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**PROTECTIVE EPITOPES IN THE LEUKOTOXIN
OF *PASTEURELLA HAEMOLYTICA***

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Elizabeth D. Fish

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DEDICATION

This thesis is dedicated to the memory of my grandfather, John Alexander Fish, who always stressed the importance of education.

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ABSTRACT

Pasteurella haemolytica A1 has been implicated as a causative agent in shipping fever pneumonia. This organism produces a potent leukotoxin which is thought to promote inflammation and potentiate bacterial proliferation in the lungs of infected animals. Although several functional regions within the leukotoxin molecule have been identified, the antigenic regions and mechanism of action of the protein are still unknown. These studies were undertaken to further characterize the location and activity of protective epitopes within the leukotoxin molecule. Two approaches were used to accomplish these goals. In the first series of experiments, activated and unactivated leukotoxin proteins were employed to determine if LKTC gene modification affected recognition of LKTA by a panel of six leukotoxin mAbs. Results indicated that the epitopes for the mAbs were not involved in activation of LKTA by LKTC. Additionally, chimeric toxin molecules, composed of domains of the leukotoxin and the serotype 5 toxin of *Actinobacillus pleuropneumoniae*, were used to definitively identify the region of the leukotoxin which contained epitopes recognized by the leukotoxin mAbs. Each mAb recognized an epitope contained within amino acids 768-939 of the leukotoxin protein. The second method of examining protective epitopes in the leukotoxin molecule was with the use of anti-idiotypic antibodies. Rabbit polyclonal anti-idiotypic antisera were generated against a leukotoxin-neutralizing mAb, mAb ltx-2. Preliminary evidence indicates that this mAb functions by preventing the leukotoxin from associating with target cells. Anti-idiotypic antibodies of the β or γ subclasses (Ab2 β or Ab2 γ) were isolated. Subsequent assays tentatively identified these as Ab2 β antibodies. Upon injection of these antibodies into mice, Ab3 antibodies were produced in several animals. The Ab3 antibodies recognized the leukotoxin by ELISA and neutralized the leukotoxin in

a neutral red cytotoxicity assay. This indicated that the antibodies isolated from the antisera were indeed Ab2 β . The Ab2 β antibodies were then used to probe for a leukotoxin receptor on leukotoxin target cells. Unfortunately, no receptor was identified using these antibodies. However, the Ab2 β antibodies were able to affinity purify antibodies from whole bovine antiserum samples which recognized the same epitope as mAb ltx-2. The results from this study indicate 1) the epitopes for the leukotoxin mAbs are not involved in LKTC-mediated activation and are located within amino acids 768-939 of LKTA; 2) Ab2 β antibodies against a leukotoxin-neutralizing mAb can induce the production of leukotoxin-neutralizing Ab3 antibodies; and 3) the mAb ltx-2 leukotoxin epitope is immunogenic in bovines and such antibodies have activity similar to that of mAb ltx-2.

TABLE OF CONTENTS

CHAPTER		PAGE
1.	INTRODUCTION	1
	The Disease	1
	Etiology.....	1
	<i>Pasteurella haemolytica</i> - The Organism.....	3
	Control of Shipping Fever	11
	Statement of Purpose	16
2.	MATERIALS AND METHODS	18
	Bacterium.....	18
	Cell Culture	18
	Leukotoxin Production	19
	Neutral Red Cytotoxicity Assay	21
	mAb Preparation.....	22
	Preparation of mAb ltx-2-HRP	23
	Immunization of Rabbits	24
	ELISA	24
	Ammonium Sulfate Precipitation of Rabbit Antisera.....	25
	Preparation of Hydrazide AvidGel™ Ax Column	25
	Purification of Rabbit Polyclonal Antisera	26
	Anti-Idiotypic Antibody Neutral Red Cytotoxicity Assay	27
	Anti-Idiotypic Antibody ELISA	28

Immunization of Mice.....	29
Glutaraldehyde Fixation of BL-3 Cells and ELISA.....	30
Plasmid Manipulations.....	31
Western Immunoblot.....	32
Bovine Serum Samples.....	35
 3. RESULTS	 36
mAb Characterization	36
mAb Domain Localization.....	52
Detection of Rabbit Anti-Mouse Antibodies.....	54
Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Whole Antisera.....	61
Binding of Fractionated Antisera to mAbs ltx-2 and ltx-35	65
Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Fractionated Antisera.....	65
Abrogation of mAb ltx-2 Protection of BL-3 Cells by Eluate 1.....	68
Recognition of Leukotoxin by Murine Antisera	68
Protection of BL-3 Cells from Leukotoxin-Mediated Lysis by Murine Sera	71
Binding of Ab2 β Antibodies to BL-3 Cells	82
Binding of Whole Bovine Antisera to Leukotoxin	83
Neutralization of Leukotoxin-Mediated Cytotoxicity by Bovine Antisera	83
Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Bovine Antisera	86
mAb ltx-2 Inhibition of mAb ltx-2-HRP Binding to Leukotoxin.....	86
Abrogation of mAb ltx-2 and Cow Anti-Lkt Antiserum Protection of BL-3 Cells by Eluate 1.....	89

Neutralization of Leukotoxin by Cow Id	94
4. DISCUSSION	96
LIST OF REFERENCES	106
VITA.....	119

LIST OF TABLES

TABLE	PAGE
1. Isotypes of mAbs reactive with <i>P. haemolytica</i> 100X concentrated leukotoxic culture filtrates.....	20
2. Neutralization titers for leukotoxin mAbs	38
3. Titers of rabbit antisera samples in ELISA determinations designed to demonstrate binding to mouse mAbs and to demonstrate ability of rabbit antisera to inhibit mAb ltx-2-HRP from binding to leukotoxin	63
4. Titers of eluates and flowthrough obtained from the mAb ltx-2 column in ELISA determinations designed to demonstrate binding to mAb ltx-2 and ability of eluates to inhibit mAb ltx-2-HRP from binding to leukotoxin	67
5. Inhibitory and neutralization titers for mice injected with eluate 1 or nonspecific rabbit IgG	76
6. Unfractionated bovine antisera samples titers for binding, neutralization and inhibition	85

7. Protein concentration of antibodies in bovine antisera samples which recognize the same leukotoxin epitope as mAb ltx-2.....	91
---	----

LIST OF FIGURES

FIGURE		PAGE
1.	Diagram of DNA encoding chimeric toxin molecules.....	33
2.	Neutralization of leukotoxin-mediated cell lysis by leukotoxin mAbs as measured by the neutral red cytotoxicity assay	37
3.	Immunoblot of mAb ltx-2 reacted with SDS-PAGE of unactivated and activated LKTA proteins.....	40
4.	Immunoblot of mAb ltx-4 reacted with SDS-PAGE of unactivated and activated LKTA proteins.....	41
5.	Immunoblot of mAb ltx-35 reacted with SDS-PAGE of unactivated and activated LKTA proteins.	42
6.	Immunoblot of mAb ltx-37 reacted with SDS-PAGE of unactivated and activated LKTA proteins.....	43
7.	Immunoblot of mAb ltx-58 reacted with SDS-PAGE of unactivated and activated LKTA proteins.....	44

8.	Immunoblot of mAb ltx-81 reacted with SDS-PAGE of unactivated and activated LKTA proteins.....	45
9.	Immunoblot of mAb ltx-2 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	46
10.	Immunoblot of mAb ltx-4 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	47
11.	Immunoblot of mAb ltx-35 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	48
12.	Immunoblot of mAb ltx-37 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	49
13.	Immunoblot of mAb ltx-58 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	50
14.	Immunoblot of mAb ltx-81 reacted with SDS-PAGE of unactivated and activated APPA proteins.....	51
15.	Immunoblot of rabbit polyclonal anti-102 kDa antiserum reacted with SDS-PAGE of chimeric toxin proteins.	53

16. Immunoblot of mAb ltx-2 reacted with SDS-PAGE of chimeric toxin proteins.	55
17. Immunoblot of mAb ltx-4 reacted with SDS-PAGE of chimeric toxin proteins.	56
18. Immunoblot of mAb ltx-35 reacted with SDS-PAGE of chimeric toxin proteins.	57
19. Immunoblot of mAb ltx-37 reacted with SDS-PAGE of chimeric toxin proteins.	58
20. Immunoblot of mAb ltx-58 reacted with SDS-PAGE of chimeric toxin proteins.	59
21. Immunoblot of mAb ltx-81 reacted with SDS-PAGE of chimeric toxin proteins.	60
22. Antibody binding to mAbs ltx-2 and ltx-35 measured by ELISA with sera from mAb ltx-2 immunized rabbits	62
23. Inhibition of mAb ltx-2-HRP binding to leukotoxin by preincubation with sera obtained from rabbits immunized with mAb ltx-2 as measured by ELISA.....	64

24. Antibody binding to mAbs ltx-2 and ltx-35 measured by ELISA with antibodies isolated by washes of ammonium sulfate precipitated antibodies over the mAb ltx-2 column	66
25. Inhibition of mAb ltx-2-HRP binding to leukotoxin by preincubation with antibodies isolated by numerous passes of ammonium sulfate precipitated antibodies over the mAb ltx-2 column as measured by ELISA.....	69
26. Abrogation of mAb ltx-2 protection of leukotoxin-mediated BL-3 cell lysis measured by a neutral red cytotoxicity assay by preincubation with antibodies isolated from passing ammonium sulfate precipitated antibodies through the mAb ltx-2 column	70
27. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum from mouse 5A2 obtained on day 63	72
28. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 5A3 on day 63	73
29. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 5B1 on day 63	74
30. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 6A3 on day 63	75

31. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5A2 on day 63 measured by the neutral red cytotoxicity assay.....	77
32. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5A3 on day 63 measured by the neutral red cytotoxicity assay.....	78
33. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5B1 on day 63 measured by the neutral red cytotoxicity assay.....	79
34. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5B2 on day 63 measured by the neutral red cytotoxicity assay.....	80
35. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 6A3 on day 63 measured by the neutral red cytotoxicity assay.....	81
36. Antibody binding to trapped leukotoxin measured by ELISA with bovine antisera samples.....	84

37. Neutralization of leukotoxin-mediated cell lysis by unfractionated bovine antisera samples measured by the neutral red cytotoxicity assay	87
38. ELISA measuring inhibition of mAb ltx-2-HRP binding to trapped leukotoxin by unfractionated bovine antisera samples.....	88
39. Inhibition of mAb ltx-2-HRP binding to trapped leukotoxin by mAb ltx-2 IgG measured by ELISA.....	90
40. Abrogation of mAb ltx-2 protection of leukotoxin-mediated BL-3 cell lysis by preincubation with antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from mAb ltx-2 immunized rabbits over the mAb ltx-2 column as measured by the neutral red cytotoxicity assay.....	92
41. Neutral red cytotoxicity assay measuring abrogation of cow anti-lkt antiserum protection of leukotoxin-mediated BL-3 cell lysis by preincubation with antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from mAb ltx-2 immunized rabbits over the mAb ltx-2 column	93

42. Neutralization of leukotoxin-mediated cell lysis by mAb ltx-2 and antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from bovines immunized with leukotoxin over the Ab2b column	95
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LIST OF ABBREVIATIONS

Ab1	antibody one
Ab2	antibody two
Ab2 α	antibody two, subclass α
Ab2 β	antibody two, subclass β
Ab2 γ	antibody two, subclass γ
Ab3	antibody three
BHI	brain heart infusion
BHV-1	bovine herpesvirus-1
BL-3	bovine lymphocyte 3 cell line
CFU	colony forming units
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HRP	horseradish peroxidase
IBR	infectious bovine rhinotracheitis
Id	idiotope, antigenic epitope
IgG	immunoglobulin G
kDa	kilodalton
LPS	lipopolysaccharide
mAb	monoclonal antibody
N-terminal	amino-terminal
nm	nanometer

O antigen	antigenic polysaccharide chain
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline with 0.05% Tween 20
PI-3	parainfluenza-3
RPMI-1640	Roswell Park Memorial Institute-1640 Medium
RTX	repeats in the structural toxin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	tris buffered saline
TBS-Tween	tris buffered saline with 0.05% Tween 20
U	unit

CHAPTER 1

INTRODUCTION

The Disease.

Shipping fever, enzootic pneumonia of calves and atypical interstitial pneumonia comprise three clinical entities of bovine respiratory disease (80). Shipping fever pneumonia is an acute disease complex often associated with stockyards, breeding facilities, and the shipment of cattle. Pneumonic pasteurellosis, a term which describes pneumonia caused by *Pasteurella* species, is often used interchangeably with shipping fever (80). Shipping fever is one of the most important causes of morbidity and mortality in cattle (116). Pathology and symptomology of the disease include fever, rhinitis, nasal discharge, diarrhea, fibrinous pleuritis, fibrinous pneumonia, and acute inflammation of the alveoli, bronchi, and airways with marked involvement of alveolar macrophages and neutrophils (80, 125, 142). Substantial weight loss invariably occurs in infected animals (142). Economic losses for consumers and beef producers result from delayed marketing of animals, insufficient feed conversion, expensive treatments, excessive shrinkage of herd size, and death of cattle (63). Such losses have been estimated at approximately 800 million dollars annually for the North American beef industry alone (42).

Etiology.

Causality of shipping fever is multifactorial (6, 41, 58, 142). During transport of cattle, exposure to dusts, sudden and extreme changes in temperature, and irregular feeding and watering, along with a variety of other factors retard the lungs' natural defense mechanisms (80, 104, 142). Such physical stresses slow the mucociliary

elevator, increase blood cortisol concentrations, and lower the resistance of calves (25, 58). In addition, an ideal opportunity for the spread of pathogenic agents exists during the convergence, shipment, and redispersal of cattle obtained from many sources (80). Infectious stresses, both viral and bacterial, inactivate cilia, suppress the cellular immune response, and further lower the resistance of the animals (25, 58).

Infection of cattle with viruses is thought to be a key factor in the development of shipping fever. Viruses such as bovine herpesvirus-1 (BHV-1), parainfluenza-3 (PI-3) virus, infectious bovine rhinotracheitis (IBR) virus, and respiratory syncytial virus alone cause clinical respiratory disease (113). They also cause direct damage to the respiratory tract and reduce bacterial clearance mechanisms (25, 37, 41, 80, 84, 124, 142). BHV-1 reduces antimicrobial functions of bovine leukocytes, increasing susceptibility to shipping fever (37). Similarly, infection of animals with bovine viral diarrhea virus causes immunosuppression, enhancing sensitivity to experimentally induced pneumonic pasteurellosis (84, 113). Also, PI-3 virus infection enhances animals' susceptibility to respiratory disease caused by *Pasteurella* species (10, 64). Although the precise role of viruses in the development of shipping fever is not clear, it is evident that their role in altering the normal immune function in the host is important.

Many bacteria have been associated with cases of shipping fever. *Pasteurella haemolytica*, *Pasteurella multocida*, *Corynebacterium* species, *Mycoplasma* species, and *Chlamydia* species have all been isolated from the lungs of animals with this disease (41, 58, 63, 142). However, *P. haemolytica*, biotype A, serotype 1, is the most common isolate from cattle with severe shipping fever (25, 41); hence the name bovine pneumonic pasteurellosis (42, 142). In calves which are healthy and unstressed, exposure to this organism under natural conditions does not result in colonization. However, any condition which promotes close contact between calves, is stressful to calves, or impairs

the health status of the animals contributes to the spread of *P. haemolytica* A1 and frequently results in the development of pneumonic pasteurellosis. Cause of death in animals which have contracted this disease is most often attributed to infection with *P. haemolytica* A1 (25)

Pasteurella haemolytica - The Organism.

P. haemolytica cells are non-motile Gram-negative pleomorphic rods which are facultatively anaerobic and display slight hemolysis when grown on agar containing 5% sheep blood (5). Fifteen serotypes of this species have been detected by indirect hemagglutination techniques (2). The serotypes have been further subdivided into two biotypes on the basis of colony morphology, carbohydrate fermentation, growth curves, and antibiotic sensitivity (127). Biotype A isolates ferment arabinose, but not trehalose, and grow as small, gray colonies, while biotype T organisms ferment trehalose, but not arabinose, and appear as larger, brown colonies on agar plates (127). Biotype A contains serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, and 14, whereas serotypes 3, 4, 10, and 15 comprise biotype T (2, 5). The most studied organism of this genus is *P. haemolytica* biotype A, serotype 1 because of its crucial role in the development of shipping fever.

P. haemolytica biotype A, serotype 1 possesses many proteins, polysaccharides, and lipids which are considered to be important virulence factors. These include fimbriae (101), a thick polysaccharide capsule (1), lipopolysaccharide (23, 70, 117), a protease (108), a neuraminidase (39), and a potent exotoxin (leukotoxin) which is produced during the log phase of growth (3, 68, 69, 91, 120, 122).

Fimbriae, which are filamentous structures present on the outer surface of the bacterial cell, are considered important virulence factors in many gram-negative bacterial infections (52, 73, 96). They provide bacteria with a mechanism of attachment to

epithelial cells (6, 26, 96), interfering with the flushing and cleansing mechanisms of the host and, thus, provide a survival advantage for these bacteria over nonfimbriated forms (73). *P. haemolytica* produces both large (12 nm wide) rigid fimbriae and smaller (5 nm wide) flexible fimbriae when grown *in vitro* (101). Fimbriae, measuring approximately 10 nm in diameter, have been visualized from *P. haemolytica* cells taken from infected calves (102). These fimbriae may be important in upper respiratory tract colonization, which is the first step in the development of shipping fever (26). However, their role in the establishment of the disease remains speculative.

In Gram-negative, as well as Gram-positive organisms, the presence of a capsule correlates with virulence (27, 40, 45, 92). Normally, the capsule protects organisms from phagocytosis by polymorphonuclear leukocytes and from antibody-mediated opsonization (28, 136). The glycocalyx capsule is responsible for the serological specificity of *P. haemolytica* (1). In addition, the capsule may play a role in protecting the bacteria from complement-mediated bactericidal activity (28). The formation of capsular material in this organism appears to be age-dependent, with the greatest amount found in 2-12 h cultures (27). The capsule may reinforce the adhesion of the cells to the epithelial surface in the upper respiratory tract and may allow the formation of microcolonies which can then be inhaled into the lungs (102). Although the precise role the capsule plays in *P. haemolytica* infections is still relatively unknown, it is known that anti-capsular antibodies are important in host resistance to this bacterium (12, 102)

The lipopolysaccharide (LPS) molecule of *P. haemolytica* A1 is similar to LPS produced by other Gram-negative bacteria and is composed of the lipid A portion, a core oligosaccharide region, and the antigenic polysaccharide chain (O antigen). This molecule makes up 12% to 25% of the dry weight of the *P. haemolytica* cell (70). LPS is important in initiating and enhancing the inflammatory response which occurs following

colonization of the lung by this bacterium (23, 141). It causes many vascular and circulatory changes (63), including alteration of normal leukocyte function (26, 32), alteration of neutrophil and whole peripheral blood leukocyte migration (23, 32), modification of cardiopulmonary hemodynamics, and elevation of circulatory prostanoids, serotonin, cAMP, and cGMP levels (86). Also, LPS has been shown to bind to neutrophils, macrophages, endothelial cells, and the alveolar lining (26). The bovine endothelium, in addition to being a target for LPS binding, is susceptible to LPS toxicity (26). Although antibodies against the LPS molecule have been used to identify serological cross-reactivity in the O antigen regions of *P. haemolytica* serotypes (32), it is questionable whether these antibodies afford a greater degree of resistance to infection with this organism (21).

Two enzymes, a neutral protease (108) and a neuraminidase (39), are also considered to be virulence determinants. Proteases have been implicated as important virulence factors in the pathogenesis of many bacterial diseases. Interestingly, proteases are found in bacteria such as *Legionella pneumophila* and *Pseudomonas aeruginosa* (40, 79) which are associated with bacterial pneumonias and which exhibit cytotoxic activity for alveolar macrophages. Neuraminidase is also produced by many strains of bacteria and is associated with virulence (99, 115, 128). Neuraminidase activity has been measured in *P. haemolytica* and is serotype associated (39). However, determining the role of each enzyme in the pathogenesis of shipping fever requires further study.

Perhaps the most important virulence determinant of *P. haemolytica* A1 is a potent cytotoxin. This cytotoxin is specific for ruminant leukocytes (3, 68, 120). Thus, the name leukotoxin has been applied to this exotoxin. Leukotoxin apparently damages target cells by causing a rapid release of intracellular potassium, which results in cell swelling, and by forming large membrane defects. This mechanism of cell damage is

analogous to that caused by the α -hemolysin of *Escherichia coli* (17). Most likely, the leukotoxin contributes to the initiation of shipping fever by impairing the primary lung defenses of the host (68, 122, 133). In addition, it may enhance the severity of lung damage caused by the bacterium by killing mononuclear leukocytes and pulmonary alveolar macrophages and neutrophils which in turn release proteolytic enzymes and/or activate the alternate complement pathway (15, 68, 69, 91, 120). Studies have shown that the leukotoxin is associated with degenerate cells in the alveolar exudate and with cells in the interalveolar septae of the infected bovine lung (141). Interestingly, studies have shown that depletion of neutrophils by hydroxyurea treatment protects calves from *P. haemolytica* induced lung damage (125). Studies have shown that the production of leukotoxin-neutralizing antibodies correlates with resistance to experimental challenge with *P. haemolytica* (46, 100).

The leukotoxin is present in supernatant fluids during the logarithmic phase of growth of *P. haemolytica* A1 with the highest concentration present 6 h post inoculation (3, 13, 122). This molecule is secreted from actively growing bacteria (122). The leukotoxin determinant is located on the bacterial chromosome, and its expression is modulated at the transcriptional level by temperature, pH, and iron concentration (134). Partial purification of the leukotoxin demonstrates that it is proteinaceous (60). Amylase treatment of supernatant fluid samples containing leukotoxic activity has shown that carbohydrate moieties are present on the molecule (13). The leukotoxin is extremely heat labile; activity is dramatically reduced following incubation of leukotoxic filtrate at 37°C for several hours (3, 13, 60). Therefore, attempts to purify the leukotoxin molecule have largely been unsuccessful. In addition, the leukotoxin is oxygen stable (3, 13), but reports concerning its sensitivity to pH extremes have varied (3, 13, 47).

Molecular weight determinations of the leukotoxin molecule have lacked

consistency. Molecular weights greater than 700 kDa have been reported, but were later concluded to be the result of multiprotein complexes (70). Sequential ultrafiltration membrane studies resulted in an estimated molecular weight of approximately 300 kDa (3). Chromatographic studies determined the molecular weight of the leukotoxin to be 160 kDa (103). Another study which used ultrafiltration and size exclusion chromatography reported the molecular weight to be 150 kDa (60). These discrepancies are most likely due to differences in purification techniques and to aggregation of leukotoxin molecules in solution. Recently, however, the leukotoxin molecule has been cloned and sequenced (81, 82). The cloned leukotoxin molecule is a protein of 101.9 kDa (82), which is readily degraded to proteins of 97 kDa and 66 kDa (14).

In recent years, several investigators have cloned and sequenced the leukotoxin molecule (59, 81, 82). Analysis of the organization of the leukotoxin determinant and the DNA sequence has revealed an extensive homology between the leukotoxin genes and those of the *E. coli* α -hemolysin (82) and various other Gram negative bacteria (74, 139). The determinant of the *E. coli* α -hemolysin includes the genes *hlyC*, *hlyA*, *hlyB*, and *hlyD* (34). Due to the similarities between the genes of the *E. coli* α -hemolysin and those of the *P. haemolytica* leukotoxin, the genes of the leukotoxin have been named *lktC*, *lktA*, *lktB*, and *lktD* in order to conform to existing convention and to avoid confusion (82, 132). It has been postulated that these toxins are members of a family of related virulence factors that are just beginning to be understood. Due to the presence of several repeat domains within the toxin molecules, the name RTX, for repeats in the structural toxin, has been given to this widely disseminated family of toxins (133). Other members of the RTX family include the *Bordetella pertussis* adenylate cyclase toxin, the hemolysins produced by *Actinobacillus pleuropneumoniae*, the *Morganella morganii* hemolysin, and the hemolysins of *Proteus mirabilis* and *P. vulgaris* (83, 132, 139).

LKTC, the gene product of *lktC*, is a protein of 19.8 kDa and is composed of 166 amino acids (82). This protein is 50.3% identical in amino acid sequence to the HLYC protein which consists of 169 amino acids and is 20 kDa in molecular weight (34, 82, 132). In addition, these proteins share homologous regions throughout the length of the protein instead of simply sharing distinct regions of homology (132). It is known that HLYC is not required for the synthesis or export of HLYA, but is responsible for covalent modification of this protein which renders it active (105). A similar relationship exists between the LKTC and LKTA proteins (82). Activation of HLYA is achieved by HLYC-directed fatty acid transfer from a cytosolic acyl carrier protein to inactive HLYA (62). Acylation of one or more amino groups in HLYA explains the increased negative charge of activated HLYA compared with inactive HLYA (105, 137). It has been suggested that this type of acylation is common to all Gram-negative pathogens which secrete membrane targeted toxins (62). If LKTC mediates this type of acylation of LKTA is not certain, but considering the degree of homology between HLYC and LKTC, it is possible that this mechanism also takes place in the activation of LKTA.

In the *E. coli* α -hemolysin system, the HLYB and HLYD proteins are involved in the transport of active hemolysin through the lipid bilayer of the bacterial membrane and into the medium (35, 53, 57, 75, 90). This process is exceptional to the normal process of bacterial transport which involves the protein products of several *sec* genes and which does not occur across the outer membrane of the bacteria (114). In addition, no leader sequence at the N-terminal end of the active hemolysin has been detected, which is also contrary to what is expected for transportable proteins (34, 57). A similar mechanism for transport also exists for the *P. haemolytica* leukotoxin. LKTB and LKTD, the protein products of the *lktB* and *lktD* genes, are proteins of 79.7 kDa and 54.7 kDa, respectively. LKTB and HLYB share 80% homology at the amino acid sequence level (59). A

hydrophobic domain, ranging from amino acids 150 to 450 in HLYB, may play a role in forming transmembrane channels which allow for activated HLYA secretion (50, 59). Due to the similarity of LKTB and HLYB in both amino acid sequence and protein structure, it is probable that LKTB functions similarly in *P. haemolytica*. LKTD shares 60% homology at the amino acid level with HLYD (59). Although no function has yet been determined for HLYD or LKTD, it is known that these proteins are essential for transport of the active toxins into the supernatant fluid (59, 75, 86).

The product of the *lktA* gene, called LKTA, is a 953 amino acid protein that is 101.9 kDa in molecular weight (59, 82). This protein is 36.4% homologous in overall amino acids to HLYA, which is 1023 amino acids long and is 107 kDa (82, 88, 89). Although this homology is relatively low compared to the other proteins of these determinants, LKTA and HLYA are highly homologous over distinct regions of the molecules (132). Several distinct domains are present within the LKTA and HLYA proteins. The extreme C-terminal 53 amino acid residues in HLYA are thought to be involved in BD-mediated secretion. Interestingly, this region is not cleaved during hemolysin export (74). It is presumed that LKTA contains a similar region that enables recognition by the BD-mediated transport system as well.

Tandemly repeating glycine-aspartic acid rich sequences make up another functional domain of these proteins (87, 132). It is due to these repeat sequences that the name RTX has been given to this family of pore-forming toxins. Each repeat is composed of nine amino acids which appear in the sequence Leu-X-Gly-Gly-X-Gly-Asn-Asp-X where X may vary among repeats. Eleven such sequences are found in HLYA, whereas LKTA contains six repeat regions. It has been suggested that a duplication event occurred in the repeat region in HLYA which may account for the fact that almost twice as many repeats are found in HLYA compared with LKTA (132).

Evidence has shown that calcium is instrumental in the binding of HLYA to target membranes (7) and that the repeat regions in the protein are responsible for binding calcium (87). Studies have suggested the similar repeat sequences in LKTA also function as calcium binding sites (8). The repeat sequences have been postulated to allow for calcium to stabilize HLYA or induce a conformational change in HLYA which promotes recognition of a hypothetical receptor on target cells (87). Deletion of a single repeat sequence leads to increased calcium dependence of HLYA (8, 87). Multiple deletions of the repeat sequences eliminate hemolytic activity altogether (8, 36, 86, 87, 132). Studies with the leukotoxin molecule have tentatively shown that a region between amino acids 768-939, which contains at least two repeat sequences, contains an epitope recognized by a leukotoxin-neutralizing mAb. This mAb neutralizes the lytic activity of the leukotoxin by preventing its association with target cell membranes (48, 49). This further strengthens the evidence that the repeat regions in LKTA, and possibly in all the RTX toxins, function in target cell binding.

Both LKTA and HLYA contain a series of hydrophobic domains in the N-terminal third of the protein (83, 86). These hydrophobic domains are thought to be involved in the interaction of the toxin molecule with target cell membranes (83). In the HLYA protein, deletions within these hydrophobic regions resulted in loss of hemolytic activity (86), even at elevated calcium concentrations (87). Studies suggest that the hydrophobic domains of HLYA act by forming a single hydrophilic transmembrane pore with a diameter of approximately 3 nm in target cell membranes (4, 29, 83, 86, 87). The hydrophobic domains in LKTA also function to form pores in target cells. However, the pores induced by LKTA in the membranes of ruminant leukocytes are much smaller, only approaching 0.9 nm in diameter (18). Experiments with LKTA have implied that this protein, and perhaps all RTX toxins, function in two stages, namely cell binding and

pore-formation (29). This implication agrees with quantitative binding studies of HLYA activity which indicated that binding and lysis are temporally separable events (33).

