposed to leukotoxin to induce cell death. Target cells exposed to leukotoxic filtrates for as little as 5 min, washed and then incubated overnight at 37°C were still sensitive to the effects of the leukotoxin (Figure 8).

Target Cell-Toxin Interactions

Dexamethasone, a known membrane stabilizer (19), was used to determine any effects that pharmacologic concentrations of corticosteroid hormones may have on interactions between the leukotoxin and target cells. Results of a 2 hour preincubation with the hormone at doses from 10^{-9} to 10^{-6} M indicated no reduction in cytotoxicity with any of the doses of dexamethasone used (Figure 9).

To further investigate whether cell-toxin interactions could be affected, concentrated leukotoxic filtrates were depleted of divalent cations by dialysis and then diluted in divalent-cation free HBSS or HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} . Leukotoxic activity appeared to require the divalent cations. A considerable reduction in cytotoxicity was observed with lower doses of leukotoxin diluted in divalent-cation free HBSS (Figure 10). In addition, leukotoxicity was abrogated in HBSS containing Ca^{2+} and Mg^{2+} in which either 1 mM EGTA, a specific Ca^{2+} chelator, or 1 mM EDTA, a chelator of both Ca^{2+} and Mg^{2+} , was present (Table 5).

Figure 8. Leukotoxin exposure time required for induction of cytotoxicity for BL-3 cells. Cells were exposed to leukotoxic culture filtrate for denoted times, washed and resuspended in fresh medium. Absorbance following neutral red exposure was measured after overnight incubation at 37°C. Groups noted c were not exposed to toxin.



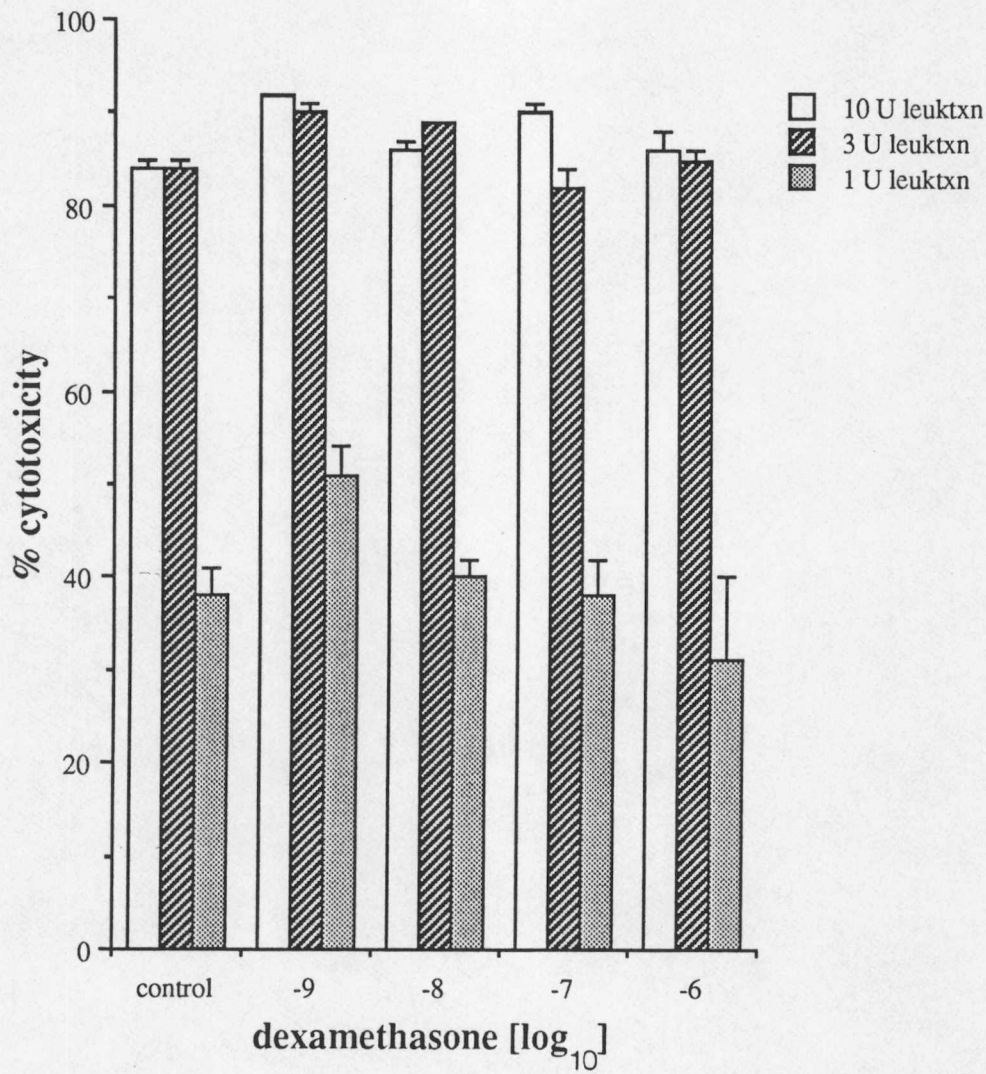


Figure 9. Lack of effect of dexamethasone pretreatment on sensitivity of BL-3 cells to *P. haemolytica* leukotoxin.

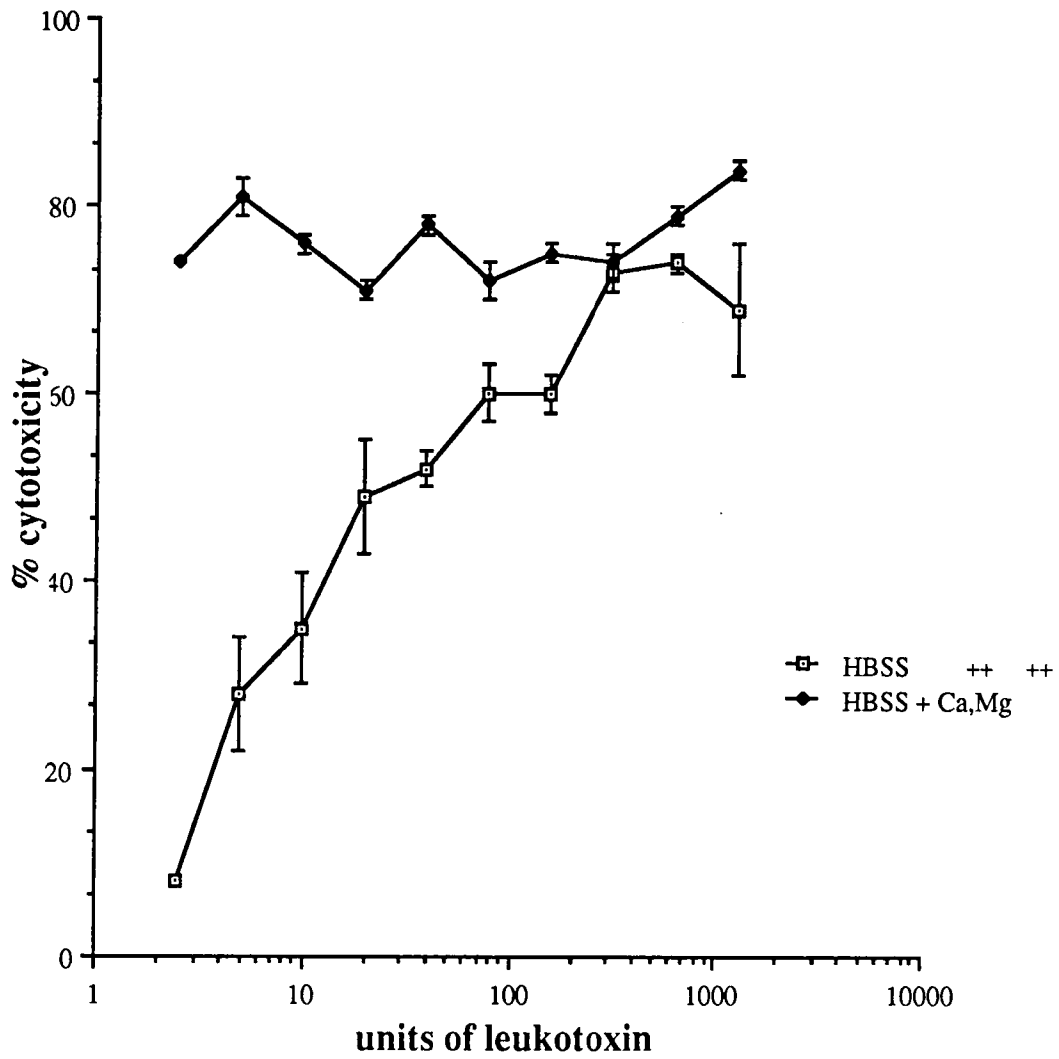


Figure 10. Influence of Ca^{2+} and Mg^{2+} on sensitivity of BL-3 cells to *P. haemolytica* leukotoxin.

Table 5. Cytotoxicity of one unit of leukotoxin in buffer containing chelating agents

Buffer	% Cytotoxicity
HBSS with Ca^{2+} and Mg^{2+}	53 $\pm$ 3
HBSS with Ca^{2+} and Mg^{2+} + EGTA	
1mM	0 $\pm$ 1
3mM	2 $\pm$ 3
HBSS with Ca^{2+} and Mg^{2+} + EDTA	
1mM	0 $\pm$ 1
3mM	5 $\pm$ 5

BL-3 cells were incubated with one unit of leukotoxin diluted in HBSS with Ca^{2+} and Mg^{2+} (each at 1mM) with the appropriate concentration of EGTA or EDTA for 1 hr at 37°C. After incubation a neutral red cytotoxicity assay was performed. Results are given as percentage of cytotoxicity plus or minus 1 standard error.

