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I am submitting herewith a thesis written by Brian S. Ranger entitled "The localization of a putative target cell binding domain of the *Pasteurella haemolytica* A1 leukotoxin using a neutralizing single chain fragment variable." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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THE LOCALIZATION OF A PUTATIVE TARGET CELL BINDING DOMAIN OF THE *PASTEURELLA HAEMOLYTICA* A1 LEUKOTOXIN USING A NEUTRALIZING SINGLE CHAIN FRAGMENT VARIABLE

A Thesis

Presented for the

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Brian Shayne Ranger

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DEDICATIONS

This thesis is dedicated to my wife, Kelly, for always being there whenever I felt that all hope was gone I am eternally grateful for your patience, encouragement, and loving support Also, I would like to dedicate this work to the memory of my grandfathers, Olen S Selby and Everett L Garver You are my constant sources of motivation and inspiration

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ABSTRACT

Bovine pneumonic pasteurellosis is the resultant acute respiratory disorder that arises from a variety of primary physical and infectious stressors with subsequent infection of the lower respiratory tract by *Pasteurella* species, the most common isolate being *Pasteurella haemolytica* serotype A1 Although this microbe possesses several virulence determinants, the primary virulence factor is a secreted, ruminant-specific, pore forming cytotoxin called leukotoxin (LKT) As the name suggests, leukotoxin executes its effector function(s) on ruminant leukocytes By reducing the population of functional infiltrating phagocytes, leukotoxin-induced damage leads to promotion of bacterial proliferation in the lower respiratory tract Furthermore, leukocyte destruction enhances tissue injury via overexposure to proinflammatory agents Given the significance of this cytotoxin, several studies have been conducted to 1) characterize this molecule on a structure/function basis and 2) devise and implement preventative measures against it This laboratory has developed a panel of hybridomas secreting LKT-neutralizing monoclonal antibodies which includes a potent neutralizer, ltx-2 Previous studies have reported that ltx-2 prevents target cell association of LKT, and that the ltx-2-specific epitope(s) map(s) to the carboxy one-third of the molecule, a region which has not been demonstrated to be directly responsible for cytolysis The primary objectives of this study were. 1) develop and characterize a leukotoxin-specific recombinant antibody molecule derived from ltx-2 mRNA and consisting of only the variable regions of the light and heavy chains covalently linked by a flexible polyglycine linker, a single-chain

fragment variable (ScFv), 2) compare the leukotoxin recognition and neutralization tendencies of the ScFv and ltx-2 using *in vitro* analyses in an effort to further define the mode of action of ltx-2, and 3) test the ability of both the ScFv and ltx-2 to recognize a panel of glutathione-S-transferase (GST) fused N-terminally deleted leukotoxin constructs generated from the carboxy one-third of the LKT molecule in order implicate the importance of this region in target cell association A leukotoxin-specific 29.5 kDa ScFv was successfully constructed, isolated, and sequenced The recombinant molecule was stably expressed in an *Escherichia coli* bacterial cell line, readily detectable in both the culture supernatant and periplasm, and purified from periplasmic concentrate Furthermore, the ScFv molecule, although five times smaller in size than the native ltx-2 antibody and without the benefit of F_c stabilization, was shown to bind to leukotoxin and the GST-fused LKT deletional constructs in a fashion comparable to ltx-2 This study also demonstrated that the ScFv has the capacity to neutralize LKT However, unlike the LKT recognition assays, the ScFv was shown to be approximately five times less efficient at LKT neutralization than its native counterpart Finally, this set of experiments also revealed that 1) the region represented by amino acids 766-939 contains all of the structural elements necessary for ltx-2/ScFv recognition and 2) *in vitro*, ltx-2/ScFv recognition of this carboxyl region closely resembles that observed for full length, native leukotoxin

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LIST OF ABBREVIATIONS

A	absorbance
ABTS	2, 2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)
ADCC	antibody-dependent cell-mediated cytotoxicity
APX	*Actinobacillus pleuropneumoniae* toxin
BCA	bicinchoninic acid
BHI	brain heart infusion
BHV-1	bovine herpesvirus-1
BL-3	bovine lymphocyte 3 cell line
BRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
BVDV	bovine viral diarrhea virus
C	constant region
°C	degrees celcius
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity determining region
CFA	complete Freund's adjuvant
CFU	colony forming units

cGMP	cyclic guanosine monophosphate
C-terminal	carboxy-terminal
D	diversity segment
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dT	deoxythymidine
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetate disodium salt
ELISA	enzyme linked immunosorbent assay
endA	endonuclease A
EtBr	ethidium bromide
Fab	fragment of antigen binding monovalent
$F(ab')_2$	fragment of antigen binding bivalent
FBS	fetal bovine serum
Fc	fragment that crystallizes
Fv	fragment variable

g	gram
Gly/Asp	glycine/aspartic acid
$(Gly_4Ser)_3$	polyglycine flexible linker
GST	glutathione-S-transferase
H	heavy chain
HEL	hen egg lysozyme
HLY	hemolysin
HRP	horseradish peroxidase
IBR	infectious bovine rhinotracheitis
ICAM-1	intercellular adhesion molecule-1
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IL	interleukin
iNOS	inducible (calcium-independent) nitric oxide synthase
ip	intraperitoneal
IPTG	isopropyl-β-D-thiogalactoside
J	joining segment
kDa	kilodalton
L	liter or light chain
LB	Luria-Bertani medium
LB-G	Luria-Bertani medium with 20mM glucose

LFA-1	lymphocyte function associated antigen-1
LKT	leukotoxin
lktA	leukotoxin A gene
LPS	lipopolysaccharide
ltx	leukotoxin-specific monoclonal antibody
M	molar
mA	milliampere
mAb	monoclonal antibody
mg	milligram
mM	millimolar
ml	milliliter
mol	moles
mRNA	message ribonucleic acid
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NFDM	nonfat dry milk
ng	nanogram
nm	nanometers
nmol	nanomoles
N-terminal	amino-terminal
O antigen	antigenic polysaccharide chain
OD	optical density

OMP	outer membrane protein
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline with 0 05% Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
PI-3	parainfluenza-3
psg	penicillin, streptomycin, L-glutamine
PVC	polyvinylchloride
PVDF	polyvinyl difluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640 Medium
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase-polymerase chain reaction
RTX	repeats in structural toxin
ScFv	single chain fragment variable
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SOBAG	selective media containing 100μg/ml ampicillin and 2M glucose

SOBAG-N	selective media containing 100μg/ml ampicillin, 2M glucose, and 100μg/ml nalidixic acid
TAE	tris, acetic acid, EDTA
Taq	*Thermus aquaticus* DNA polymerase
TBE	tris, borate, EDTA
Tbp	iron-regulated outer membrane protein
TBS	tris buffered saline
TES	tris-HCl, EDTA, sucrose
TNF-α	tumor necrosis factor alpha
U	unit
μg	microgram
μl	microliter
μm	micrometer
μmol	micromoles
UV	ultraviolet
V	variable region
V_H	variable region of the heavy chain
V_L	variable region of the light chain
v/v	volume to total volume
x g	times gravity
2N	ltx-2
2xYT	double strength yeast extract tryptone medium

2xYT-AG	double strength yeast extract tryptone medium containing 100μg/ml ampicillin and 2% glucose
2xYT-AI	double strength yeast extract tryptone medium containing 100μg/ml ampicillin and 1mM IPTG
2xYT-AK	double strength yeast extract tryptone medium containing 100μg/ml ampicillin and 50μg/ml kanamycin
2xYT-G	double strength yeast extract tryptone medium containing 2% glucose

CHAPTER 1

INTRODUCTION

The Disease

The comprehensive investigation of bovine diseases, especially those caused by infectious agents, is of primary importance in minimizing substantial losses to the beef cattle industry The bovine respiratory disease complex has recently received considerable attention due to its significant detrimental impact on the health of cattle and the subsequent welfare of the industry This disease complex consists of three distinct clinical entities enzootic pneumonia of calves, atypical interstitial pneumonia, and shipping fever (162) Shipping fever, a bane to beef producers in North America and Northern Europe for several years (237, 285), is an acute respiratory disorder that is often associated with stockyards, feedlots, and the logistical practices of the cattle industry Since most of the cattle are exposed to at least one of these situations, shipping fever tends to be the most common, serious, and costly clinical syndrome within the complex (31, 88, 183, 261) Shipping fever is also known as pneumonic pasteurellosis due to its strong correlation with *Pasteurella* species in diseased animals (88, 162, 219) The disease is characterized clinically by fever up to 42° C, depression, anorexia, diarrhea, rhinitis, nasal discharge, coughing, increased lacrimation, dyspnea, increased heart rate, and referred pleuritic sounds in the anteroventral regions of the lungs (116, 126, 162, 285) Pathologically, the disease is marked by acute airway inflammation, massive fibrin

exudate resulting in fibrinous pleuritis and alveolitis, intravascular capillary thrombosis, lobular necrotic foci, and extensive macrophage and neutrophil involvement (126, 219, 263, 267, 279, 285) In infected animals, these clinical and pathological factors invariably lead to substantial weight loss, deterioration of overall fitness, and an increase in mortality (285) Death may result from hypoxemia, pulmonary necrosis, endotoxemia, shock, heart failure, or any combination of these factors (126) Because of significant reductions in marketable cattle, shipping fever is a tremendous liability to both beef producers and consumers who suffer severe economic repercussions and inflated prices respectively (84, 140, 285) Economic losses result from insufficient feed conversion, excessive shrinkage of herd populations, delayed marketing of animals, expensive treatment costs, and deaths (126) In North America alone, these losses may exceed one billion dollars annually (11, 107)

Etiology

The causes of bovine respiratory disease complex in general, and shipping fever particularly, have been extensively investigated Many contributing factors have been identified, but the mechanistic dynamics between these factors in causing the disease remain poorly understood In fact, McKercher once noted that there was "still no agreement as to the precise cause or causes of the syndrome, or general acceptance that it had been reproduced by experimental means"(183) Even though research has revealed much about this disease since McKercher's pessimistic conclusion, the precise etiological agents and their respective roles in pathogenesis as well as methods to

accurately reproduce the disease remain elusive and widely debated The general consensus, however, is that shipping fever is multifactorial and generally requires immunosuppression of the host. Therefore, any agent(s) that jeopardizes the immune status of the host may play an important role in induction of the disease state (285) Many factors, either independently or in conjunction with other predisposing factors have been shown to be involved in disease development, including stresses involved in transportation and animal management, viral infections, and opportunistic bacterial infections (84, 285)