Recent evidence has suggested the existence of a fourth functional domain within LKTA and HLYA. Target cell specificity is determined by the LKTA, not the LKTC, protein (38). Results have suggested that LKTC-dependent modification of LKTA may be required for target cell binding (29). Similar evidence has been presented for HLYA (8). A mAb directed against HLYA has identified the region for HLYC activation to be in the 100 amino acids immediately proximal to the start of the repeat sequences (amino acids 626-726) (8, 110). This mAb recognizes the activated HLYA protein only and neutralizes the lytic activity of the hemolysin against erythrocytes (110). Thus, for the HLYA protein, it is possible that there is an interaction between the HLYC mediated modification in HLYA and the repeat region which allows for productive binding to target membranes (8). It is also possible that this type of interaction occurs in the leukotoxin.

Control of Shipping Fever.

Ideally, protecting cattle from agents typically associated with shipping fever is the way to prevent animals from contracting the disease. However, in situations where cattle are shipped to feedlots from various sources and are combined into large herds, it is almost impossible to prevent cattle from acquiring the viral and bacterial factors which contribute to the development of the disease from infected herd-mates. Therefore, the most effective and practical control for this disease is a prophylactic vaccine (104). An effective vaccine must provide protection of the cattle against all the infectious agents which are involved in the development of shipping fever. Numerous approaches to this problem have been studied.

Viral vaccines have been used to prevent shipping fever. This approach has been

used because a large number of cases of bacterial pneumonias are secondary infections which result after viral infections (80). Variable success has been achieved with this method. According to experimental studies and vaccine field trials, a correlation between high PI-3 virus titers and protection against shipping fever does not exist. In fact, vaccination of cattle for PI-3 virus may enhance susceptibility to the disease (104). Vaccination of animals for IBR virus reduced the incidence of disease development following experimental challenge with IBR and *P. haemolytica* (64, 130, 131). However, these vaccines did not afford complete protection and calves stressed by transport exhibited an even lesser degree of protection (131). Vaccines consisting of multiple viruses have demonstrated some protection in challenge infection studies, but these vaccines have not shown any effect in vaccine field trials (93, 94, 95).

Since *P. haemolytica* A1 is the most common bacterial isolate found in cattle with shipping fever (25, 41), research has focused heavily on the control of this organism. The goals of these studies have been to determine the immune responses required to provide resistance to *P. haemolytica* infection, to investigate potential *P. haemolytica* antigens involved in stimulating immunity, and to develop a vaccine to combat the spread of this organism. Vaccines were developed which contained various combinations of *P. haemolytica* and viral vaccines. In experimental studies, these types of vaccines generally provided protection, but vaccine field trials did not consistently provide protection. Occasionally, such viral and *P. haemolytica* based vaccines yielded increased susceptibility to vaccinated animals (25).

Results from vaccination of cattle with bacterins have been variable and somewhat confusing. It appears that the type of adjuvant incorporated with the bacterin is significant in the generation of a protective response (25, 104). One study indicated that although a bacterin containing aluminum hydroxide gel did induce a serum antibody

response to *P. haemolytica*, it did not induce protection against *P. haemolytica* challenge exposure (20). Several studies reported increased susceptibility of cattle to *P. haemolytica* infection following subcutaneous or aerosol vaccination with formalin killed bacteria (43, 140). This may be due to the formation of opsonizing antibodies to surface antigens of *P. haemolytica* which allow for enhanced phagocytosis of the organism by leukocytes. Cell lysis and lung lesions would result following elaboration of the leukotoxin by phagocytosed bacteria (121, 123). In another study, calves were not protected following BHV-1/*P. haemolytica* challenge when given an intradermal vaccination of formalin killed *P. haemolytica* with aluminum hydroxide gel as the adjuvant. However, resistance was enhanced when calves were given the same vaccine in a mineral oil adjuvant followed by the same challenge (9). Results from a similar study indicated that bacterins containing Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA) enhanced resistance to experimentally induced *P. haemolytica* infection, while bacterins which used aluminum hydroxide gel or trehalose dimycolate did not (24). Overall, however, bacterin vaccines proved ineffective and many of these bacterins are no longer commercially available (58).

Vaccination of animals with live *P. haemolytica* cells has also been used in an attempt to prevent shipping fever. Following administration of this type of vaccine, organisms replicate within the animal and antigens associated with growth are produced. The animal generates a response against the antigens associated with live organisms (123). Vaccination with approximately 10^9 CFU/ml live *P. haemolytica* bacteria yielded increased resistance compared with controls on the basis of severity of lung lesions following transthoracic challenge (20, 22, 24, 109). In addition, other investigators reported increased protection after vaccination with live *P. haemolytica* followed by BHV-1/*P. haemolytica* challenge (9, 11, 77, 126). This enhanced resistance may be due

to the stimulation of antibodies to antigens associated with live, but not killed organisms (109). It may also be due to stimulation of cell-mediated immunity (109). One study, however, presented data which did not agree with the previous findings. The investigators reported that aerosol vaccination with live *P. haemolytica* did not induce protection against BHV-1/*P. haemolytica* challenge (65). Failure of this vaccine to induce immunity may be due to the prophylactic or therapeutic administration of antibiotics throughout the injection period or from improper handling, storage, or administration of the vaccine itself (123). It has been found that cattle vaccinated with logarithmic phase cultures were afforded greater resistance to transthoracic challenge with *P. haemolytica* (19). Both a capsule (27) and the leukotoxin (3) are elaborated in logarithmic phase cultures and antibodies to these antigens may provide enhanced resistance.

Due to the observation that resistance to shipping fever correlated with high serum antibody levels to certain *P. haemolytica* antigens, such as the leukotoxin and capsular antigens (104), another method of protecting cattle from acquiring shipping fever has been developed. In this procedure, cattle are vaccinated with bacterial components or with bacterial culture supernatant fluid. Investigations have demonstrated that high antibody titers against the leukotoxin, induced either by natural infection with *P. haemolytica* or by vaccination with supernatant fluid containing the leukotoxin, are associated with increased resistance to shipping fever (46, 123). Additionally, antibodies against bacterial surface components which are released from actively growing bacteria are important in protection against experimental challenge (24). Most studies utilizing specific antigen extracts in vaccine preparations have met with success (44, 51, 97, 129, 143). The value of vaccines of this nature will grow as a greater understanding of antigens which provide protection become further characterized.

Another alternative which could be used to prevent shipping fever is the use of synthetic vaccines which confer protective immunity in susceptible animals and impart increased safety when compared to live vaccines (30). In particular, anti-idiotypic vaccines may induce resistance to shipping fever. The use of anti-idiotypic antibodies as vaccine candidates is based on the network theory of the immune response put forth by Jerne (66). Jerne postulated that the regulation of the immune response when it encounters antigen is due to a series of idiotypic-anti-idiotypic reactions. It is these immune regulatory networks which control the production of antibodies. Anti-idiotypic antibodies represent viable alternatives for vaccines in numerous circumstances. They are often used when there is difficulty obtaining safe, purified antigen or when a conventional, live, attenuated vaccine has a high propensity for reverting to a virulent form (31, 71).

In this system, anti-idiotypic antibodies (Ab2) may be prepared against a mAb (Ab1) directed against an important epitope in the antigen (106). Three major categories of anti-idiotypic antibodies may result. Ab2 α antibodies are idiotope-induced antibodies which have combining site paratopes directed against the framework idiotopes of the Ab1. Ab2 γ antibodies recognize antigen-inhibitable idiotypes which are near the paratope region of the Ab1. Ab2 β antibodies are paratope-induced antibodies which are steric mimics, or internal images of the epitope (30, 31, 67, 106) and which compete with the antigen for binding to the Ab1 (71). These Ab2 β antibodies, since they bear structural similarity to the antigen, can either prime the immune system of a host against a specific antigenic epitope or stimulate the production of neutralizing antibodies against the antigenic epitope (106).

Anti-idiotypic antibodies have been used to induce immune responses against infectious agents or their products in several studies. The first investigation that utilized

anti-idiotypes produced antibodies against a surface glycoprotein in *Trypanosoma*, the organism which is the causative agent in African sleeping sickness (119). Additional investigations have supported the protective potential of anti-idiotypic vaccines. Ab2 β antibodies against a surface antigen of the hepatitis B virus have been used to protect chimpanzees from challenge with this virus (72). Idiotope vaccines have induced protective immunity against the LPS portion of Gram-negative bacterial cells (135). Rabbit Ab2 β antibodies have been used to protect mice against challenge with tetanus toxin (111, 112). Serum generated by immunization of anti-idiotypic antibodies which resembled diphtheria toxin was able to protect Vero cells from diphtheria toxin mediated cell lysis (118). Finally, Ab2 β antibodies which mimicked an epitope of cholera toxin protected rats against cholera toxin challenge (85). This laboratory has generated a panel of leukotoxin-specific mAbs, one of which is a potent neutralizer of leukotoxic activity (48, 49). Previous investigations in this lab attempted to produce an anti-idiotypic mAb which could induce leukotoxin-neutralizing Ab3 antibodies in recipient animals. Although these experiments were unsuccessful, the possibility that the leukotoxin-neutralizing mAb could be used to generate Ab2 β and, in turn, leukotoxin-neutralizing Ab3 antibodies still exists.

Statement of Purpose.

The purpose of this study has been twofold. The first goal of this study was to identify a small portion of the leukotoxin molecule which could be used in a subunit vaccine against shipping fever. The preliminary location for mAb ltx-2 binding on the leukotoxin molecule has been published (48, 49). However, the methods for determining the location of this epitope were based on negative rather than positive data using deletion proteins. The alkaline phosphatase fusion proteins also used in the

previous study introduced large protein moieties on to the molecule. These large proteins may have interfered with mAb binding to the epitope or they may have caused the epitope to become buried and thus remain inaccessible for mAb binding. The present investigation used chimeric leukotoxin molecules formed between LKTA and APPA to positively localize the region which contained epitope for mAb ltx-2 recognition. Additionally, evidence for the location of the epitopes for the remaining ltx mAbs was obtained.

The second goal of this study was to produce Ab2 β antibodies in rabbits by immunization with mAb ltx-2. These antibodies may have the capability of inducing leukotoxin-neutralizing Ab3 antibodies if injected alone or as a component of a subunit vaccine. Preliminary evidence in this laboratory has shown injection of mAb ltx-2 into rabbits stimulates the production of rabbit anti-mouse antibodies, some of which may be Ab2 β antibodies. The initial experiments were designed to isolate possible Ab2 β antibodies from polyclonal rabbit antiserum. Next, the ability of these antibodies to induce leukotoxin-neutralizing antibodies in mice was examined.

CHAPTER 2

MATERIALS AND METHODS

Bacterium.

Pasteurella haemolytica (isolate 881165, biotype A, serotype 1) was used in this study. Dr. Robert D. Walker (Michigan State University, East Lansing, MI) provided this bacterium which was originally isolated from the pneumonic lung of a calf. The organism was passed two times in calves by intrapulmonic inoculation, then isolated, and stored in skim milk at -70°C. The organism was grown at 37°C on blood agar, which consisted of brain heart infusion agar (BHI, Grand Island Biological Company, GIBCO, Grand Island, NY) and 5% defibrinated sheep blood (138).

Cell Culture.

The BL-3 cell line, a bovine leukemia-derived B lymphocyte cell line, was supplied by Dr. George H. Theilen (University of California, Davis, CA). The cells were maintained in Roswell Park Memorial Institute-1640 Medium (RPMI-1640, Sigma Chemical Co., St. Louis, MO) supplemented with 10% defined Fetal Bovine Serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 1000 U penicillin/1.0 mg streptomycin solution (Sigma) per 500 ml of medium, 40 mM L-glutamine (Sigma), and 0.2% sodium bicarbonate (NaHCO₃, Mallinckrodt Inc., Paris, KY). The cells were grown in 25 cm² tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were split at a 1:5 dilution every five days.

Hybridoma clones ltx-2, ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81, produced by Mark Cameron and Donald Gerbig of this laboratory (48, 49), were grown in Dulbecco's

Modified Eagle's Medium (DMEM, Sigma) supplemented with 20% defined FBS, penicillin, streptomycin, L-glutamine, and NaHCO_3 . The cells were grown in 25 cm² tissue culture flasks and were split at a 1:20 dilution every four days. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. Immunoglobulin G (IgG) subclasses of the various mAbs are shown in Table 1 (48).

Leukotoxin Production.

Supernatant fluid containing leukotoxin was prepared by a modification of the method described by Shewen *et al.* (122). Briefly, ten colonies from an overnight culture of *P. haemolytica* grown on a blood agar plate were inoculated into 500 ml of BHI broth (BBL, Cockeysville, MD) and incubated for 4.5 h at 37°C in a shaking water bath (Orbit Environ-Shaker, Lab-Line Instruments, Inc., Melrose Park, IL). The broth culture was then centrifuged (Beckman JA-21 centrifuge, Beckman Instruments, Inc., Palo Alto, CA) at 10,000 x g for 20 min at 4°C. The supernatant medium was discarded and the pellet was resuspended in buffer comprised of 0.026 M sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, Mallinckrodt, Paris, KY), 2.25 M sodium phosphate, dibasic, heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$, Mallinckrodt), 8.8% sodium chloride (NaCl, Mallinckrodt) (PBS, pH 7.0). The bacteria were again centrifuged for 20 min at 10,000 x g at 4°C. Following centrifugation, the supernatant medium was discarded and the bacterial pellet was resuspended to a spectrophotometric determination of 4×10^8 CFU/ml in RPMI-1640 medium supplemented with 0.2% NaHCO_3 . This concentration was determined using a linear regression line prepared by Donald Gerbig of this laboratory in which an optical density at 600 nm of 1.2 was equal to 4×10^8 CFU/ml. This bacterial suspension was incubated for an additional 1.5 h in a shaking water bath at 37°C. The bacteria were then centrifuged at 10,000 x g for 20 min at 4°C and the supernatant fluid

Table 1. Isotypes of mAbs reactive with *P. haemolytica* 100X concentrated leukotoxic culture filtrates.

mAb	IgG Subclass
ltx-2	IgG ₁
ltx-4	IgG ₁
ltx-35	IgG _{2b}
ltx-37	IgG _{2b}
ltx-58	IgG ₁
ltx-81	IgG ₁

was filtered through a 0.2 μ m filter (Nalge Company, Rochester, NY). Culture filtrates were stored at -70°C. A Pellicon concentrator, equipped with four 30 kDa exclusion membranes (Millipore, Bedford, MA), was used to concentrate the culture filtrates 100-fold. The concentrated filtrates were then aliquoted and stored at -70°C.

Neutral Red Cytotoxicity Assay.

Cytotoxicity of the bacterial culture filtrate was measured by uptake of the vital stain neutral red in a modification of the neutral red cytotoxicity assay described by Greer and Shewen (54). In this assay culture filtrates were adjusted to an appropriate dilution in 100 μ l of RPMI-1640 in a sterile, 96-well round bottom microtiter plate (Corning). BL-3 cells were adjusted to a concentration of 5.7×10^6 cells/ml in serum free RPMI-1640 medium and 35 μ l was added to each appropriate well. The plate was incubated for 1 h. One hundred μ l of warm (37°C) 0.036% neutral red (GIBCO) in 0.85% NaCl was added to each well and the plate was incubated for an additional 20 min. All incubations were in a humidified atmosphere of 5% CO₂ in air at 37°C. The plate was then centrifuged (Omnifuge RT, American Scientific Products, McGaw Park, IL) at 1000 rpm for 9 min at 22°C. Free neutral red in the supernatant fluid was removed and the cells were washed multiple times with a 0.85% NaCl solution. One hundred μ l of lysis buffer consisting of 50% v/v ethanol (Midwest Grain Products Co., Weston, MO) in 0.02 M sodium phosphate, monobasic, anhydrous (NaH₂PO₄, Sigma), was added to each well following the final wash. The contents of the wells were mixed and the plate was frozen at -70°C. The plate was then thawed, centrifuged for 9 min at 1000 rpm at 22°C, and 75 μ l of the supernatant fluid was removed and placed into a 96-well flat bottom microtiter plate (Corning). Absorbance of individual wells was determined with an automated microplate reader (Bio-Tek Instruments Inc., Burlington, VT). Percent cytotoxicity was

determined by comparing the mean optical density at A_{540} in test wells which contained cells and leukotoxin to the mean optical density at A_{540} in control wells which contained cells only (54, 81). One unit (U) of leukotoxic activity was defined as the dilution of concentrated leukotoxin which yielded 50% cytotoxicity of BL-3 cells (48, 49).

The neutralizing capacity of the various mAbs and antiserum samples was determined by a similar assay, with necessary modifications. Briefly, 50 μ l of a predetermined concentration of antibody fluid or a predetermined dilution of antiserum was added to wells of a sterile, 96-well round bottom microtiter plate and two-fold dilutions of the antibody were performed. Fifty μ l of leukotoxin adjusted to the desired number of units was then added to all appropriate wells. All dilutions were performed in RPMI-1640. The plate was incubated at 4°C for 1 h. Next, 35 μ l of BL-3 cells adjusted to 5.7×10^6 cells/ml in RPMI-1640 was added to appropriate wells and the assay was continued as described above.

mAb Preparation.

Leukotoxin mAb IgG was generated by the production of ascitic fluid in mice. Four to six week old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were injected with 0.2 ml of 2,6,10,14-tetramethyldecanoic acid (pristane, Sigma) by the intraperitoneal route (ip). Seven days later mice were again injected with 0.2 ml pristane ip. On day 21 mice were injected ip with 5×10^5 to 5×10^6 hybridoma cells/ml in 0.5 ml PBS (56). Ascitic fluid developed within seven to ten days post injection and was collected by peritoneal puncture while animals were under methoxyflurane (metofane, Pitman-Moore, Inc., Mundelein, IL) anesthesia. One and one half volumes of ascitic fluid was mixed with one volume of SeroClear® Reagent (Calbiochem Corp., La Jolla, CA). The mixture was centrifuged for 10 min at 3000 x g (International Instrument Co.,

Boston, MA). Following centrifugation, the top layer of lipid free ascitic fluid was collected. The pH of the ascites was adjusted to 6.0-7.5 with 0.01 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.15 M NaCl (wash buffer, pH 7.0).

The lipid free ascitic fluid was then applied to a GammaBind® PLUS Prepack column (Genex Corp., Gaithersburg, MD) for purification of IgG. The column was washed with wash buffer until the OD_{280} fell to less than 0.050. The bound IgG was eluted from the column with 0.5 M ammonium acetate ($\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, Mallinckrodt), pH 3.0. The eluted fractions were immediately adjusted to pH 6.5-7.5 with 1M trizma base ($\text{C}_4\text{H}_{11}\text{NO}_3$, Sigma). The IgG was dialyzed against PBS and the protein concentration determined with a Bradford Method Kit containing BioRad Protein Reagent (BioRad Laboratories, Richmond, CA). The IgG was then aliquoted and stored at -70°C until use.

Preparation of mAb ltx-2-HRP.

Horseradish peroxidase (HRP) enzyme was coupled to mAb ltx-2 according to the technical instructions provided with the ActiZyme-HRP Kit (Zymed Laboratories, South San Francisco, CA). Briefly, mAb ltx-2 was adjusted to 2-4 mg/ml in 0.1 M NaHCO_3 , pH 9.5, and then dialyzed extensively against 0.1 M NaHCO_3 . One mg of mAb ltx-2 was then added to 2 mg of activated HRP and the final volume was adjusted to 0.5 ml with 0.1 M NaHCO_3 . The mixture was gently stirred and was then incubated overnight at 4°C . The remaining active sites were blocked by the addition of 0.2 ml of 1 M L-lysine (Sigma). Following a 1 h incubation at room temperature, the conjugate was dialyzed extensively against PBS. Finally, two parts of conjugate stabilizer was added to one part of conjugate. The conjugate was stored at 4°C and was stable for six months.

Immunization of Rabbits.

Three female, New Zealand white rabbits (Hazelton, Denver, PA), designated D44, D46, and D48, were immunized with 1.0 ml of mAb ltx-2 adjusted to 500 µg/ml. The mAb was emulsified in an equal volume of Freund's Complete Adjuvant (FCA, Sigma) and injected subcutaneously (sc) in four sites, one above each limb. Boosting immunizations at four week intervals were identical to the initial immunization except Freund's Incomplete Adjuvant (FIA, Sigma) was used. The rabbits were bled from the central ear artery seven days following each injection. Blood from each of the three rabbits was stored overnight at 4°C and was then centrifuged for 20 min at 3000 x g. The antisera were tested for the presence of anti-idiotypic antibodies against mAb ltx-2 using a variety of assays.

ELISA.

The basic assay used to determine the presence of anti-murine mAb IgG antibodies in the rabbit antisera was a capture ELISA. mAb ltx-2, as well as mAb ltx-35 and mAb ltx-58, was adjusted to 10 µg/ml in PBS and 50 µl was applied to appropriate wells of a 96-well polyvinyl chloride plate (PVC plate, Dynatech Laboratories, Chantilly, VA). The plate was incubated for 2 h at room temperature with shaking. The wells were washed and dilutions of the unfractionated rabbit antisera were performed in the appropriate wells by serially transferring 50 µl. The plate was again incubated for 2 h at room temperature on a shaker. Following 3 washes of the wells, 50 µl of a 1:1500 dilution of goat anti-rabbit HRP (Cappel, Organon Teknika Corp., West Chester, PA) was added to all appropriate wells. The plate was then incubated overnight at room temperature on a shaker. The plate was washed 3 times and 50 µl of ABTS [2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)] and H₂O₂ (Pierce, Rockford, IL) (HRP

substrate) was added to all wells. Following a 30 min incubation, the absorbance at 405 nm was determined. All dilutions and washes were performed with PBS-Tween.

In order to calculate the approximate percentage of mAb ltx-2 idiotopes contained within various bovine antiserum samples, another ELISA determination was performed. The assay was carried out as described above with the exception that the secondary antibody was HRP conjugated rabbit anti-bovine IgG (Zymed) used at a 1:2000 dilution. The mean absorbance at 405 nm was calculated for each dilution of antiserum.

Ammonium Sulfate Precipitation of Rabbit Antisera.

Further determination and use of rabbit anti-mAb ltx-2 antibodies required at least partial purification of IgG antibodies from the antiserum. The antisera was placed in a beaker and mixed while one-half the antisera volume of saturated ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$, Sigma) was added. The solution was then incubated overnight at 4°C with stirring. The solution was centrifuged for 30 min at 3000 x g and the supernatant fluid was transferred to another beaker. Again, one-half the original antisera volume of saturated ammonium sulfate was added with stirring. The solution was incubated overnight at 4°C with stirring. The antisera was centrifuged for 30 min at 3000 x g. The supernatant fluid was discarded and the pellet was resuspended in 0.3-0.5 the original antisera volume PBS. The resuspended pellet was dialyzed extensively against PBS and then frozen at -20°C until run over affinity columns (56).

Preparation of Hydrazide AvidGel™ Ax Column.

The purification of rabbit antisera necessitated the generation of several immunoaffinity columns. Hydrazide AvidGel Ax columns were prepared according to the technical instructions provided with the Hydrazide AvidGel Ax gel (BioProbe

International, Inc., Tustin, CA) Briefly, purified IgG from mAbs ltx-4 (6.6 mg), ltx-35 (6.64 mg), ltx-37 (1.45 mg), ltx-58 (6.64 mg), and ltx-81 (9.6 mg) was pooled and dialyzed extensively against 0.05 M sodium acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, Fisher Scientific Co., Fairlawn, NJ), pH 5.0 (coupling buffer). The IgG was then oxidized by adding 90 μl of 0.1 M sodium periodate (NaIO_4 , Sigma) to 9.0 ml of antibody. The reaction vessel was mixed, wrapped in foil, and then incubated for 2-4 h at 4°C. Unreacted NaIO_4 was removed by dialyzing the reaction mixture against coupling buffer. The gel slurry was washed three times with coupling buffer and the supernatant fluid was decanted. One to two volumes of the oxidized IgG solution was added to each volume of gel and the mixture was tumbled gently for 8-16 h at 4°C. The gel was then washed three times with coupling buffer, three times with PBS, pH 7.4 with 0.5 M NaCl (wash buffer), and three times with PBS, pH 7.4 with 0.02% sodium azide (Sigma) (storage buffer). The IgG coupling efficiency was determined as the ratio of mg IgG coupled to mg IgG applied based on the protein determination method supplied in the instructions from BioProbe International.

Three other columns were prepared in a manner identical to that previously described with the exception that mAb ltx-2, rabbit Ab2 β antibodies, or nonspecific, purified rabbit IgG, each, was the only antibody coupled to the gel.

Purification of Rabbit Polyclonal Antisera.

Both negative depletion and positive purification techniques were used to obtain rabbit antibodies specific for mAb ltx-2 idiotopes. Ammonium sulfate purified rabbit IgG in PBS was passed exhaustively over the affinity column containing mAbs ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81. The column was initially washed with approximately 50 ml of PBS at a flow rate of 2.0 ml/min and concentrated antibodies were applied to the column.

Approximately 10.0 ml of antisera with a protein concentration of 5 mg/ml was mixed into the gel matrix and was allowed to remain on the column for 1 h. The antibodies were then allowed to flow through the column and the flowthrough was collected. The column was then washed with PBS containing 0.05% polyethylene glycol (PEG, Sigma) until the absorbance at 280 nm fell to less than 0.05. The antibodies remaining on the column following this wash were eluted with 0.1 M glycine (pH 2.5, BioRad). One ml eluates were collected in 10 x 75 mm glass tubes (Fisher) and were immediately neutralized to a pH of approximately 7.0 with 50 μ l of 1M trizma base (pH 9.5). Following elution of the antibodies from the column, the column was washed with approximately 100 ml of PBS and then with 50 ml of PBS with 0.02% azide. The column was stored at 4°C in PBS with 0.02% azide. The flowthrough was concentrated to approximately 10 ml and this process was continued until the absorbance of the eluates was less than 0.2.

The flowthrough was concentrated and then passed over the column containing mAb ltx-2. This process was performed exactly as described previously. The eluates from this column were dialyzed extensively against PBS and then frozen until future use.

With regard to the use of the other columns, procedures followed were identical to those described above. For each sample, a negative depletion of antibodies was performed with the nonspecific, purified rabbit IgG column, followed by isolation of desired antibodies with the Ab2 β column.

Anti-Idiotypic Antibody Neutral Red Cytotoxicity Assay.

The neutral red cytotoxicity assay was modified slightly in order to demonstrate the ability of eluate 1 rabbit antibodies obtained from the ltx-2 column to neutralize mAb ltx-2 protection of BL-3 cells. One hundred μ l of eluate 1 antibody containing fluids was

added to appropriate Eppendorf tubes (Brinkman Instruments, Co., Westbury, NY) and two-fold dilutions were performed. One hundred μ l of a predetermined concentration of mAb ltx-2 was then added to each appropriate tube. All dilutions were performed in RPMI-1640. The tubes were mixed and incubated overnight at 4°C with shaking (S/P Rotator V, Baxter Diagnostics, Inc., Scientific Products Division, McGaw Park, IL). Fifty μ l of the contents of each tube was added to the wells of a sterile, 96-well round bottom microtiter plate. Fifty μ l of leukotoxin diluted to 3 U in RPMI-1640 was then added to appropriate wells. The plate was incubated for 1 h at 4°C and 35 μ l of BL-3 cells at 5.7×10^6 cells/ml in RPMI-1640 was added to the wells and the assay was performed as previously described.

The ability of eluate 1 antibodies to prevent leukotoxin lysis of BL-3 cells was also determined by a similar assay. Fifty μ l of various concentrations of eluate 1 antibodies were preincubated with 35 μ l of BL-3 cells adjusted to 5.7×10^6 cells/ml in RPMI-1640 in the wells of a sterile, 96-well round bottom microtiter plate. The plate was incubated at 4°C for 1 h. A visual examination of the cells was performed in order to determine if clumping of the cells had occurred. Fifty μ l of leukotoxin adjusted to 1 U was then added to all appropriate wells and the plate was incubated for 1 h at 37°C. Neutral red was added to the plate and the assay was continued as previously described.

Anti-Idiotypic Antibody ELISA.

The ELISA capture assay described previously was used with the fractionated rabbit antibodies obtained from the affinity columns to determine if rabbit anti-mouse IgG antibodies had been removed from the antiserum samples and to determine the specific binding of the samples.

A modification of the above capture assay was performed in order to determine if

antiserum samples could prevent mAb ltx-2-HRP from binding to the leukotoxin. This assay was performed with unfractionated and fractionated rabbit antiserum. mAb ltx-35 was used as the capture antibody. This mAb was adjusted to 20 µg/ml in PBS and 50 µl was added to the wells of a PVC plate. Following an incubation at room temperature for 2 h with shaking, the wells were washed 3 times. Residual protein binding sites were blocked by adding 100 µl of 3% bovine serum albumin (BSA, Sigma) in PBS to each well. The plate was incubated overnight at room temperature with shaking. One hundred µl of antisera was added to Eppendorf tubes and serial dilutions were performed. One hundred µl of a 1:3000 dilution of mAb ltx-2-HRP was then added to appropriate tubes. The tubes were mixed and incubated overnight at 4°C with shaking. The wells of the PVC plate were then washed 3 times and 50 µl of leukotoxin adjusted to 5 µg/ml was added to appropriate wells. The plate was incubated for 2 h at room temperature on a rotator. The plate was washed 3 times and fifty µl of the contents of each Eppendorf tube was then added to appropriate wells of the plate. The plate was incubated for 2 h at room temperature on a rotator. The plate was washed 3 times and 50 µl of HRP substrate was added to all wells. Following a 30 min incubation, the absorbance at 405 nm was determined. All dilutions and washes were performed with PBS-Tween. Percent inhibition was calculated by comparing the mean optical density in test wells containing unfractionated or fractionated antisera to the mean optical density in control wells containing mAb ltx-2-HRP alone.