Calcium as a Requirement for Leukotoxicity

Data obtained in the preceding experiments suggested a requirement for divalent cations, and Ca^{2+} in particular, for expression of leukotoxicity by *P. haemolytica* culture filtrates. To further investigate this apparent requirement, dialyzed, concentrated filtrates diluted in HBSS with 1 mM Ca^{2+} , but not Mg^{2+} , restored cytotoxicity to equivalent values seen with both divalent cations present (Figure 11). Apparently, Ca^{2+} was required for optimal cytotoxicity as measured by these cytotoxicity assays. Other divalent cations were used to determine if they could substitute for Ca^{2+} in the restoration of cytotoxicity of dialyzed filtrates. Ba^{2+} , Co^{2+} and Sr^{2+} (each at 1 mM) could restore the leukotoxic effects of dialyzed concentrated filtrates, but Mn^{2+} could not (Figure 12).

To further investigate the role Ca^{2+} was playing in cytotoxicity, a time course was performed with the BL-3 cells exposed to the leukotoxin for a 6 hr period. At indicted time points, viability was assessed using thymidine incorporation, chromium⁵¹ release, trypan blue exclusion and neutral red cytotoxicity assays. The incubations were performed in tandem with treated cells either in complete medium or in complete medium containing 1 mM EGTA. Complete medium was used since the BL-3 cells die if incubated for periods longer than one hr in HBSS. Using these assays, target cells were protected for one hr when Ca^{2+} was bound by EGTA in complete medium (Figures 13, 14, 15). However, these target cells died after the one hr period, indicating that they are likely damaged by the leukotoxin despite the viability observed at one hr by the neutral red

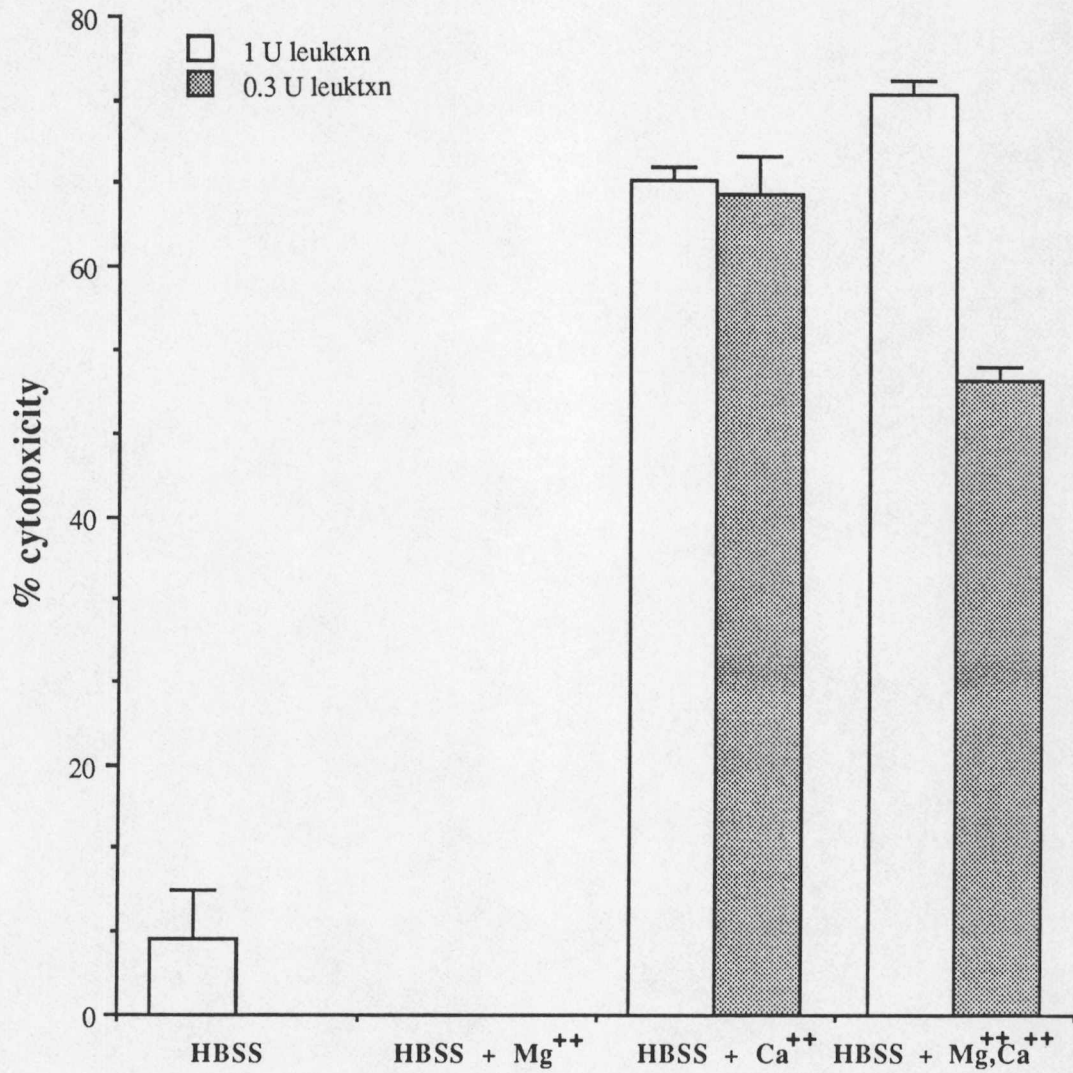


Figure 11. Restoration of leukotoxin sensitivity of BL-3 cells by addition of Ca²⁺ but not Mg²⁺.

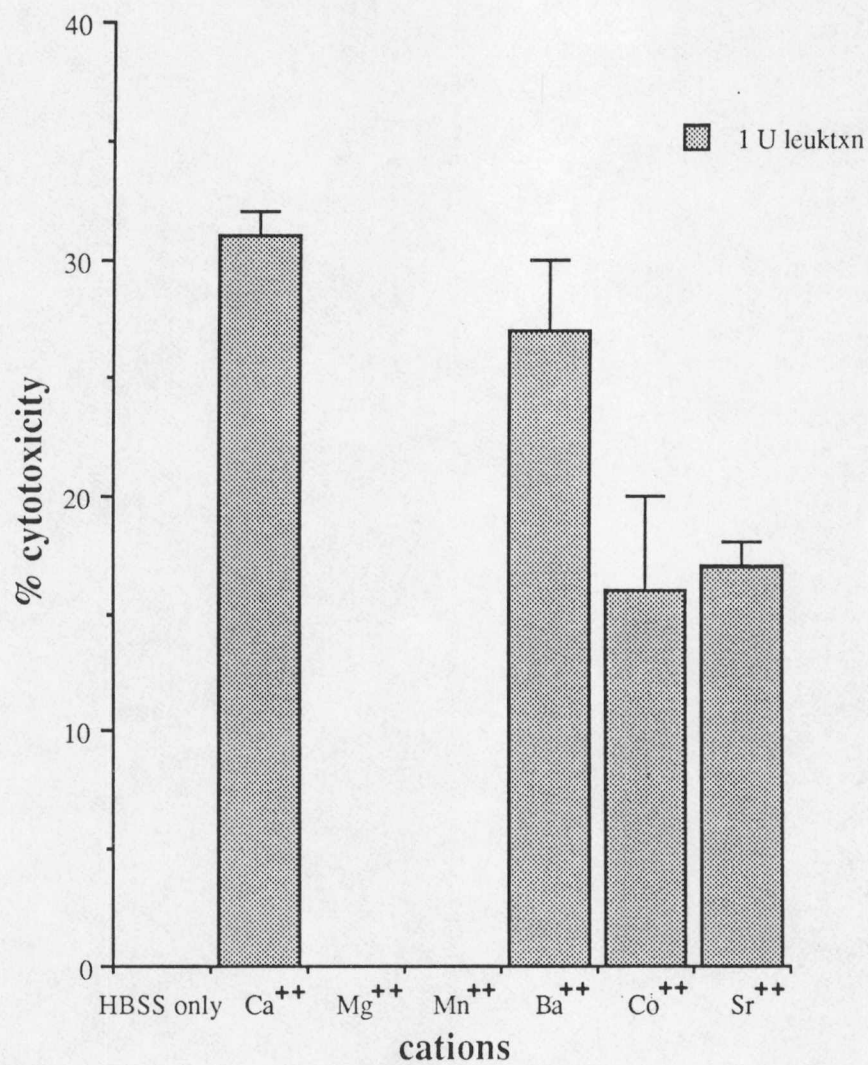


Figure 12. Restoration of leukotoxin sensitivity of BL-3 cells by addition of Ca^{2+} , Ba^{2+} , Co^{2+} and Sr^{2+} . Magnesium²⁺ and Mn²⁺ did not restore sensitivity in divalent cation-depleted buffer.