First, both physical and environmental stresssors alter host immunity Due to the rigors of the shipping process, physical stresses are generally unavoidable Practices such as bleeding, vaccination, restraint, constant handling, dehorning, castration, and herding often impose excessive stress on calves (86, 126, 162) Additionally, the inconsistency involved with sudden herding exertion followed by close-quartered restraint results in anxiety, fatigue, and a state of altered immunity (25, 26, 141, 272) Even though physical stresses can compromise immunity in the host, sudden and radical changes in the immediate environment of cattle are often more devastating and exacerbate any physically-derived immunosupression These environmental shifts include irregular feeding and watering schedules, weaning, exposure to extremes in temperature, overexposure to dust, overcrowding, and intermingling with foreign individuals/herds (84, 132, 162, 285) The fact that these forms of stress compromise host immunity has been evidenced by a marked reduction in innate effector cells and increased susceptibility to systemic infection in recently shipped calves (161, 289)

In addition to physical and environmental stressors, infectious agents play a key role in altering the immunological state of the host Several viral agents have been implicated in compromising the host's nonspecific and specific immune defenses The result is a reduced capacity to efficiently clear bacteria from the lower respiratory tract accompanied with an increased risk of developing bacterial pneumonia (125) The virus which has been most associated with shipping fever is parainfluenza-3 virus (PI-3), which is highly correlated with pneumonic animals (35, 50, 89, 99, 127) Additionally, bovine respiratory syncytial virus (BRSV), bovine herpesvirus-1 (BHV-1, also known as infectious bovine rhinotracheitis virus, IBR (285)), bovine viral diarrhea virus (BVDV), adenoviruses, rhinoviruses, enteroviruses, bovine papular stomatitis virus, and respiratory coronavirus have all been implicated in the disease process (12, 80, 137, 213, 214, 244, 285)

Although the intricacies involved in the development of shipping fever may be highly variable from one affected individual to the next, it is widely accepted that viral-bacterial synergism is a common denominator A number of investigations have led to several plausible mechanisms involved with this synergy (33, 84, 285) First, viral infection (either productive or nonproductive) often results in direct damage to the respiratory tract, including the upper tract, lower tract, and alveolar epithelium, and associated immune effector cells (224). This damage is often manifested as impairment and/or death of ciliated epithelial cells, thus reducing the clearing efficiency of the mucociliary escalator BHV-1 (80), BRSV (12), BVDV (214), and especially PI-3 (35, 127, 137) all negatively affect ciliated epithelia of the respiratory tract and subsequent

bacterial clearance. Furthermore, both BVDV and BHV-1 cause generalized immunosuppression by altering immune effector cells BHV-1 inhibits the antimicrobial actions of bovine leukocytes, thereby affecting the ability of the host to clear microbes (80) Additionally, BVDV is thought to cause immunosuppression by affecting the gastrointestinal tract and lymphoid tissues, which leads to an overall impairment of peripheral immunity and a subsequent decrease in pulmonary resistance (213, 214) Second, viruses have been shown to enhance bacterial adherence and/or proliferation by altering levels of factors important to proper immunological function, especially fibronectin, a host substance that enhances phagocytosis by acting as an opsonin and/or macrophage stimulant (11) This disturbance is via release of viral proteases and/or host cell-derived proteases from virally infected regions In fact, viral infection has been correlated to an overall decrease in fibronectin levels (11) Additionally, destruction of cellular fibronectin may expose binding sites necessary for bacterial adherence, thereby paving the way for increased colonization and subsequent proliferation (11, 281) This is supported by the fact that colonization by Gram-negative bacteria coincides with reduced levels of fibronectin (281) Finally, viruses can often exert immunosuppressive effects by indirect means This is accomplished by inducing inflammation and the respective cytokine milieu The resultant cytokine profile elicited by viral infections may lead to an aberrant host response by reducing leukocyte activity, altering leukocyte migration, promoting tissue destruction, or diverting the responses necessary for bacterial clearance (11, 285)

With rare exception, neither viral infection nor stresses associated with the practices of the industry directly cause the high rates of morbidity/mortality associated with shipping fever. Rather their role(s) in the etiology of this disease is correlated with immunological impairment of the host, a prerequisite for bacterial colonization in the lower respiratory tract This bacterial opportunism is reiterated by three lines of evidence First, those microbes most often implicated in the development of pneumonia tend to be commensals with normal, healthy individuals, colonizing only the mucosal surfaces of the upper respiratory tract (84, 85) Second, unstressed calves inoculated, via aerosol or intratracheally, with a physiological dose of *P haemolytica* or other bacteria commonly isolated from pneumonic calves tend to remain subclinical or display short-lived symptomology only (83) In fact, several attempts to naturally reproduce the disease with bacterial preparations alone have proven unsuccessful (237, 285) Finally, contrary to the poor success of bacterially-induced reproduction of shipping fever, several experimental models have succeeded in recreating the disease, adhering to physiological realities, using stress-induced or virally-mediated approaches (83, 237, 239, 285)

In addition to the several viruses mentioned, many bacteria have also been implicated in the acute onset of shipping fever pneumonia *Pasteurella haemolytica*, *Pasteurella multocida*, *Haemophilus somnus*, *Actinomycetes pyogenes*, *Mycoplasma* species, *Corynebacterium* species, and *Chlamydia* species have all been isolated from the lungs of sick animals (59, 88, 111, 126, 285, 286) Because so many different species have been isolated, and often concurrently, the exact causative agent is enigmatic However, the most common isolate from pulmonary lesions of pneumonic calves

displaying shipping fever symptomology is *P haemolytica* A1 (51, 59, 85, 86, 88, 194, 285) Because A1 is so often isolated from pneumonic individuals, it is certainly implicated as a principal elicitor of the disease Typically, A1 is found in the nasopharynx and tonsils of healthy individuals where it exists as a commensal and does not detrimentally affect the host (84) Physical and environmental stresses compounded by opportunistic viral infections jeopardize the immune systems of transported cattle, thereby allowing massive proliferation of A1 in the upper respiratory tract As a result, the lungs are seeded by the aspiration of infected droplets into the lower respiratory tract, eventually resulting in pulmonary pathology and death if left untreated (59, 86) The disease is transmitted to other immunocompromised individuals via infected nasal secretions and/or aerosolized droplets (285) Because of the common isolation of this organism from diseased animals and its correlation to losses due to morbidity/mortality, contemporary research efforts have attempted to control the proliferation and spread of *P haemolytica* A1 and alleviate/eliminate its detrimental consequences.

Characterization of *Pasteurella haemolytica*

All organisms within the genus *Pasteurella* share the following characteristics: Gram-negative, pleomorphic (coccobacilli), non-motile, facultatively anaerobic, oxidase positive, catalase positive, and able to ferment several carbohydrates (3, 16, 22) Additionally, *P haemolytica*, the key focus of this investigation, exhibits weak alpha hemolysis when grown of 5% sheep blood agar (243). *P haemolytica* is divided into two biotypes based on carbohydrate fermentation, colony morphology, growth dynamics, and

antibiotic sensitivity (3) These two biotypes are categorized into seventeen distinct serotypes according to indirect hemagglutination techniques (3, 70) Biotype A is classified by the ability to ferment arabinose and consists of serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16, and 17, all appearing as small grayish colonies (3, 70, 288) Biotype T is characterized by the ability to ferment trehalose but not arabinose and consists of serotypes 3, 4, 10, and 15, all appearing as medium to large brown colonies (3, 21, 243) However, more contemporary serotype studies, based on 16s rRNA sequencing and DNA-DNA hybridization, have raised questions about this traditional classification scheme (7, 8) It has recently been proposed that the trehalose negative *P haemolytica* serotypes 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, and 16 should comprise a new genus called *Mannheimia* Within this genus, species are defined according to host specificity, serological reactivity, and genetic composition (7, 8) Additionally, it has been suggested that all of the serotypes which comprise the T biotype of *P haemolytica* be reclassified as a single new species, *P trehalosi* (70, 245) Despite this more contemporary classification, the traditional classification system has been adopted for this investigation

As with all potential pathogens, the ability to colonize and proliferate in the host is directly related to the number of virulence factors possessed by the invading microbe Virulence factors assist in evasion of host immunity, overcoming physical barriers, and providing nutritional support Accordingly, *P haemolytica* A1 possesses several virulence determinants, which have been characterized to varying degrees These virulence determinants include lipopolysaccharide (LPS)/endotoxin (61, 139, 222), capsular polysaccharide (4), adhesive fimbrae (191, 192), various outer membrane

proteins (70, 123, 204), a neuraminidase (87, 256, 260), a neutral sialoglycoprotease (157, 207), an IgG_1-specific protease (157), and a well-characterized potent leukotoxin (13, 135, 174, 234, 235)

Like the lipopolysaccharide possessed by other Gram-negative organisms, the LPS of *P haemolytica* consists of three components· a highly conserved glycophospholipid, lipid A, a core oligosaccharide, and the O antigen, an antigenic polysaccharide chain comprised of species/strain-specific sugar residues This multi-component molecular complex can account for up to 25% of the dry weight of the cell (139) Following colonization of the lower respiratory tract by *P haemolytica*, LPS elicits a potent inflammatory response by damaging vascular endothelium, inducing the formation of microvascular thrombi, and activating immune effector cells The magnitude of this inflammatory response is dependent on the dose of LPS At low doses, mononuclear phagocytes produce the acute-phase, pyrogenic cytokines IL-1 and TNF-α These two cytokines can elicit fever independently, but can also enhance synergistically *P haemolytica* LPS-induced endothelial cell damage (232) Additionally, LPS at higher doses can elicit a strong inflammatory response and alter vascular hemodynamics by causing microvascular and lymphatic thromboses (61, 278), which result from clotting cascades and platelet aggregation due to endothelial cell damage Pathologically, endothelial cell damage is marked by shape change, cell retraction, and increased membrane permeability (30, 210) Furthermore, endothelial cell damage results in the production of several other inflammatory mediators via activation of neutrophils, macrophages, platelets, and endothelial cells, including· thromboxane A_2,

prostaglandins, leukotrienes, serotonin, cAMP, cGMP, histamine, and nitric oxide (61, 77, 78, 226, 287) This cocktail of inflammatory molecules can exacerbate endothelial damage. Both histamine produced by mast cells and nitric oxide derived from alveolar macrophage inducible nitric oxide synthase (iNOS) in response to LPS may result in the rapid influx of neutrophils and serum proteins due to increased capillary permeability (5, 287) This rapid infiltration of circulating inflammatory factors contributes to respiratory edema and septic shock Finally, LPS enhances phagocyte migration patterns by activating the alternative pathway of the complement system, inducing the production of the potent chemokine IL-8, and causing the upregulation of ICAM-1 expression on endothelial cells Complement activation results in the production of the chemotaxin C5a which leads to extravasation of neutrophils into the LPS-affected tissues along a chemotactic gradient (229) IL-8 is a potent neutrophil chemoattractant which is expressed by a variety of effector cells especially during acute pneumonic episodes, and it has been shown to play a direct role in neutrophil recruitment and extravasation in individuals suffering from pneumonic pasteurellosis (37) One study concluded that IL-8 expression by alveolar macrophages is linked to TNF-α production (149) Lastly, upregulation of ICAM-1 expression by LPS-damaged endothelial cells correlates with increased neutrophil infiltration into infected tissues because this cellular adhesin serves as the receptor site for β_2 integrins found on circulating neutrophils Association of β_2 integrins with ICAM-1 molecules initiates neutrophil diapedesis and extravasation during the course of pneumonic pasteurellosis (217) These mechanisms which enhance neutrophil migration into the tissues not only contribute to the inflammatory milieu, but

also provide a potential target cell rich environment for the potent leukotoxin produced by *P haemolytica* (30, 240) Despite the multifaceted role of LPS in the induction and sustenance of inflammation during pulmonary infection, circulating antibodies against *P haemolytica* LPS do not generally correlate with disease resistance (54, 60, 84)