Immunization of Mice.

Immunizations of mice were performed to determine if mice could produce leukotoxin-neutralizing Ab3 antibodies and to determine conclusively if the rabbit eluate 1 IgG contained Ab2β antibodies. Female, four to six week old BALB/c mice (Harlan

Sprague Dawley, Indianapolis, IN) were immunized with 50 µg or 25 µg of eluate 1 IgG obtained from the ltx-2 column. A group of mice were also immunized with 50 µg rabbit IgG as a control. Immunoglobulin was emulsified in FCA and injected by the ip route in 0.2 ml. Two weeks later, mice were immunized with 50 µg or 25 µg of eluate 1 IgG or 50 µg rabbit IgG emulsified in FIA. The injection volume remained 0.2 ml. Seven days later, mice were bled from the retroorbital plexus while mice were under metofane anesthesia. The sera from each group was pooled. Immunization of eluate 1 IgG or rabbit IgG in PBS then continued at biweekly intervals. Mice were bled seven days after each injection. On day 63, mice were sacrificed and the serum from each mouse was collected and frozen at -20°C. Sera from each group was not pooled prior to being frozen. The serum samples were assayed for leukotoxin-neutralizing activity and for anti-leukotoxin titers by ELISA as described previously.

Glutaraldehyde Fixation of BL-3 Cells and ELISA.

BL-3 cells were affixed to the wells of a 96-well Immulon II microtiter plate (Dynatech) for use in ELISA determinations. Briefly, poly-L-lysine (Sigma) was adjusted to 1 mg/ml in PBS and 50 µl was added to all appropriate wells. The plate was incubated 30 min at room temperature. Excess poly-L-lysine was removed from the wells and 50 µl of BL-3 cells, resuspended to 2×10^6 cells/ml in RPMI-1640 was added to all wells. The plate was centrifuged at 2500 rpm for 5 min at room temperature. Fifty µl of 0.5% glutaraldehyde (Sigma) in PBS was then added to each well. Incubation of the plate for 15 min at room temperature followed. The plate was then washed twice with PBS and 200 µl of 100 mM glycine in 0.1% BSA in PBS was added to all wells. The plate was incubated at room temperature for 30 min. The wells were again washed twice with PBS and 100 µl of 1% BSA in PBS was added to all wells. The plate was

incubated for 1 h at room temperature. Following this incubation, the contents of the wells were dumped and 150 µl of 1% BSA in PBS was added to each well and the plates were then frozen at -20°C until future use.

When an ELISA determination was performed, each plate was thawed as needed and the wells were washed three times and fifty µl of serial two-fold dilutions of antibody was added to appropriate wells. The plate was incubated for 2 h at room temperature on a rotator. The wells were washed three times and the desired dilution of goat anti-rabbit HRP was added to each well. The plate was incubated overnight at room temperature on a rotator. The wells were again washed and 50 µl of HRP substrate was added to all wells. Incubation of the plate for 30 min followed. Forty µl of supernatant fluid was removed and placed into a clean, nonsterile, flat-bottom, 96-well microtiter plate. The absorbance at 403 nm was determined. All dilutions and washes were performed using PBS-0.05% Tween.

Plasmid Manipulations.

All TCA precipitated protein samples were the gift of Dr. Douglas K. Struck (Texas A & M University, College Station, TX). Plasmids containing the *lktA*, *lktC* plus *lktA* genes, *AppA*, or *AppC* plus *AppA* genes were derived from plasmid pYFC19 and pYFC37 (14, 16, 29) in which the genes are expressed under *lacPO* control. Methods for constructions of these plasmids have been reported in other investigations (29). Inactive LKTA and APPA (LKTA and APPA) and LKTC gene activated LKTA and APPA (*LKTA and *APPA) molecules were prepared by growing *E. coli* TB1, which harbored the various plasmids, in BHI broth to an OD₅₅₀ of >2.0. Six ml of late logarithmic phase cultures were washed and precipitated with 10% trichloroacetic acid (TCA) (29). The samples were resuspended in 500 µl 2X sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Aliquots of the samples were boiled for five minutes prior to separation by SDS-PAGE.

Additionally, plasmids were constructed which contained chimeric toxin molecules composed of *P. haemolytica* A1 and *A. pleuropneumoniae* serotype 5 (98). The plasmids were derived from pYFC19 and pYFC37 (14, 16). The leukotoxin molecule was divided into three domains based on restriction endonuclease cuts. Domain 1 extended from amino acids 1-492, domain 2 contained amino acids 493-767, while domain 3 ranged from amino acids 768-939. These domains were fused with domains from similar regions in the toxin molecule from *A. pleuropneumoniae*. Domains were designated L if the domain was obtained from *P. haemolytica* and A if the domain was obtained from *A. pleuropneumoniae*. The plasmids generated were as follows: pYFC19, LLL; pYFC37, AAA; pDS10, LLA; pDM32, LAA; pDM15, AAL, pDM28, ALL; pDM30, LAL; and pDM34, ALA (Figure 1). Procedures performed with these chimeric toxin plasmids were identical to those performed with unactivated and activated toxins.

Western Immunoblot.

SDS-PAGE was performed using a Mini Protein II Gel Apparatus (BioRad) according to the method described by Laemmli (78). Stacking and separating gels of 4% and 8% (acrylamide/bisacrylamide 30:0.8, National Diagnostics, Atlanta, GA), respectively, were used to separate the TCA precipitated constructs and chimeric proteins. Twenty five μ l of 10% TCA precipitate samples were electrophoresed for 150 min with constant amperage of 14 mA.

The proteins were transferred for 90 min with a constant amperage of 175 mA to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) using a semi-dry

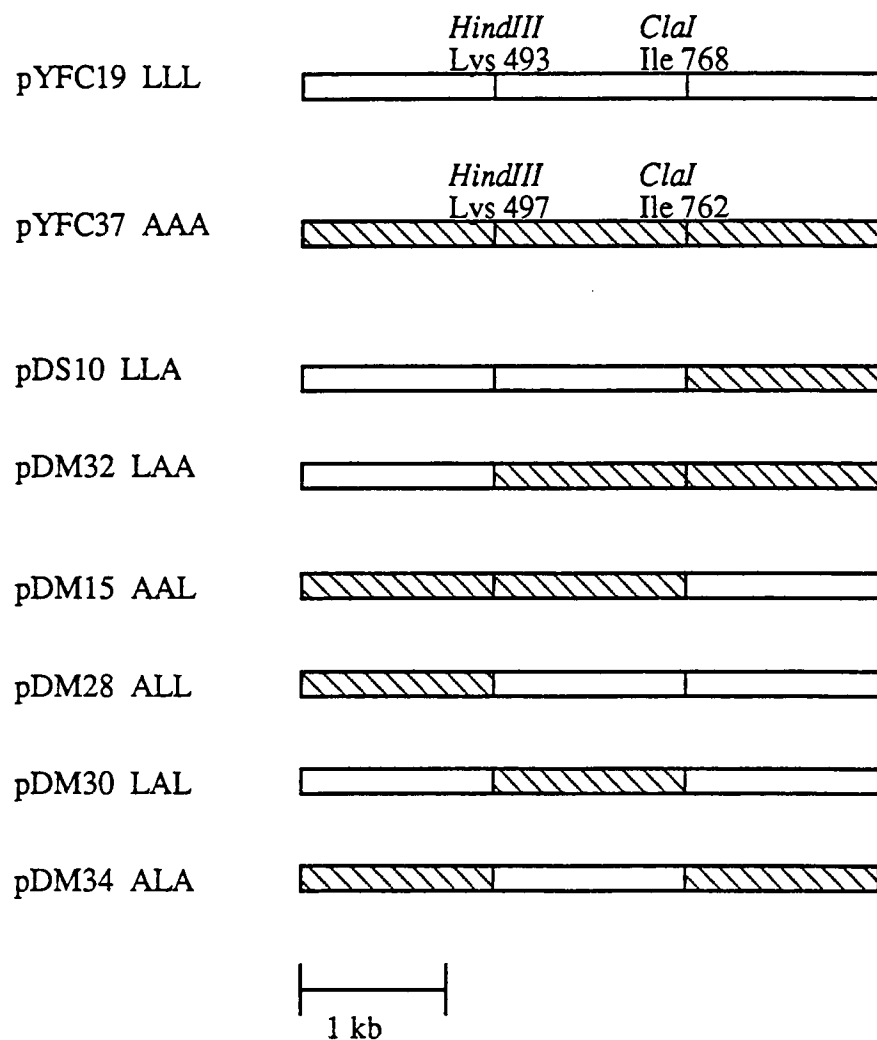


Figure 1. Diagram of DNA encoding chimeric toxin molecules. The open boxes represent sequences derived from the *P. haemolytica* A1 leukotoxin. The crosshatched boxes represent sequences from the *A. pleuropneumoniae* serotype 5 toxin. The parental toxins are represented by the plasmids pYFC19 (LLL) and pYFC37 (AAA).

protein apparatus (Gelman, Ann Arbor, MI). Transfer was accomplished using protein transfer buffer which consisted of 0.025 M trizma base (pH 8.2) containing 0.19 M glycine and 0.1 M SDS (BioRad). Residual protein binding sites were blocked by incubating the membranes for 30 min at 37°C on a rotator with trizma buffered saline (TBS) plus 3% Nonfat Dry Milk (Carnation Co., Los Angeles, CA). Membranes were rinsed with deionized water, followed by 10 min washes with TBS with 0.05% Tween 20 (TBS-Tween). Primary antibody was diluted to 40 µg/ml in TBS-Tween and incubated with the membrane strips overnight at room temperature on a rotator. If antiserum was used as the primary antibody, a 1:50 dilution was made in TBS-Tween and incubation then followed as previously described. The membranes were then washed with deionized water and TBS-Tween as previously described. HRP recombinant Protein G (Zymed) was diluted to an appropriate dilution in TBS-Tween and incubated with the membranes at 37°C for 90 min with shaking in order to detect bound primary antibody. When rabbit antiserum was the primary antibody, detection of bound antibody was made with goat anti-rabbit HRP. Following this incubation, membranes were washed as previously described and were then developed with 4-chloro-1 naphthol and 30% H₂O₂ (Sigma). Development was terminated by washing the membranes with several changes of deionized water.

The india ink staining methods of Hancock and Tsang (55) was used to visualize protein bands transferred to the PVDF membranes. Briefly, membrane strips were rinsed with deionized water, followed by several 10 min washes with PBS-0.3% Tween 20. India ink (Pelikan AG, Hannover, Germany), diluted to 1 µl/ml in PBS-0.3% Tween 20, was added to the membrane strips. Development was terminated by washing the membrane strips with several changes of deionized water.

Bovine Serum Samples.

Bovine serum samples used in this study were provided by Dr. Philip N. Bochslar (The University of Tennessee, Knoxville, TN) and by Dr. Douglas K. Struck. The samples were assayed by the neutral red cytotoxicity assay and the ELISA determinations as previously described using characterized FBS (cFBS, Hyclone) as a control.

CHAPTER 3

RESULTS

mAb Characterization.

Mark Cameron and Donald Gerbig of this laboratory derived six monoclonal antibodies against the 102 kDa leukotoxin of *P. haemolytica*. The mAbs were named as follows: ltx-2, ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81. Several studies have been performed with these antibodies and a preliminary localization of the leukotoxin epitope for these mAbs has been published (48, 49). Mapping with deletion mutant proteins provisionally positioned all of the epitopes in the carboxyl end of the protein between amino acids 768-939. The original classification of the mAbs as protective or nonprotective was based on results obtained with hybridoma supernatant fluid in the neutral red cytotoxicity assay. When purified IgG from the mAbs was tested in this assay, it was found that mAb ltx-4, ltx-35, and ltx-37, as well as mAb ltx-2, have leukotoxin-neutralizing capabilities as shown in Figure 2. Neutralization titers are displayed in Table 2.

Several mAbs reactive with the *E. coli* α -hemolysin have also been derived (110). Pellet et. al. (110) developed an interesting mAb which recognized only the activated form of the *E. coli* α -hemolysin. This type of restriction has not been studied with the leukotoxin mAbs. Therefore, the ability of the leukotoxin mAbs to recognize unactivated and activated LKTA and APPA proteins was investigated. Dr. Douglas K. Struck created individual plasmids containing the *lktC*, *appC*, *lktA*, and *appA* genes. The plasmids containing the *lktA* or *appA* genes were expressed in the *E. coli* TB1 host either alone or with the plasmids containing the corresponding *lktC* or *appC* genes. Plasmids

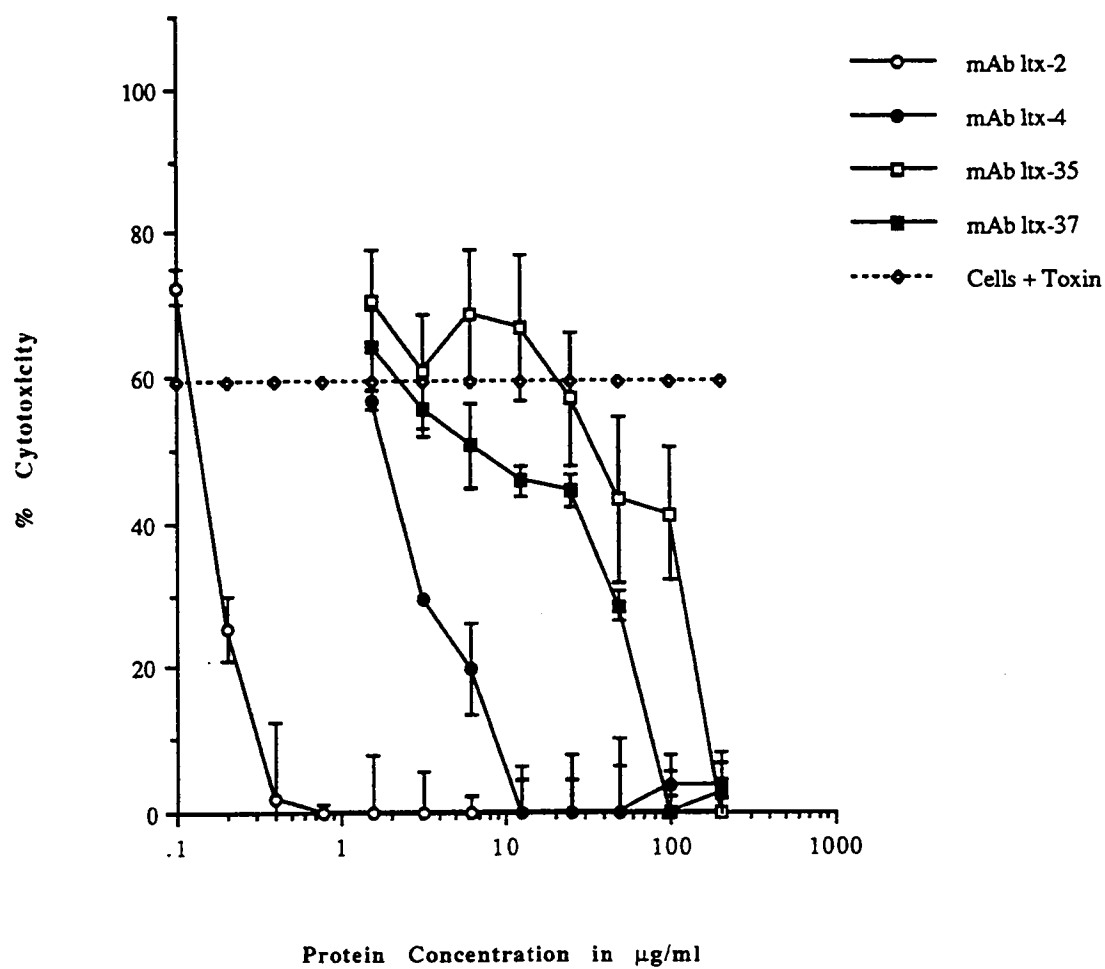


Figure 2. Neutralization of leukotoxin-mediated cell lysis by leukotoxin mAbs as measured by the neutral red cytotoxicity assay.

Table 2. Neutralization titers for leukotoxin mAbs.

mAb	Neutralization Titer ($\mu\text{g/ml}$) ^a
ltx-2	0.2
ltx-4	2
ltx-35	120
ltx-37	50
ltx-58	>200
ltx-81	>200

^aTiter was equivalent to the protein concentration at which 50% cytotoxicity was observed with 1 U leukotoxin.

carrying only the *lktA* and *appA* genes produced unactivated toxin molecules designated LKTA or APPA. Plasmids possessing the *lktA* and *lktC* or *appA* and *appC* genes produced activated toxin proteins designated *LKTA or *APPA. The *E. coli* cells were grown and intact cells were precipitated with 10% TCA. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with each leukotoxin mAb, followed by detection of mAb binding with protein G-HRP.

Each mAb, ltx-2 (Figure 3), ltx-4 (Figure 4), ltx-35 (Figure 5), ltx-37 (Figure 6), ltx-58 (Figure 7), and ltx-81 (Figure 8), reacted with a band of approximately 102 kDa in both unactivated and activated leukotoxin samples. The mAbs demonstrated no reactivity with the *E. coli* TB1 host alone. The lanes which received protein G-HRP alone also showed no reactivity. India ink stains of the unactivated and activated leukotoxin samples showed a smear of protein bands ranging from <30 kDa to >200 kDa, which would be expected in a sample of intact TCA precipitated *E. coli* cells. The immunoblot performed with mAb ltx-35 (Figure 5) did not contain an activated leukotoxin lane probed with protein G-HRP alone. A mistake was made during the development of this lane and the lane was not included in the photograph. Based on the results from identical lanes developed with protein G-HRP alone, it can be assumed that the results of this lane would be negative.

None of the mAbs tested reacted with either the unactivated or activated form of the *A. pleuropneumoniae* serotype 5 toxin (Figures 9-14). Rabbit polyclonal antiserum prepared against the 102 kDa leukotoxin protein reacted with both forms of the *A. pleuropneumoniae* toxin (data not shown) as well as with both forms of the *P. haemolytica* leukotoxin. This indicated that although none of the mAbs tested recognized the *A. pleuropneumoniae* toxin, this toxin was detectable in our immunoblot protocol.

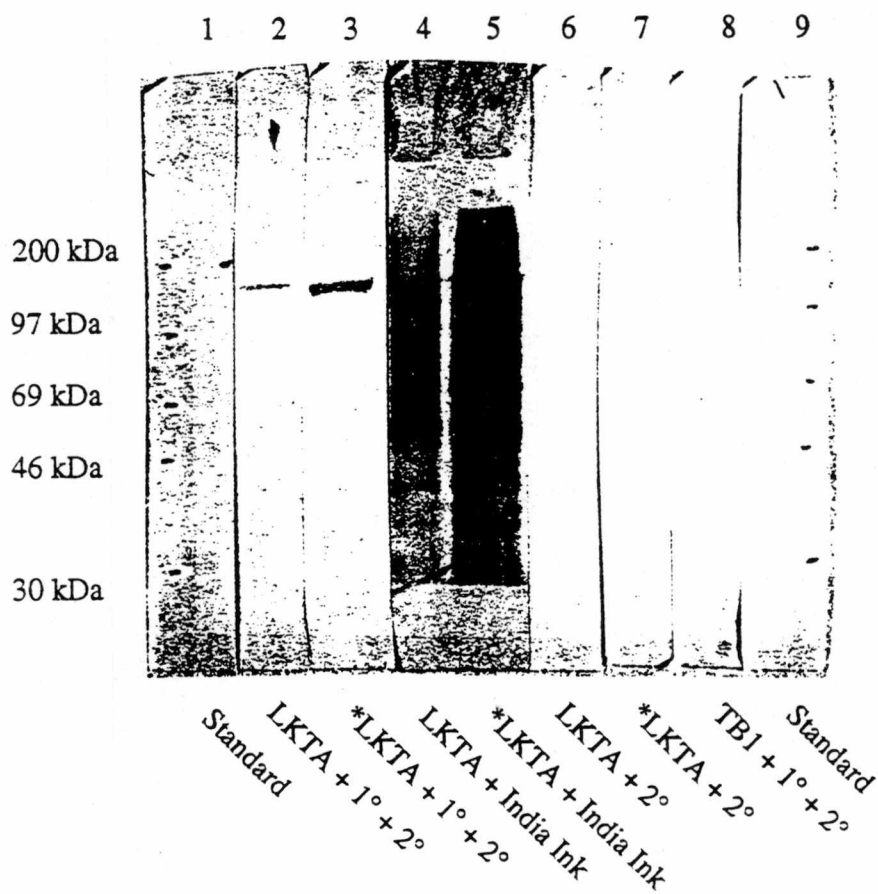


Figure 3. Immunoblot of mAb ltx-2 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

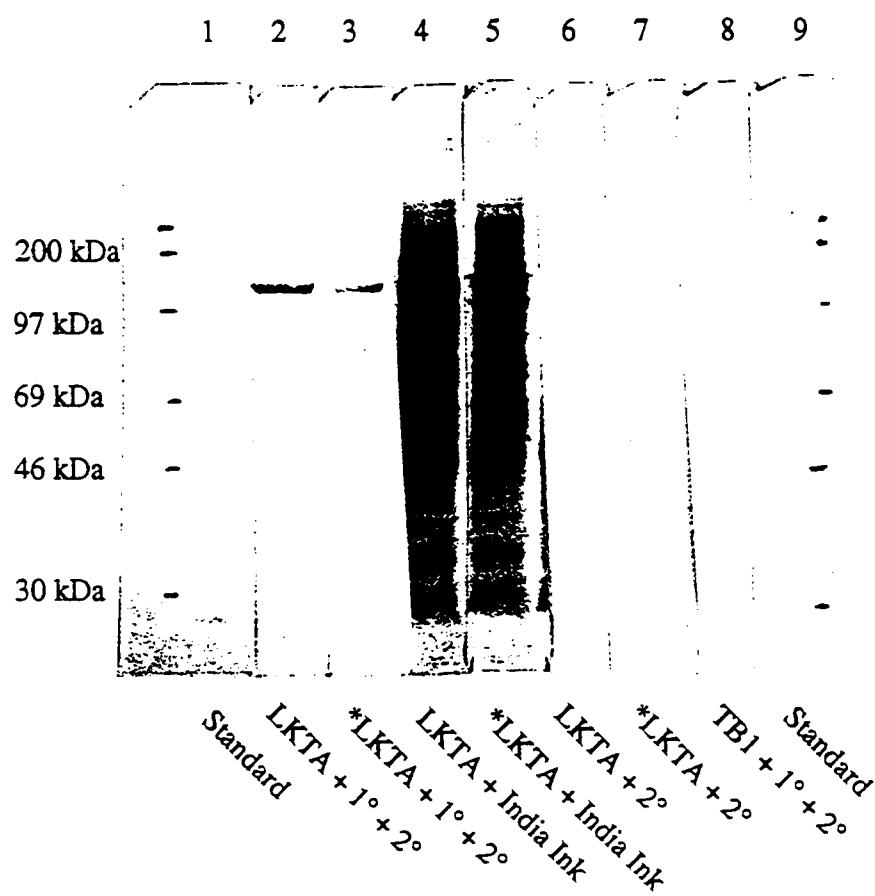


Figure 4. Immunoblot of mAb ltx-4 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

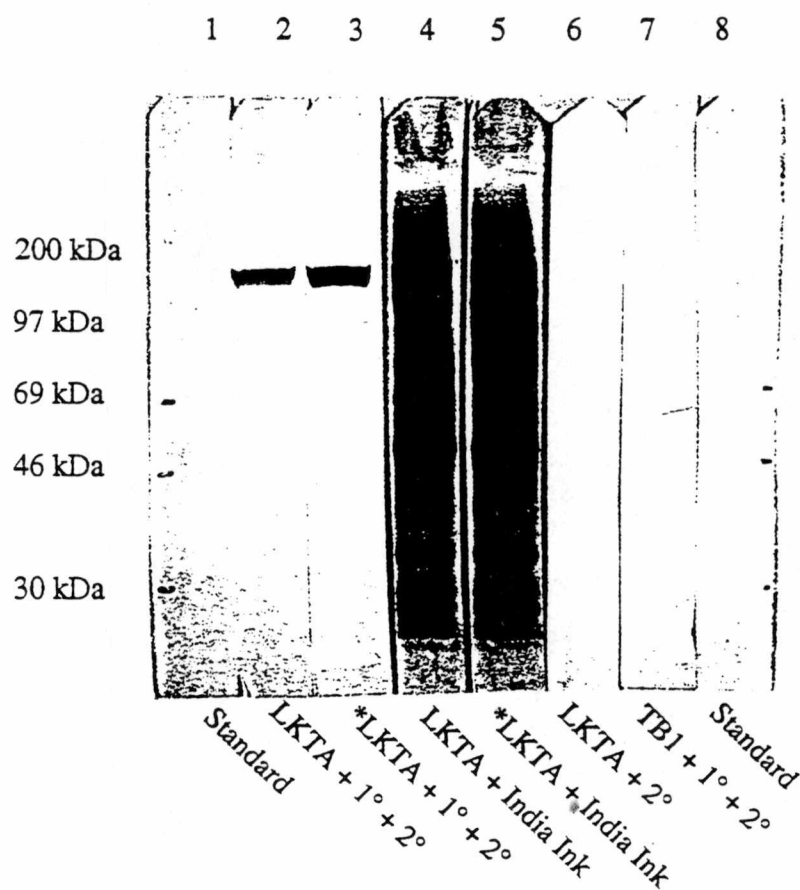


Figure 5. Immunoblot of mAb ltx-35 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

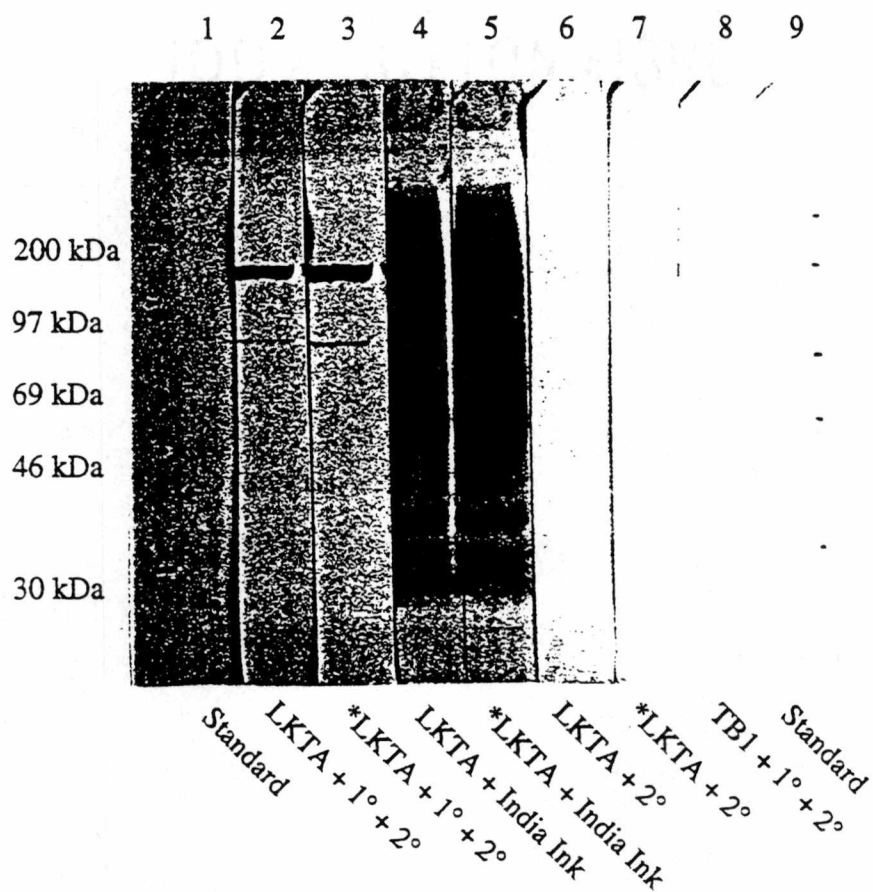


Figure 6. Immunoblot of mAb ltx-37 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

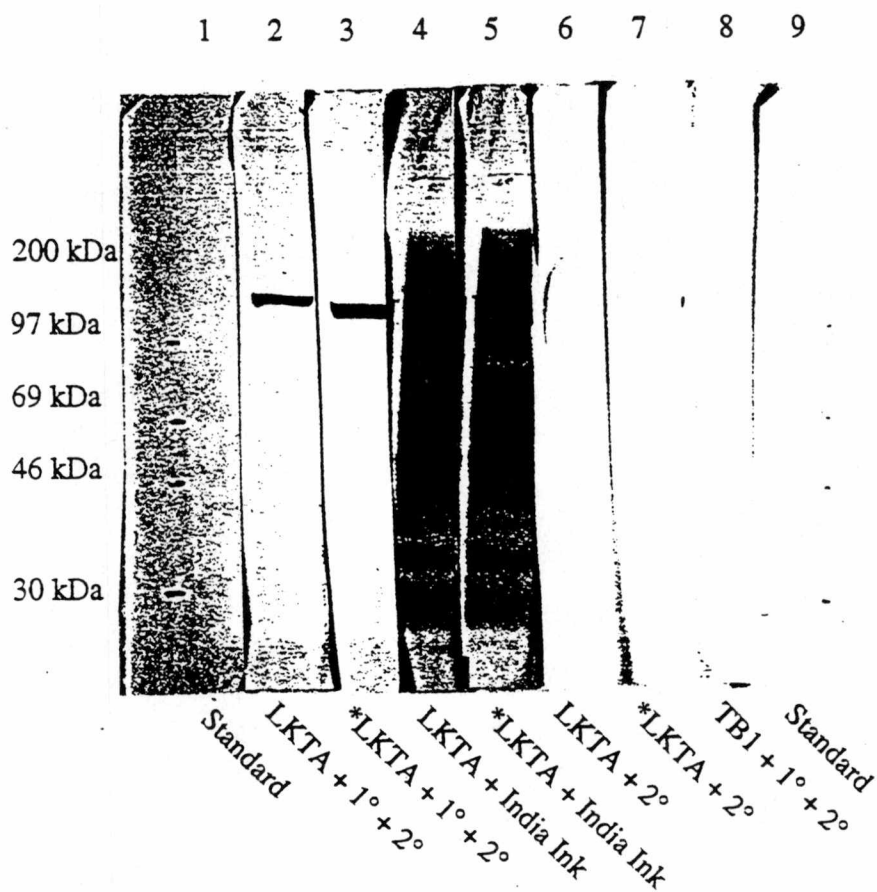


Figure 7. Immunoblot of mAb ltx-58 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