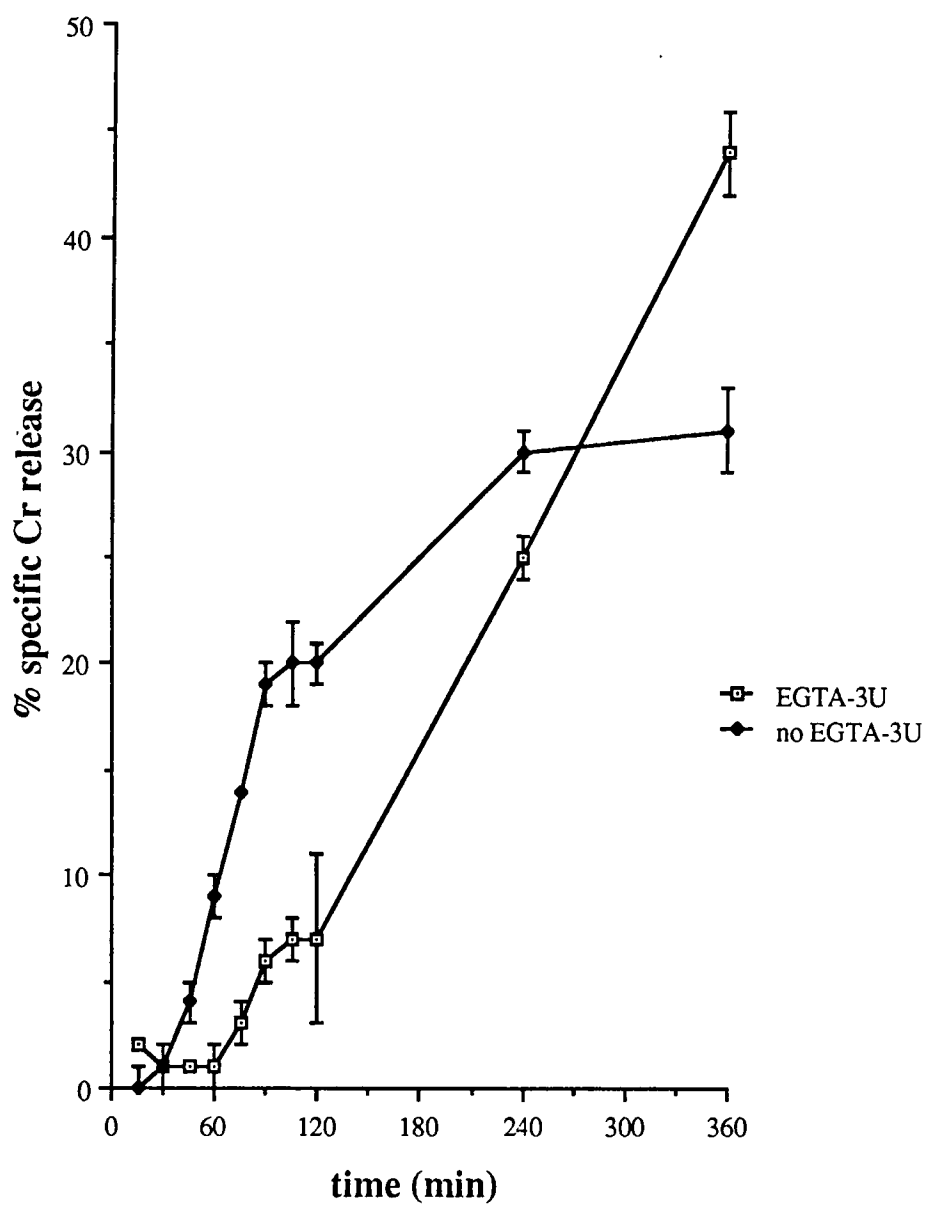


Figure 13. Leukotoxin cytotoxicity time course for BL-3 cells in the presence or absence of EGTA assessed by the release of ^{51}Cr .

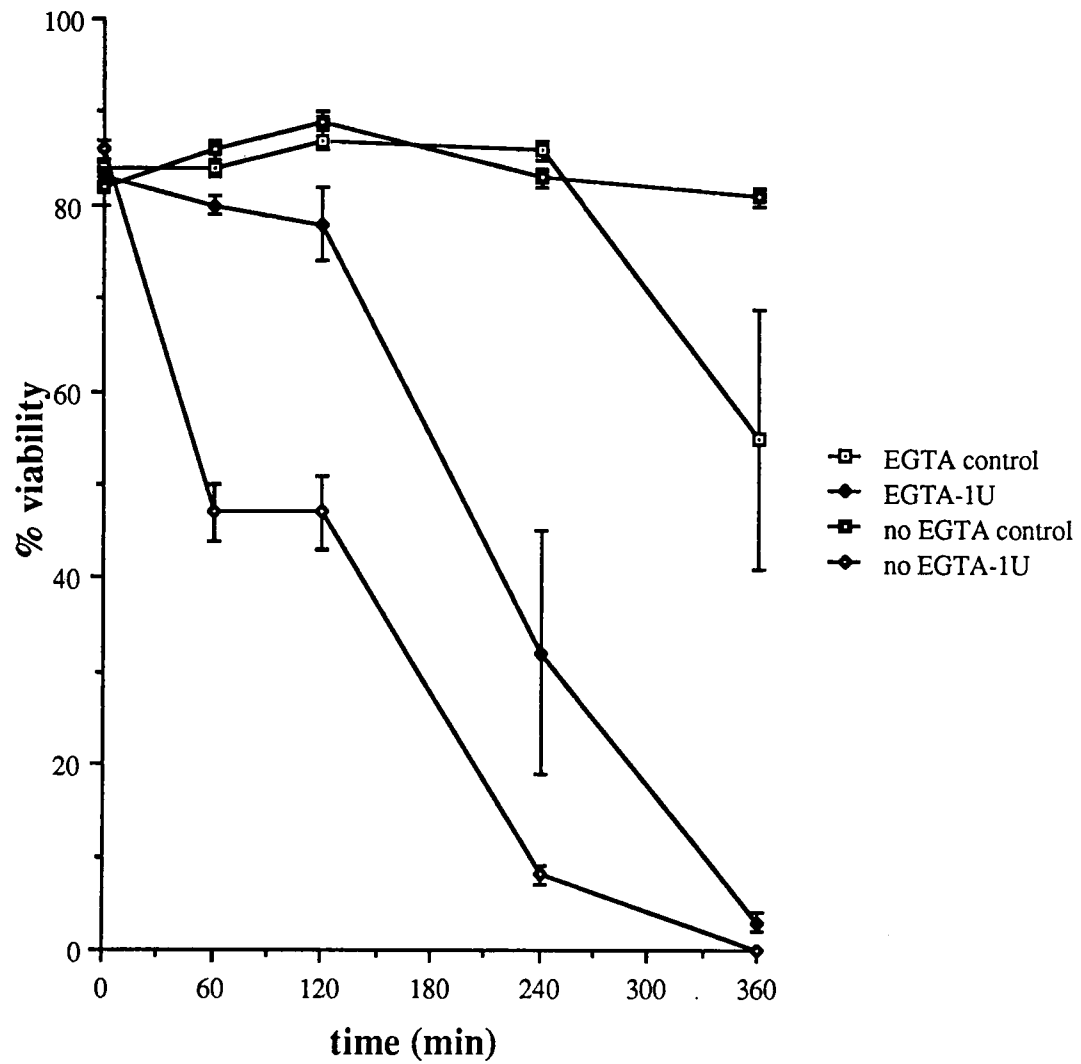


Figure 14. Leukotoxin cytotoxicity time course for BL-3 cells in the presence or absence of EGTA assessed by exclusion of trypan blue.

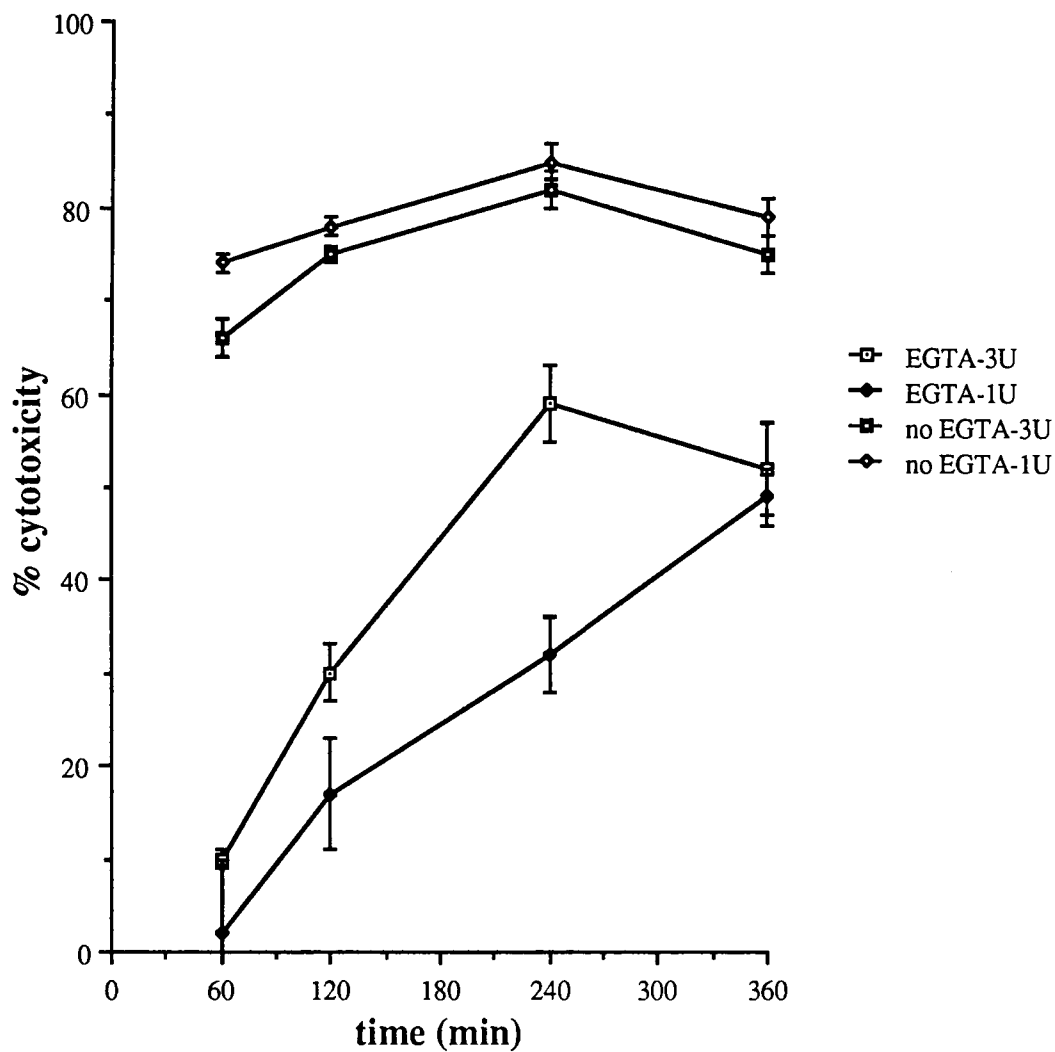


Figure 15. Leukotoxin cytotoxicity time course for BL-3 cells in the presence or absence of EGTA assessed by the uptake of neutral red.