Another important virulence mechanism associated with *P haemolytica* A1 is a polysaccharide capsule produced during log-phase growth (3, 65, 86) It is widely accepted that organisms, both Gram-positive and Gram-negative, that produce capsules are better protected from phagocytosis, antibody-mediated opsonization, and complement-mediated bactericidal activity (39, 66, 265) Furthermore, the capsule of *P haemolytica* is thought to play a role in nonspecific adherence to nasopharyngeal, bronchiolar, and alveolar surfaces (32) The role of capsular polysaccharide as an important virulence determinant of *P haemolytica* is supported by two lines of evidence First, experimentally derived capsule deficient strains of *P haemolytica* show enhanced susceptibility to phagocytosis, opsonization, and complement-mediated killing, compared to those with capsular material (39) Additionally, *P haemolytica* recovered from the tracheas of infected calves tend to have capsular material much thicker than that seen for organisms grown in culture, which suggests that this structure is important for *in vivo*, but not *in vitro*, survival (192) The second line of evidence is that antibodies against capsular material are correlated to resistance during experimental challenge with *P haemolytica* (62)

Although the capsule may provide a mechanism of nonspecific adherence to mucosal surfaces, it has been demonstrated that *P haemolytica* possesses filamentous

structures called fimbrae (or sometimes called pili) which allow for more specific adherence Although fimbrae are not found on all bacterial species, they have been widely reported as important virulence factors in many Gram-negative infections (105, 142, 180) Because fimbrae tend to be highly specific for eukaryotic cell surface ligands, especially those on epithelial cells, bacteria that possess them may more readily resist innate mechanisms of clearance in the upper and lower respiratory tracts (22, 54, 191). In *P haemolytica*, two distinct fimbrial stuctures recovered from pulmonary lavage fluid of live infected calves and *in vitro* cultures have been identified, each being 10-12 nm in length and 5 nm in diameter (191, 192) Although the discovery of these structures implicates a specific attachment to respiratory epithelium, no specific cellular ligand(s) has been identified As a result, the functional significance of *P haemolytica's* fimbrae remains undefined

In addition to the cell-associated virulence factors previously discussed, *P haemolytica* possesses several outer membrane proteins (OMPs) which may also assist in virulence either directly or indirectly (70, 123, 204) Although several different OMPs have been implicated as potential virulence determinants, seemingly the most important are the 105kDa and 70kDa bovine-specific transferrin receptors, TbpA and TbpB (204, 215) Because of the necessity of iron for normal cellular/metabolic processes, both the host and any microbial flora present compete for free iron Typically the host produces iron-scavenging proteins, such as transferrin, to chelate free iron and shuttle it to host cells via specific receptor interactions However, bacteria that have devised strategies to pirate free and transferrin-bound iron, including siderophore production and molecular

mimicry, have the capacity for increased growth rates and virulence (274) Both TbpA and TbpB have the ability to scavenge iron from 30% saturated bovine transferrin, but not from transferrin derived from other species including pigs, horses, and humans (71, 204) Loss or alteration of these transferrin receptors *in vivo* may reduce the ability of *P haemolytica* to elicit disease (204) Furthermore, these OMPs collectively provide some protection against experimentally induced bovine pneumonic pasteurellosis, suggesting that either TbpA, TbpB, or both enhances virulence (215) Although these two OMPs are the best characterized, it has been suggested that several other OMPs isolated via membrane preparation/purification may play roles in neutrophil modification/chemotaxis (123), host specificity (70), and/or nonspecific adhesion to mucosal surfaces (166, 247) However, like several other potential virulence factors manipulated *in vitro*, OMPs may undergo modified expression and/or alteration of function (52, 71) As a result, the precise role(s) of OMPs and their contribution to virulence are unclear

Although *P haemolytica* has several virulence determinants attributed to cellular ultrastructure, several soluble proteins which are suspected to play a role in virulence have been isolated and identified These soluble virulence factors include a neuraminidase, a neutral sialoglycoprotease, an IgG_1-specific protease, and a potent cytotoxin specific for bovine macrophages and neutrophils called leukotoxin First, the neuraminidase of *P haemolytica* is believed to facilitate the adherence of the bacterium to respiratory epithelium via cleavage of fibronectin, a component of the eukaryotic extracellular matrix which may shield putative binding sites (87, 260) The fact that neuraminidase activity is substantially higher in serotype A1 than in a less virulent

serotype, A2, suggests that this enzyme is correlated to virulence (87) The second soluble virulence determinant associated with *P. haemolytica* A1 is a neutral sialoglycoprotease, an enzyme that may be responsible for platelet activation, platelet clumping, and the subsequent fibrin deposition observed in diseased cattle (202) Furthermore, other Gram-negative bacteria associated with pneumonia, including *Legionella pneumophilia* and *Pseudomonas aeruginosa*, produce proteases which exhibit cytotoxic activity for alveolar macrophages (84, 156) This macrophage targeting by the sialoglycoprotease of *P haemolytica* A1 has not been directly demonstrated However, it has been established that this protease is highly specific for proteins having O-linked glycosides attached to serine or threonine (2) Lastly, a recent study has revealed that *P haemolytica* A1 may secrete an IgG-specific protease (157) This protease, isolated from partially purified culture supernatant, was shown to hydrolyze IgG_1 into three distinct fragments of 39, 12, and 7 kDa after six hours of exposure Furthermore, partial hydrolysis of IgG_2 was noted However, neither IgM nor IgA were hydrolyzed (157) Nevertheless, proteolysis of these IgGs, both major secretory immunoglobulins, could certainly quench the potency of the host's specific immune response Despite isolation and/or partial characterization of this set of soluble virulence determinants, little is known about their relative importance during the course of pneumonic pasteurellosis

The most important virulence factor produced by *P haemolytica* is a potent, chromosomally encoded leukotoxin (LKT) Leukotoxin is an exotoxin which is remarkably specific for ruminant leukocytes including alveolar macrophages, blood monocytes, neutrophils, and to a lesser extent lymphocytes and platelets (13, 17, 48, 135,

174, 234) As a cytotoxin, LKT damages the membranes of susceptible cells by forming small transmembrane pores 0 9-1 2 nm in diameter (47) This aspect of LKT activity is highly dependent on the availability of calcium, and toxin/calcium association is a prerequisite for target cell binding (27, 28) After initial association of LKT with the target cell, the cation-selective pores are quickly formed in the membrane Subsequently, these pores act as channels for the rapid efflux of potassium ions and influx of calcium ions However, the small pore size prevents the leakage of cytoplasmic proteins (47) Affected cells swell and eventually rupture due to the loss of cytoplasmic organization and nuclear integrity (13, 17)

Although the pore formation mechanism is regarded as the principal means of cytolysis, recent studies have revealed that LKT may also induce apoptosis at sublytic concentrations further contributing to the depletion of immune effector cells (257, 259, 268) Apoptosis is evidenced by target cell membrane blebbing, phosphotidylserine translocation from the inner leaflet to the outer leaflet of the cell membrane, chromatin condensation, internucleosomal DNA fragmentation, and poly(ADP-ribose) polymerase cleavage (250, 268) It is believed that LKT-induced apoptosis may prolong bacterial survival and allow for sustained colonization (292)

Regardless of the mechanism of cytolysis, be it pore formation or apoptosis, it is believed that LKT exerts its cytotoxic effects via the β_2 integrin LFA-1 (lymphocyte function associated antigen-1) (131, 160, 268). LFA-1 is composed of two subunits called CD11a and CD18 Studies using mAbs directed against each subunit have shown that blocking of either subunit diminishes toxin association, however, CD18 is seemingly

of greater importance for LKT association (131, 160) The importance of this cellular ligand in LKT binding is reiterated by the fact that the magnitude of target cell cytolysis is directly related to LFA-1 expression (131) Furthermore, a recent report has concluded that recombinant IL-1β upregulates LFA-1 expression, and that this enhanced integrin expression amplifies the effects of partially purified *P haemolytica* leukotoxin on bovine neutrophils (158) Because LFA-1 is a common mammalian leukocyte ligand, LKT associates with leukocytes derived from a number of mammalian species including non-ruminant equine, porcine, canine, and human cells (153, 258) However, only ruminant leukocytes seem to be susceptible to LKT intoxication and subsequent cytolysis (258) The mechanisms which govern the remarkable specificity of LKT's effector function(s) remain enigmatic

Because leukotoxin-induced destruction of respiratory tract leukocytes impairs primary phagocytic lung defenses and diminishes immune-mediated clearance of *P haemolytica*, this molecule certainly promotes survival and proliferation of the organism. However, the pathological implications of LKT-induced cytolysis are much more comprehensive The rapid influx of calcium following pore formation is associated with increased, unregulated neutrophilic degranulation, superoxide generation, leukotriene synthesis, proteolytic enzyme production (elastase and collagenase),and phospholipase activation (110, 206, 240, 269, 270). The extracellular presence of these molecules may activate the alternative complement pathway and/or induce severe inflammation, resulting in extensive damage to the respiratory epithelia (240) Although LKT acts on several types of cells, studies show that respiratory tract damage is primarily caused by

neutrophils (240) The role of LKT-induced neutrophil cytolysis in respiratory injury is substantiated by three lines of evidence First, calves depleted of neutrophils by preliminary hydroxyurea treatment tend to be resistant to the effects of intratracheal challenge with live *P haemolytica* A1, while normal calves develop severe necrotizing pneumonia (240, 241) Second, mutant strains of *P haemolytica* deficient only in the production of functional LKT fail to establish clinical disease (91) Finally, the significance of this molecule in the disease process is underscored by the fact that animals resistant to experimental challenge with *P haemolytica* (as well as those recovering from pneumonic pasteurellosis) have detectable anti-leukotoxin antibody titers in lung secretions (84, 94)