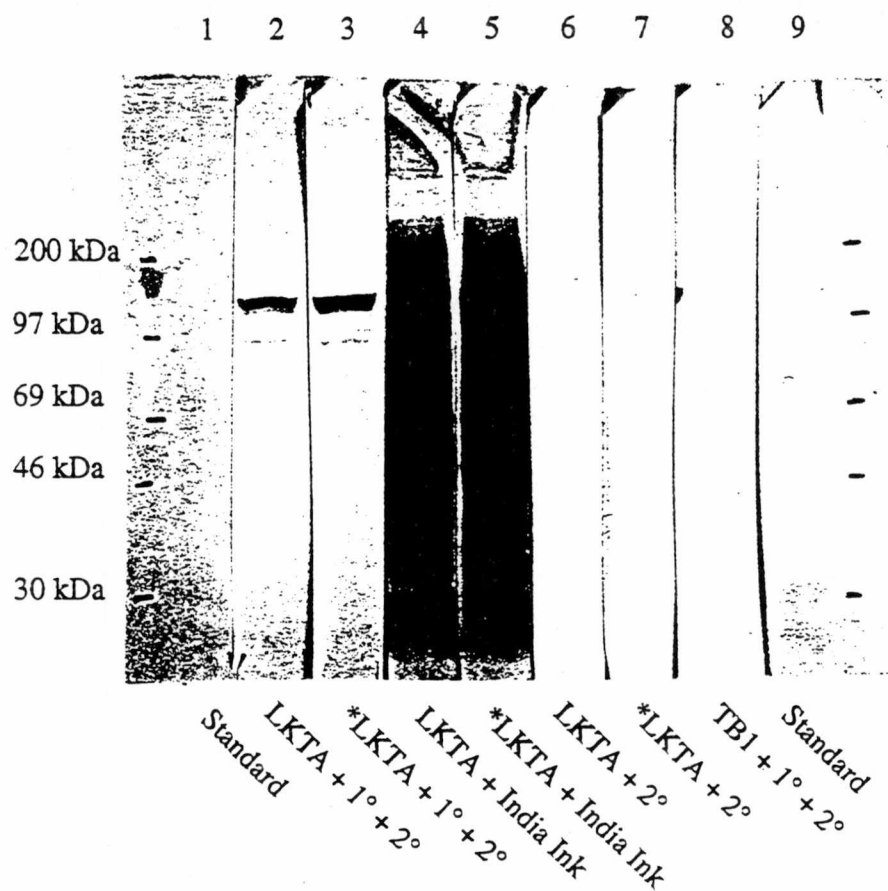


Figure 8. Immunoblot of mAb ltx-81 reacted with SDS-PAGE of unactivated and activated LKTA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

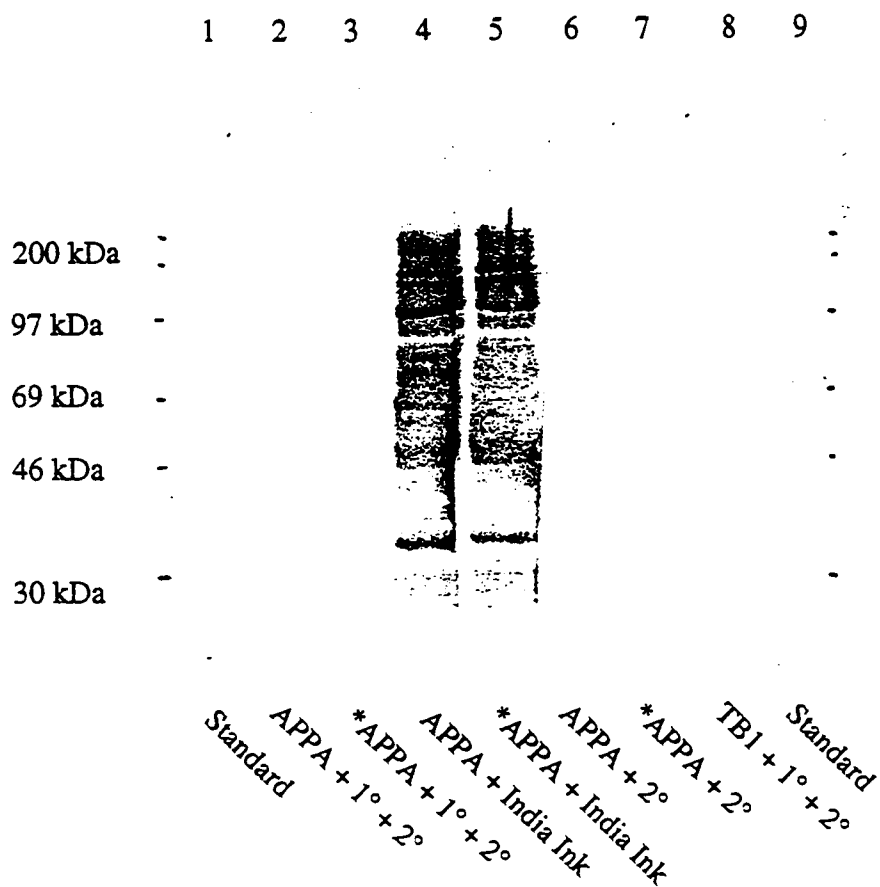


Figure 9. Immunoblot of mAb ltx-2 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

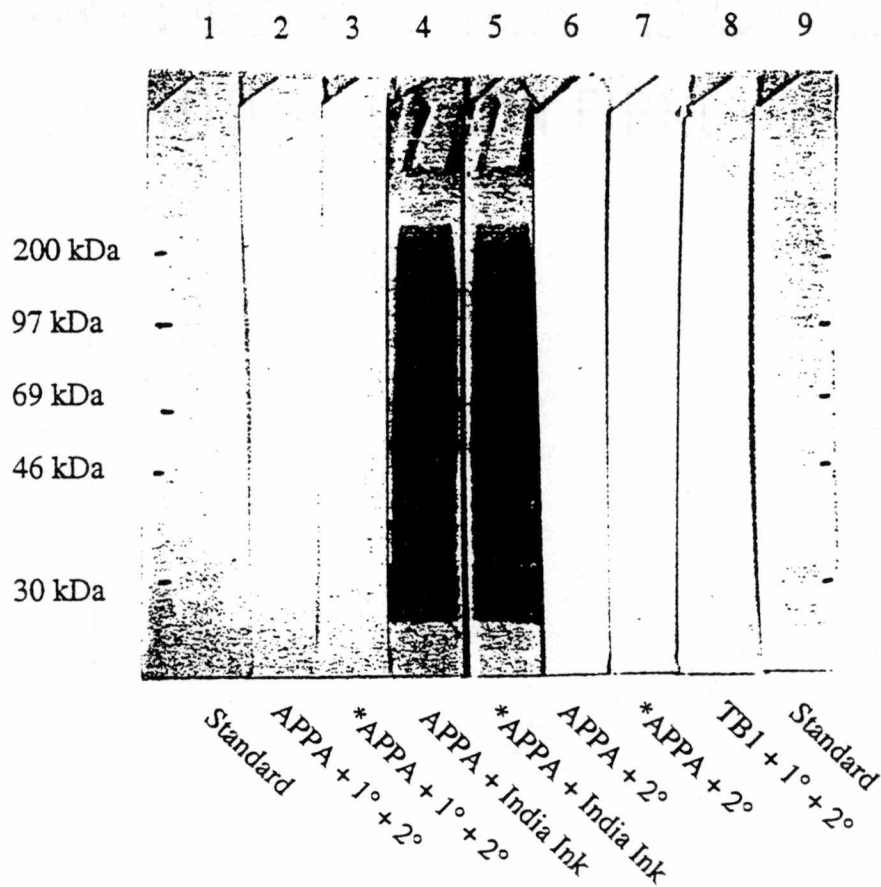


Figure 10. Immunoblot of mAb ltx-4 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

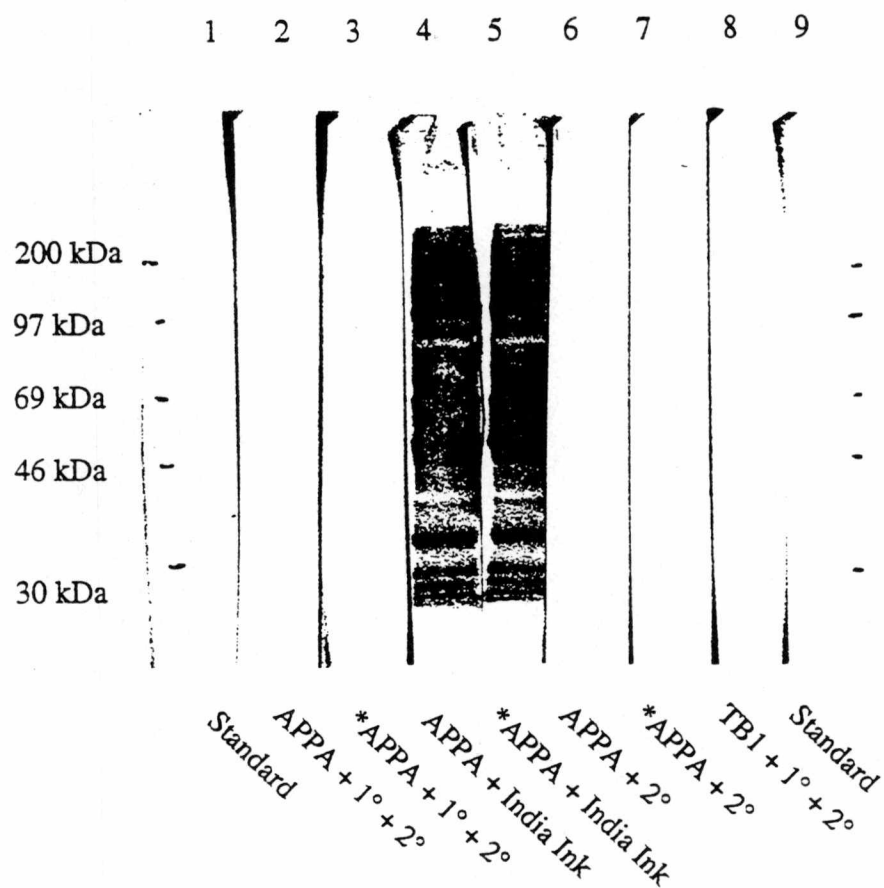


Figure 11. Immunoblot of mAb ltx-35 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

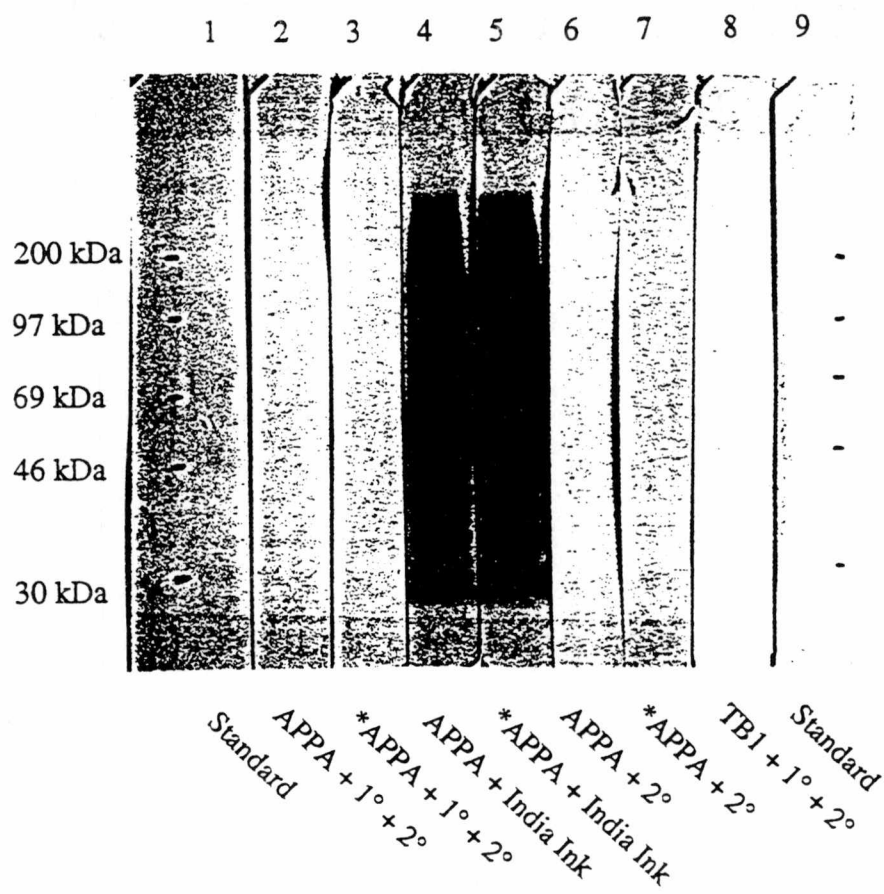


Figure 12. Immunoblot of mAb ltx-37 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

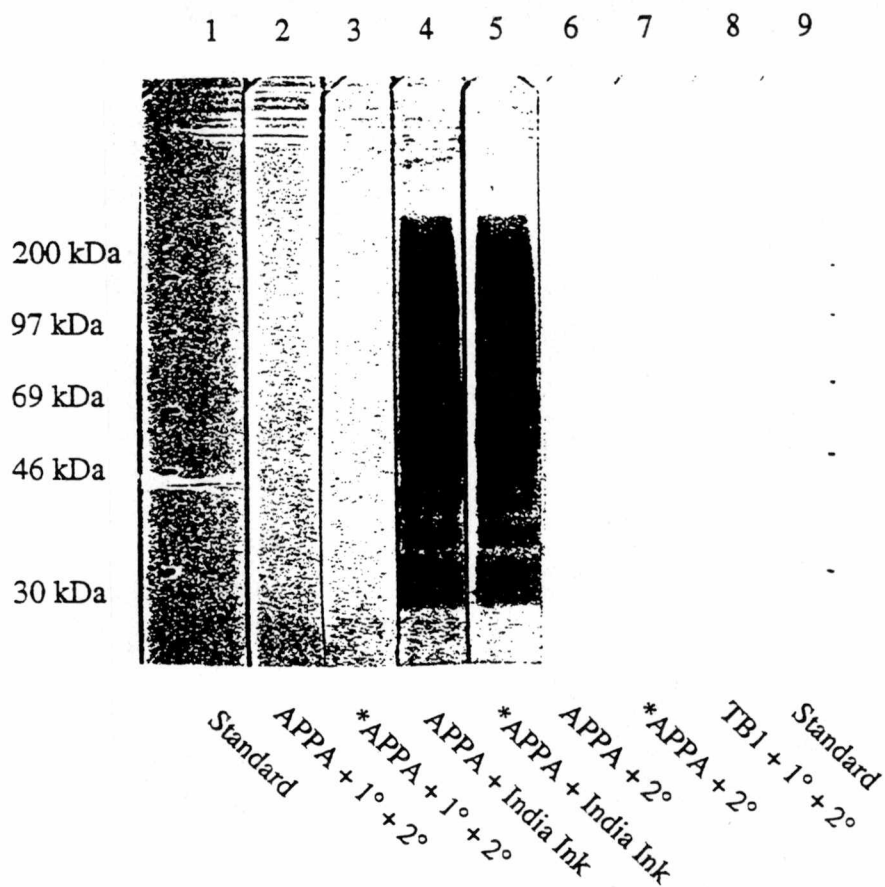


Figure 13. Immunoblot of mAb ltx-58 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

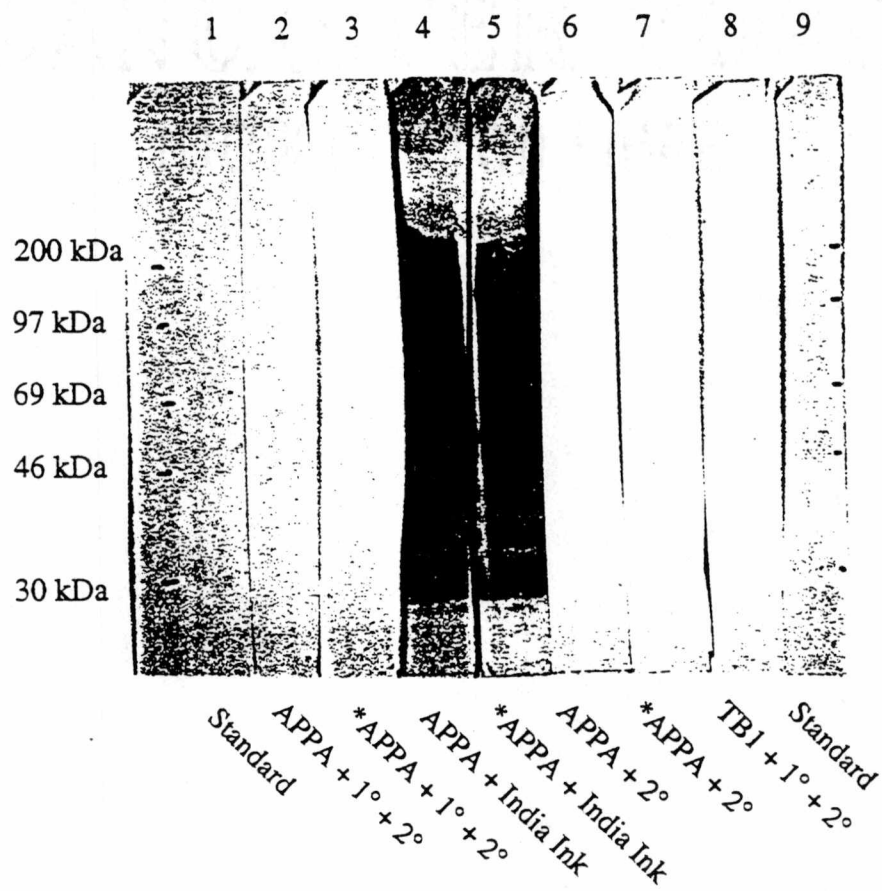


Figure 14. Immunoblot of mAb ltx-81 reacted with SDS-PAGE of unactivated and activated APPA proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

mAb Domain Localization.

Chimeric protein molecules were created by Dr. Douglas K. Struck in an attempt to identify the region in the *P. haemolytica* leukotoxin and the *A. pleuropneumoniae* serotype 5 toxin responsible for target cell specificity (98). In this study the chimeric proteins were used to identify the region in the leukotoxin molecule which contained epitopes recognized by the leukotoxin mAbs. The *A. pleuropneumoniae* serotype 5 toxin was an ideal negative control in these experiments because results had previously shown that it was not recognized by any of the leukotoxin mAbs. Therefore, binding of the mAbs to leukotoxin domains only could be detected. Unique restriction endonuclease sites within the genetic domain encoding the leukotoxin molecule allowed for the localization of domains in the protein. In this manner, the DNA encoding for the leukotoxin molecule was divided into three domains. Domain 1 encoded amino acids 1-492, domain 2 encoded amino acids 493-767, while domain 3 encoded amino acids 768-939. These domains were fused with domains obtained from similar regions encoding for the toxin molecule of *A. pleuropneumoniae* serotype 5 as shown in Figure 1. The fusion products were inserted into plasmid vectors and then transfected into competent *E. coli* TB1 host cells. Each TCA precipitated sample containing the plasmid pYFC19 (LLL), pYFC37 (AAA), pDS10 (LLA), pDM32 (LAA), pDM15 (AAL), pDM28 (ALL), pDM30 (LAL), or pDM34 (ALA) was separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with rabbit polyclonal anti-leukotoxin antiserum as a positive control and with each of the six leukotoxin mAbs.

As expected, the rabbit polyclonal antiserum reacted with all the chimeric toxins, which demonstrated that each domain was capable of being recognized (Figure 15). Each mAb was then tested for its ability to recognize chimeric toxin molecules. All of the mAbs reacted with a protein of approximately 102 kDa in the lanes containing plasmids



Figure 15. Immunoblot of rabbit polyclonal anti-102 kDa antiserum reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

pYFC19, pDM15, pDM28, and pDM30 (Figures 16-21). The common feature in each of these plasmids was that amino acids 768-939 were derived from the *P. haemolytica* leukotoxin. This indicated that the epitope for each mAb was located within amino acids 768-939. Lane 10 contained plasmid pYFC19 and received secondary antibody only. This lane did not show reactivity in any of the immunoblots.

Detection of Rabbit Anti-Mouse Antibodies.

Donald Gerbig previously showed that mAb ltx-2 neutralizes the leukotoxin by preventing its association with BL-3 target cells (48, 49). Additionally, mapping studies have indicated the epitope for mAb ltx-2 lies within amino acids 768-939 of the LKTA protein. It is interesting that this region contains at least two glycine-aspartic acid rich repeat regions which are thought to function in linking the leukotoxin to target cells. In addressing the question of whether the mAb ltx-2 leukotoxin epitope is involved in target cell binding, a major factor must be considered. Purified leukotoxin is not commercially available and attempts to purify the leukotoxin result in an excessive loss of activity. Therefore, leukotoxin itself cannot be used to answer this question. However, anti-idiotypic antibodies, especially Ab2 β antibodies, represent an alternative means of investigating this potentially important epitope. Additionally, Ab2 β antibodies may also be used to identify a leukotoxin receptor in target cells. In an attempt to generate anti-idiotypic antibodies which resembled the mAb ltx-2 epitope of the *P. haemolytica* leukotoxin, three rabbits were injected at monthly intervals with 500 μ g mAb ltx-2 (a mouse IgG_{2b} mAb). Rabbits were bled seven days following injection and the antisera was collected. Unfractionated antisera were tested by ELISA to ascertain both reactivity for mAb ltx-2 as well as with murine IgG using mAb ltx-35 as the IgG control. This assay was performed by incubating mAb ltx-2 and mAb ltx-35, which had been affixed

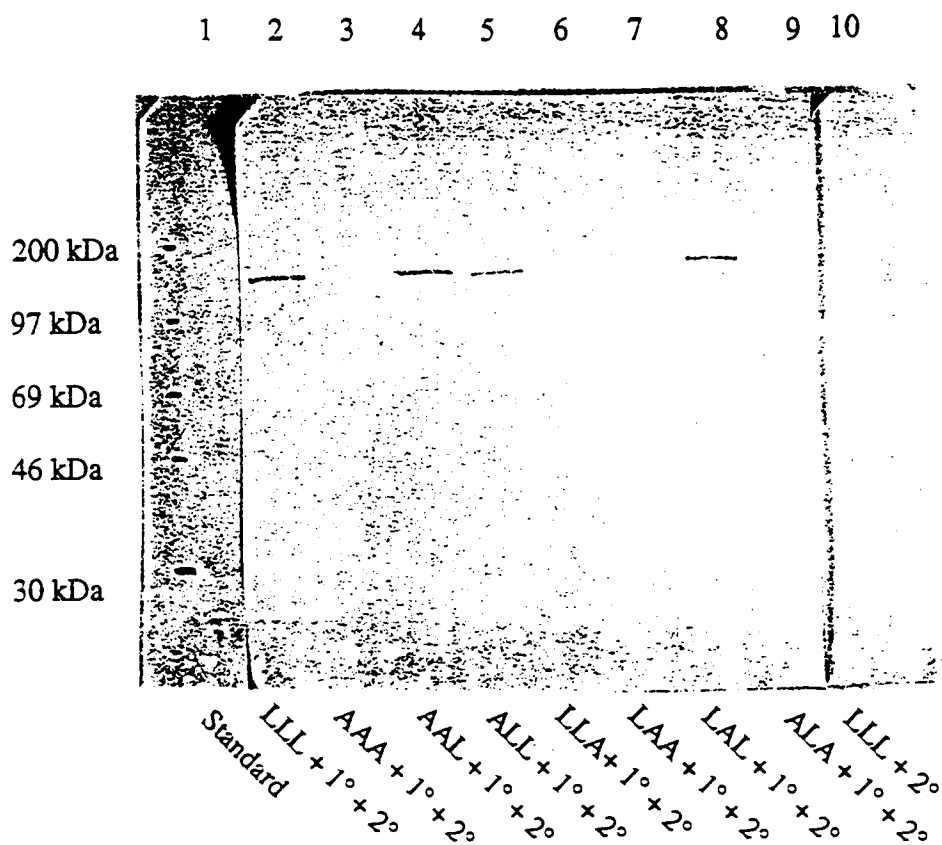


Figure 16. Immunoblot of mAb ltx-2 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

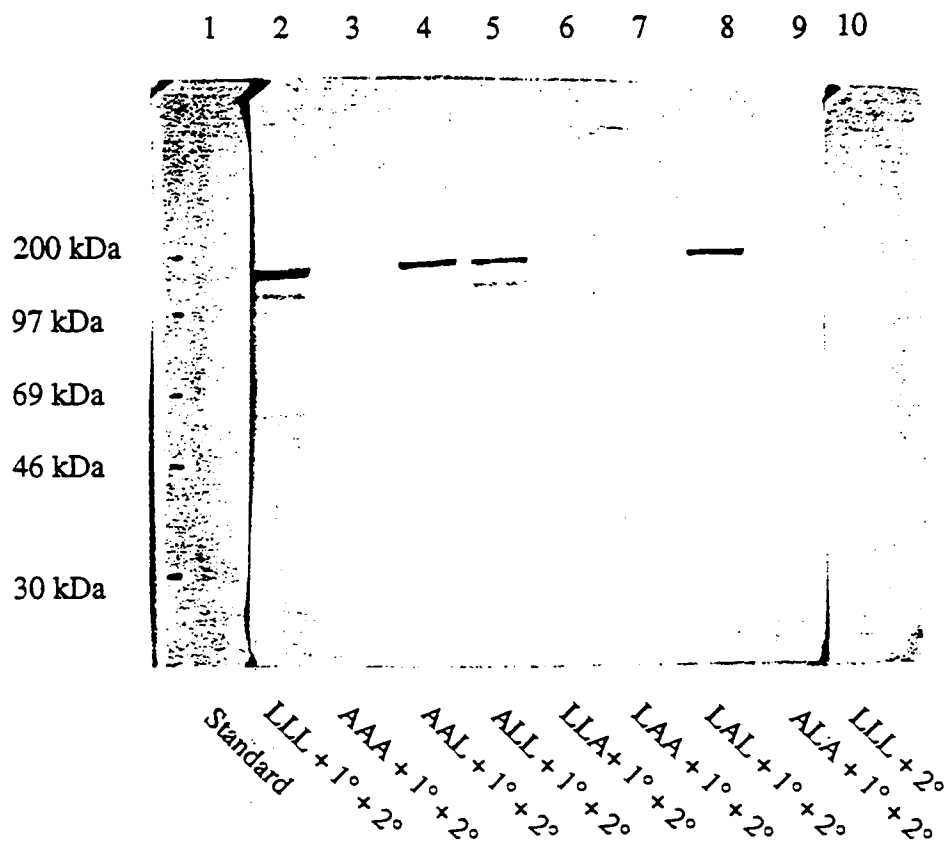


Figure 17. Immunoblot of mAb ltx-4 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

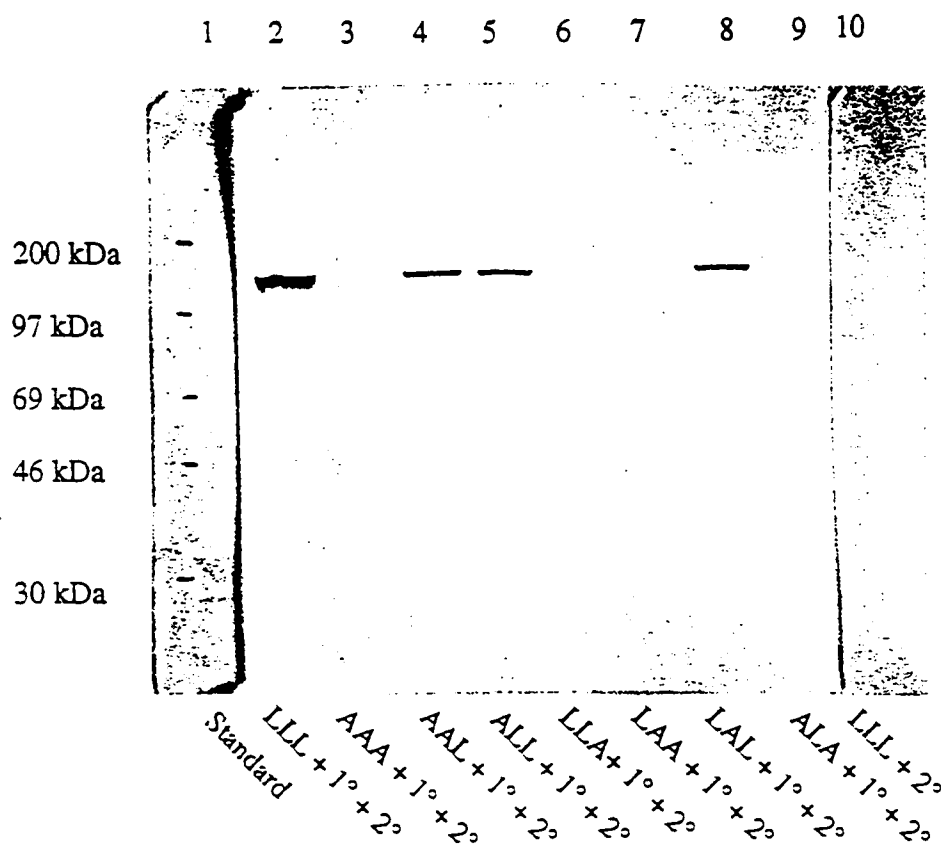


Figure 18. Immunoblot of mAb ltx-35 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