cytotoxicity assay, trypan blue exclusion assay and the chromium release assay. Thymidine incorporation over the 6 hr time period indicated that cells incubated with leukotoxin in the presence of EGTA are damaged, as noted by the low cpm (Figure 16). Additionally, the cells in complete medium with EGTA, when examined microscopically at the one hr period, appeared damaged, as they were enlarged and rounded compared to control cells.

Effects of Verapamil and the Ionophore A23187 on Leukotoxicity

Since Ca^{2+} appeared to have a role in the action of the leukotoxin, another aspect of its requirement was investigated. Verapamil and the ionophore A23187, both known to influence membrane Ca^{2+} channels (1, reviewed in 4), were used. Target cells were incubated for one hr with leukotoxin in HBSS with Ca^{2+} , Mg^{2+} (both at 1 mM) and Verapamil at concentrations ranging from 3.3 μM to 100 μM . Results from this cytotoxicity assay indicated that Verapamil exerted a dose dependent protective effect. As shown in Figure 17, 33 μM Verapamil afforded 50% protection against 5U of leukotoxin while 100 μM Verapamil reduced cytotoxicity to less than 10%. The protective effect seen with Verapamil at concentrations of 10 μM and 33 μM against 1U of leukotoxin were overcome by addition of exogenous 3 mM Ca^{2+} but not 1 mM Ca^{2+} (Figure 18).

The ionophore at concentrations of 10^{-8} , 10^{-7} , and 10^{-6} M protected the target cells in a dose dependent manner compared to the control without ionophore (Figure 19) under conditions similar to those described for Verapamil using 5U of leukotoxin. The effect of

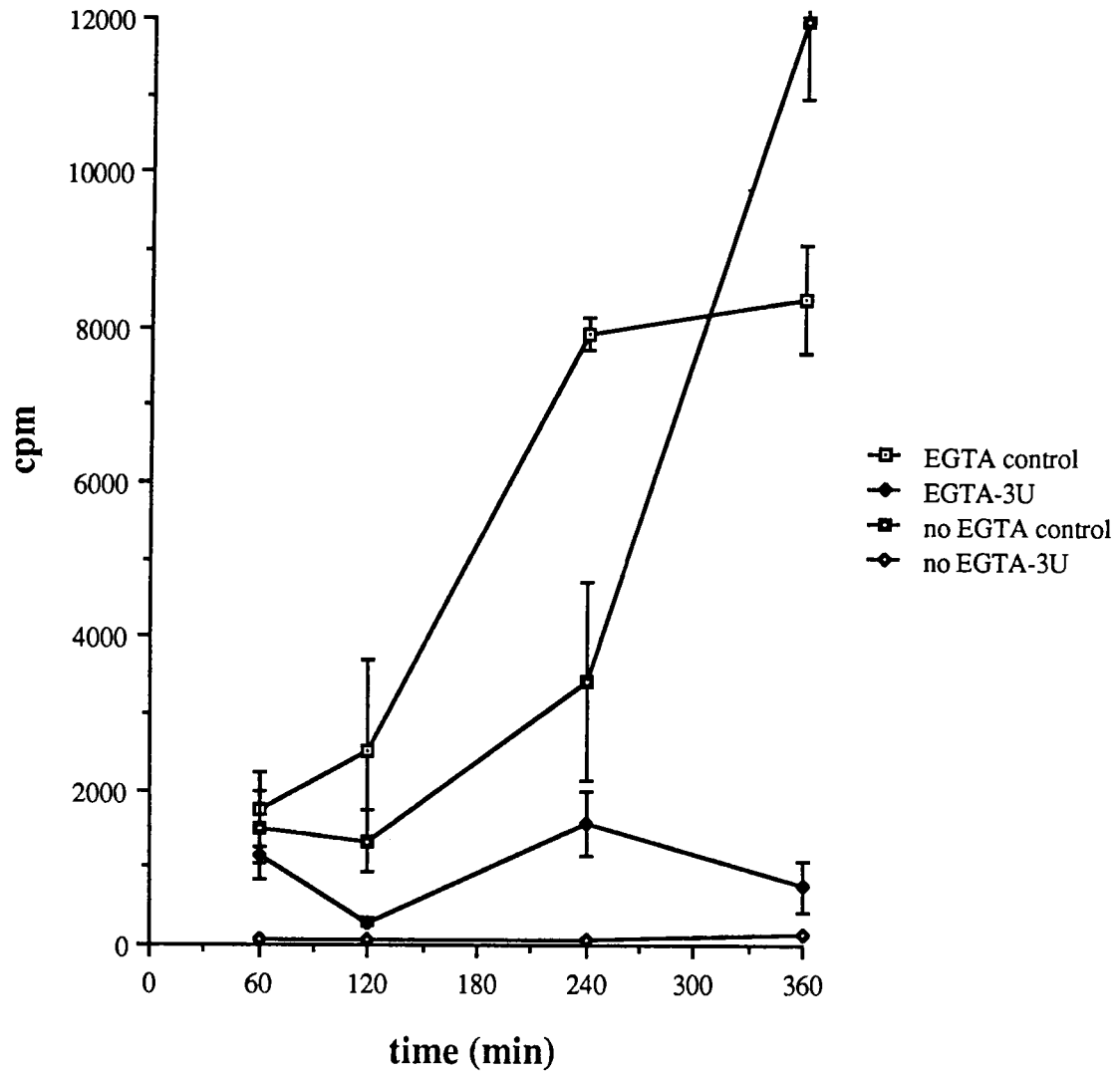


Figure 16. Leukotoxin cytotoxicity time course for BL-3 cells in the presence or absence of EGTA assessed by incorporation of $^3\text{HTdR}$.

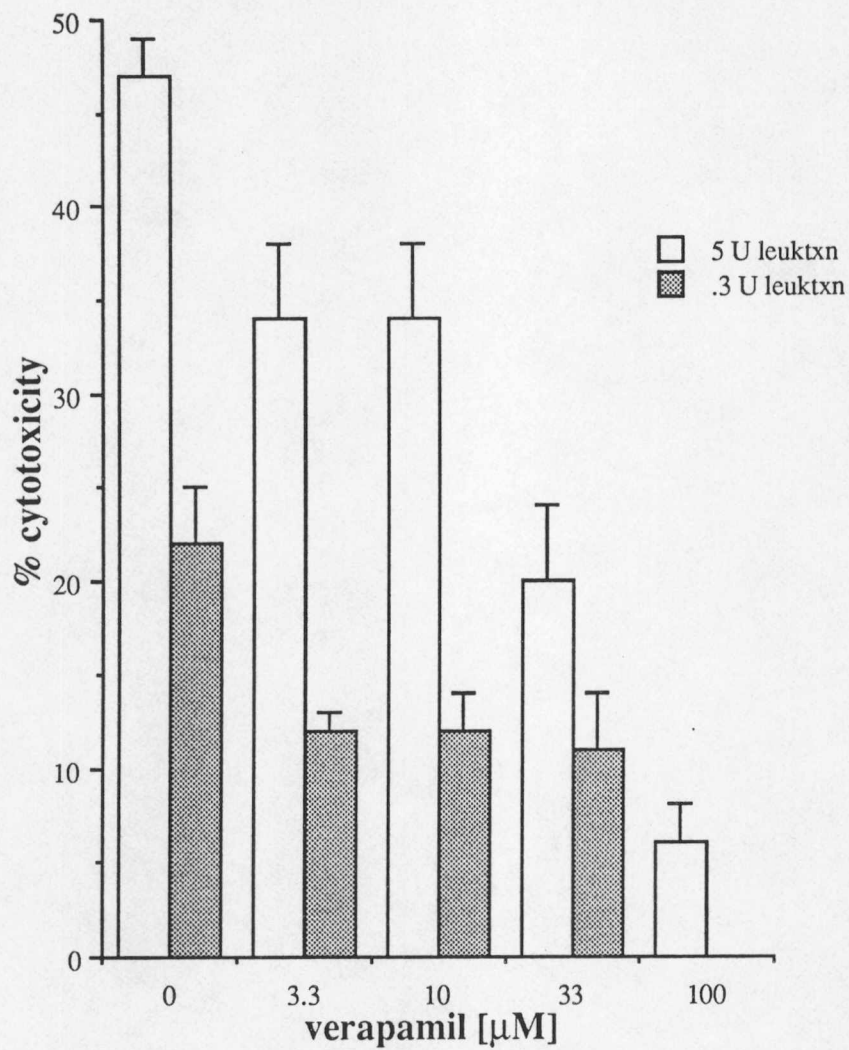


Figure 17. Protective effect of Verapamil for BL-3 cells exposed to *P. haemolytica* leukotoxin.