Leukotoxin is secreted from actively growing cells during logarithmic growth of *P haemolytica*, reaching peak concentrations at 6-8 hours post-inoculation (13, 41, 235) Although LKT is a protein (115), carbohydrate moieties are implicated since amylase treatment of cellular supernatant abrogates LKT activity (41) Like any other macromolecule, there are a number of environmental parameters which influence the stability of LKT's structure/function LKT is oxygen stable but extremely heat labile, with complete loss of cytolytic activity occurring within 2-3 hours at room temperature (13, 41, 115) Given the remarkable sensitivity to temperatures above 4° C, purification and advanced structural characterizations have not been possible Reports on sensitivity to pH extremes vary (13, 41) Furthermore, lipopolysaccharide may contribute to LKT stability Reportedly, LKT/LPS complexes tend to exhibit enhanced thermal stability and

leukolytic activity over LKT independently (159) Finally, bovine serum albumin has been shown to enhance leukotoxic activity (271)

In order to develop a more complete understanding of LKT and the mechanisms by which it works, several research endeavors have focused in on the molecular characterization of the molecule including transcriptional regulation, DNA sequencing, and homology studies The gene encoding LKT has been cloned and sequenced, the gene product composed of 953 amino acids with a molecular weight of 101 9 kDa (163, 164) However, due to a strong tendency to aggregate and different purification techniques, molecular weight determinations of LKT have been highly variable, ranging from 150 kDa to greater than 700 kDa (13, 115, 139, 196) Analysis of the genetic organization of the leukotoxin-encoding sequence suggests that this molecule is a member of the RTX (Repeats in Structural ToXin) family of toxins (112, 164) Furthermore, in a fashion typical of the RTX toxins, the LKT gene cluster is arranged into a polycistronic operon in the order C, A, B, D, where A is the structural gene (112, 164) Transcription of this operon, and subsequently expression of LKT, is tightly regulated and is dependent on several environmental factors including temperature, pH, and iron concentration (95, 254) In addition to genetic organization, RTX toxins all share some common characteristics including Gly/Asp-rich nine amino acid tandem repeats which number from six to forty-one depending on the toxin (101), mode of toxin activation, and mechanisms of secretion (276) Other members of the RTX family include the prototypical α-hemolysin of *E coli* (19, 38, 255); the leukotoxins of *Actinobacillus pleuropneumoniae* (43) and *Actinobacillus actinomycetemcomitans* (146, 151, 152), the

adenylate cyclase of *Bordetella pertussis* (102), and the hemolysins produced by *Morganella morganii* (76, 144), *Proteus mirabilis* (144, 275), and *Proteus vulgaris* (144, 165, 255, 275)

Because the alpha-hemolysin of *E coli* is the best characterized member of the RTX family, it serves as the prototype for defining the mechanisms associated with the secretion, activation, and cytolytic activity of the RTX toxins (255) Likewise, due to the apparent similarity in toxin mechanisms (164), the α-hemolysin convention is adopted for LKT despite some outstanding differences such as target cell specificity Unlike LKT, HLY is cytotoxic for a wide variety of cell types including erythrocytes, leukocytes, and renal tubular cells and is not species specific (18, 19, 138) The four genes which make up the LKT operon are designated *lktC*, *lktA*, *lktB*, and *lktD* to conform to the α-hemolysin nomenclature (*hlyC*, *hlyA*, *hlyB*, and *hlyD*) Respectively, these genes encode protein products designated LktC, a posttranslational acylase, LktA, the structural toxin, LktB and LktD, both of which are involved in transporting the toxin out of the cell

First, the 101 9 kDa LktA structural toxin shares 36 4% homology with HlyA, which is composed of 1,023 amino acids and has a molecular weight of 107 kDa (164, 169, 170) Despite the low overall homology, the two molecules are highly homologous over two of the four functionally defined regions (255) It is presumed that these regions of greater homology account for the ability of HlyB and HlyD to modify and secrete LktA as an active molecule in an *E coli* background (42) The first of the related regions is the N-terminal one-third of each protein For each molecule, this region contains

clusters of hydrophobic domains thought to be involved in the intimate association of the toxin with the target cell membrane and subsequent pore formation (47, 165). Studies have indicated that this region of HlyA acts by forming a single hydrophilic transmembrane pore 3 nm in diameter in the target cell membrane (20, 67, 165, 167, 168) The aforementioned 0 9-1 2 nm pores formed by LKT have been attributed to the respective hydrophobic-rich domain of LktA (47)

The carboxyl one-third of both HlyA and LktA comprises the other highly homologous functional domain This region contains the tandemly repeating Gly/Asp sequences, with the sequence Leu-X-Gly-Gly-X-Gly-Asn-Asp-X repeated eleven times in HlyA and six times in LktA (167, 255) Calcium binding studies indicate that these repeats form a putative motif which strongly binds calcium, resulting in a conformational change that promotes target cell association (79, 167) Furthermore, studies on α-hemolysin have indicated that deletion of a single repeat sequence causes an increase in calcium dependence (27, 28, 167), and that multiple deletions completely abrogate cytolysis (27, 28, 79, 167, 168, 255) Finally, it has been demonstrated that the region of LktA consisting of amino acids 768-939, an area inclusive of most of the repeats, contains an epitope recognized by a leukotoxin-neutralizing mAb This mAb is believed to neutralize the leukotoxin by preventing association with the target cell membrane (96, 97)

The remaining two functional domains found within both HlyA and LktA share markedly reduced homology The first of these domains is located in the central region of each molecule, just proximal (amino) to the repeats In HlyA this region contains the

HlyC acylation sites (249) It is assumed that LktC acylates a similar site(s) present in the respective region of LktA (188, 225) The fourth and final functional domain of LktA and HlyA is the carboxy-terminal 70 amino acids This region of HlyA contains the secretion signal which is not cleaved during hemolysin export as discussed below (145, 201) This region shows the least homology between the two proteins, however, circular dichroism analyses have shown that both molecules exhibit similar biophysical properties in this region (290) Nevertheless, the precise role(s) that this region plays during secretion has not been clearly defined (113, 201, 290) Interestingly, some studies have also implicated this region in cell association domain formation (150, 184)

Secondly, LktC and HlyC are proteins that exhibit 50 3% homology and are 19 8 kDa (166 amino acids) and 20 kDa (169 amino acids) respectively (164) HlyC posttranslationally modifies HlyA via fatty acid acylation of lysine residues at positions 564 and 690 This process requires both HlyC and a fatty acid charged acyl carrier protein (120, 124, 249) This modification is believed to result in a conformational change of the toxin, inducing the formation of the active toxin (120, 124, 225) The exact mechanism(s) of LktC is/are not yet fully understood, but LktA modification by LktC may proceed in a similar fashion (82) Lastly, the importance of *C*-gene modification of LktA is underscored by the fact that in the absence of *C*-gene function, the *A* gene product is secreted but has no lytic activity (114, 200)

Finally, HlyB (46 kDa) and HlyD (53 kDa) are proteins involved in transporting active hemolysin through the membrane and into the medium (76, 106, 109, 144, 145, 169-171) This secretion pathway, designated type I, is poorly understood. Unlike

proteins secreted by type II mechanisms, the RTX toxins lack a cleavable signal sequence at the N-terminus Furthermore, unlike the type II pathway, type I secretion does not involve a periplasmic intermediate. The export of α-hemolysin across the cytoplasmic and outer membranes requires the C-terminus of HlyA, the two translocator proteins HlyB and HlyD, and the outer membrane protein TolC (145, 264) A mechanism has been postulated which suggests that the combination of TolC and HlyD forms a pore in the outer membrane through which the hemolysin is released This model also proposes that the C-terminal sequence of HlyD interacts with HlyB to form a closed channel spanning the periplasm (230) LktB (79 7 kDa) and LktD (54 7 kDa) are 80% and 60% homologous to their hemolysin counterparts respectively (112, 255) Due to the significant homology between the respective proteins, it is presumed that both systems proceed via a similar mechanism, a notion supported by the fact that HlyB and HlyD are capable of secreting LktA (42)

Control of Pneumonic Pasteurellosis

Given the tremendous economic burden of pneumonic pasteurellosis, the beef industry has diligently pursued strategies for the prevention and control of this disease Perhaps the best method of prevention is to prohibit the overcrowding and intermingling of calves from different sources during the shipping process and at feedlots This would appreciably reduce the incidence of epidemics from horizontal transmission by infected herds or small groups But, due to the economics of the industry and the inability to easily revamp the system, this proposal isn't feasible. Another management approach

that has been suggested is widespread administration of antimicrobial drugs at feedlots However, concerns about high costs, promotion of bacterial resistance (40), and the poor overall success of this strategy have led to industrial as well as public disapproval. Given the downfalls of these two proposals, many efforts have been made to develop and refine prophylactic vaccines against the infectious agents implicated in the disease process (195) These strategies have included viral vaccines (93, 127, 130, 176, 177, 195, 251, 252, 285), bacterins (56, 89, 94, 197), live bacterial vaccines (56, 89, 94, 147, 190, 197, 208, 285), supernatant subunit vaccines (193, 233, 286), and DNA-based approaches (155) While many of these vaccine preparations have been promising during laboratory trials, field trials have been disappointing, incongruent, and often inconclusive

First, because bacterial pneumonic pasteurellosis seems to be a secondary consequence of viral infection, many investigations have focused on viral vaccines as a means of shipping fever prevention (195, 285) Experimental and commercial viral vaccines have included PI-3, BHV-1, BRSV, and BVDV (93, 127, 130, 176, 177, 195, 251, 252, 285) The results have invoked doubts about this method of protection (195) Individuals vaccinated with PI-3 showed no appreciable protective difference than unvaccinated controls (127, 282-284) Furthermore, PI-3 vaccination has been shown to enhance disease susceptibility in some animals (195) Alternatively, vaccination with BHV-1 provided some protection against shipping fever, however, this protection varied widely among cattle (128, 130, 251, 252) Stress and transport seemingly compromise the effectiveness of the BHV-1 vaccine (251) Several other multiple virus vaccine

cocktails have provided limited protection against experimental challenge, but these have generally been ineffective in field vaccine trials (119, 176-178, 253)