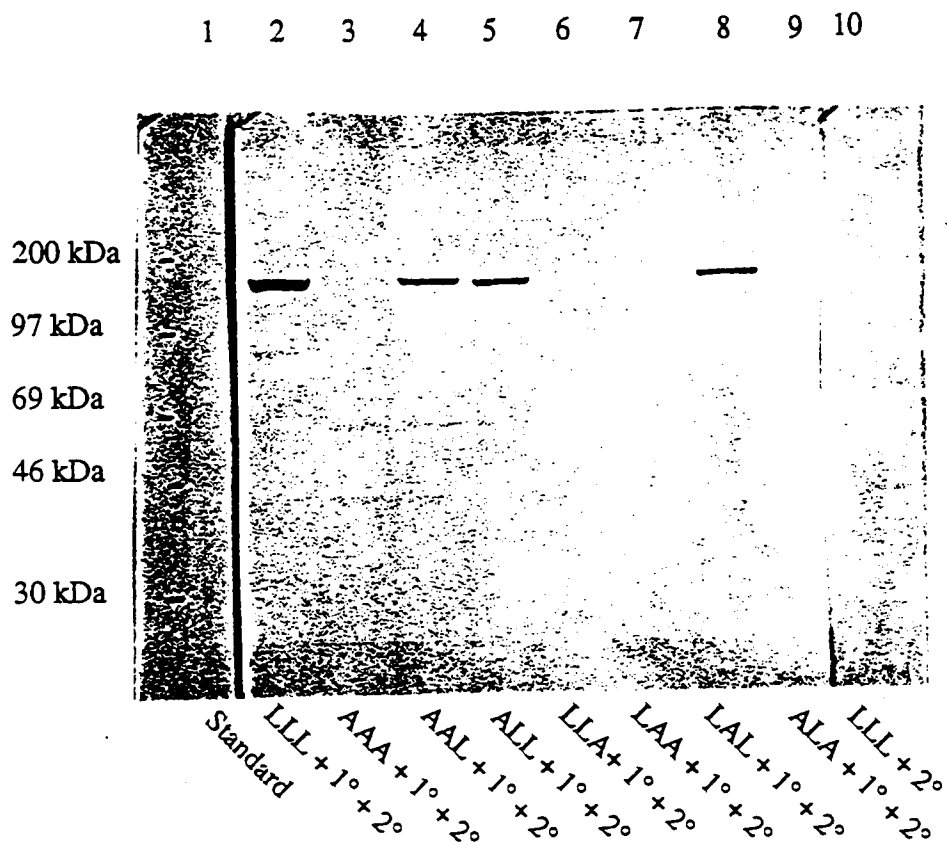


Figure 19. Immunoblot of mAb ltx-37 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

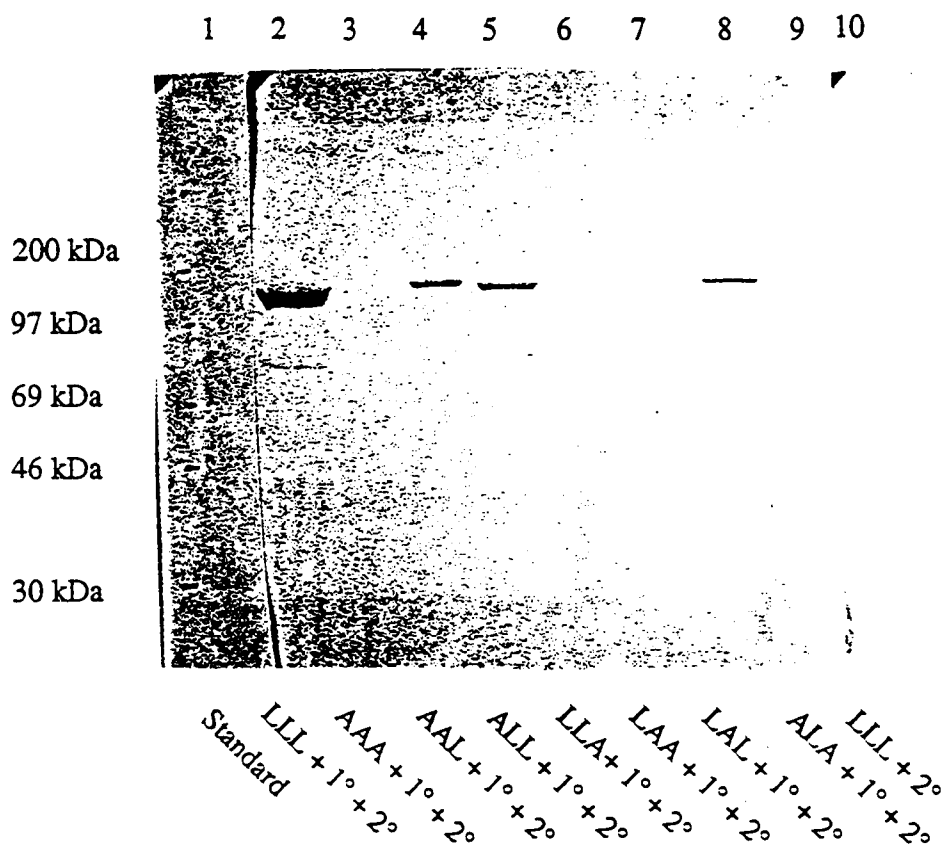


Figure 20. Immunoblot of mAb ltx-58 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

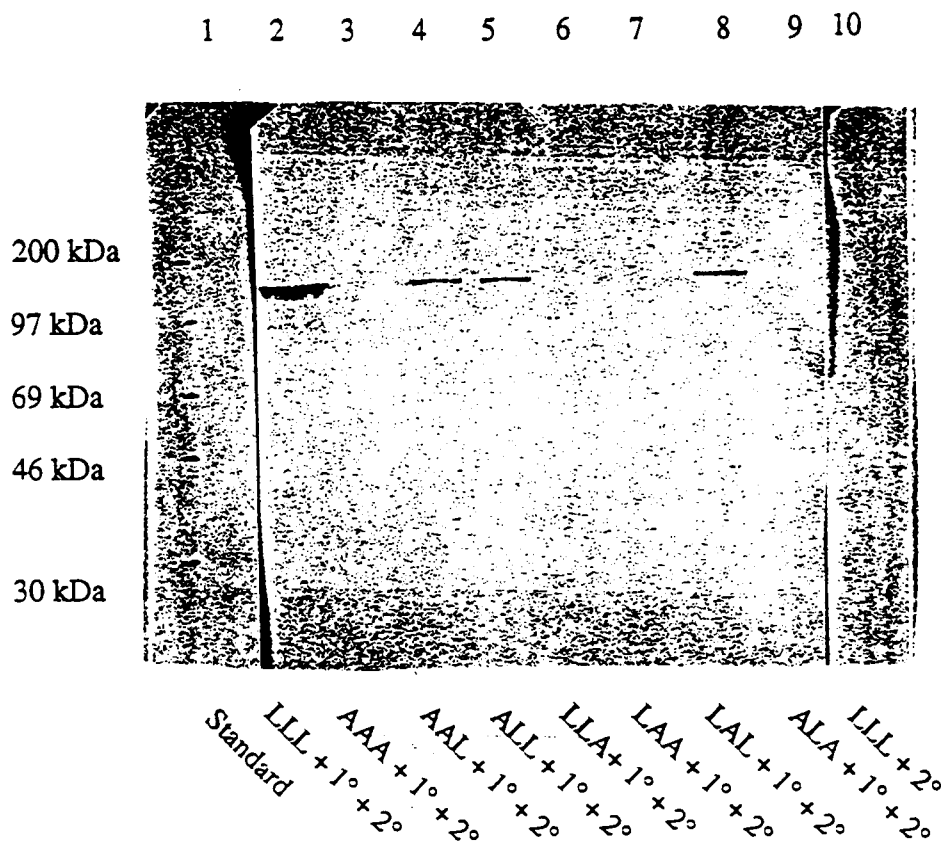


Figure 21. Immunoblot of mAb ltx-81 reacted with SDS-PAGE of chimeric toxin proteins. The samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids.

to the wells of a PVC plate, with dilutions of rabbit antisera. The addition of goat anti-rabbit IgG HRP allowed for the detection of rabbit antibodies which remained bound to the mAbs. The absorbance was calculated at each antiserum dilution and comparisons were made between each mAb for binding specificity. Each antiserum sample recognized both mAbs, but slightly elevated binding of antisera to mAb ltx-2 was observed (Figure 22). Normal rabbit antiserum was also tested in an identical assay. This antiserum did not exhibit elevated binding to mouse mAbs as did the test antisera. Unfractionated antisera titers for binding to mAb ltx-35 and mAb ltx-2 are shown in Table 3.

Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Whole Antisera.

Unfractionated antisera were tested for the ability to inhibit mAb ltx-2-HRP recognition of leukotoxin. This competitive ELISA would provide initial evidence for the presence of Ab2 γ or Ab2 β antibodies in the rabbit antisera. Ab2 γ antibodies would prevent mAb ltx-2-HRP binding to leukotoxin due to steric interactions. Ab2 β antibodies would inhibit mAb ltx-2-HRP recognition of leukotoxin by binding to the antigen binding regions of mAb ltx-2 and would prevent these regions from associating with the leukotoxin. Dilutions of antisera were incubated overnight at 4°C with a preselected dilution of mAb ltx-2-HRP. Incubation of these antibodies with trapped leukotoxin followed. Percent inhibition of ltx-2-HRP binding compared to control wells containing mAb ltx-2-HRP alone was measured. Results demonstrated each of the antisera samples inhibited mAb ltx-2-HRP binding to leukotoxin (Figure 23). Inhibitory titers for each rabbit are shown in Table 3.

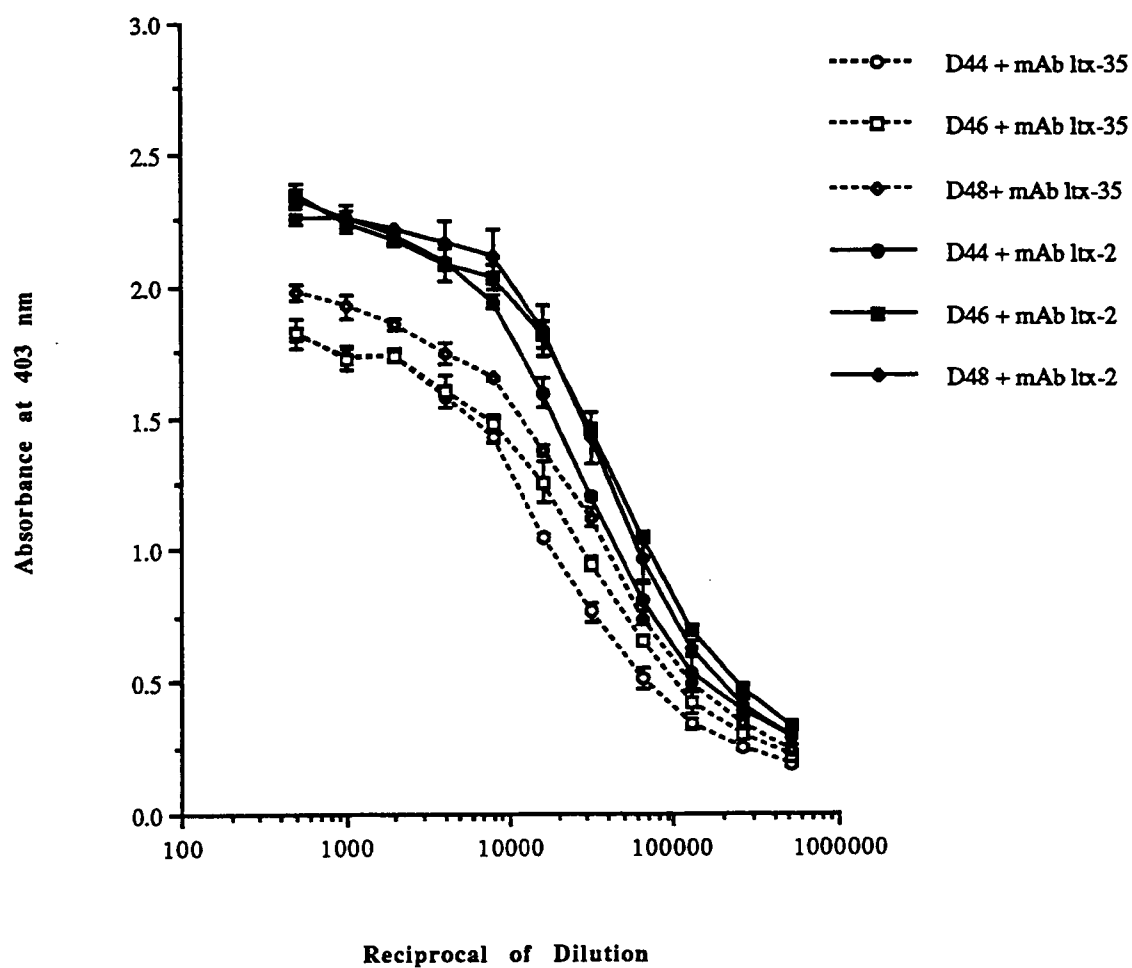


Figure 22. Antibody binding to mAbs ltx-2 and ltx-35 measured by ELISA with sera from mAb ltx-2 immunized rabbits.

Table 3. Titers of rabbit antisera samples in ELISA determinations designed to demonstrate binding to mouse mAbs and to demonstrate ability of rabbit antisera to inhibit mAb ltx-2-HRP from binding to leukotoxin.

Rabbit Number	Binding Titer ^a		Inhibitory Titer ^b
	ltx-35	ltx-2	
D44	60,000	150,000	20,200
D46	90,000	200,000	40,000
D48	120,000	160,000	40,000
Preimmune Serum	8,000	8,000	<400

^aReciprocal dilution of antisera yielding twice the A_{403} of the background binding to each mAb.

^bReciprocal dilution of antisera resulting in 50% inhibition of mAb ltx-2-HRP binding to leukotoxin.

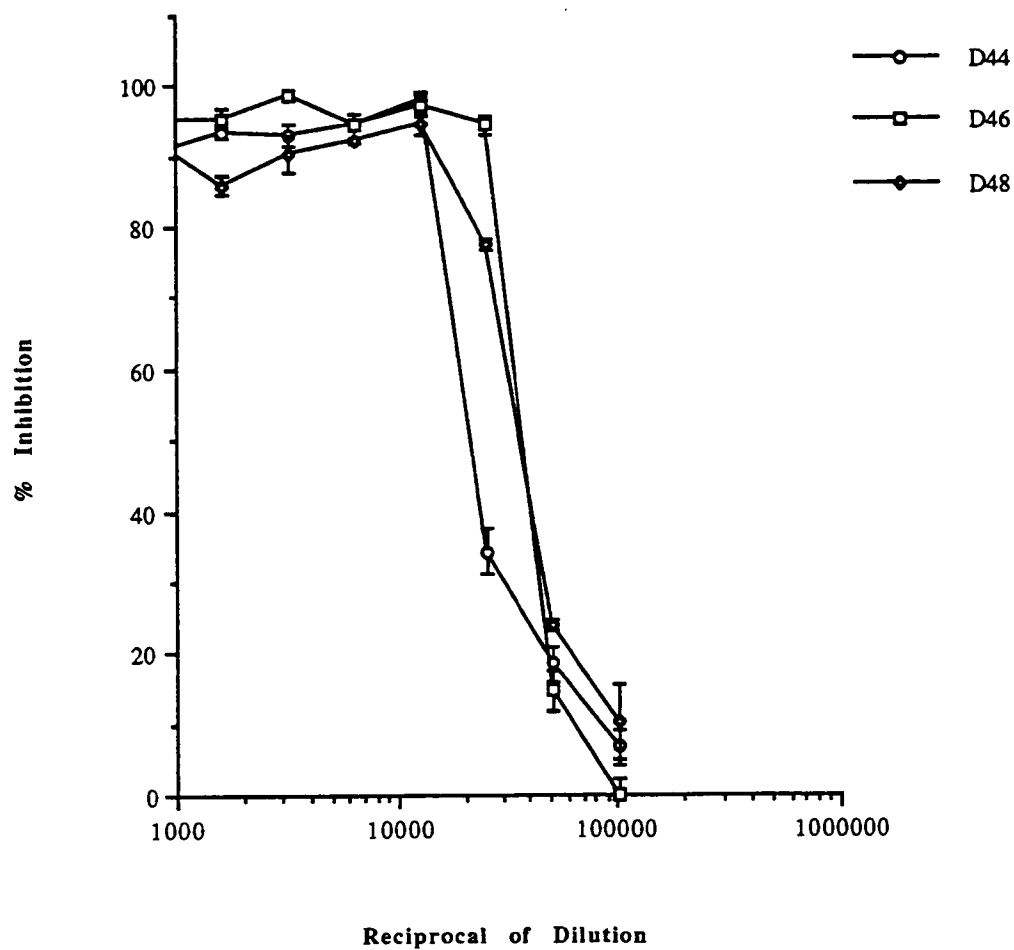


Figure 23. Inhibition of mAb ltx-2-HRP binding to leukotoxin by preincubation with sera obtained from rabbits immunized with mAb ltx-2 as measured by ELISA.

Binding of Fractionated Antisera to mAbs ltx-2 and ltx-35.

Results from the initial screening assays indicated the rabbit antisera contained anti-idiotypic antibodies. To concentrate and attempt to purify the anti-idiotypic antibodies, the antisera were pooled and the IgG collected and concentrated following 50% ammonium sulfate precipitation. The concentrated IgG was then sequentially passed over nonspecific and specific murine mAb affinity columns. The IgG was passed multiple times over a column containing mAbs ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81 to remove nonspecific rabbit anti-mouse antibodies. In order to isolate the possible Ab2 β antibodies, the concentrated flowthrough was then passed over a column containing mAb ltx-2. The antibodies which were isolated from this column following several washes of the column were called the eluate, while the antibodies which washed through the column were called the flowthrough. Each respective eluate from the mAb ltx-2 column was tested for binding to mAbs ltx-2 and ltx-35 affixed to wells of a PVC plate. Detection of bound rabbit antibodies was with goat anti-rabbit IgG HRP. This ELISA determined the specificity of the antibodies contained in the eluates for each mAb. The results indicated that the eluate antibodies from the mAb ltx-2 column bound to mAb ltx-2 more specifically and that IgG reactive antibodies, i.e., mAb ltx-35 reactive antibodies, had been largely removed (Figure 24). Titers for each eluate and the flowthrough are given in Table 4.

Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Fractionated Antisera.

To quantify the Ab2 β or Ab2 γ antibodies in the eluates from the mAb ltx-2 column, the eluate and flowthrough antibodies were tested by competitive ELISA for inhibition of mAb ltx-2-HRP binding to leukotoxin. This assay was performed as previously described. Results showed that the eluates were able to bind to mAb ltx-2-

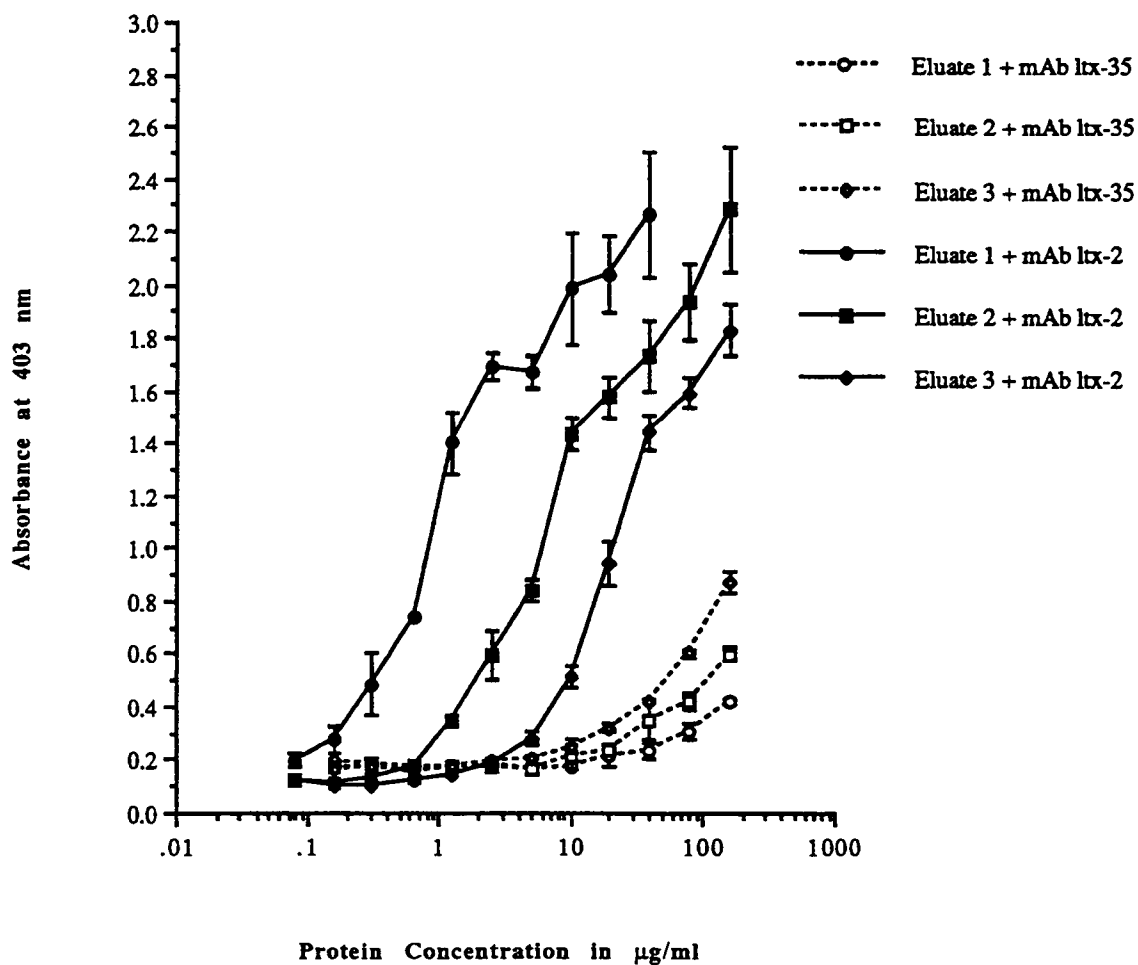


Figure 24. Antibody binding to mAbs ltx-2 and ltx-35 measured by ELISA with antibodies isolated by washes of ammonium sulfate precipitated antibodies over the mAb ltx-2 column.

Table 4. Titers of eluates and flowthrough obtained from the mAb ltx-2 column in ELISA determinations designed to demonstrate binding to mAb ltx-2 and ability of eluates to inhibit mAb ltx-2-HRP from binding to leukotoxin.

Fraction	Binding ($\mu\text{g/ml}$) ^a		Inhibitory ($\mu\text{g/ml}$) ^b	Abrogation ($\mu\text{g/ml}$) ^c
	ltx-35	ltx-2		
Eluate 1	33	0.23	3	11
Eluate 2	55	1.5	33	ND ^d
Eluate 3	140	7	>200	ND
Flowthrough	ND	ND	0	0

^aTiters were equivalent to the protein concentration at which the A_{403} of the test sample was twice the A_{403} of the background.

^bTiters were determined as the protein concentration at which 50% inhibition of mAb ltx-2-HRP binding to leukotoxin was observed.

^cTiters were the protein concentration at which 50% cytotoxicity was observed.

^dNot Determined.

HRP and prevent this mAb from binding the leukotoxin (Figure 25). In fact, eluates 1 and 2 demonstrated complete inhibition at elevated protein concentrations (greater than 5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$, respectively). As expected, the flowthrough from the mAb ltx-2 column did not show any inhibition of mAb ltx-2-HRP binding. Titers for each fraction are given in Table 4.

Abrogation of mAb ltx-2 Protection of BL-3 Cells by Eluate 1.

In order to determine if the antibodies present in eluate 1 were capable of blocking mAb ltx-2 protection of BL-3 cells, a neutral red cytotoxicity assay was performed. Eluate 1 was the only fraction tested because the protein concentrations of the remaining eluates were not high enough to be compatible with this assay. Eluate 1 antibodies were incubated overnight at 4°C with a final concentration of 2 $\mu\text{g/ml}$ of mAb ltx-2 (a concentration known to protect BL-3 cells from 3U of leukotoxin). An aliquot of this mixture was incubated with leukotoxin for 1 h at 4°C, then BL-3 cells were added and percent cytotoxicity in test wells was determined. Control wells containing mAb ltx-2 plus 3U leukotoxin contained 0% cytotoxicity (data not shown). Results demonstrated that eluate 1 was able to block mAb ltx-2 protection of leukotoxin mediated cell lysis (Figure 26). The titer was equivalent to the reciprocal dilution at which 50% cytotoxicity was observed and was determined to be 11 $\mu\text{g/ml}$.

Recognition of Leukotoxin by Murine Antisera.

Groups of mice were injected with 50 μg or 25 μg of eluate 1 to determine conclusively if Ab2 β antibodies were present in this fraction. By definition Ab2 β antibodies should evoke an Ab3 antibody response with neutralizing capacity similar to the original Ab1 or mAb ltx-2 in this case. An additional group of control mice were

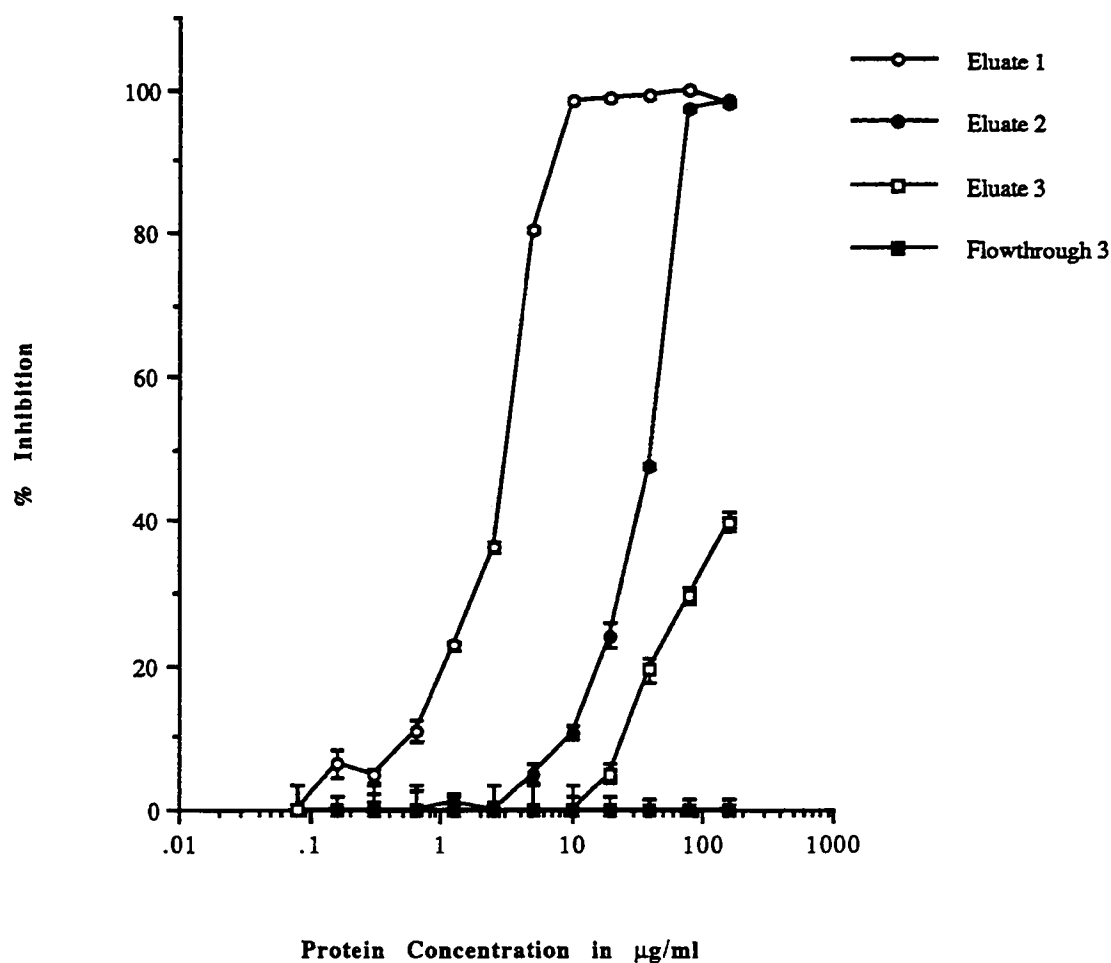


Figure 25. Inhibition of mAb ltx-2-HRP binding to leukotoxin by preincubation with antibodies isolated by numerous passes of ammonium sulfate precipitated antibodies over the mAb ltx-2 column as measured by ELISA.