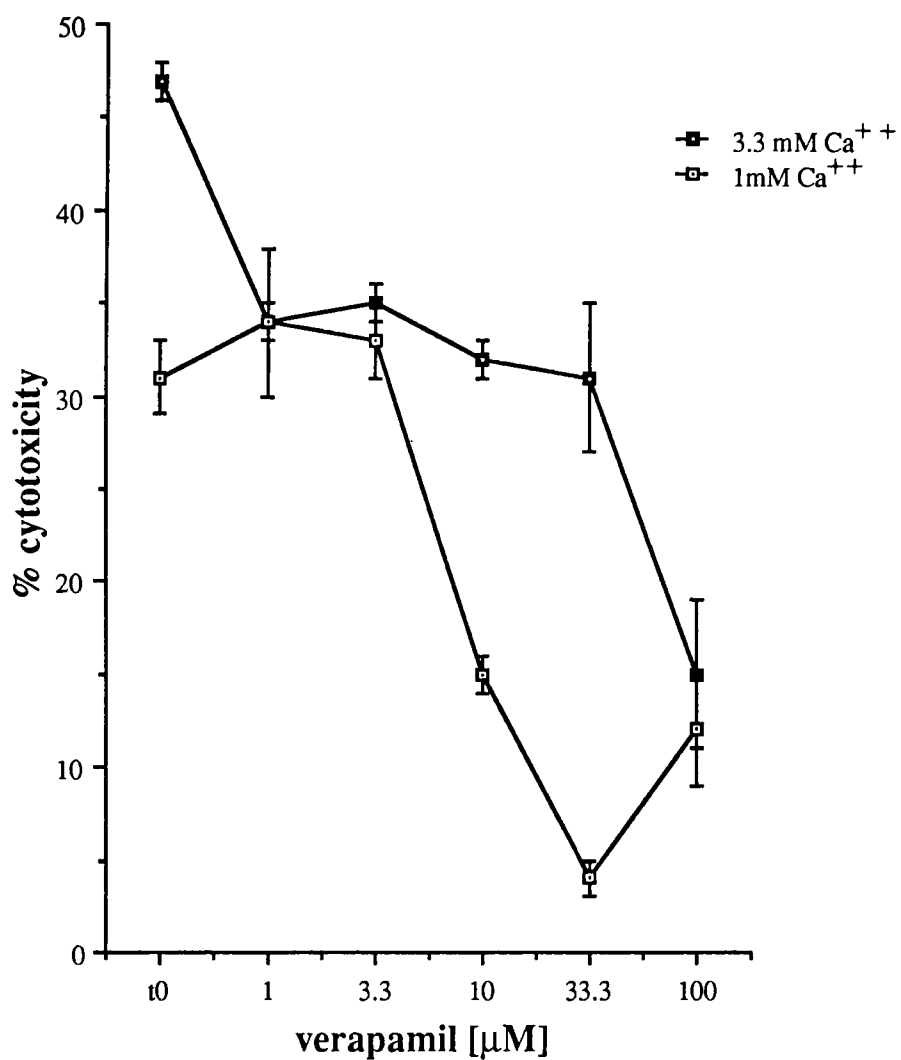


Figure 18. Reversal of Verapamil protection of leukotoxin-treated BL-3 cells by exogenously added Ca^{2+} .

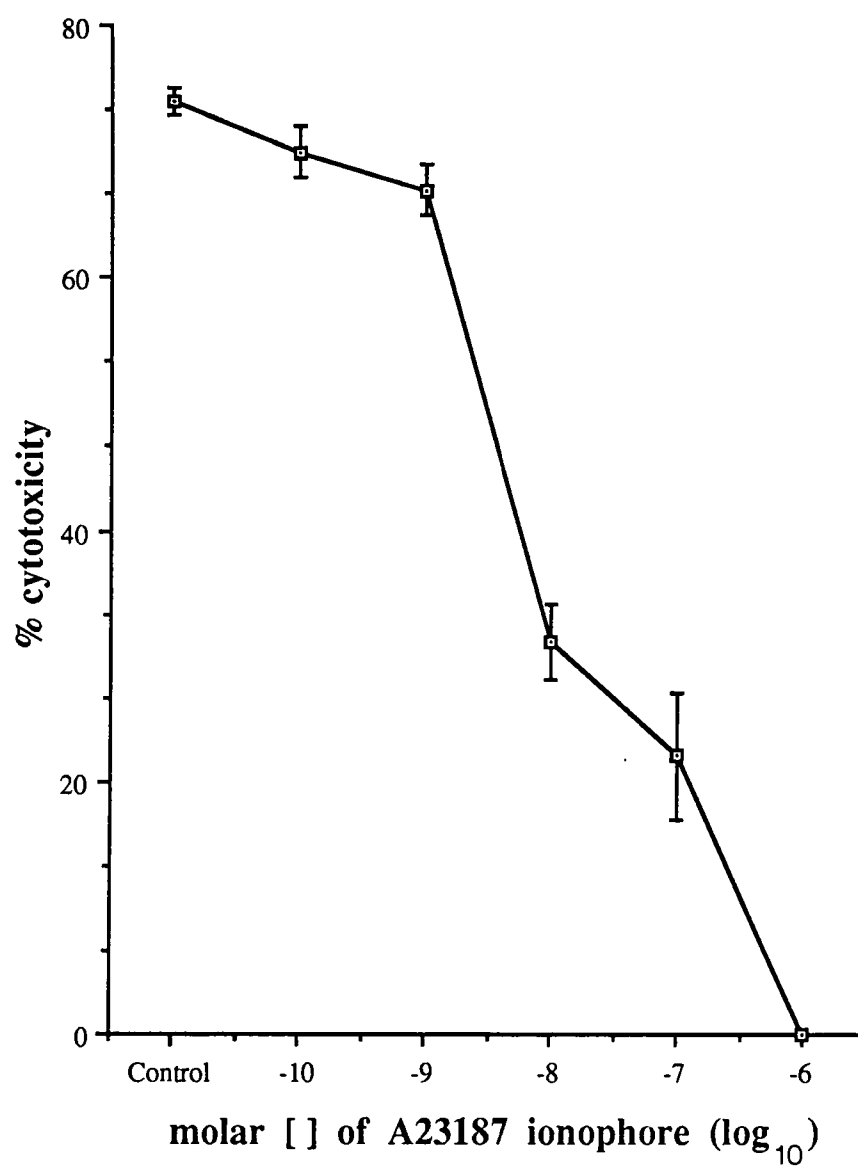


Figure 19. Protective effect of A23187 ionophore for BL-3 cells exposed to P. haemolytica leukotoxin.

the ionophore could result from an increase in the concentration of intracellular Ca^{2+} . Since Ca^{2+} is a secondary intracellular messenger, increasing its concentration could have many effects, most notably increasing Ca^{2+} activated phospholipid-dependent protein kinase C (PK-C). PK-C is known to induce changes in membranes of cells. For example, PK-C activation in B cells induces changes in receptor and antigen expression (36). Therefore, one effect of the ionophore, mediated through PK-C, might be modification of membrane receptors or substrates for the leukotoxin. To test this possibility, target cells were incubated with phorbol myristate acetate (PMA), a phorbol ester that activates PK-C directly (7), for one hr and then exposed to the leukotoxin. The results indicated that PMA at concentrations from 1 ng/ml to 100 ng/ml did not evoke protection as seen previously with the ionophore A23187 (Figure 20).