Due to the disappointing results of the indirect prevention of shipping fever with viral vaccines, many efforts were made to derive successful vaccines from preparations of the principal causative agent, *P haemolytica* Initial preventative preparations were composed of bacterins or killed organisms However, these attempts led to discouraging results (195) First, formalin-killed *P haemolytica* bacterins administered either by aerosol or subcutaneous routes proved to yield more severe lesions in experimental subjects than in controls following experimental challenge (89) It is believed that bacterins elicit a humoral response to bacterial surface antigens, which allows for increased opsonization and phagocytosis of the live organism during subsequent challenge Consequently, bacterial cells come into contact with phagocytes more readily, allowing for increased destruction of leukocytes by active leukotoxin and increased disease severity (203, 233) Second, the success of bacterins seems to depend on the use of adjuvants, with different adjuvants yielding disparate results For example, bacterins combined with Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) give slight protection, but those combined with aluminum hydroxide gel and trehalose dimycolate do not enhance protection (58) Finally, because bacterins are metabolically inactive, they do not produce leukotoxin Therefore, there is not a protective humoral response to leukotoxin produced by live organisms This may have a negative impact on the efficacy of this approach since high antibody titers to LKT have been correlated with resistance to pneumonic pasteurellosis (84, 94). Overall, bacterins

have consistently failed to provide any appreciable protection against the disease (111, 195)

In addition to bacterin preparations, both live and live-attenuated *P haemolytica* cultures have been tested in vaccination trials Theoretically, this approach holds more merit than bacterins because it exposes the host to immunogenic virulence determinants produced by metabolically viable cells However, this approach has also experienced variable and conflicting results (233, 236) This may be due to vaccine mismanagement or the concurrent use of prophylactic antibiotics, both of which have the capacity to diminish this approach's effectiveness (233) Nevertheless, those vaccines which have shown promise in preventing the disease during experimental challenge tend to do so in an age, route, and dose-dependent way (55, 57, 127, 147, 285) A study designed to reveal the effect of culture age on eliciting a protective response revealed that log-phase cultures were more efficacious than stationary-phase cultures (55) Log-phase cultures showed reduced lesion size and severity following experimental challenge, an effect that is presumably related to an elevated humoral response to the peak production of the capsular polysaccharide and leukotoxin virulence determinants (13, 55, 65) In terms of immunization route, it has been found that live *P haemolytica* administered by either intratracheal or subcutaneous routes displayed enhanced protection compared to controls upon subsequent challenge (56, 199, 208). Aerosol exposures of *P. haemolytica*, either independently or in tandem with viral preparations, have not shown appreciable protection when compared to unvaccinated controls (127, 128). Also, dose size seems to be important in determining the level of potential protection. In one study, it was shown

that cultures at $1x10^8$ CFU/ml delivered subcutaneously resulted in enhanced protection when compared to calves immunized with only $1x10^6$ CFU/ml (57) Finally, because of the field trial variability and poor results associated with several live *P haemolytica* vaccines, many researchers sought to determine if adding viral agents to live organism vaccines might boost the response Although some studies have reported enhanced protection with *P haemolytica*/BHV-1 mixtures (34, 147, 208, 242), other studies have indicated that this approach either does not offer protection against live organism challenge or gives inconclusive results (127, 129, 285)

So far, the strategy that has shown the greatest promise is based on multicomponent vaccines derived from *P haemolytica* A1 culture supernatant (100, 193, 215, 233, 236, 286) These vaccine cocktails, typically harvested from log-phase cultures, may include one or several of the following virulence determinants· leukotoxin, and/or other soluble proteins, capsular polysaccharide, LPS, OMPs, and the transferrin receptors TbpA and TbpB (39, 53, 54, 60, 62, 94, 186, 193, 204) Although the exact antigen or antigenic combination which yields maximal protection is vague, the best results seem to be achieved with combinations of LKT and capsular polysaccharide (233, 236) Although leukotoxin is a key component of this bivalent preparation, studies have shown that recombinant LKT alone does not offer significant protection (63) Interestingly, spiking culture supernatant with recombinant LKT offers better protection than supernatant alone (233). Also, the transferrin receptor tandem of TbpA and TbpB seems to offer increased protection (204). Outer membrane proteins added to carbohydrate subunit preparations have been demonstrated to provide partial protection

only (193) These experiments suggest that the specific virulence determinants that comprise a particular vaccine may not be as important as the number of different antigens present (185, 195) Presumably, more antigenic determinants lead to a more comprehensive immune response against *P haemolytica's* armaments, thus increasing the chances of alleviating one or more of the factors important to the organism's virulence/survival As with live vaccines, the route of immunization seems to be an important variable in the level of protection established, with intramuscular and subcutaneous delivery generally giving better results than aerosols (195) However, despite some promising results arising from this facet of protective strategies, some reports indicate that the success rate of filtrate-derived, multivalent vaccines is no better than other strategies (100, 233, 285) Nevertheless, this aspect of vaccination research probably holds the greatest promise for future control and prevention, especially as more information is gathered on *P haemolytica's* antigenic determinants and their roles in virulence

Upon efforts to evaluate and rank the efficiency of each vaccination approach (248), it has been noted that attempts to control pneumonic pasteurellosis have experienced poor to moderate success at best Thus far, the maximum protective efficiency has peaked at about 65% (233) And, despite the intensive efforts at diminishing the prevalence of this disease, bovine pneumonic pasteurellosis remains a legitimate concern for the cattle industry. As a result, financial losses to the industry continue to escalate due to treatment costs, marketing delays, and animal morbidity/mortality Until a more definitive and acceptable treatment protocol is

adopted, further research into *P haemolytica*, its virulence attributes, and the host response that it invokes remains a top priority

Immunoglobulins: Structure, Function, and Application

As is the case with many infections, humoral immunity is an essential component of the bovine host response to pneumonic pasteurellosis This aspect of acquired immunity is mediated primarily by antibodies, glycoproteins which can execute a variety of effector functions depending upon the foreign antigen bound, the anatomic location of the antibody, and the isotype of the antibody (1) Although several isotypes of antibodies exist including IgA, IgD, IgE, IgG, and IgM (1), the major antibodies involved in pneumonic pasteurellosis seem to by IgG and IgA (190) In general, IgA is typically involved in the mucosal immune response and is responsible for antigen neutralization, prevention of antigen binding to mucosal receptors, and antibody-dependent cell-mediated cytotoxicity (ADCC) (1) IgG is the major isotype of soluble, circulating antibody involved in memory responses The functions of IgG include opsonization, ADCC, complement activation, and antigen neutralization (1, 108) The latter, which is of primary importance to this study, can proceed by 1) steric hindrance, which prevents the antigen from binding to its receptors/cell surfaces, 2) conformational distortion elicited by the formation of the antigen/antibody complex, and, 3) depending on the presence of multivalent epitopes within the antigen, agglutination, which is the result of antigen crosslinking and the formation of large insoluble lattices of antigen/antibody complexes (1, 108)

In order to facilitate the discussion of this study, the structure of murine IgG needs to be addressed Secreted IgG is a heterodimeric glycoprotein of approximately 150-160 kDa which consists of four polypeptide chains, two heavy chains (H, 450-575 amino acids) and two light chains (L, approximately 220 amino acids) (1, 108, 228) Both the heavy and light chains can be divided into constant (C) and variable (V) regions depending on the presence/absence of highly conserved amino acid sequences Furthermore, the V regions of both chains can be subdivided into four moderately conserved framework domains and three hypervariable complementarity determining regions (CDR) Based on crystallographic studies, the CDRs from the H and L chains associate to form a highly specific antigen binding pocket, or paratope (1, 69, 108, 228) This antibody specificity is determined by somatic recombination of germline antibody DNA, mutations, allelic exclusion, and alternate RNA splicing (1, 108, 228, 262) Antibodies can associate, via noncovalent interactions, with a variety of macromolecules including proteins, polysaccharides, and nucleic acids Also antibodies can bind complex structures such as pollens, viruses, bacteria, and tissue cells (1, 228) The size and shape of the specific antibody bound is largely dependent on the amino acid residues present in the paratope (1) In addition to the variable domains, both H and L chains possess constant regions of isotype-specific, highly conserved amino acid composition (1) In fact, it is the constant region of the heavy chain that determines the isotype of the antibody (1) Finally, structural diversity is augmented by the presence of diversity (D) and joining (J) segments within the heavy chain and a single J segment within the light chain (1)

X-ray crystallographic studies as well as computer-generated modeling have helped to elucidate the structure of antibodies (1, 6, 108) Based on these studies, antibodies are composed of three distinct regions with two arms extending away from a central axis, thus forming a "Y"-shaped structure The two arms consist of the amino half of the heavy chain complexed with the entire light chain This interchain association is mediated and stabilized by disulfide bridges which form between sulfur-containing amino acids present in each chain (1) The N-termini of both the heavy and light chains align at the distal end of each arm and form the antigen-specific paratope Therefore, IgG is bivalent, meaning it can potentially bind two molecules of the same antigen At the carboxy termini of the light chains, the heavy chain bends to form a versatile hinge region which allows lateral rotation and flexion of the two arms (108) Finally, the carboxyl one-half of each heavy chain associates to form the base of the structure Again this association is stabilized by the presence of disulfide bridges

By using proteolytic enzymes to digest IgG, the molecule can be separated into its three structurally and functionally distinct regions (1, 108, 209, 212) Treatment of IgG with the enzyme papain results in complete separation of the three regions due to digestion just above the hinge region two identical monovalent antigen binding fragments (Fragments of antigen binding, Fab) and a single fragment consisting of the two associated H chain carboxyl domains Because of this single fragment's strong propensity to self associate and form large, insoluble complexes, it is referred to as the Fragment that crystallizes (Fc) Furthermore, the Fc region is responsible for many of the effector functions mediated by antibodies including ADCC, opsonization, and

complement activation (1) Treatment with the enzyme pepsin, which digests just below the hinge region, results in the formation of a bivalent antigen binding molecule consisting of both arms linked together, or the bivalent antigen binding fragment ($F(ab')_2$) The $F(ab')_2$ displays greater avidity than the monovalent species derived from papain digestion However, this enzyme also results in the severe fragmentation of the Fc portion of the molecule (1) It has been determined that the different isotypes of murine IgG have variable susceptibilities to enzymatic digestion with pepsin and papain, with the order of susceptibility being IgG2b>IgG2a>IgG1 (209) Because some species of IgG are more resistant to digestion with these enzymes, other enzymatic treatments have been used including elastase, trypsin, and chymotrypsin (209) However, these treatments often result in the formation of incomplete, unstable fragments (209)