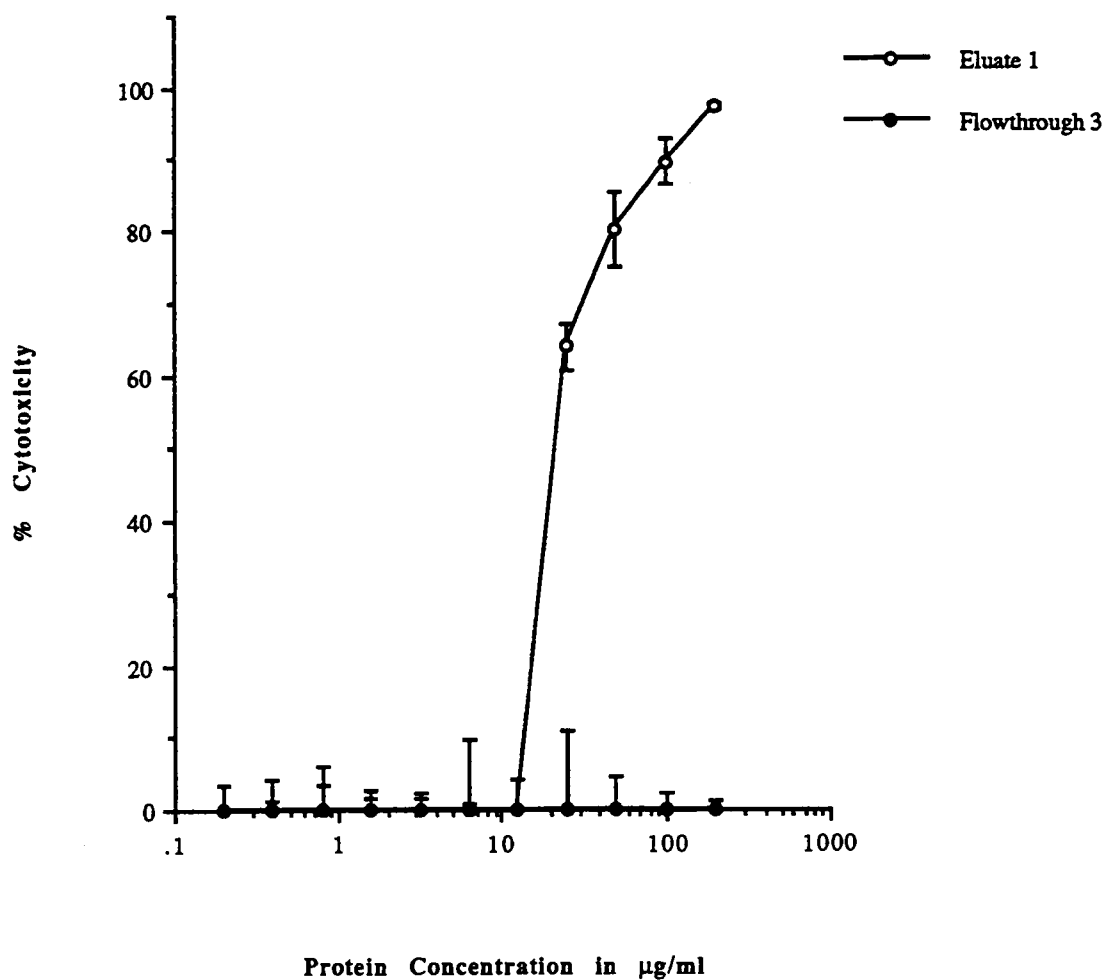


Figure 26. Abrogation of mAb ltx-2 protection of leukotoxin-mediated BL-3 cell lysis measured by a neutral red cytotoxicity assay by preincubation with antibodies isolated from passing ammonium sulfate precipitated antibodies through the mAb ltx-2 column.

injected with 50 µg of nonspecific rabbit IgG. Antisera obtained from the mice on days 21, 35, and 49 was pooled so that each group had only one large serum sample. Antisera obtained from the final bleed on day 63 were not pooled in this manner. The antisera were tested by competitive ELISA to determine if antibodies contained within the samples could bind to the mAb ltx-2 epitope and thus prevent mAb ltx-2-HRP from binding to the leukotoxin. Trapped leukotoxin was preincubated with dilutions of murine antisera followed by the addition of a constant dilution of mAb ltx-2-HRP. Inhibition of mAb ltx-2-HRP binding by murine antisera was not detectable until the day 63 bleed. As can be seen in Figures 27-30, serum from mouse 5A2, mouse 5A3, mouse 5B1, and mouse 6A3 inhibited mAb ltx-2-HRP from binding to the leukotoxin. The inhibitory serum titer for each mouse is shown in Table 5.

Protection of BL-3 Cells from Leukotoxin-Mediated Lysis by Murine Sera.

The putative Ab3-containing murine sera were further tested for their ability to protect BL-3 cells from leukotoxin-mediated cell lysis. Dilutions of the murine sera were incubated with leukotoxin for 1 h at 4°C and then BL-3 cells were added. After a 1 h incubation, a visual determination of cell viability and cell clumping was made and the assay was continued as described in the materials and methods. A visualization of protection of BL-3 cells by the murine sera was seen with that obtained on days 35 and day 49. However, the neutral red assay performed with the day 35 and day 49 sera was inconclusive because the controls did not work properly. The sera obtained from the day 63 bleed showed visual protection of BL-3 cells, and the neutral red assay data obtained also showed protection. Antiserum obtained from mouse 5A2 (Figure 31), mouse 5A3 (Figure 32), mouse 5B1 (Figure 33), mouse 5B2 (Figure 34), and mouse 6A3 (Figure 35), displayed complete neutralization of cytotoxicity at low dilutions. Titers for each

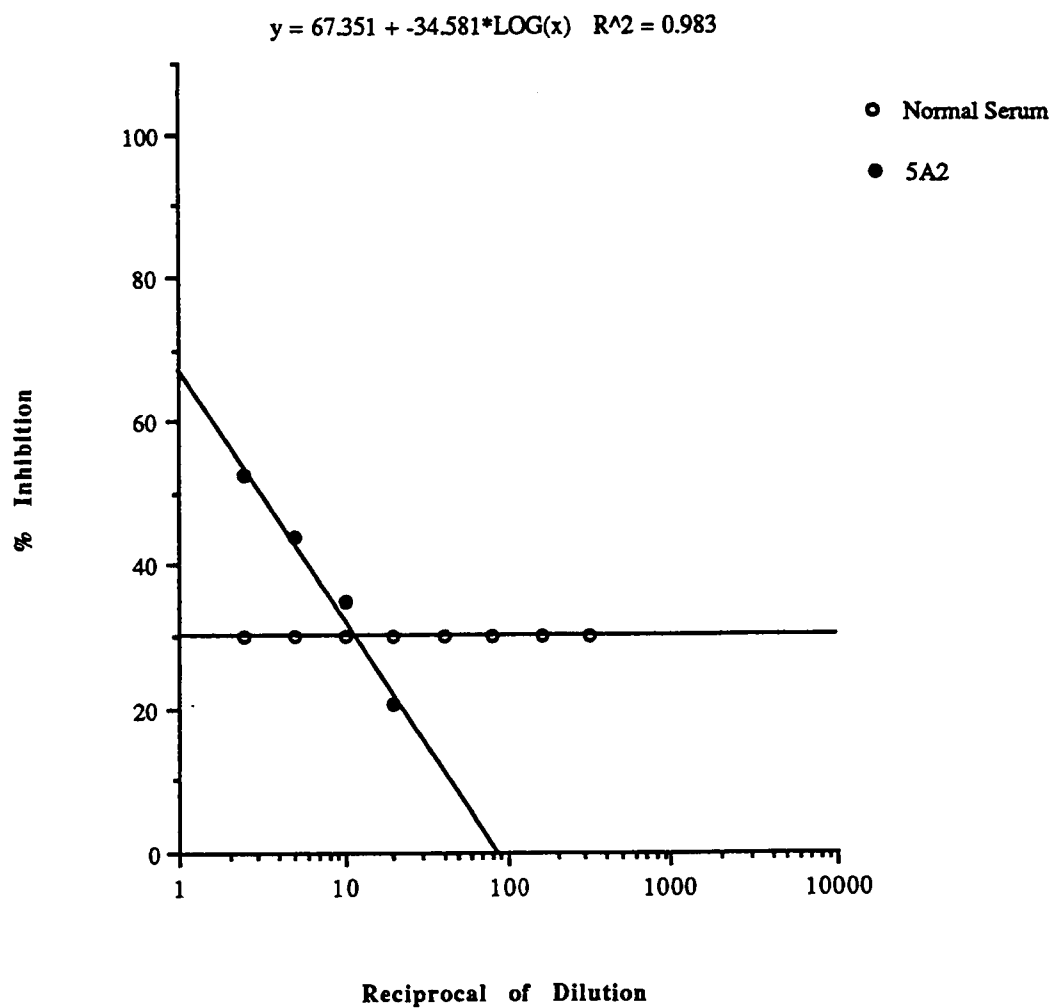


Figure 27. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum from mouse 5A2 obtained on day 63.

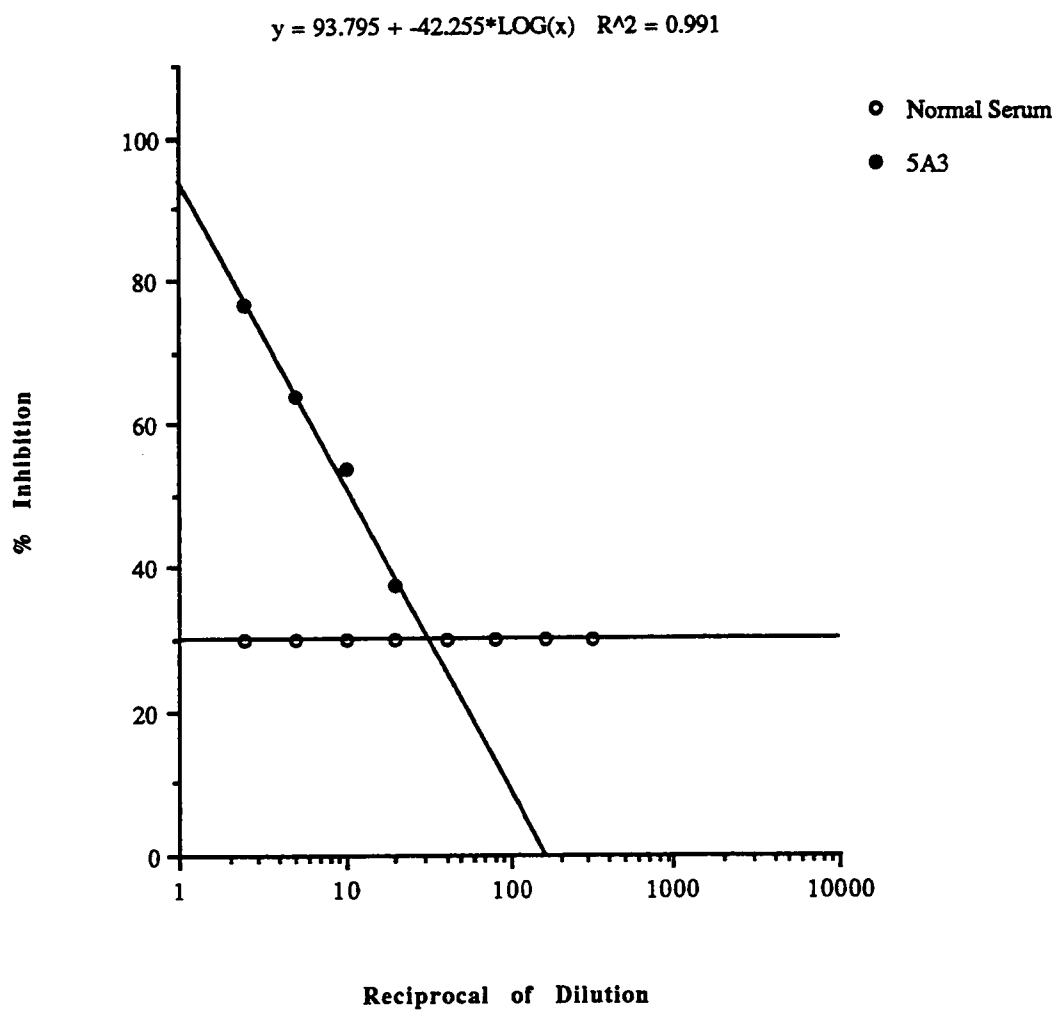


Figure 28. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 5A3 on day 63.

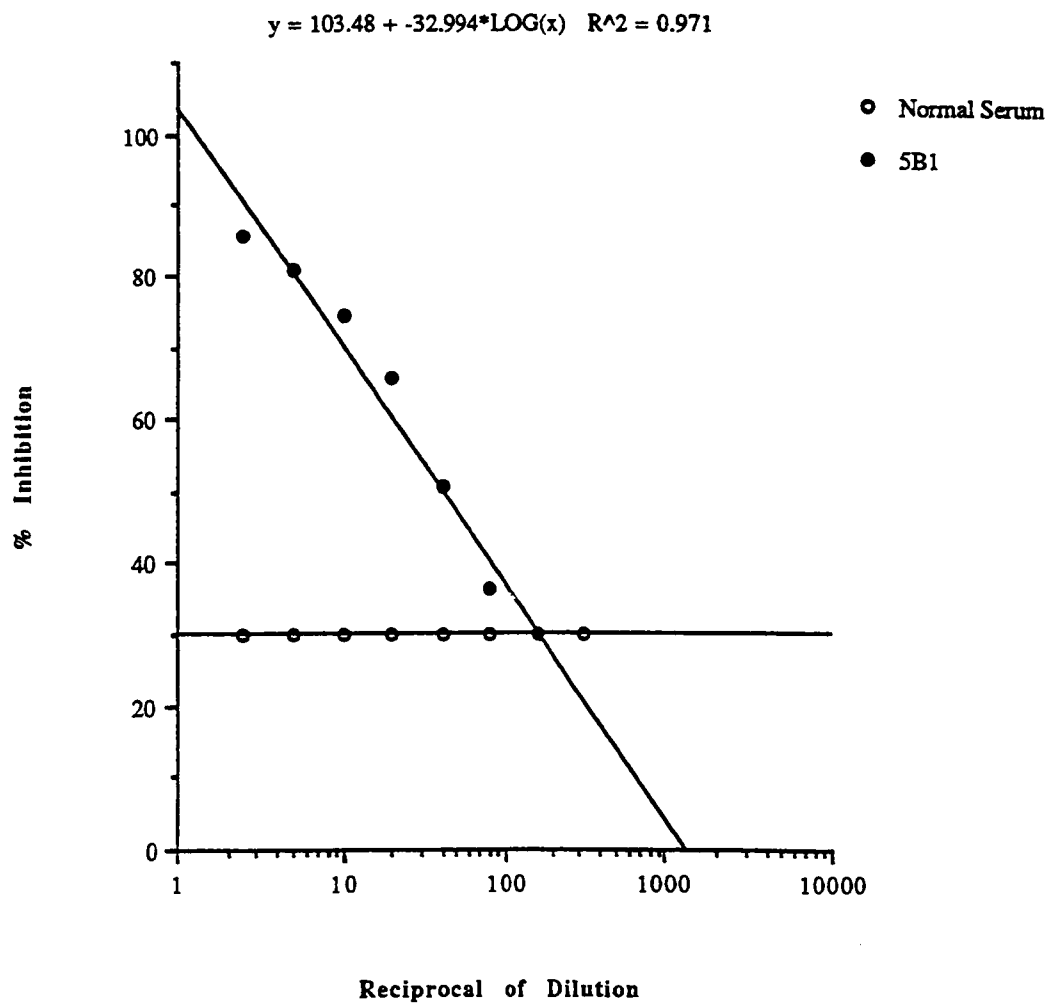


Figure 29. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 5B1 on day 63.

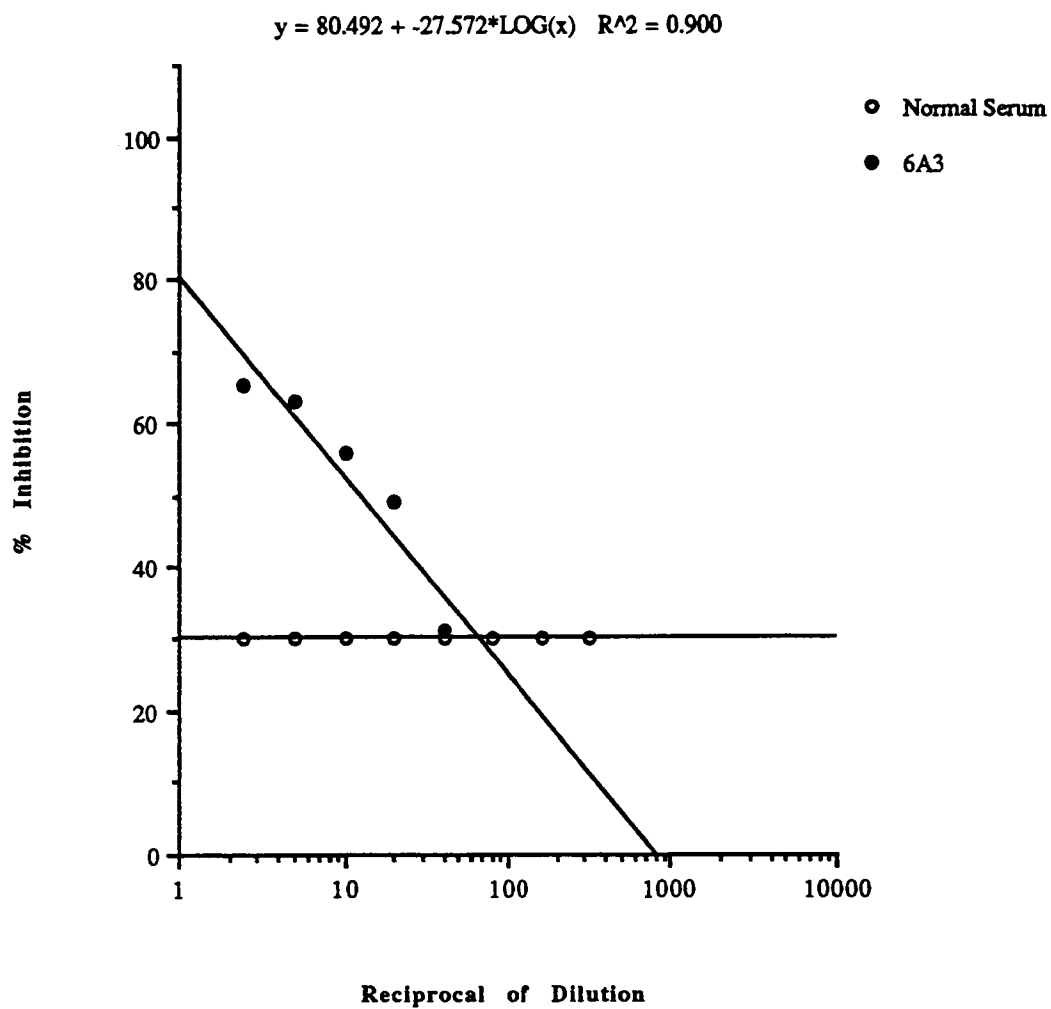


Figure 30. ELISA measuring percent inhibition of mAb ltx-2-HRP binding to leukotoxin by antiserum obtained from mouse 6A3 on day 63.

Table 5. Inhibitory and neutralization titers for mice injected with eluate 1 or nonspecific rabbit IgG.

Mouse Injected With	Mouse Number	Inhibitory Titer ^a	Neutralization Titer ^b
50 µg Eluate 1	5A1	<2.5	<5
	5A2	13	17
	5A3	30	380
	5B1	10	400
	5B2	<2.5	28
25 µg Eluate 1	6A1	<2.5	<5
	6A2	<2.5	<5
	6A3	75	280
	6B1	<2.5	<5
	6B2	<2.5	<5
50 µg Rabbit IgG	7A1	<2.5	<5
	7A2	<2.5	<5
	7A3	<2.5	<5
	7B1	<2.5	<5
	7B2	<2.5	<5

^aTiters were the reciprocal dilution of antiserum equal to the background inhibition displayed by normal mouse serum plus two standard deviations.

^bTiters were equivalent to the reciprocal dilution of antiserum which gave the identical percent cytotoxicity as the control normal mouse serum less two standard deviations.

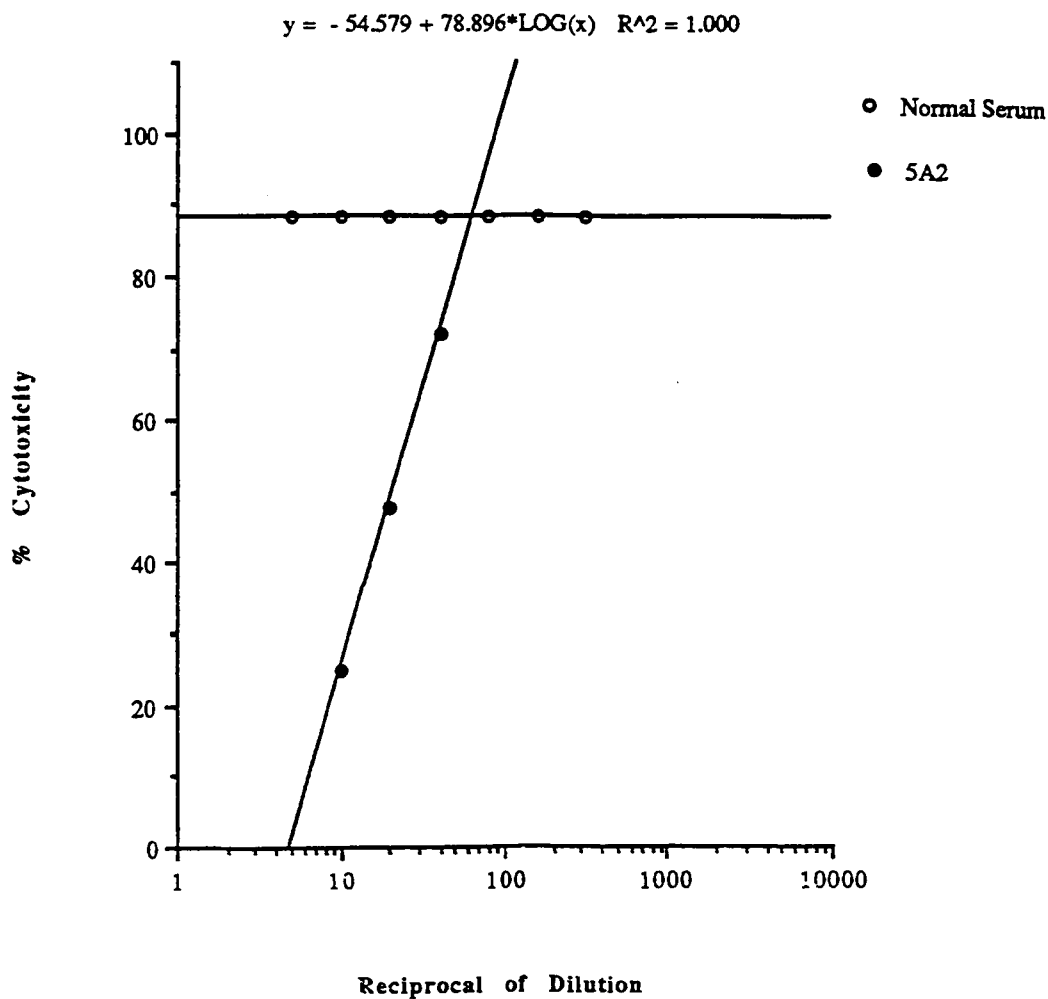


Figure 31. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5A2 on day 63 measured by the neutral red cytotoxicity assay.

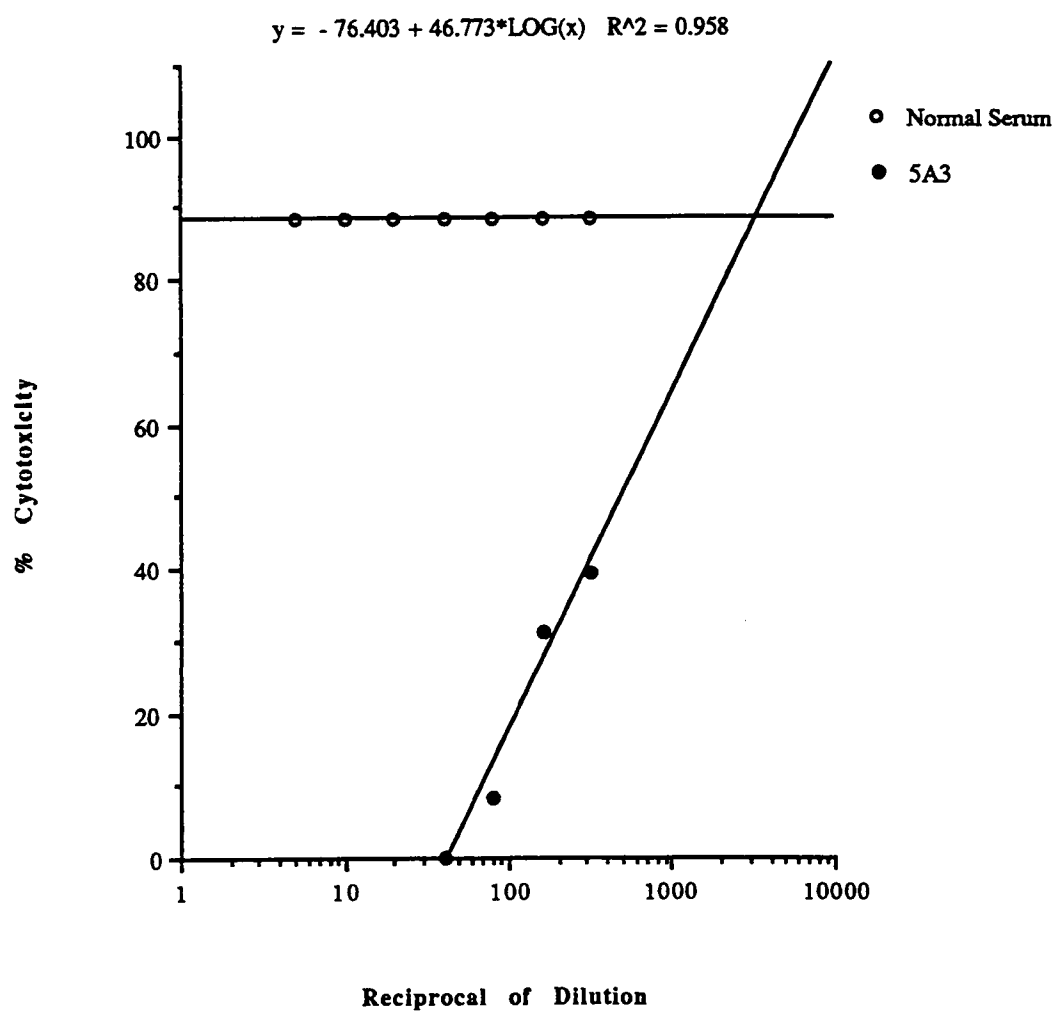


Figure 32. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5A3 on day 63 measured by the neutral red cytotoxicity assay.

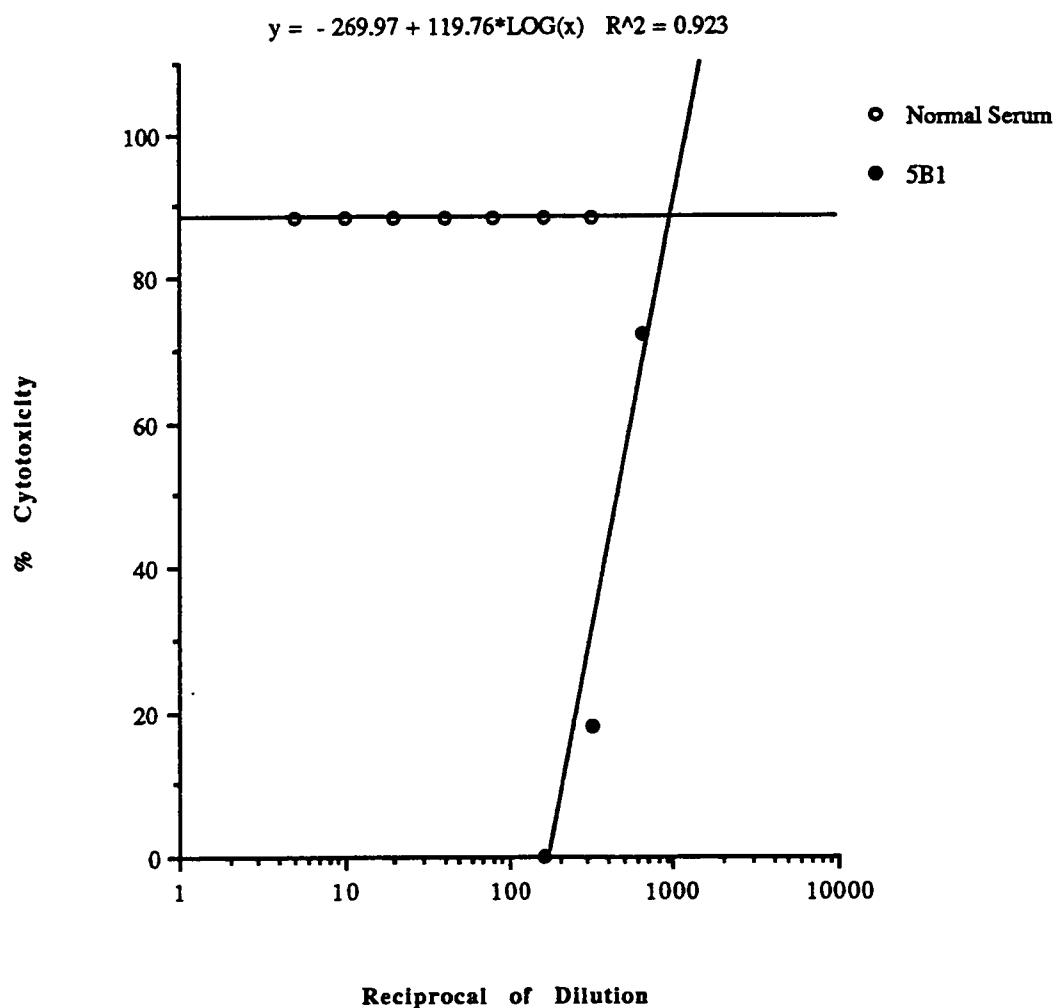


Figure 33. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5B1 on day 63 measured by the neutral red cytotoxicity assay.