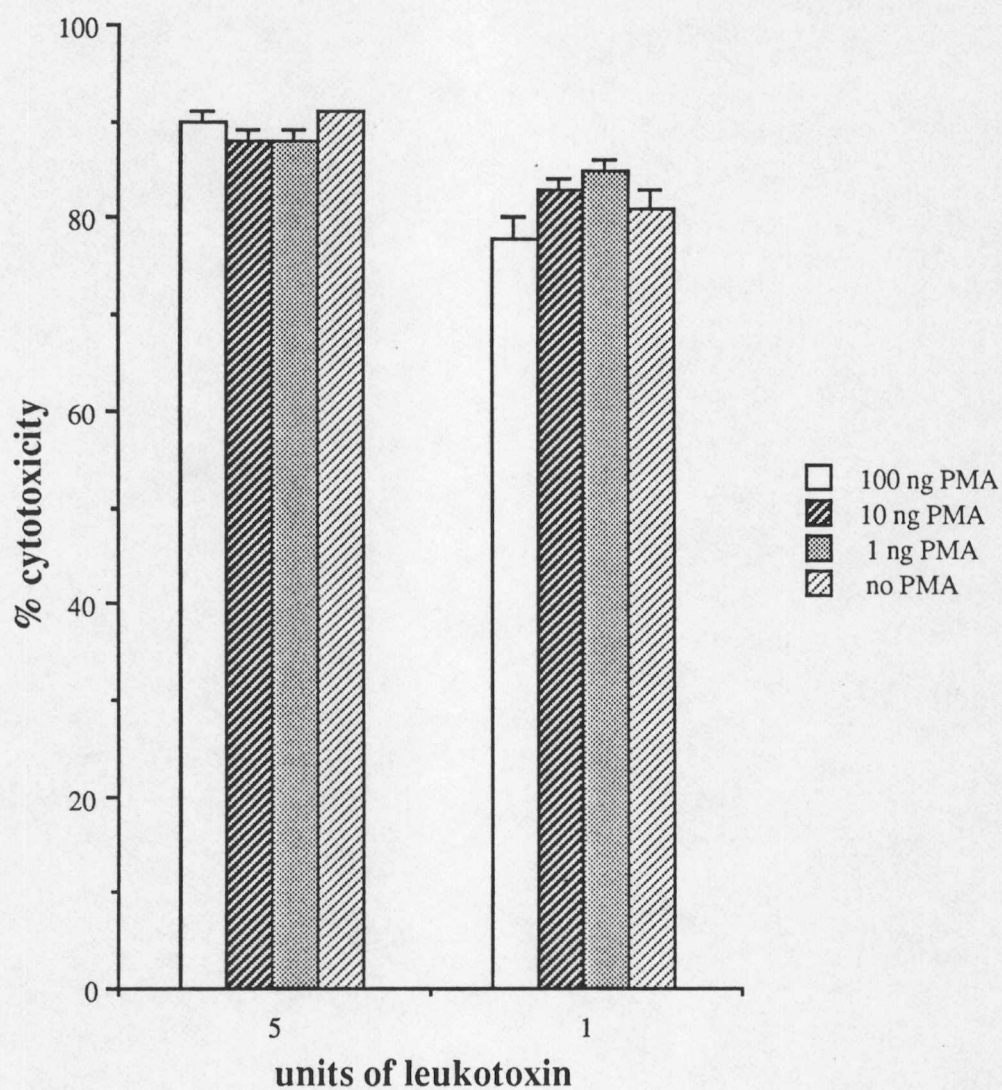


Figure 20. Failure of phorbol myristate acetate (PMA) to protect BL-3 cells from *P. haemolytica* leukotoxin.

CHAPTER 4

DISCUSSION

The leukotoxin of Pasteurella haemolytica is poorly understood despite research attention focused on the molecule. The instability of this toxin (47) has prevented a thorough understanding of the nature of the molecule. As has been the case in a majority of investigations, the present study used concentrated culture filtrates containing leukotoxin. While purified toxin would be desirable in elucidating the nature and mode of action of the toxin, such preparations are not available, and the techniques for purification of the leukotoxin have not been established. Use of the BL-3 cell line as the targets for the leukotoxin, however, did help to minimize variability which was encountered with cells freshly derived from bovine sources.

The leukotoxin was produced by actively growing bacteria as determined by bacterial growth curves (Figure 5, page 27). This observation was consistent with findings of others (8,47). In addition, other investigators have observed that the leukotoxin is probably secreted rather than cell associated (47,52). No assessment of this point was attempted in the present study.

The methods for production of leukotoxin have varied considerably (2,14,51). The method used herein was a modification of that reported by Shewen and Wilkie (47). A serum dependency for production of the leukotoxin was observed (Table 2, page 28) and concentration-dependent in that, as the serum concentration of the

growth medium increased, the amount of leukotoxin detected increased (Table 2). Furthermore, an increase in the amount of leukotoxin was detected when bovine serum was used in the growth medium instead of horse serum. The leukotoxin's lability could not be overcome by the addition or deletion of serum once the leukotoxin was produced (Table 3). Thus, the serum dependency is probably involved in production rather than stabilization.

The toxicity of the leukotoxin was influenced by temperature. Target cells chilled in an ice water bath and exposed to the leukotoxin were killed but the extent of killing was not equal to that observed at 37°C (Table 4, page 34). Sutherland et al. noted that P. haemolytica leukotoxin was not active at 4°C or at 22°C (53). However, in that study ovine strains of P. haemolytica were used to generate leukotoxin while ovine bronchoalveolar macrophages were used as target cells. Although suggestive of an active temperature dependent process, the significance of the observed temperature effect remains uncertain. It is possible that the slower rate of cytotoxicity in the cold is related more to the rate of cell death than to some activity of the toxin.

Molecular weight estimations were consistent with those of Moore et al. (unpublished). A major toxin peak at approximately 75,000 d was observed, and minor peaks at approximately 46,000 d and 16,000 d were also detected (Figure 7, page 32). The multiple peaks suggested that the leukotoxin may be in monomeric units aggregating to give the higher molecular weight values observed. Another possibility is that the major peak could be smaller monomeric units interacting with serum

albumin. However, since the molecular weight of the leukotoxin has been found by molecular cloning to be 100,000 d (29), it is conceivable that the leukotoxin is produced intact and cleaved into the smaller fragments detected by gel filtration.

The leukotoxin acted rapidly on target cells in agreement with previous reports (37,63). Cells exposed to the leukotoxin for as little as 5 min, washed, suspended in complete medium and incubated at 37°C died after approximately 6 hr (Figure 8, page 36). It is possible that some toxin remained after the wash; however, Moore et al. (37) reported that the leukotoxin exerted its effect within 20 min. Thus, it appears that the leukotoxin acts rapidly on target cells regardless of the mechanism.

Little is actually known about target cell-leukotoxin interactions. Dexamethasone treatment was attempted to, perhaps, gain insight on how the leukotoxin interacts with the target cells. This cortisteroid hormone is known to be a membrane stabilizer (19,27). However, pharmacological concentrations of the hormone had no effect on cytotoxicity (Figure 9, page 38) indicating that membrane "stability" did not influence action of the toxin.

Divalent cations are required for the action of several bacterial toxins (33,44,45,49). Results obtained in this investigation indicated that divalent cations, and Ca^{2+} in particular can influence the activity of P. haemolytica leukotoxin. Dialyzed, concentrated filtrates diluted in divalent cation-free HBSS reduced cytotoxicity considerably when compared to concentrated culture filtrates diluted in HBSS containing Ca^{2+} and Mg^{2+} . Abrogation of

rapid (less than 1 hr) cytotoxicity by the inclusion of either EGTA or EDTA (Table 5, page 40) further indicated that divalent cations were playing a role in the cytotoxicity observed. More specifically, Ca^{2+} was found to be the cation necessary for the cytotoxic effect observed (Figure 11, page 42). Such a role for Ca^{2+} is consistent with other toxins including staphylococcal Pantone-Valentine leucocidin (68,70), Shigella's shiga toxin (44) and Pseudomonas leucocidin (15). These toxins require Ca^{2+} to exert maximal cytotoxic effects on target cells. Woodin and Wieneke (70) demonstrated that when medium is depleted of Ca^{2+} , neutrophils exposed to leucocidin do not extrude vesicles. Shiga toxin requires Ca^{2+} for entry into the cytoplasm (44). Hirayama and Kato (15) demonstrated that Pseudomonas leukocidin requires Ca^{2+} , but requires the cation intracellularly to induce the cytotoxicity observed.

The mechanism by which Ca^{2+} is acting to promote cytotoxicity observed with P. haemolytica leukotoxin remains speculative. Results from time course experiments indicated that cell death does occur, regardless of the presence of Ca^{2+} , but occurs slower in medium depleted of Ca^{2+} (Figures 13, 14, 15, pages 44, 45, 46). This could be interpreted that the divalent cation is not involved in binding but may be involved in promoting cell death as observed with staphylococcal Pantone-Valentine leucocidin (68,69,70). Conversely, Ca^{2+} may be involved in leukotoxin target cell binding, and only after a period of prolonged incubation, is enough Ca^{2+} present on the cell surface to allow leukotoxin to bind. This last point is probably unlikely since thymidine was incorporated only at very low levels when

EGTA was included in the growth medium (Figure 16, page 48). The target cells are likely damaged by the leukotoxin regardless of the presence of Ca^{2+} . However, not all Ca^{2+} can be excluded from an assay of this nature. Cells bind Ca^{2+} on the surface of their membrane (reviewed in 5), and this Ca^{2+} , although not available to be bound by EGTA, may play a role in the cytotoxicity observed in prolonged incubation times.

Verapamil and the ionophore A23187 provided some insight into the role of Ca^{2+} in the cytotoxicity observed with leukotoxin. Verapamil is known to "block" Ca^{2+} channels, as well as decrease cell membrane associated Ca^{2+} and inhibit Ca^{2+} activated phospholipid-dependent protein kinase C (PK-C) (5,41). The ionophore A23187 causes a rapid influx of Ca^{2+} and other divalent cations into the cytosol from the extracellular medium and intracellular Ca^{2+} stores (1). Verapamil and the ionophore A23187 protected target cells from the effects of the leukotoxin (Figures 17, 19, pages 49, 51). In addition, the effects of Verapamil were overcome with increased extracellular Ca^{2+} (Figure 18, page 50) indicating that the protective effect of the drug probably involved Ca^{2+} .

The mechanisms by which Verapamil and the ionophore A23187 protect from the leukotoxin can only be speculated. Since Verapamil causes a decrease in the Ca^{2+} associated with the cell membrane (5), it is plausible that the leukotoxin, if it has a binding requirement for Ca^{2+} , is not binding to its membrane target. However, the leukotoxin may be binding at a decreased rate. Additionally, it is possible that phospholipid dependent protein kinase-C (PK-C) activity

is decreased with Verapamil, contributing to the protective effect seen. PK-C is associated with activation of B and T cells (16,17,36). It is unlikely, however, that the effect of Verapamil involved depression of PK-C. Similar results were observed with the ionophore A23187. The ionophore protected BL-3 cells against the leukotoxin in a dose dependent manner. Although the mechanism of ionophore protection probably involves blocking of Ca^{2+} channels, it is possible that the ionophore protected the cells by raising intracellular Ca^{2+} levels and activating Ca^{2+} -activated PK-C. Activation of PK-C is associated with antigen and receptor expression, such as I-A expression, on B cells (50,64). Incubating BL-3 cells with various pharmacological concentrations of phorbol myristate acetate (PMA), a phorbol ester known to directly activate PK-C (7, reviewed in 17), did not reduce or enhance cytotoxic activity due to the leukotoxin. Results from this experiment indicated, at least indirectly, that protection afforded by Verapamil and the ionophore does not involve activation of PK-C.