Because of the importance of antibodies to the humoral response, it is often prudent to investigate antibodies specific for a given antigenic determinant and the mechanisms by which these antibodies elicit their immune effector function This is especially true in the context of antibody-mediated responses to infectious agents *in vivo* Additionally, antibodies are important for other areas biotechnical research including X-ray crystallography, fluorescence microscopy, and protein detection/isolation (1, 108) However, given the relative instability and short half-lives of antibodies, they are difficult to study over long periods of time *in vitro* Fortunately, a way to immortalize B-cell lines secreting monoclonal antibodies (*i e*, antibodies with specificity for a single antigen) was developed in 1975 (143) This technique, known as hybridoma technology, involves fusing antibody-producing B-cell clones derived from the spleen of an animal (typically a

mouse) immunized with the antigen of interest to a nonantibody-producing, immortal myeloma cell line such as SP2/0 This fusion can be mediated via Sendai virus envelope protein or any other suitable chemical fusing agent Fused hybridomas are then isolated by HAT (hypoxanthine, aminopterin, thymidine) selection Because aminopterin blocks *de novo* nucleotide biosynthesis, the only cells which survive for periods of greater than 14 days in HAT media are B-cell/myeloma fusions The myeloma component of the fusion allows for the extended period of survival (immortalization), and the B-cell component provides the salvage pathway enzymes necessary for nucleotide biosynthesis from hypoxanthine and thymidine, enzymes not present in myelomas The specificity of each hybridoma clone for the antigen of interest can then be determined using radioimmunoassays (RIAs) or enzyme-linked immunoassays (ELISAs) (1, 143)

This laboratory has generated a panel of murine-derived hybridomas which secrete leukotoxin-specific antibodies ltx-2, ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81 Three of these antibodies, ltx-2, ltx-4, and ltx-35 consistently neutralize the cytolytic effects of the toxin to varying degrees (96, 97) Of these, ltx-2 has proven to be the most potent neutralizer Neutralization studies have shown that only 30 ng/ml of ltx-2 is necessary to neutralize one unit of LKT, an amount equivalent to 50% target cell lysis (81, 96, 97) Flow cytometry has revealed that ltx-2 prevents LKT association with the cell surface of bovine leukocytes (97). Several methods of epitope mapping have shown that the protective LKT epitope recognized by ltx-2 resides in the region of amino acids 768-939 (42, 96, 187) A more recent study has narrowed the epitope domain to amino acids 876-939 (184)

ScFv Technology

Within the last fifteen years, a new technology has been developed which circumvents the laborious nature of antibody production by repeated immunizations and/or hybridoma generation and allows for "immortalization" of rearranged immunoglobulin genes in bacterial systems (181, 205, 280) This approach utilizes polymerase chain reaction (PCR) to amplify the variable domains from both the heavy (V_H) and light (V_L) chains of immunoglobulins derived from hybridomas or spleen cells Because the DNA sequences within the first framework of the variable domain and the carboxy-terminal portion of the J region are relatively conserved for all classes of antibodies derived from a given animal, this method of cloning is feasible Subsequently, the amplified variable domains can be linked together by a 15-amino acid synthetic oligonucleotide linker to form a single open reading frame This synthetic linker is comprised of a repeating hydrophilic motif, $(Gly_4Ser)_3$, which consists of neutral amino acids The flexible nature of this peptide linker was specifically designed to bridge the 3 5 nm gap between the C-terminus of the V_H chain and the N-terminus of the V_L chain (98, 121) Furthermore, this construction facilitates chain pairing and minimizes refolding and aggregation problems encountered when the two chains are expressed individually Studies have indicated that the affinity and stability of ScFv antibodies which contain the $(Gly_4Ser)_3$ motif are comparable to those of the native antibody (98, 121) The single polypeptide derived from expression of this construct is called a Single-chain Fragment variable (ScFv) (98, 103, 121) Furthermore, this recombinant molecule retains the antigen specificity of its full-sized native counterpart

Subsequent to variable chain amplification and linking, the constructed single-gene product can be cloned into any stable expression vector However, in order to confirm the specificity of the derived ScFv, specialized vectors known as phagemids, which utilize the M13 coliphage genome, have been developed (189, 218, 266) These hybrid vector systems carry both the nonlytic bacteriophage M13 (single-stranded) and plasmid (double-stranded) origins of replication (189, 218) As a result, phagemids transformed into bacterial systems, usually *E coli*, can be grown as plasmids or alternatively packaged as recombinant nonlytic M13 phage ScFv technology employs this advantageous system, expressing the linked antibody variable domains as M13 tail fiber fusion proteins These fusion proteins are subsequently displayed on the surface of bacterial cells as part of the M13 life cycle (189, 218) Because the displayed ScFv retains antigen recognition properties, it is possible to select and enrich for recombinant phage expressing antigen-specific ScFv via affinity selection Depending on the strain of *E coli* used and the versatility of the phagemid vector, it is possible to prevent tail fiber-coupled expression by terminating protein synthesis at the end of the ScFv gene. In this scenario, a stop codon introduced upstream of the phage tail fiber gene is heeded in some bacterial strains but not in others Therefore, phage-displayed ScFv can be expressed as soluble entities merely by tranducing the ScFv gene construct into a strain which adheres to the stop codon Resulting soluble ScFv accumulates in the periplasm and eventually leaks into the supernatant (117) Subsequently, soluble ScFv can be harvested, concentrated, and purified from either the periplasm or the supernatant Although high

levels of cytoplasmic ScFv exist, the strong reducing environment of the cytoplasm results in insoluble, inactive aggregates and inclusion bodies (179)

Compared to other means of monoclonal antibody production, there are several benefits associated with ScFv technology These benefits include an easier, more time-efficient approach to cloning and screening from hybridoma/spleen cells, genetic stability, decreased usage of experimental animals, monetary economy, and an enhanced capacity for genetic manipulation (280) Furthermore, ScFvs are versatile immunological reagents conducive to affinity maturation studies, antigen detection and characterization, and antibody function studies Nevertheless, ScFv technology does have some drawbacks First, the *E coli* periplasmic export approach commonly used gives very low ScFv yield, often well below 1 mg/L and sometimes only in trace amounts (221) This is due to the limited amount of protein exported into the periplasm, which never exceeds 10% of all soluble proteins in *E coli* systems (10, 36) Attempts at enhancing ScFv yields have included altering the bacterial host (68), enhancing the thermodynamic stability of the molecules to cope with the cytoplasmic reducing environment (90, 103, 104, 179), and utilizing eukaryotic expression systems (72, 74, 133, 221, 273) Although many of these approaches have given increased ScFv yields, some of them have undesirably resulted in the partial or complete destruction of antigen specificity/binding (68, 228) To date, eukaryotic expression seems to hold the most promise (74, 221, 273) Another drawback is that bacterial systems do not carry out the glycosylating modifications that some ScFv need to be functional, resulting in inactive molecules (228). Again, eukaryotic expression systems can be used to circumvent this (133)

Although ScFv technology can be greatly appreciated in laboratory settings, its clinical application is still in its infancy Despite this, ScFvs are clinically appealing for several reasons First, because ScFvs lack highly antigenic constant domains as well as the inflammatory-mediating Fc portion of the antibody molecule, they are not as immunogenic as their native counterparts (154, 175, 228) Therefore, ScFvs aren't as prone to induce xenoreactivity Also, ScFvs display rapid extravascular space diffusion, increased volume distribution, and rapid renal clearance (49, 228, 277) However, rapid clearance has also been shown to reduce the clinical efficiency of ScFvs relative to Fab fragments (154)

Despite the clinical merit of ScFvs, the aforementioned limitations of the prokaryotic systems in which ScFvs have been developed have retarded the progression of clinical application However, some studies have confirmed that prokaryotic-derived molecules can have clinical utility including cancer cell targeting, viral neutralization, and immunopharmacology (121, 154, 277) Nevertheless, eukaryotic-derived ScFvs have proven to be more versatile and have greater therapeutic impact (221) These ScFvs have been used for intracellular molecular targeting (14, 15, 23, 24, 73, 220), diminishing HIV-1 replication (75, 172, 173, 223, 231, 291), and tumor cell targeting (14, 15, 73, 277)

Obviously, the development of ScFv technology has provoked numerous and diverse investigations The versatility and benefit of this relatively young facet of research is indisputable Certainly, as ScFv systems are progressively modified and refined, their applications and merit will continue to increase. Hopefully, ScFvs will

prove to be a successful approach to preventing and controlling pneumonic pasteurellosis in the future

Statement of Purpose

The purpose of this study has been twofold First, this study aimed at generating a ScFv derived from a potent LKT-neutralizing hybridoma, ltx-2, in order to further understand this mAb's mechanism of neutralization This was conducted using a phage-display recombinant antibody expression system Furthermore, this facet of the investigation also entailed preliminary characterization of the ScFv construct, including the nucleotide/amino acid sequence, antigen specificity, the ability to bind LKT relative to native ltx-2, and the capacity to neutralize active LKT compared to native ltx-2 Because the ScFv represented the ltx-2 paratope, it was predicted that this construct's LKT-association character would be comparable to that of ltx-2

Secondly, this study sought to map the LKT epitope(s) recognized by the ScFv construct This was determined by the ScFv's ability to recognize a panel of glutathione-S-transferase (GST)-fused amino-deleted LKT fragements (184) The primary goal of this aspect of the investigation was to confirm the presence of LKT epitopes implicated in target cell association, which have been reported to reside in the carboxyl one-third of the molecule (96, 97, 184) Based on previous epitope mapping of ltx-2 (184), it was predicted that the ScFv would bind to the region of LKT corresponding to amino acids

876-939 By indirect inference, the results of this research would provide some insight into the regions of LktA which are involved in the formation of a potential target cell binding domain

CHAPTER 2

MATERIALS AND METHODS

Bacterium

Pasteurella haemolytica biotype A serotype 1 (isolate 881165), provided by Dr Robert D Walker (Michigan State University, East Lansing, MI), was isolated from the lung of a pneumonic calf The organism was passed two times in calves by intrapulmonary inoculation, re-isolated, and stored in skim milk at –80° C *In vitro* growth of the organism was carried out at 37° C on brain heart infusion agar (BHI agar, Grand Island Biological Company, GIBCO, Gaithersburg, MD) containing 5% defibrinated sheep blood (267)