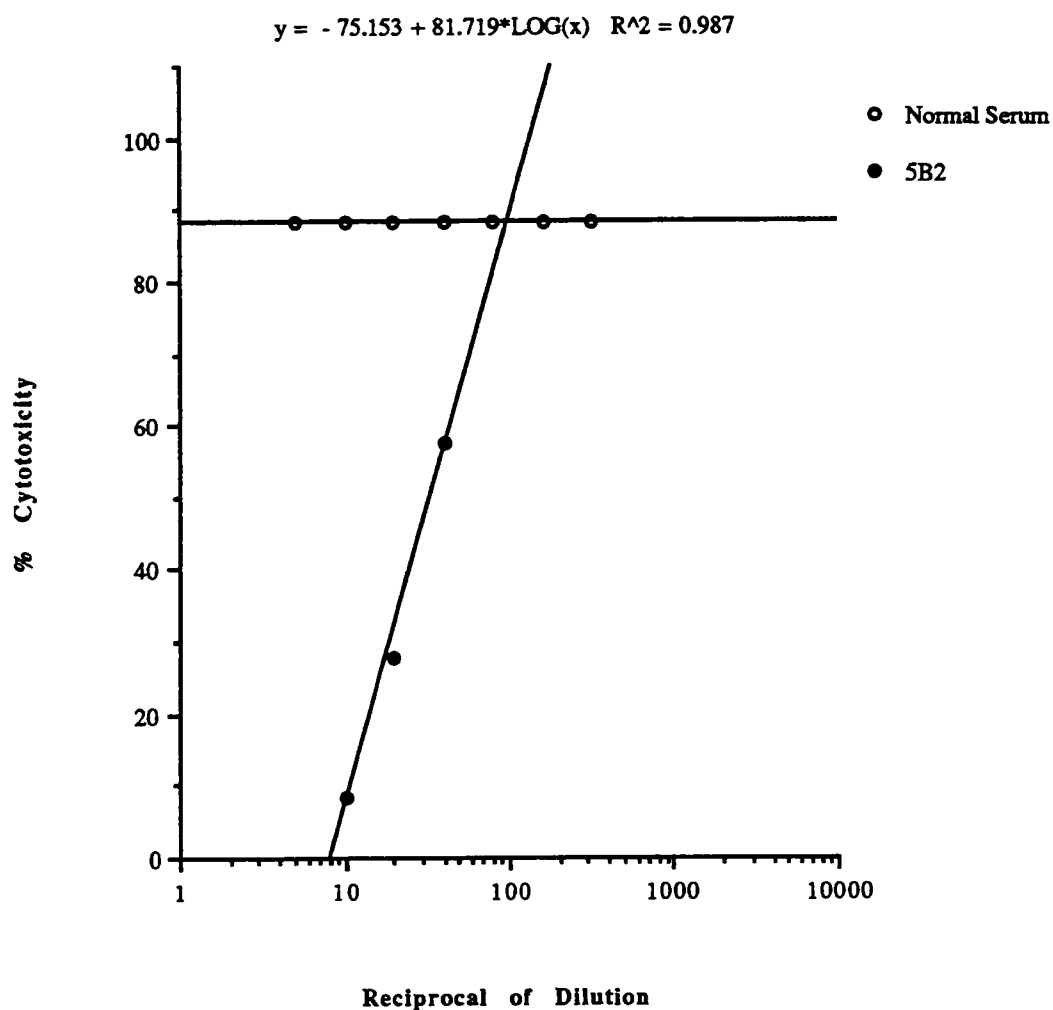


Figure 34. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 5B2 on day 63 measured by the neutral red cytotoxicity assay.

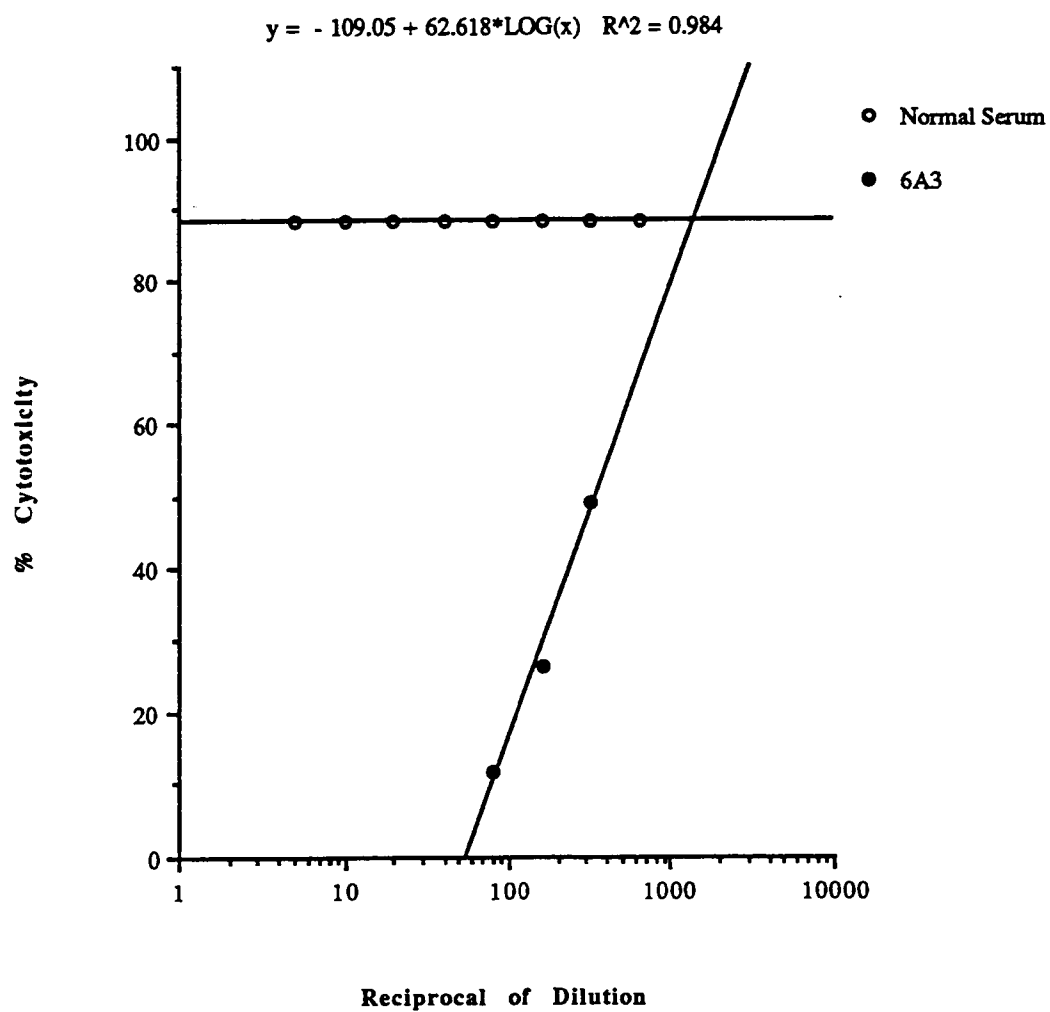


Figure 35. Percent cytotoxicity of BL-3 cells following incubation of leukotoxin with antiserum obtained from mouse 6A3 on day 63 measured by the neutral red cytotoxicity assay.

mouse are given in Table 5.

Binding of Ab2 β Antibodies to BL-3 Cells.

The generation of Ab3 antibodies following immunization of mice with eluate 1 antibodies indicated that Ab2 β antibodies were present in this fraction. Inherent in the definition of Ab2 β antibodies is that they should have the same biological activities as the original antigenic epitope and should therefore have similar receptor-ligand interactions compared with the antigenic epitope. Previous results with mAb ltx-2 have shown this mAb neutralizes leukotoxin by preventing its association with BL-3 cells (48, 49). It appears evident that the mAb ltx-2 leukotoxin epitope is involved in binding of the leukotoxin to target cells, possibly through a receptor. Experiments were performed which tested the ability of Ab2 β antibodies to bind to BL-3 cells. In a modified neutral red cytotoxicity assay, various concentrations of Ab2 β antibodies were preincubated with BL-3 cells for 1 h at 4°C. The plate was then screened visually for clumping of BL-3 cells. Leukotoxin was added and the plate was incubated for 1 h at 37°C. Percent cytotoxicity of each well was determined. No positive data resulted from this experiment (data not shown).

Another assay was performed in order to determine if Ab2 β antibodies recognized a moiety on the surface of BL-3 cells. BL-3 cells were glutaraldehyde fixed to the wells of a PVC plate and dilutions of Ab2 β were added for 2 h at room temperature. Binding was detected by the addition of goat anti-rabbit HRP. Again, no positive data resulted from this experiment (data not shown).

Binding of Whole Bovine Antisera to Leukotoxin.

The ability of bovines to produce antibodies which recognized the mAb ltx-2 leukotoxin epitope was then examined. Although the isolated Ab2 β antibodies did not appear to recognize a leukotoxin receptor on BL-3 cells, these antibodies were used to construct a column which was used to isolate antibodies from ammonium sulfate precipitated antibodies from bovine antiserum. These isolated antibodies should recognize the same leukotoxin epitope as mAb ltx-2. Bovine antibodies isolated in this manner were tested for their ability to react similarly to mAb ltx-2 in a variety of assays. The bovine antiserum sample supplied by Dr. Douglas K. Struck was obtained following immunization of cattle with leukotoxin and was designated Cow Anti-Lkt. Those samples obtained from Dr. Philip N. Bochsler were from cattle which may or may not have been exposed to *P. haemolytica* (Cow 20, Cow 51, Cow 8916, Cow 800 and Pooled Sera). The antisera were tested by ELISA to determine if antibodies were present in the antisera which would recognize leukotoxin. Dilutions of antisera were added to trapped leukotoxin and detection of bound antibodies with rabbit anti-bovine IgG HRP followed. Absorbance at each dilution was calculated and the results are shown in Figure 36. Antibodies which recognized the leukotoxin were present in each sample tested, with the exception of cFBS which served as a negative control. Titers for binding were high in each sample, especially in the antiserum sample obtained from animals immunized with leukotoxin. Titers for each sample are displayed in Table 6.

Neutralization of Leukotoxin-Mediated Cytotoxicity by Bovine Antisera.

The neutral red assay was used to determine if the bovine antisera samples contained antibodies which would protect BL-3 target cells against leukotoxin-mediated cell lysis. Leukotoxin was incubated with dilutions of bovine antisera for 1 h. BL-3

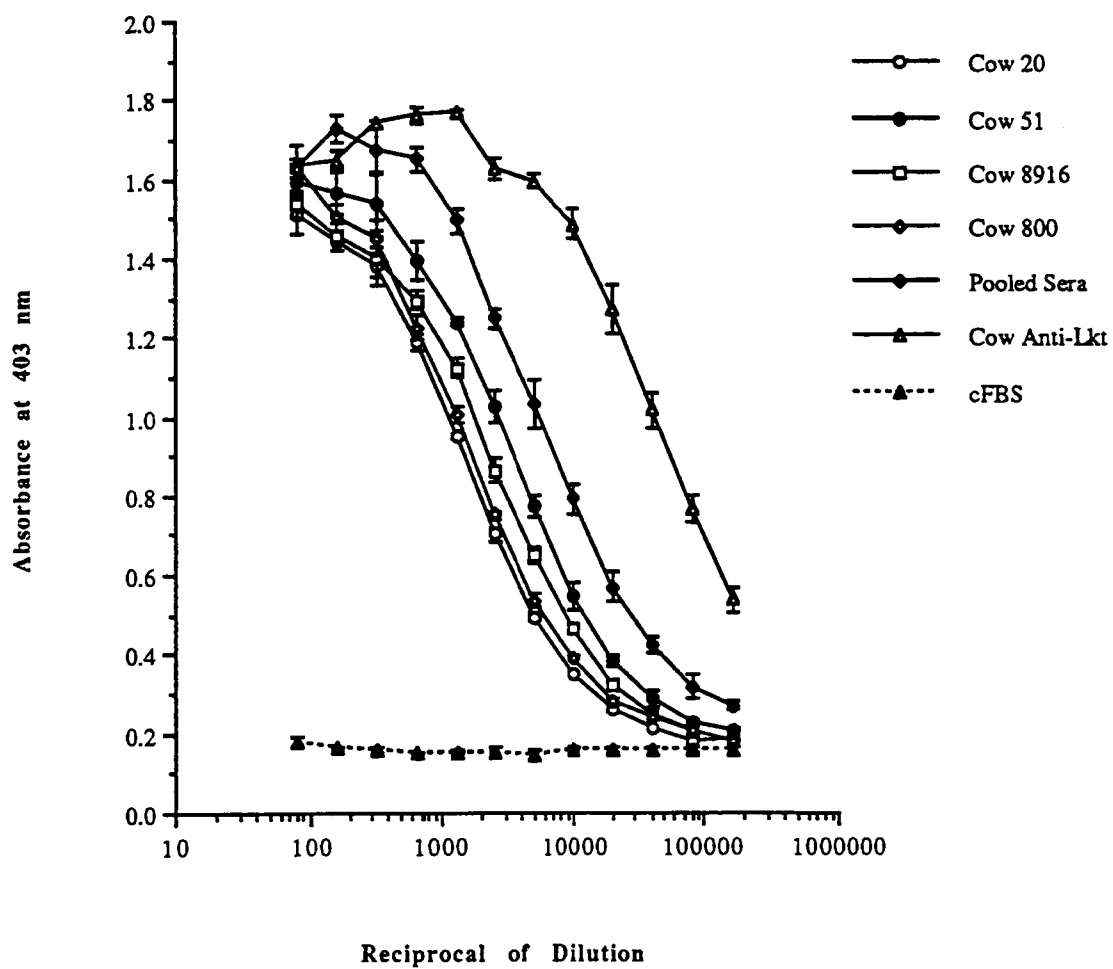


Figure 36. Antibody binding to trapped leukotoxin measured by ELISA with bovine antisera samples.

Table 6. Unfractionated bovine antisera samples titers for binding, neutralization and inhibition.

Antiserum Sample	Binding ^a	Neutralization ^b	Inhibitory ^c
Cow 20	10,200	5,000	6
Cow 51	30,500	>10,000	20
Cow 8916	20,000	400	22
Cow 800	10,800	2,500	18
Pooled Sera	80,000	>10,000	40
Cow Anti-Lkt	>200,000	>10,000	110
cFBS	<80	ND ^d	<5

^aTiters were equivalent to the reciprocal dilution of antisera at which twice the A₄₀₃ of the background was observed.

^bTiters were the reciprocal dilution of antisera equivalent to 50% cytotoxicity.

^cTiters were the reciprocal dilution of antisera equivalent to 50% inhibition of mAb ltx-2-HRP binding to leukotoxin.

^d Not Determined

cells were added and incubation for 1 h followed. Percent cytotoxicity of cells in each well compared with control wells containing cells only was determined. All of the antisera samples provided protection for the BL-3 cells (Figure 37), and a general trend was noted in that antisera with the greatest leukotoxin-reactivity titers contained the greatest leukotoxin-neutralizing titers (Table 6).

Inhibition of mAb ltx-2-HRP Binding to Leukotoxin by Bovine Antisera.

The bovine antisera samples were tested for their ability to inhibit mAb ltx-2-HRP binding to leukotoxin. If inhibition of mAb ltx-2-HRP was observed, this would indicate the serum samples contained antibodies which recognized the same epitope as mAb ltx-2. Dilutions of antisera were incubated with trapped leukotoxin. mAb ltx-2-HRP was then added and percent inhibition was calculated based on control wells which contained mAb ltx-2-HRP only. Results showed that each of the samples inhibited mAb ltx-2-HRP binding to leukotoxin (Figure 38). Complete inhibition was observed with the antisera obtained from cattle immunized with leukotoxin and with antisera pooled from cattle which may or may not have been exposed to *P. haemolytica*; however, these titers were markedly less than both the binding and neutralization titers measured for the same samples. Titers for all of the samples are shown in Table 6.

mAb ltx-2 Inhibition of mAb ltx-2-HRP Binding to Leukotoxin.

To determine the concentration of mAb ltx-2 required to cause 50% inhibition of mAb ltx-2-HRP binding to leukotoxin, an ELISA determination was performed. An approximation of the concentration of antibodies present in the bovine antisera samples which recognized the mAb ltx-2 epitope could then be made using this information. Various concentrations of mAb ltx-2 were incubated with trapped leukotoxin. A constant

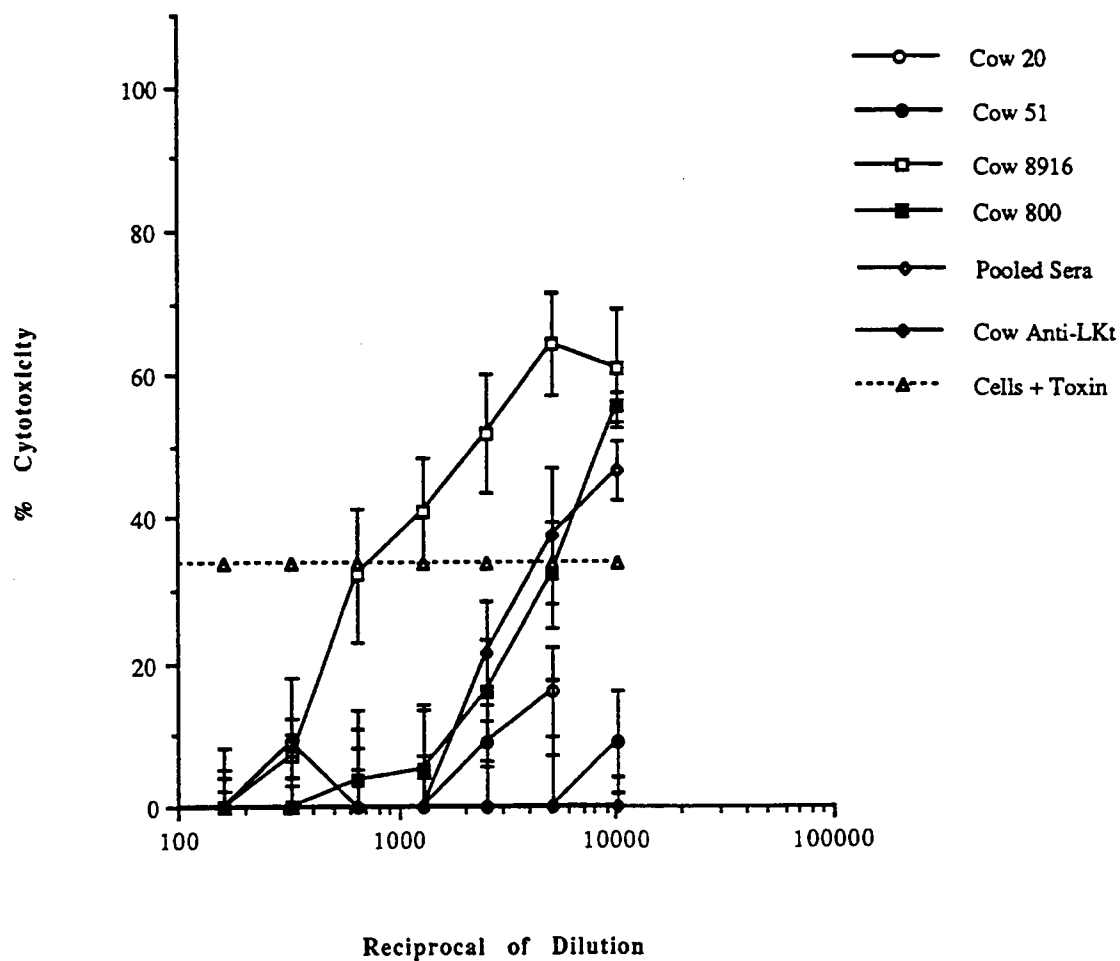


Figure 37. Neutralization of leukotoxin-mediated cell lysis by unfractionated bovine antisera samples measured by the neutral red cytotoxicity assay.

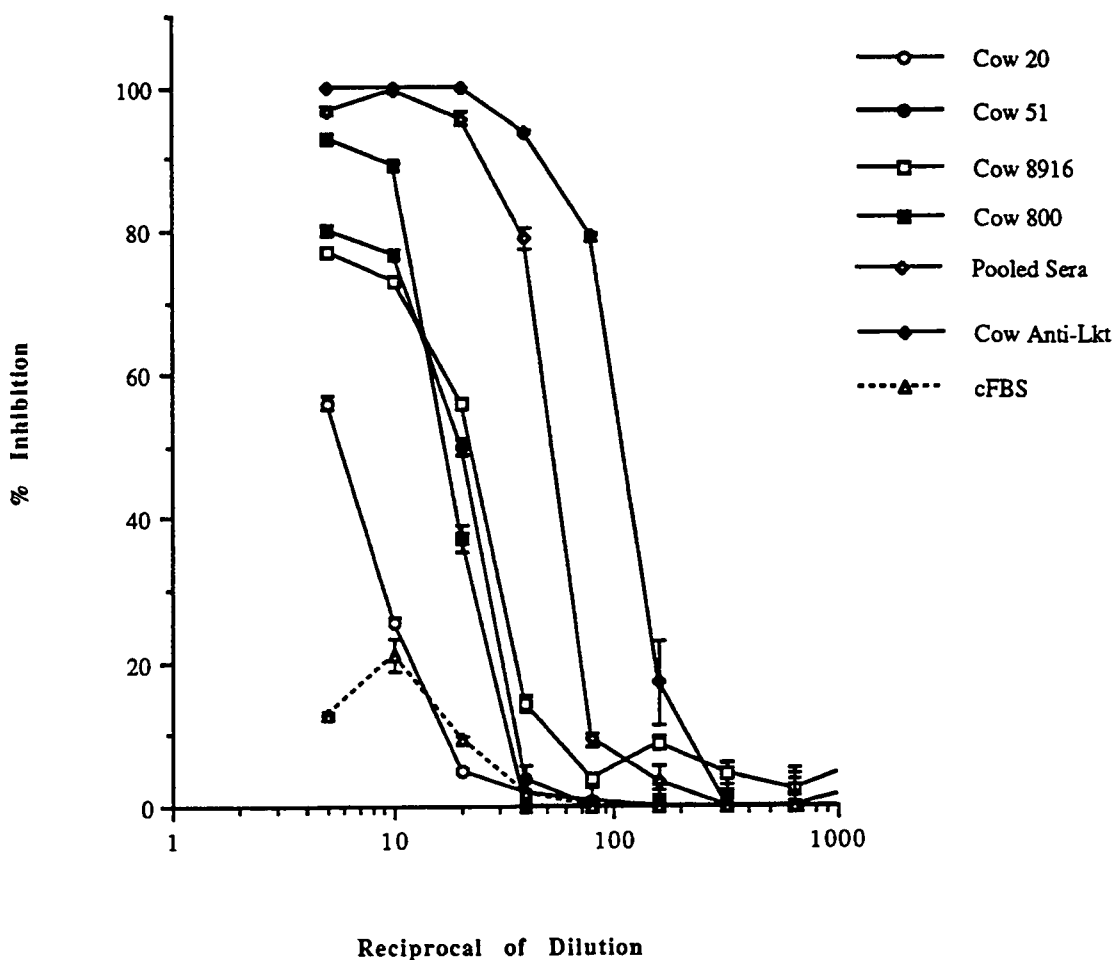


Figure 38. ELISA measuring inhibition of mAb ltx-2-HRP binding to trapped leukotoxin by unfractionated bovine antisera samples.

dilution of mAb ltx-2-HRP was then applied to each well. Percent inhibition at each protein concentration was calculated and results are shown in Figure 39. Fifty percent inhibition of mAb ltx-2-HRP binding occurred at 2 $\mu\text{g/ml}$ of mAb ltx-2. By extrapolating the reciprocal dilution at which the various bovine antisera inhibited 50% mAb ltx-2-HRP binding and multiplying this dilution by 2 $\mu\text{g/ml}$, the relative concentration of antibodies which recognized the mAb ltx-2 epitope was determined. These concentrations are listed in Table 7.

Abrogation of mAb ltx-2 and Cow Anti-Lkt Antiserum Protection of BL-3 Cells by Eluate 1.

A neutral red assay was performed to determine if preincubation of mAb ltx-2 or cow anti-lkt antiserum with eluate 1 antibodies would abolish protection of BL-3 cells. Comparison of the results obtained from this experiment may help to indicate the percentage of antibodies which recognize the mAb ltx-2 leukotoxin epitope in the bovine this antiserum sample. Both mAb ltx-2 and the cow anti-lkt antiserum were adjusted to the appropriate concentration or dilution which gave 50% inhibition of mAb ltx-2-HRP binding. These samples were incubated with varying dilutions of eluate 1 obtained from the mAb ltx-2 column. After an overnight incubation, 1 U of leukotoxin was added and a 1 h incubation on ice followed. BL-3 cells were added and percent cytotoxicity in each well was determined. Eluate 1 antibodies abolished mAb ltx-2 protection of BL-3 cells and had a titer of 6 $\mu\text{g/ml}$ (Figure 40). However, no abolishment of cow anti-lkt antiserum protection of BL-3 cells was observed following incubation with eluate 1 as can be seen in Figure 41.

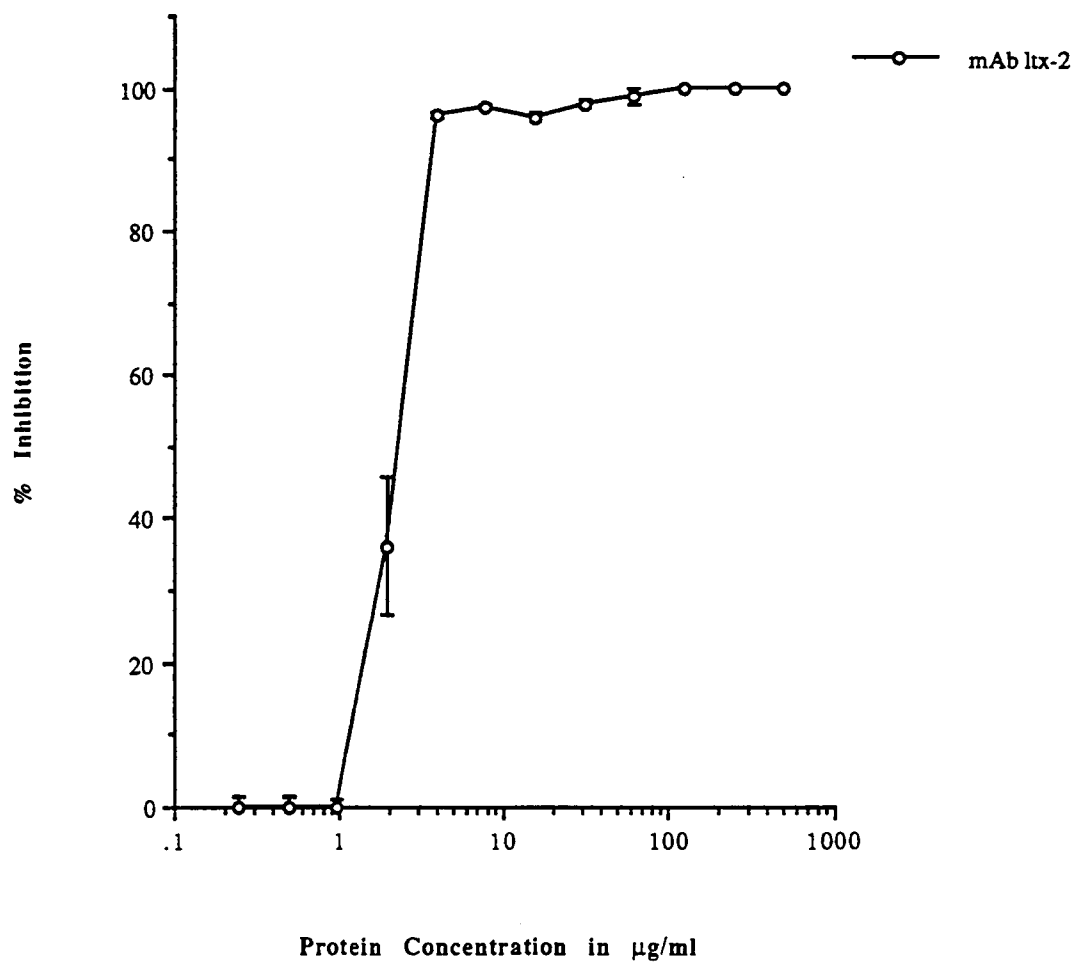


Figure 39. Inhibition of mAb ltx-2-HRP binding to trapped leukotoxin by mAb ltx-2 IgG measured by ELISA.