Eukaryotic cells are sensitive to changes in intracellular Ca^{2+} . The Ca^{2+} concentration in cytoplasm is approximately 10^{-7} M, while extracellular Ca^{2+} is approximately 10^{-3} M (1). To maintain this difference, a variety of mechanisms can be used by a cell. Calcium can be actively pumped out of the cytosol via plasma membrane bound Ca^{2+} - ATPase or Na^{+} -driven antiports (1). Calcium can also be removed from the cytosol by Ca^{2+} binding molecules or taken up by the endoplasmic reticulum or mitochondria (1). A rapid influx of Ca^{2+} , therefore, is followed by the cell closing Ca^{2+} channels and rapidly

pumping the cation out (1). This fact may explain the results observed with the ionophore A23187. Verapamil also closes Ca^{2+} channels in target cells (reviewed in 41). Dying cells are known to accumulate Ca^{2+} (reviewed in 70). Therefore, the decrease in cytotoxicity observed with these two Ca^{2+} influencing agents probably involves natural Ca^{2+} channels. Cytotoxicity with this leukotoxin may be influenced by altering the influx of Ca^{2+} into cells.

Other reported observations with Shiga toxin support this conclusion. Shiga toxin is a protein cytotoxin that requires entry into Vero target cells to inhibit protein synthesis (35). Vero cells could be protected from shiga toxin using either Verapamil or the ionophore A23187 indicating that Ca^{2+} through physiologically occurring Ca^{2+} channels is required for entry of toxin into cells (44). These results with shiga toxin appear to parallel the results observed with P. haemolytica leukotoxin, that is, cells are protected with Verapamil and the ionophore A23187.

BL-3 target cells treated with Verapamil or the ionophore A23178 in the presence of leukotoxin still appear to be damaged when observed microscopically at the end of the one hr cytotoxicity assays. The protection observed with these two agents is probably short term. As discussed previously, target cells incubated with leukotoxin in the presence of complete medium with EGTA remained viable after one hr. However, these cells were damaged by the leukotoxin and died over a period of time as indicated by thymidine incorporation and neutral red cytotoxicity assays.

The mechanisms by which the leukotoxin kills target cells remain unknown. For example, it is not known whether the leukotoxin acts at the membrane or is transported to exert its effects. Because of the rapid death observed when treating target cells with the leukotoxin, it is unlikely that the leukotoxin requires transport. Due to the rapid death induced by the leukotoxin, it is likely that the toxin is membrane active. The protective effects observed with Ca^{2+} channel blockers may be prolonging death as opposed to preventing toxin entry into target cells. Thus, death from the leukotoxin may be observed in one of two ways: a rapid death requiring Ca^{2+} or a prolonged death occurring independent of Ca^{2+} in the medium. Further work will be necessary to clarify the exact mechanism of action of this leukotoxin. However, this study has demonstrated that Ca^{2+} is required for the leukotoxin to exert its optimal cytotoxic action and that this action can be blocked by Verapamil and the ionophore A23187, two agents known to influence Ca^{2+} channels. Thus, Ca^{2+} and Ca^{2+} channels appear to play a role in the cytotoxicity observed with P. haemolytica leukotoxin.

LIST OF REFERENCES

LIST OF REFERENCES

1. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson. Molecular Biology of the Cell. Garland Publishing, Inc., New York. 1983. p. 301, 740, 741.
2. Baluyut, C. S., R. R. Simonson, W. J. Bemrick, W. J. and S. K. Maheswaran. 1981. Interaction of Pasteurella haemolytica with bovine neutrophils: Identification and partial characterization of a cytotoxin. Am. J. Vet. Res. 42: 1920.
3. Benson, M. L., R. G. Thomson, and V. E. O. Valli. 1978. The bovine alveolar macrophage II. In vitro studies with Pasteurella haemolytica. Can. J. Comp. Med. Vol. 42, p. 368.
4. Berggren, K. A., C. S. Baluyut, R. R. Simonson, W. J. Bemrick and S. K. Maheswaran. 1981. Cytotoxic effects of Pasteurella haemolytica on bovine neutrophils. Am. J. Vet. Res. 42: 1383.
5. Bianca, V. D., M. Grzeskowiak, P. D. Togri, M. Cassatella and F. Rossi. 1985. Inhibition by verapamil of neutrophil responses to FMLP and PMA. Mechanisms involving calcium changes, cyclic amp and protein kinase C. Biochem. Biophys. Acta 845: 223.
6. Carter, G. R. 1956. Some remarks on shipping fever in Canada. Can. J. Comp. Med. 20: 289.
7. Castagna, M. Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka. 1982. Direct activation of calcium activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847.
8. Chang, Y. F., H. W. Renshaw and A. B. Richards. 1986. Pasteurella haemolytica leukotoxin: Physiochemical characteristics and susceptibility of leukotoxin to enzymatic treatment. Am. J. Vet. Res. 47: 716.
9. Collier, J. R. 1968. Significance of bacteria in bovine respiratory disease. Am. Vet. Med. Assoc. 153: 1645.
10. Collins, M. F. 1977. Mechanisms of acquired resistance to Pasteurella multocida in action: a review. Cornell Vet. 67: 103.
11. Confer, A. W., R. J. Panciera, R. E. Corstvet, J. A. Rummage and R. W. Fulton. 1984. Bovine pneumonic pasteurellosis: Effect of culture age of Pasteurella haemolytica used as a live vaccine. Am J. Vet Res. 45: 2543.

12. Confer, A. W., R. J. Panciera, R. W. Fulton, M. J. Gentry and J. A. Rummage. 1985. The effect of vaccination with live or killed Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. Am. J. Vet. Res. 46: 342.
13. Henry, C. W. 1984. Shipping fever pneumonia: a new look at an old enemy. Vet. Med. 79: 1200.
14. Himmel, M. E., M. D. Yates, L. H. Lauerman and P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from Pasteurella haemolytica. Am. J. Vet. Res. 43: 764.
15. Hirayma, T. and I. Kato. 1984. Mode of cytotoxic action of Pseudomona leukocidin on phosphatidylinositol metabolism and activation of lysosomal enzyme in rabbit leukocytes. Infect. Immun. 43: 21.
16. Isakov, N., M. I. Mally, W. Scholz and A. Altman. 1987. T-lymphocyte activation: the role of protein kinase C and bifurcating inositol phospholipid signal transduction pathway. Immunol. Rev. G. Moller (ed). 95: 89.
17. Isakov, N., W. Scholz and A. Altman. 1986. Signal transduction and intracellular events in T lymphocyte activation. Immunol. Today. 7: 271.
18. Jakab, G. J. and G. M. Green. 1973. Immune enhancement of pulmonary bactericidal activity in murine virus pneumonia. J. Clin. Invest. 52: 2878.
19. Janoff, A., G. Weissman, B. Zweifach and L. Thomas. 1962. Pathogenesis of experimental shock. IV. Studies of lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxaemia. J. Exp. Med. 116: 451.
20. Jensen, R. and D. R. Mackay Eds. Shipping fever pneumonia. In Diseases of Feedlot Cattle. Third ed. Lea and Febiger, Philadelphia. 1979. p. 65.
21. Jensen, R., R. E. Pierson, P. M. Braddy, D. A. Saari, L. H. Lauerman, J. J. England, H. Keyvanfar, J. R. Collier, D. P. Horton, A. E. McChesney, A. Benitez and R. M. Christie. 1976. Shipping fever pneumonia in yearling feedlot cattle. J. Am. Vet. Med. Assoc. 169: 500.
22. Jericho, K. W. F., C. le Q. Darcel and E. V. Lanford. 1982. Respiratory disease in calves produced with aerosols of parainfluenza-3 virus and Pasteurella haemolytica. Can. J. Comp. Med. 46: 293.

23. Jericho, K. W. F. and E. V. Langford. 1982. Aerosol vaccination of calves with Pasteurella haemolytica against experimental respiratory disease. Can. J. Comp. Med. 46: 287.
24. Jericho, K. W. F., W. D. G. Yates and L. A. Babiuk. 1982. Bovine herpesvirus-1 vaccination against experimental bovine herpesvirus-1 and Pasteurella haemolytica respiratory tract infection: onset of infection. Am. J. Vet. Res. 43: 1776.
25. Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat and D. W. Johnson. 1980. Evidence of cytotoxic effects of Pasteurella haemolytica on bovine peripheral blood mononuclear leukocytes. Am. J. Vet. Res. 41: 1690.
26. Kaehler, K. L. R. J. F. Markham, C. C. Muscoplat and D. W. Johnson. 1980. Evidence of species specificity in the cytotoxic effects of Pasteurella haemolytica. Infect. Immun. 30: 615.
27. Kapur, B. M. L., B. N. Balkrishna Rao and P. K. Purkayastha. 1972. Evaluation of different doses of dexamethasone in canine endotoxin shock. Indian J. Med. Res. 60: 1205.
28. Kim, J. D. S. 1977. Immunological injury in shipping fever pneumonia of cattle. Vet. Rec. 100: 109.
29. Lo, R. Y. C., P. E. Shewen, C. A. Strathdee and C. N. Greer. 1985. Cloning and expression of the leukotoxin gene of Pasteurella haemolytica A1 in Escherichia Coli K-12. Infect. Immun. 50: 667.
30. Lopez, A., R. G. Thomson and M. Savan. 1976. The pulmonary clearance of Pasteurella haemolytica in calves infected with bovine parainfluenza-3 virus. Can. J. Comp. Med. 40: 385.
31. Maheswaran, S. K., T. R. Leininger, T. R. Ames, and E. L. Townsend. 1984. Immune response to Pasteurella haemolytica leukotoxin. Abstracts of Papers Presented at the 65th Annual Meeting of the Conference of Research Workers in Animal Disease. p. 28.
32. Martin, W. V. 1984. Neutrophils kill pulmonary endothelial cells by a hydrogen-peroxide dependent pathway as a model of neutrophil-mediated lung injury. Am. Rev. Resp. Dis. 130: 209.
33. McClane, B. A. and A. P. Wnek. 1986. Divalent cation involvement in the action of Clostridium perfringens type A enterotoxin. Abstracts of the Annual Meeting of the American Society for Microbiology. p. 61.