Escherichia coli strains TG1 (Amersham-Pharmacia Biotech Inc , AP Biotech, Piscataway, NJ) and HB2151 (AP Biotech) were used for phage display and soluble protein expression of the Single chain Fragment variable (ScFv) respectively Lyophilized cultures were resuspended in 2x yeast extract tryptone (2x-YT) broth composed of 17g tryptone (Fisher Scientific, Pittsburgh, PA), 10g yeast extract (Fisher), and 5g sodium chloride (NaCl, Mallinckrodt, Paris, KY) per liter of medium, pH 7 0 Cells were grown overnight at 37° C with shaking at 250 revolutions per minute (rpm) in a shaking water bath (Orbit Environ-Shaker, Lab-Line Instruments Inc , Melrose Park, IL) Each culture was streaked for isolation on minimal media agar composed of· 6g of sodium phosphate, dibasic, anhydrous (Na_2HPO_4, Fisher), 3g of potassium phosphate,

monobasic (KH_2PO_4, Mallinckrodt), 1g of ammonium chloride (NH_4Cl, Fisher) per liter of medium, pH 7 4, combined with 1ml of 1M magnesium chloride, hexahydrate ($MgCl_2$ 6 H_2O, Fisher), 1ml of 1M calcium chloride, dihydrate ($CaCl_2$ 2 H_2O, Fisher), 1ml of 1M thiamine hydrochloride (Fisher), and 5ml 20% glucose (D-(+)-glucose, anhydrous, Fisher) solution Long term maintenance for each strain was achieved by adding 20% v/v (200µl) of sterile 80% glycerol (Fisher) to every 800µl of stationary phase culture (grown from an isolated colony on minimal media agar) and storing at -80° C

Escherichia coli strain JM109 (Promega, Madison, WI) was used for transformation, plasmid propagation, and protein expression of leukotoxin A gene (*lktA*)-glutathione-S-transferase (GST) fusion constructs, prepared in this laboratory by Jackson McPherson (184) Briefly, cell stocks were prepared from cultures grown in Luria-Bertani (LB) broth (10g tryptone, 5g yeast extract, and 10g NaCl per liter of medium, pH 7 5) to an optical density at 595nm (OD_{595}) of 0 5. The cells were harvested by centrifugation (Beckman J-21 centrifuge, Beckman Instruments Inc , Palo Alto, CA) at 5,000 x g for 10 minutes The cell pellet was resuspended in glycerol-based medium (50ml glycerol, 2g tryptone, 1g yeast extract, 1g NaCl, and 150ml deionized water (dH_2O)), aliquoted, and stored at -80° C

Cell Culture

The leukotoxin-sensitive BL-3 cell line, a bovine leukemia-derived B lymphocyte cell line (American Type Culture Collection #CRL 8037), was used as the target cell line for all cytotoxicity / neutralization assays (198) The cells were maintained in Roswell

Park Memorial Institute-1640 medium with phenol red (RPMI-1640, Sigma Chemical Co , St Louis, MO) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2000 U penicillin / 2mg streptomycin per liter of medium, 40mM L-glutamine (psg, Gibco), and 0 2% sodium bicarbonate ($NaHCO_3$, Sigma) The cells were grown in tissue culture flasks (Corning Glass Works, Corning, Corning, NY) at 37° C in a humidified atmosphere of 5% carbon dioxide (CO_2) and were split at a 1.10 dilution every 3-4 days

Mark Cameron and Donald Gerbig of this laboratory produced the mouse-derived hybridoma clones ltx-2, ltx-35, and ltx-81 (96, 97) The cells were maintained in Dulbecco's Modified Eagle's Medium with phenol red (DMEM, Sigma) supplemented with 10% FBS, 2000 U penicillin / 2mg streptomycin per liter of medium, 40mM L-glutamine, and 0 37% $NaHCO_3$ The cells were grown in tissue culture flasks at 37° C in a humidified atmosphere of 5% CO_2 and were split at a 1 10 dilution every 3-4 days Immunoglobulin G (IgG) subclasses of the monoclonal antibodies (mAbs) produced by these hybridomas are shown in Table 2 1 (96)

Leukotoxin Production

Leukotoxin contained in the supernatant of log phase cultures was prepared by a modified protocol of the method described by Shewen *et al* (235) Briefly, several colonies from an overnight culture of *P haemolytica* A1 grown on a blood agar plate (BBL, Cockeysville, MD) were inoculated into 500ml BHI broth (BBL)

Table 2.1. Isotypes of mAbs reactive with *P. haemolytica* 100x concentrated leukotoxic culture filtrates

mAb	IgG Subclass
ltx-2	IgG_1
ltx-35	IgG_{2b}
ltx-81	IgG_1

and incubated for approximately 4 5 hours at 37° C in a shaking water bath at 225 rpm The culture was centrifuged at 10,000 x g for 20 minutes at 4° C, the supernatant discarded, and the remaining pellet resuspended in sterile 1x phosphate buffered saline, pH 7 0 (PBS), which consists of 137mM NaCl, 2 7mM potassium chloride (KCl, Fisher), 1 3mM Na_2HPO_4, and 1 8mM KH_2PO_4. The resuspension was centrifuged again as previously indicated, and the supernatant was discarded again The remaining pellet was resuspended in RPMI-1640 (Sigma) at a concentration of $4x10^8$ colony forming units per milliliter (CFU/ml) This value was predetermined based on linear regression performed by Donald Gerbig in which an OD_{595} of 1 2 is equivalent to $4x10^8$ CFU/ml This bacterial suspension was subsequently incubated in a shaking water bath at 225 rpm and 37° C for 1 5 hours The suspension was then centrifuged as before, and the supernatant was filtered through a 0 2μm filter (Millipore, Bedford, MA) and stored at -80° C Cultured filtrates were concentrated 100-fold using a Pellicon concentrator equipped with four 30 kDa exclusion membranes (Millipore), aliquoted, and stored at -80°C

Monoclonal Antibody Production

Two monoclonal antibodies (mAbs) directed against the leukotoxin of *P haemolytica* A1, ltx-35 and ltx-81, were 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 2ml of 2,6,10,14-tetramethyl decanoic acid (Pristane, Sigma)

intraperitoneally (ip) on day 0 On day 14, mice were injected ip with hybridoma cells at a concentration of $5x10^6$ cells/ml in 0 5ml 1x PBS, pH 7 4 Within 7-10 days, ascitic fluid developed and was collected by peritoneal puncture using an 18 gauge needle (Becton- Dickinson, Franklin Lakes, NJ) while animals were anesthetized with methoxyflurane (metofane, Pitman-Moore, Inc , Mundelein, IL) The collected ascitic fluid was then pooled and mixed at a ratio of 4 1 with Liposorb PHM-L™ (Calbiochem, Inc , San Diego, CA) and centrifuged at 500 x g for 10 minutes at 25° C in a HN-SII swinging bucket clinical centrifuge (Damon/IEC, Needham Heights, MA) The lipid-free supernatant was decanted, and the pH was adjusted to 6 0-7 5 with wash buffer, pH 7 0 (0 01M Na_2HPO_4, 0 15M NaCl) For purification of IgG , the lipid-free ascitic fluid was passed over a recombinant ProteinG / Sepharose® 4B coupled affinity matrix (Zymed, South San Francisco, CA) The column was washed extensively with wash buffer until the absorbance at 280nm (A_{280}) fell to less than 0 05 The bound IgG was eluted from the column with 0 5M ammonium acetate ($NH_4C_2H_3O_2$, Mallinckrodt), pH 3 0 The eluted fractions were immediately adjusted to pH 6 5-7 5 with 1M Tris base (Tris, Sigma), pH 9 5 All fractions with an A_{280} greater than 0 1 were pooled and dialyzed at 4° C against three changes of 1x PBS in 4L The protein concentration was determined by colorimetric analysis using the bicinchoninic acid (BCA, Pierce, Rockford, IL) method and measuring the A_{562} on a SpectraMax microtiter plate reader (Molecular Devices, Corp., Sunnyvale, CA) Purified antibodies were aliquoted and stored at -80° C

In order to obtain pure stock of ltx-2, this mAb was purified from hybridoma supernatant 0 5ml of ltx-2-producing hybridoma freezer stock was placed in 7ml of

DMEM with phenol red +10% FBS + psg + 0.37% $NaHCO_3$ (DMEM mix) as previously described in a 25cm^2 tissue culture flask Upon medium yellowing of the culture (visual estimation), indicated by phenol red conversion to yellow when pH < 6 8, the entire 7ml were added to 30ml of fresh DMEM mix in a 75cm^2 tissue culture flask on day 0 and incubated as previously described for 24 hours 30ml of fresh DMEM mix were added to the flask on day 1 and incubated an additional 24 hours On day 2, this 60ml culture was placed into 90ml of fresh DMEM in a 850 cm^2 tissue culture roller bottle (Corning), the lid tightened, and incubated on rollers at 37° C without CO_2 for 48 hours Each day subsequent to placing the culture in the roller bottle, the hybridoma cells were infused with CO_2 for 1 hour at 37° C in a 5% CO_2 atmosphere On each of days 4, 5, and 6 150ml of fresh DMEM mix were added to the culture and incubated at 37° C for 24 hours After the addition of fresh DMEM mix on day 6, the culture was allowed to incubate for an additional 24 hours On each of days 8 and 9, 300ml of fresh DMEM were added to the bottle, and the culture was incubated at 37° C for 24 hours On day 10, 600ml of fresh DMEM mix were added, and the culture was allowed to grow for an additional 4 days at 37° C, until there was medium yellowing and appreciable turbidity of the culture The entire 1 8L of cell culture were centrifuged at 5,000 x g, and the supernatant was collected and filtered with a 0 2μm filter (Millipore) The supernatant was passed over a rat anti-mouse IgG (kappa) / Sepharose® coupled affinity matrix provided by Dr Habib Zaghouani (University of Tennessee; Knoxville, TN) to purify the culture-derived IgG The column was washed extensively with 1x PBS until the A_{280} fell to less than 0 05 The bound IgG was eluted into fractions with 0 1M sodium citrate