Table 7. Protein concentration of antibodies in bovine antisera samples which recognize the same leukotoxin epitope as mAb ltx-2.

Antiserum Sample	Protein Concentration ($\mu\text{g/ml}$)
Cow 20	13
Cow 51	40
Cow 8916	44
Cow 800	36
Pooled Sera	80
Cow Anti-Lkt	220

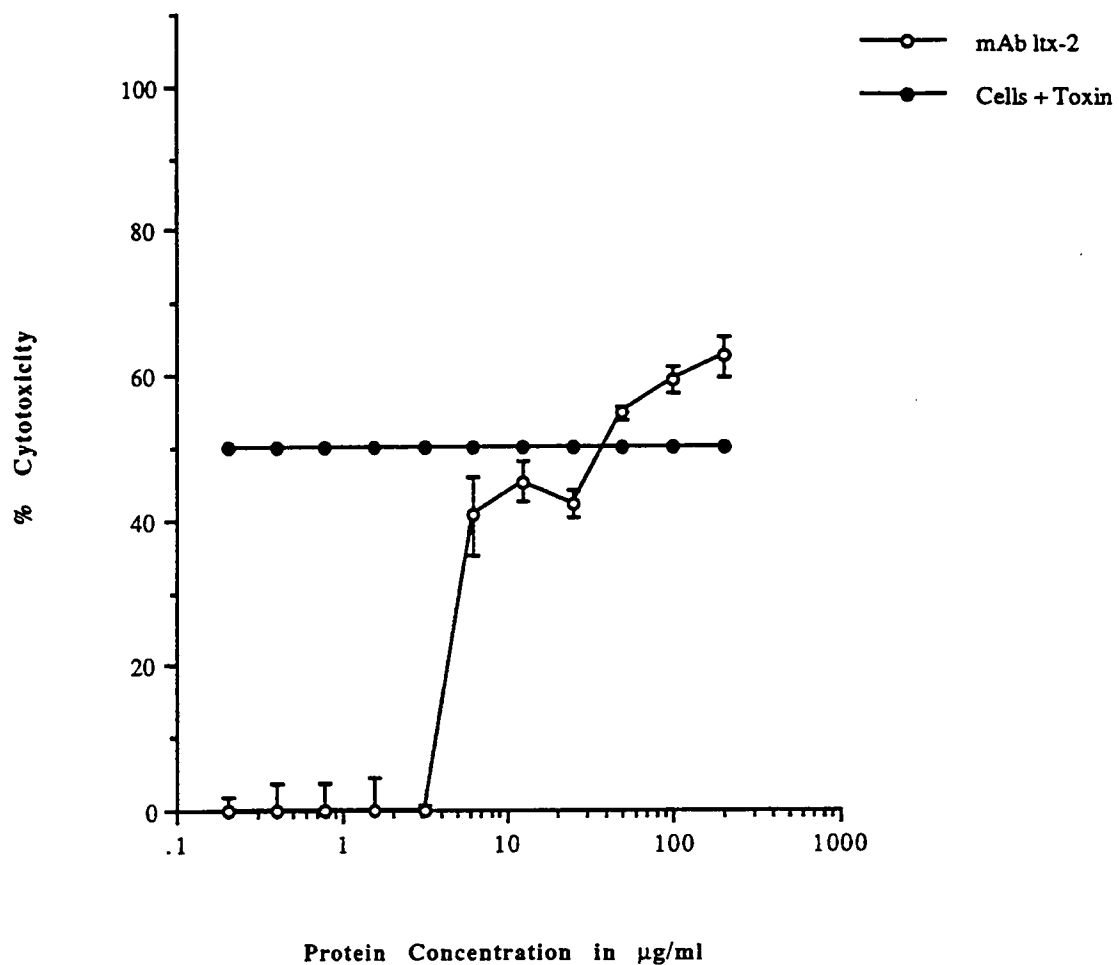


Figure 40. Abrogation of mAb ltx-2 protection of leukotoxin-mediated BL-3 cell lysis by preincubation with antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from mAb ltx-2 immunized rabbits over the mAb ltx-2 column as measured by the neutral red cytotoxicity assay.

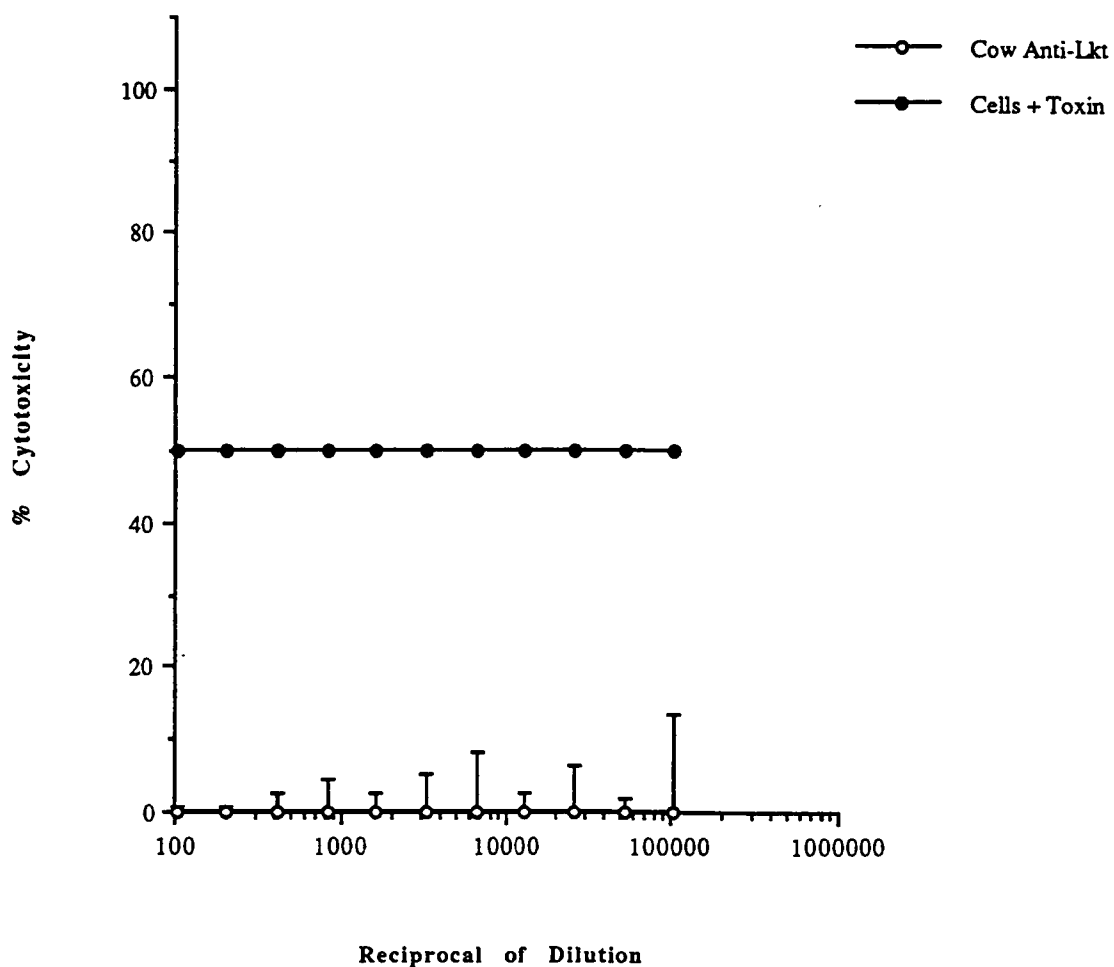


Figure 41. Neutral red cytotoxicity assay measuring abrogation of cow anti-lkt antiserum protection of leukotoxin-mediated BL-3 cell lysis by preincubation with antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from mAb ltx-2 immunized rabbits over the mAb ltx-2 column.

Neutralization of Leukotoxin by Cow Id.

Cow anti-lkt antiserum was 50% ammonium sulfate precipitated and antibodies which recognized the mAb ltx-2 leukotoxin epitope were purified following sequential passage of the immunoglobulins over a nonspecific rabbit IgG column and over an Ab2 β column. The antibodies contained in the eluate (cow id) were tested for their ability to neutralize leukotoxin-mediated cell lysis by a neutral red assay. Cow id and mAb ltx-2 were preincubated with leukotoxin and BL-3 cells were added. Percent cytotoxicity in all wells was determined. Results indicated that although neutralization of the leukotoxin was observed with the cow id, the specific activity for these antibodies was much lower (300 μ g/ml) than that obtained with mAb ltx-2 (0.35 μ g/ml) (Figure 42).

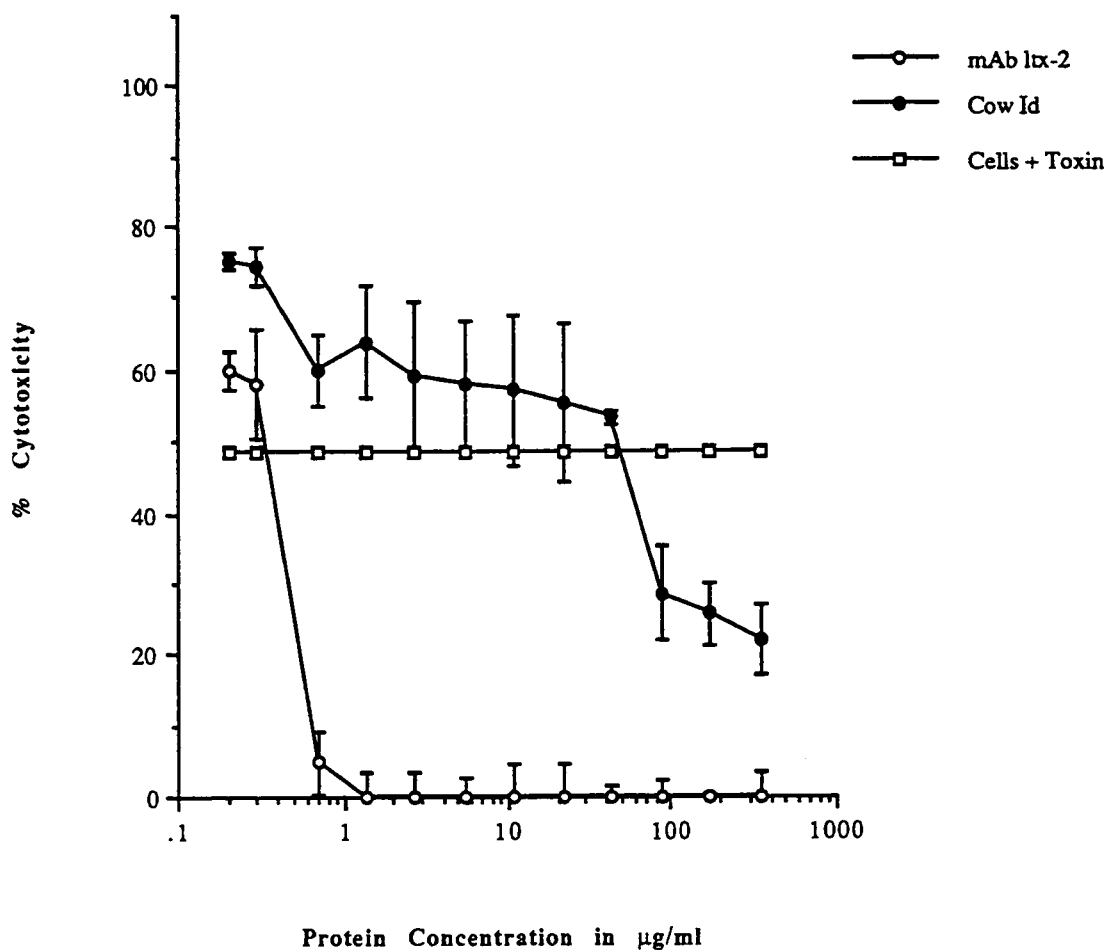


Figure 42. Neutralization of leukotoxin-mediated cell lysis by mAb ltx-2 and antibodies isolated by passing ammonium sulfate precipitated antibodies obtained from bovines immunized with leukotoxin over the Ab2 β column.

CHAPTER 4

DISCUSSION

In recent years, monoclonal antibodies have been used to characterize functional domains and three-dimensional structures within bacterial protein toxins (61, 76). Included as members of this family are the α -hemolysin of *E. coli* and the leukotoxin of *P. haemolytica*. Monoclonal antibodies, along with genetic studies, have localized regions within the α -hemolysin which are important for the activity of this protein (107, 110). In addition, deletion analysis of the leukotoxin molecule has helped to identify functional regions within this protein (29). The use of monoclonal antibodies may help to further characterize and understand the leukotoxin protein.

Mark Cameron and Donald Gerbig of this laboratory generated a panel of mAbs against the 102 kDa leukotoxin protein. Six mAbs resulted from this fusion. All the mAbs recognized the leukotoxin protein in concentrated leukotoxic filtrate by ELISA and Western immunoblot determinations. Initial neutral red cytotoxicity assays which assessed leukotoxin-neutralizing potential of these mAbs were performed with hybridoma supernatant fluid. One mAb, designated mAb ltx-2, was found to be a potent neutralizer, acting apparently by preventing the leukotoxin from associating with target cells (48, 49). In the present study, however, when purified IgG from the remaining mAbs were tested in the cytotoxicity assay, three additional mAbs were identified as leukotoxin neutralizers. Most likely, the initial assays with these mAbs did not show neutralizing activity because the protein concentrations of the mAbs in the hybridoma supernatant fluid were not sufficient to provide protection against leukotoxin-mediated cell lysis. Therefore, although mAb ltx-2 is the most potent neutralizer of leukotoxic activity, mAbs ltx-4, ltx-

35, and ltx-37 should also be classified as neutralizing mAbs.

Pellett et. al. (110) recently described the identification of a mAb which recognized only the activated form of the *E. coli* α -hemolysin. Binding of this mAb to the activated α -hemolysin completely eliminated hemolytic activity of the protein. The epitope for this mAb was located between amino acids 626-726. Consequently, it was concluded that HLYC-mediated activation of HLYA occurred within this region. The location for LKTA activation has not yet been identified. Initial experiments in this study were designed to determine if LKTC modification of LKTA was necessary for recognition by the leukotoxin mAbs. The unactivated and activated *A. pleuropneumoniae* serotype 5 toxins were also tested in these experiments. As demonstrated by Western immunoblots, all of the leukotoxin mAbs, including mAb ltx-2, recognized both the unactivated and the activated forms of the leukotoxin molecule. Therefore, the epitopes recognized by these mAbs are not dependent upon the activation of LKTA by LKTC.

Neither form of the *A. pleuropneumoniae* serotype 5 toxin was recognized by the leukotoxin mAbs. This suggested that epitopes present in the leukotoxin were either not available for antibody recognition or were not present within the *A. pleuropneumoniae* serotype 5 toxin. This was interesting because this toxin is also a member of the RTX family of toxins and has a structure similar to that of the leukotoxin. Although the two toxins share many similar structural features, it is clear that they also possess sequence or conformational variations which differ. While the leukotoxin is specific for ruminant leukocytes, the serotype 5 toxin of *A. pleuropneumoniae* lyses both erythrocytes and a variety of nucleated cells, including ruminant leukocytes (98). Perhaps the sequence or conformational variations which contain epitopes recognized by the leukotoxin mAbs contribute to the target cell specificity of the leukotoxin molecule.

Results have suggested that productive target cell interaction requires LKTC

modification of LKTA (29). Therefore, it may be possible to immunize cattle with a subunit vaccine containing the unactivated LKTA protein, which would not bind effectively to target cells. Thus, antibodies could be formed against epitopes of the leukotoxin molecule without exposing animals to the crude leukotoxin. One concern with this method, however, is that activation of the leukotoxin may induce a conformational change in the protein or may result in an increased negative charge of the molecule, which does occur in the α -hemolysin system (62). Potentially important epitopes may become buried within the molecule or increased charge may prevent the immune system from interacting with an immunogenic epitope. The result may be that effective leukotoxin-neutralizing antibodies may not be formed. This, however, does not appear to be the case with either of the epitopes recognized by mAbs ltx-2 and ltx-4 which react with both activated and unactivated leukotoxin. A key future experiment will be to determine if an immune response can be elicited to these epitopes using unactivated leukotoxin molecules or epitope containing polypeptide fragments.

Previous studies with leukotoxin deletion mutants resulted in the preliminary localization of the epitopes recognized by the leukotoxin mAbs between amino acids 768-939 (48, 49). Additional studies utilizing cyanogen bromide cleavage and alkaline phosphatase fusion proteins indicated the epitope neutralized by mAb ltx-2 was located between amino acids 918-939 (48). Three drawbacks to these studies exist, however. One is that deletion of large portions of the leukotoxin molecule may have influenced the folding of the protein such that the mAb ltx-2 leukotoxin epitope was no longer accessible for binding. Similarly, the fusion of a large enzyme to the leukotoxin molecule may have sterically inhibited mAb ltx-2 from binding to the epitope. Also, the addition of the enzyme to the leukotoxin protein may have altered the tertiary structure of the leukotoxin so that critical epitopes were no longer exposed. An alternative approach for mapping the

epitopes recognized by the leukotoxin mAbs was needed.

Previous results indicated none of the leukotoxin mAbs recognized the *A. pleuropneumoniae* serotype 5 toxin. Therefore, chimeric toxin molecules, composed of the *P. haemolytica* leukotoxin and the *A. pleuropneumoniae* serotype 5 toxin, were used to identify the domain of the leukotoxin recognized by the leukotoxin mAbs. According to Western immunoblot analysis, mAb ltx-2, as well as the other leukotoxin mAbs, recognized epitopes contained only within domain 3 of the leukotoxin molecule. This domain was represented by pYFC19, LLL; pDM15, AAL; pDM28, ALL; and pDM30, LAL. Domain 3 contains amino acids 768-939 and it is noteworthy that at least two glycine-aspartic acid rich repeat sequences, which have been implicated in target cell interaction, are included within this region. The results obtained with these mAbs substantiate results previously reported which tentatively identified the region between amino acids 768-939 as containing the epitopes recognized by each mAb (48, 49).

It can be inferred from these results that immunization of cattle with amino acids 768-939 of LKTA may induce leukotoxin-neutralizing antibodies. The antibodies formed against the mAb ltx-2 leukotoxin epitope would function by preventing crude leukotoxin from associating with ruminant leukocytes. This approach may not be successful, however. The peptide corresponding to amino acids 768-939 may assume a completely different conformation by itself in solution than does the intact leukotoxin molecule. Thus, the epitopes may not be presented in an optimal manner or even at all to the immune system of the vaccinated animals. Additionally, the entire leukotoxin molecule may be required for maximal stimulation of an immune response to this protein.

Additional studies need to be performed with the leukotoxin in order to further characterize and understand functional domains in the molecule. For example, studies have indicated HLYC-mediated activation of HLYA is due to acylation of the inactive

hemolysin molecule (62). Although it is believed that activation of all RTX toxins occurs by acylation, it is not known with certainty if LKTC activation proceeds in a similar manner. Binding of the leukotoxin mAbs to LKTA was not dependent upon activation by LKTC. In order to more fully investigate the region activated by LKTC, additional mAbs could be generated or proteins with deletions in the region N-terminal to the glycine-aspartic acid rich repeats could be used. The actual mechanism of action of the leukotoxin protein at each of the functional domains needs to be investigated.

Another question which needs to be examined is related to the target cell specificity of the leukotoxin. While the leukotoxin is specific for ruminant leukocytes (3, 68, 120), other members of the RTX toxin family are not as restricted with regard to the cells they lyse. It is not known what allows for the specificity of the leukotoxin. It is known, however, that this specificity is controlled by the LKTA protein, not the LKTC protein. Perhaps ruminant leukocytes possess a leukotoxin receptor on the cell membrane which non-ruminant cells lack. Alternatively, non-ruminant leukocytes may have cell surface determinants which block leukotoxin attachment (38). In each scenario, however, LKTA must possess a domain which functions in target cell recognition. It has been demonstrated that the mAb ltx-2 epitope is involved in target cell binding (48, 49). Although it is tempting to speculate that this epitope is involved in imparting target cell specificity to LKTA, it has been shown that the epitope for mAb ltx-2 recognition is present in other members of the RTX family. mAb ltx-2 reacts with the *E. coli* α -hemolysin and the toxins of *A. pleuropneumoniae* serotypes 1, 6, and 7. These toxins are not specific for a cell type of a specific species and lyse both red blood cells as well as leukocytes. This suggests that the epitope for mAb ltx-2 recognition is not critical in defining the target cell specificity of LKTA. However, further investigations with the remaining leukotoxin mAbs may provide some insight into the region which is

responsible for target cell specificity.

It is essential that the mAb ltx-2 leukotoxin epitope be identified. This epitope appears to be involved in binding of the leukotoxin to target cells and is present in the toxins from other RTX family members. Several methods could be utilized to accomplish the task of localizing the mAb ltx-2 epitope on the leukotoxin. It is possible that additional hybrid proteins could be constructed which would be varied only in the region of amino acids 768-939. Additionally, epitope mapping kits are available which localize protein epitopes based on recombinant DNA technology. Cloning of the amino acid sequence 768-939 into a plasmid and then sequentially removing amino acids from either the N-terminal or C-terminal ends until reactivity with mAb ltx-2 by Western immunoblot is lost may also help to identify the mAb ltx-2 epitope. The possibility exists, however, that mAb ltx-2 recognizes a conformational epitope within amino acids 768-939. Although mAb ltx-2 recognized the leukotoxin following SDS-PAGE analysis, it is possible that full denaturation of the molecule did not occur prior to separation. If mAb ltx-2 does recognize a conformational epitope, localization of an exact amino acid sequence may never be accomplished.

One possible method of further examining the mAb ltx-2 leukotoxin epitope is with the use of anti-idiotypic antibodies. Three types of anti-idiotypic antibodies are commonly discussed. Ab2 α antibodies are directed against epitopes located distal to the combining site of the Ab1. Ab2 γ antibodies are antibodies which recognize epitopes close to the combining site of the Ab1, but are not actually against the combining site. These antibodies may sterically hinder the Ab1 from binding to antigen. Ab2 β antibodies are antibodies which are made against the combining site of the Ab1. These antibodies are internal images of the antigenic epitope which the Ab1 recognizes. The combining site of these antibodies may have similar actions as the antigen and may have similar

ligand-receptor interactions as the antigen (30, 31, 67, 71, 106). In our system, Ab2 β antibodies against mAb ltx-2 could be used in place of the leukotoxin because, unlike the leukotoxin, they are heat stable, do not lose activity, and can be generated in relatively large quantities. Experiments were designed to generate polyclonal anti-idiotypic antibodies against a leukotoxin-neutralizing mAb, to isolate Ab2 β antibodies, and to subsequently produce leukotoxin-neutralizing Ab3 antibodies.

Following the immunization of rabbits with mAb ltx-2 (Ab1) and collection of antisera, unfractionated antisera were tested for the ability to recognize mAb ltx-2 and mAb ltx-35 and for the ability to block mAb ltx-2-HRP binding to leukotoxin. Results indicated that Ab2 γ antibodies or Ab2 β antibodies were present in the samples. Using affinity chromatography, these Ab2 γ or Ab2 β antibodies were isolated from the antisera by both negative and positive selection techniques. Several experiments were performed in order to determine if the isolated antibodies were actually Ab2 γ or Ab2 β antibodies. Following these assays, it was concluded that eluate 1 from the mAb ltx-2 column most likely contained Ab2 β antibodies. However, the possibility that sterically hindering Ab2 γ antibodies were present still existed. The presence of Ab2 β antibodies could be proven if, following injection of these antibodies into mice, leukotoxin-neutralizing antibodies were generated.

Mice were injected with eluate 1 antibodies or rabbit IgG, were bled seven days after injection, and were sacrificed on day 63. Sera were tested for direct binding to leukotoxin and for protection of BL-3 cells from leukotoxin-mediated cell lysis. Following the ELISA determinations and the neutral red assays with the sera, it was concluded that injection of mice with eluate 1 antibodies had induced the production of leukotoxin-neutralizing Ab3 antibodies. Therefore, eluate 1 contained Ab2 β antibodies which resembled a neutralizable epitope on the leukotoxin molecule.

Previous evidence demonstrated that mAb ltx-2 neutralized the leukotoxin by preventing its association with BL-3 target cells (48, 49). This implied that the mAb ltx-2 leukotoxin epitope was involved in target cell binding. If that was the case, then the isolated Ab2 β antibodies should also be involved in this target cell interaction. If these isolated antibodies bound to target cells, then we could conclude that the mAb ltx-2 leukotoxin epitope was responsible for binding of the leukotoxin to target cells. These isolated antibodies could then be used to identify the presence of a leukotoxin receptor on target cells. The Ab2 β antibodies were tested in several different assays to determine if they were capable of interacting with BL-3 target cells. None of the experiments showed conclusively that the Ab2 β antibodies bound to target cells. This may indicate that mAb ltx-2 prevents leukotoxin from interacting with target cells simply by steric forces or by inhibiting possible conformational phase-shifts required for binding.

Subunit vaccines containing anti-idiotypic antibodies are an alternative method of generating immunity to predominantly protein antigens, such as the leukotoxin. In several studies, anti-idiotypic vaccines against bacterial protein toxins have been shown to induce protection following challenge with the protein toxin (85, 111, 112). Presumably, protection in these investigations was mediated by toxin-neutralizing antibodies, but the presence of these antibodies was never demonstrated. In another study, anti-idiotypic antibodies resembling diphtheria toxin did induce the production of toxin-neutralizing Ab3 antibodies (118). Since antibodies to the leukotoxin have been shown to correlate with resistance to experimental challenge with *P. haemolytica* (46, 100), it is possible that the Ab2 β antibodies generated in this study could be included as a component of a subunit vaccine against shipping fever.

Inclusion of these antibodies in a subunit vaccine could induce the production of leukotoxin-neutralizing antibodies in cattle. Based on available information (46, 100),

such leukotoxin-neutralizing antibodies should provide animals with increased resistance following challenge exposure with *P. haemolytica*. Typically, the injection of anti-idiotypes requires the use of a carrier protein in order to generate a maximal immune response (71). In this study, mice were injected with xenogeneic Ab2 β antibodies in FCA and FIA and a response was generated. It has been suggested that coupling of Ab2 β antibodies to a carrier may be unnecessary when immunizing xenogeneic species (135). While an immune response against xenogeneic epitopes on the Ab2 β antibodies may appear detrimental, these antibodies also serve a carrier and self-adjuvanting function and may enhance the immune response against the Ab2 β antibodies (111). Since the Ab2 β antibodies in this investigation were generated in rabbits, it is likely that xenoimmunizations of cattle with these antibodies would also generate an Ab3 response. A model in which mice demonstrate protection following challenge exposure to leukotoxin is not applicable in this study. Because ruminants are the only species susceptible to the leukotoxin, field trial vaccinations of these animals followed by *P. haemolytica* challenge are the only way to determine if these Ab2 β antibodies could provide enhanced protection against the development of shipping fever.

Numerous assays were performed with unfractionated bovine antisera samples to determine if antibodies which recognized the mAb ltx-2 epitope and which were therefore equivalent to mAb ltx-2 could be detected. These studies were designed to indicate if the mAb ltx-2 leukotoxin epitope was antigenic in bovines and if this epitope induced the production of antibodies which were predominant in the antisera. Each of the antiserum samples contained antibodies which recognized the leukotoxin and high titers were recorded for each. Each antiserum sample neutralized leukotoxin when tested in a neutral red cytotoxicity assay, which indicated that leukotoxin-neutralizing antibodies were present. When tested by competitive ELISA, each sample contained antibodies which

recognized the mAb ltx-2 epitope and which were thus equivalent to mAb ltx-2. This implied that the bovines had previously been exposed to the leukotoxin. However, this may also indicate that the antiserum contained antibodies which cross-reacted with the leukotoxin in the ELISA determination. The protein concentrations of these antibodies were then quantified. Preincubating the cow anti-lkt antiserum with Ab2 β failed to abolish cow anti-lkt antiserum mediated protection of BL-3 cells. Based on these results, it was concluded that the antibodies which recognized the mAb ltx-2 leukotoxin epitope made up less than 1% of the total leukotoxin-neutralizing antibodies in the antiserum. However, such antibodies could be isolated from the cow anti-lkt antiserum using an Ab2 β column. These isolated antibodies reacted in a manner similar to mAb ltx-2, although with a lower specific activity. Therefore, bovines are capable of generating antibodies which resemble mAb ltx-2 but these antibodies are clearly not a major portion of the overall anti-leukotoxin response.

In order to develop an effective vaccine against *P. haemolytica* and shipping fever pneumonia, more studies must be performed in order to identify which antigens and what structures of the antigens induce antibodies which provide resistance following challenge. Many in the field believe that vaccines which only induce the production of leukotoxin-neutralizing antibodies are sufficient in providing immunity against the disease. Others contend that vaccines consisting of multiple antigens, such as leukotoxin and LPS, reduce animals' susceptibility to infection. Finally, subunit vaccines composed of important antigenic determinants of a whole antigen have also been suggested. The information gained from this investigation may be useful when determining which components of the leukotoxin could be included in such subunit vaccines against shipping fever pneumonia.

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LIST OF REFERENCES

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