34. McIntire, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley and A. Y. Lee. 1967. Chemical, physical, and biological properties of a lipopolysaccharide from Escherichia coli k-235. Biochem. 6: 2363.
35. Middlebrook, J. L. and R. B. Dorland. 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. 48: 199.
36. Monroe, J. G., J. E. Nidel and J. C. Cambier. 1984. B cell activation. IV. induction of cell membrane depolarization and hyper-IA expression by phorbol diesters suggest a role for protein kinase C in murine B lymphocyte activation. J. Immunol. 132: 1472.
37. Moore, R. N., R. D. Walker, G. A. Shaw, F. M. Hopkins and E. P. Shull. 1985. Antileukotoxin activity produced in the bovine lung after aerosol exposure to viable Pasteurella haemolytica. Am J. Vet. Res. 46: 1909.
38. Mosier, D. A., A. W. Confer, M. Hall, M. J. Gentry and R. J. Panciera. 1986. Enzyme-linked immunosorbant assay for detection of serum antibodies to Pasteurella haemolytica leukotoxin in cattle. J. Clin. Microbiol. 24: 218.
39. Mosier, D. A., B. A. Lessley, A. W. Confer, S. M. Antone and M. J. Gentry. 1986. Chromatographic separation and characterization of Pasteurella haemolytica leukotoxin in cattle. J. Clin. Microbiol. 24: 218.
40. Noda, M., T. Hirayama, J. Kato and F. Matsuda. 1980. Fixation and inactivation of staphylococcal leukocidin by phosphotidylcholine and ganglioside GM₁ in rabbit polymorphonuclear leukocytes. Infect. Immun. 29: 678.
41. Norris, D. K. and H. F. Bradford. 1985. On the specificity of verapamil as a calcium channel blocker. Biochem. Pharm. 34: 1953.
42. Otulakowski, G. L., P. E. Shewen, A. E. Udoh, A. Mellars and B. N. Wilkie. 1981. Proteolysis of sialoglycoprotein by Pasteurella haemolytica cytotoxic culture supernatant. Infect. Immun. 42: 64.
43. Pillemer, L., M. D. Schoenberg, L. Blum and L. Wurz. 1985. Properdin system and immunity. II. Interaction of the properdin system with lipopolysaccharides. Science. 122: 545.
44. Sandvig, K. and E. Brown. 1987. Ionic requirements for entry of shiga toxin from Shigella dysenteriae 1 into cells. Infect. Immun. 55: 298.

45. Sandvig, K. and S. Olsnes. 1982. Entry of toxin proteins abrin, modeccin, ricin and diphtheria toxin into cells. J. of Biol. Chem. 257: 7495.
46. Shewen, P. E. and B. N. Wilkie. 82. Cytotoxin of Pasteurella haemolytica acting on bovine leukocytes. Infect. Immun. 35: 91.
47. Shewen, P. E. and B. N. Wilkie. 1985. Evidence for Pasteurella haemolytica cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. 96: 1212.
48. Slocombe, R., J. Malurk and R. Ingeroll. 1985. Importance of neutrophil and pathogenesis of acute pneumonic pasteurellosis. Am. J. Vet. Res. 46: 2253.
49. Snyder, I. S. and P. Zwadyk. 1969. Some factors affecting production and assay of Escherichia coli haemolysins. J. Gen. Microbiol. 55: 139.
50. Strassmann, G. S., S. D. Somers, T. A. Springer, D. O. Adams and T. A. Hamilton. 1986. Biochemical models of interferon gamma mediated macrophage activation: independent regulation of lymphocyte function associated antigen and I-A antigen on murine peripheral macrophages. Cell. Immunol. 97: 110.
51. Sutherland, A. D. and W. Donachie. 1986. Cytotoxic effect of serotype of Pasteurella haemolytica on sheep bronchoalvolar macrophages. Vet Microbiol. 11: 331.
52. Sutherland, A. D., W. Donachie and J. Redmond. Studies on Pasteurella haemolytica cytotoxin. In Bacterial Protein Toxins. Alout, J. E., F. J. Fehrenbach, J. H. Freer and J. Jeljaszewicz. eds. Academic Press, London. 1984 p. 374.
53. Sutherland, A. D. and J. Redmond. 1986. Cytotoxin from an ovine strain of Pasteurella haemolytica: characterization studies and partial purification. Vet. Microbiol. 11: 337.
54. Tabatabui, L. B. and G. H. Frank. 1981. Neuraminidase from Pasteurella haemolytica. Curr. Microbiol. 5: 203.
55. Taichman, N. S., R. T. Dean and C. S. Sanderson. 1980. Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from Actinobacillus actinomycetemcomitans. Infect. Immun. 28: 258.
56. Thomas, R. G. General Veterinary Pathology. W. B. Saunders Co., Philadelphia. 1978. p. 148.

57. Thomson, R. G., S. Chander, M. Savin and M. L. Fox. 1975. Investigation of factors of probable significance in the pathogenesis of pneumonic pasteurellosis in cattle. *Can. J. Comp. Med.* 34: 194.
58. Thomson, R. G. and F. Gilka. 1974. A brief review of pulmonary clearance of bacterial aerosols emphasizing aspects of particular relevance to veterinary medicine. *Can. Vet. J.* 15: 99.
59. Thomson, R. F., B. J. McSherry and V. E. D. Valli. 1974. Endotoxin induced disseminated intravascular clotting in cattle. *Can. J. Comp. Med.* 38: 457.
60. Tsai, C. C., B. J. Shenker, J. M. DiRienzo, D. Malamud and N. S. Taichman. 1984. Extraction and isolation of a leukotoxin from Actinobacillus actinomycetemcomitans with polymyxin B. *Infect. Immun.* 43: 700.
61. Wadstrom, T. Bacterial neuraminidase. In Bacterial Toxins and Cell Membranes. Jeljaszwics, J. and T. Wadstrom eds. Academic Press: London. 1978. p. 27.
62. Walker, R. D., F. M. Hopkins, T. W. Schultz, M. D. McCracken and R. N. Moore. 1985. Changes in leukocyte populations in pulmonary lavage fluids after inhalation of Pasteurella haemolytica. *Am. J. Vet. Res.* 46: 2429.
63. Walker, R. D., T. W. Shultz, F. M. Hopkins and M. J. Bryant. 1984. Growth phase dependent phagocytosis of Pasteurella haemolytica by bovine pulmonary macrophages. *Am. J. Vet. Res.* 45: 1230.
64. Weiel, J. E., D. O. Adams and T. A. Hamilton. 1985. Biochemical models of interferon gamma action: altered expression of transferrin receptors on murine peritoneal macrophages after treatment in vitro with PMA or A23187. *J. Immunol.* 134: 293.
65. Wilkie, B. N. 1982. Respiratory tract immune responses to microbial pathogens. *J. Am. Vet. Med. Assoc.* 181: 1074.
66. Wilkie, B. N. and R. J. F. Markham. 1981. Bronchoalveolar washing cells and immunoglobulins of clinically normal calves. *Am. J. Vet. Res.* 42: 241.
67. Wilkie, B. N., R. J. F. Markham and P. E. Shewen. 1980. Response of calves to lung challenge exposure with Pasteurella haemolytica after parenteral or pulmonary immunization. *Am. J. Vet. Res.* 41: 1773.

68. Woodin, A. M. Staphylococcal Leucocidin. In The Staphylococci. J. Cohen ed. Wiley-Interscience, New York. 1972. pp. 281-299.
69. Woodin, A. M. Staphylococcal Leukocidin. In Microbial Toxins. Montie, T. C., S. Kadis and S. J. Aji. eds. Academic Press, London. 1970. pp. 327-355.
70. Woodin, A. M. and A. A. Wieneke. 1962. The accumulation of calcium by the polymorphonuclear leukocyte treated with staphylococcal leukocidin and its significance in the extrusion of protein. Biochem. J. 47: 487.
71. Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46: 225.
72. Zambon, J. J., C. Deluca, J. Slots and R. J. Genco. 1983. Studies of leukotoxin from Actinobacillus actinomycetemcomitans using the promyelocytic HL-60 cell line. Infect. Immun. 40:205.

VITA

Donald G. Gerbig, Jr. was born in Barberton, Ohio on May 15, 1962. He attended schools in that city and graduated from Barberton High School in May 1980. The following September he entered the University of Akron, Akron, Ohio. In December of 1984, he was graduated cum laude with a Bachelor of Science in Biology. He began graduate studies in the Department of Microbiology at The University of Tennessee, Knoxville in September of 1984. He received the Master of Science degree in August 1987.

The author is a member of Phi Kappa Phi and the American Society of Microbiology.