(Fisher), pH 2 6 All fractions with an A_{280} greater than 0 1 were pooled and the pH was adjusted to 6 5-7 5 with 1M potassium phosphate, dibasic (K_2HPO_4, Fisher), pH 8 0 The IgG was then dialyzed at 4° C against 3 changes of 1x PBS in 4L, and the protein concentration was determined with BCA as previously indicated The purified IgG was aliquoted and stored at -80° C

mRNA Isolation

mRNA from the Itx-2-producing hybridoma was isolated using the MicroPoly(A) Pure™ mRNA isolation kit (Ambion, Austin, TX) (136) The protocol followed that provided with the kit Briefly, cells were removed from a culture of greater than 90% viability and placed in a sterile Falcon™ tube (Becton-Dickinson) The cells were centrifuged at 500 x g for 5 minutes at 25° C in a swinging bucket clinical centrifuge The supernatant was discarded and the pellet was resuspended in a volume of sterile DMEM mix that yielded approximately $5x10^6$ cells/ml. A 1ml aliquot of this cell resuspension was placed in a 1 5ml eppendorf tube (Eppendorf, Westbury, NY) and spun in a Biofuge® B microcentrifuge (Biofuge, Inc , McGaw Park, IL) for 5 minutes at 1,500-2,000 x g and 25° C to pellet the cells The supernatant was discarded, and 250µl of Lysis solution (Ambion) were added to the pellet and vortexed vigorously for 1 minute (44, 45, 216) 500µl of Dilution buffer (Ambion) were added to the homogenate, mixed briefly, and centrifuged at 12,000 x g at 4° C for 15 minutes in a refrigerated microcentrifuge (Centrifuge 5402, Eppendorf) The supernatant was transferred to a clean 1 5ml eppendorf tube and 20mg of oligo dT cellulose resin were added to the

supernatant This was subsequently gently rocked at 25° C for 1 hour to allow for mRNA binding to the resin The oligo dT / mRNA resin mix was pelleted by centrifugation at 2,000-4,000 x g for 3 minutes at 25° C and the supernatant discarded The resin pellet was resuspended in 1.2ml of Binding buffer for high salt washes, mixed, and centrifuged as before This step was repeated two more times using 1 2ml of Binding buffer each time The resin was resuspended in 1 2ml of Wash buffer (Ambion) for low salt washes, mixed, and centrifuged as before This step was repeated twice After the third wash, the resin was resuspended in 400µl of Wash buffer and transferred to a spin column inserted into a 2ml microfuge tube (Ambion), which was centrifuged at 5,000 x g at 25° C for 10 seconds The eluent liquid was discarded, and the resin pellet was rinsed two times with 500µl of Wash buffer, centrifuging as before each time to remove the rinse The mRNA was eluted from the resin into a sterile, Rnase-free 2ml microfuge tube by adding 100µl of 70° C Elution buffer (Ambion) and immediately centrifuging at 5,000 x g at 25° C for 30 seconds This step was repeated once more The mRNA was precipitated using 20µl of 5M ammonium acetate (Ambion), 2µl of 5mg/ml glycogen (Ambion), 2 5 volumes of ice cold 100% ethanol (AAPER Alcohol and Chemical Co , Shelbyville, KY) and placing on dry ice for 1 hour The precipitated mRNA was recovered by centrifugation at 12,000 x g for 20 minutes at 4° C, the supernatant discarded, and the pellet dried under a vacuum (Savant Instruments, Inc , Farmingdale, NY) The dessicated pellet was resuspended in 10µl of nuclease-free dH_2O / 0 1mM disodium ethylenediamine tetraacetate (EDTA, Ambion) The purity of the mRNA was assessed by ultraviolet (UV) asorbance (A_{260}/A_{280} of 1 7-2 1) and gel electrophoresis on a native 1% agarose gel with 1µg/ml

ethidium bromide (EtBr, Mallinckrodt) for UV visualization in 1x TBE buffer (90mM Tris, 90mM boric acid (Fisher), 2mM EDTA, Fisher), pH 7 8 The purified mRNA was immediately used for cDNA synthesis

Molecular Cloning of ScFv from Mouse-Derived Hybridoma

The ScFv construct was generated using the Mouse ScFv Module / Recombinant Phage Antibody System (RPAS™, AP Biotech) (9, 118, 122, 182, 227). The protocol followed the module instruction manual with some modifications Briefly, purified ltx-2 mRNA was obtained as previously described and subjected to first-strand cDNA synthesis This reaction was carried out in duplicate and consisted of 5µl mRNA, 11µl Primed First Strand Mix (FPLC*pure*™ Cloned Murine Reverse Transcriptase, random hexadeoxyribonuclotides, RNAguard™, RNase / DNase-free Bovine Serum Albumin (BSA), dATP, dCTP, dGTP, and dTTP in aqueous buffer, AP Biotech), 1µl 200mM dithiothreitol solution (DTT, AP Biotech), and 16µl nuclease-free dH_2O (AP Biotech) in a 0 5ml eppendorf tube These reactions were incubated at 37° C for 1 hour

In order to amplify the appropriate heavy and light chain variable fragments from the obtained cDNA, the First Strand reaction mix was subjected to polymerase chain reaction (PCR) The light chain was amplified using the following reaction mix 33µl of First Strand reaction mix, 10µl 10x PCR buffer (Gibco), 3µl 50mM magnesium chloride ($MgCl_2$, Gibco), 2µl Light Primer Mix (10 variable light chain primers, both 5' and 3', in water, AP Biotech), 5µl deoxynucleosidetriphosphate (dNTP) mix (20mM of each, Gibco), 1µl DNA *Taq* polymerase (*Taq*, Gibco), and 46µl nuclease-free dH_2O in a 0.5ml

eppendorf tube The heavy chain was amplified using the following mix 33µl of First Strand reaction mix, 10µl 10x PCR buffer, 3µl 50mM $MgCl_2$, 2µl Heavy Primer 1 (5', AP Biotech), 2µl Heavy Primer 2 (3', AP Biotech), 5µl dNTP mix (20mM each), 1µl *Taq*, and 44µl nuclease-free dH_2O To prevent evaporation, both reactions were overlaid with mineral oil (Sigma) Both reactions were subjected to the hot start PCR technique in which the dNTP mix and *Taq* were added beneath the mineral oil after the remainder of the mix was heated to 95° C for 5 minutes The PCR protocol was carried out in a DNA Thermocycler (Perkin Elmer Cetus, Norwalk, CT) under the following conditions 1 cycle 95°C for 5 minutes, 30 cycles of 94° C for 1minute, 55° C for 2 minutes, and 72° C for 2 minutes, and 1 cycle of 72° C for 10 minutes

Following amplification, the entire reaction mixes were electrophoresed on a 1 5% agarose gel with 1µg/ml EtBr in 0 5x TAE buffer (20mM Tris-acetate (Tris adjusted to pH 8 0 with glacial acetic acid {Mallinckrodt}), 0 5mM EDTA, pH 8 0) The appropriate bands representing the light and heavy fragments were excised, and the DNA was eluted by spinning the excised gel pieces into respective clean 1 5ml eppendorf tubes using MicroSpin columns (AP Biotech) The columns were spun at 735 x g at 25° C for 2 minutes in a microcentrifuge Purified DNA concentrations were determined by assaying small aliquots of the purified products by gel electrophoresis as before The band intensities of the heavy and light fragments were compared with a standard of known concentration (V_H Marker, AP Biotech)

In order to assemble the purified light and heavy fragments into a single chain, the following fill-in reaction was used· 50ng of heavy chain fragment (12µl), 50ng of light

chain fragment (24µl), 5µl 10x PCR buffer, 2.5µl dNTP mix (20mM each), 1 5µl 50mM $MgCl_2$, 4µl Linker-Primer Mix (equimolar 5' and 3' linking primers in water, AP Biotech), and 1µl *Taq* in a 0 5ml eppendorf tube The reaction was overlaid with mineral oil and exposed to the following Thermocycler conditions. 7 cycles of 94° C for 1 minute, 63° C for 4 minutes. The assembled product was then amplified and engineered for restriction digestion by adding the following mix to the fill-in reaction beneath the mineral oil 5µl 10x PCR buffer, 1µl dNTP mix (20mM each), 1 5µl 50mM $MgCl_2$, 4µl RS Primer Mix (mixture of 5' heavy chain primer with *Sfi* I site and 3' light chain primer with *Not* I site in water, AP Biotech), 1µl *Taq*, and 37 5µl nuclease-free dH_2O This mix was amplified using the following Thermocycler conditions 30 cycles of 94° C for 1 minute, 55° C for 2 minutes, 72° C for 2 minutes, 1 cycle of 72° C for 10 minutes. The entire sample was electrophoresed and the single chain fragment purified as previously described The concentration and correct size of the ScFv were determined with gel electrophoresis as previously indicated and confirmed using a standard of known concentration and correct size (ScFv Marker, AP Biotech)

ScFv Manipulation

DNA manipulation of the ScFv construct followed the protocols outlined in the RPAS™ Mouse Module and the RPAS™ Expression Module (AP Biotech) with minor modifications. The purified ScFv DNA was subjected to restriction endonuclease digestion with *Sfi* I (Gibco) and *Not* I (Gibco). Briefly, the *Sfi* I digest was set up in a 0 5ml eppendorf tube as follows 0.4µg ScFv (74.5µl), 8.5µl REACT® 2 (Gibco), 20U

Sfi I (2µl) The reaction was overlaid with mineral oil and incubated at 50° C for 3 hours Following equilibration of the mix to 25° C, the *Not* I reaction mix was added to the tube beneath the mineral oil as follows. 1 5µl REACT® 3 (Gibco), 40U *Not* I (3µl), and 10 5µl nuclease-free dH_2O The mix was incubated at 37° C for 3 hours Following digestion, enzymes and salts were removed by adding 100µl phenol chloroform isoamyl alcohol (25 24 1, Sigma) to the 100µl mix in a 1 5ml eppendorf tube, vortexing vigorously for 1 minute, and spinning at 14,000 x g for 3 minutes at 25° C The aqueous layer was aspirated and transferred to a clean 1 5ml eppendorf tube In order to separate the cleaved ScFv product from the smaller products created during the digestion, the entire sample was electrophoresed, the ScFV excised, and eluted through a Microspin column as indicated previously To determine the concentration, a small aliquot of the cleaned product was compared to the ScFv Marker via gel electrophoresis as previously described

Ligation reactions were performed with the purified *Sfi* I / *Not* I-digested ScFv product and the pCANTAB 5 E™ phagemid vector (246, 266) pre-cut with the same restriction endonucleases (RPAS Expression Module, AP Biotech), illustrated in Figure 2 1 The ligation reaction was set up as follows 150ng (30µl) ScFv gene fragment, 5µl OPA+ Buffer (AP Biotech), 250ng (5µl) pCANTAB 5 E, 5µl 10mM adenosine triphospate (ATP, Gibco), and 5U (5µl of 1U/µl) T4 DNA ligase (Gibco) in a 1 5ml eppendorf tube. The ligation mix was incubated at 16° C for 3 hours

The ScFv-religated pCANTAB 5 E phagemid was used to transform competent *E coli* TG1cells

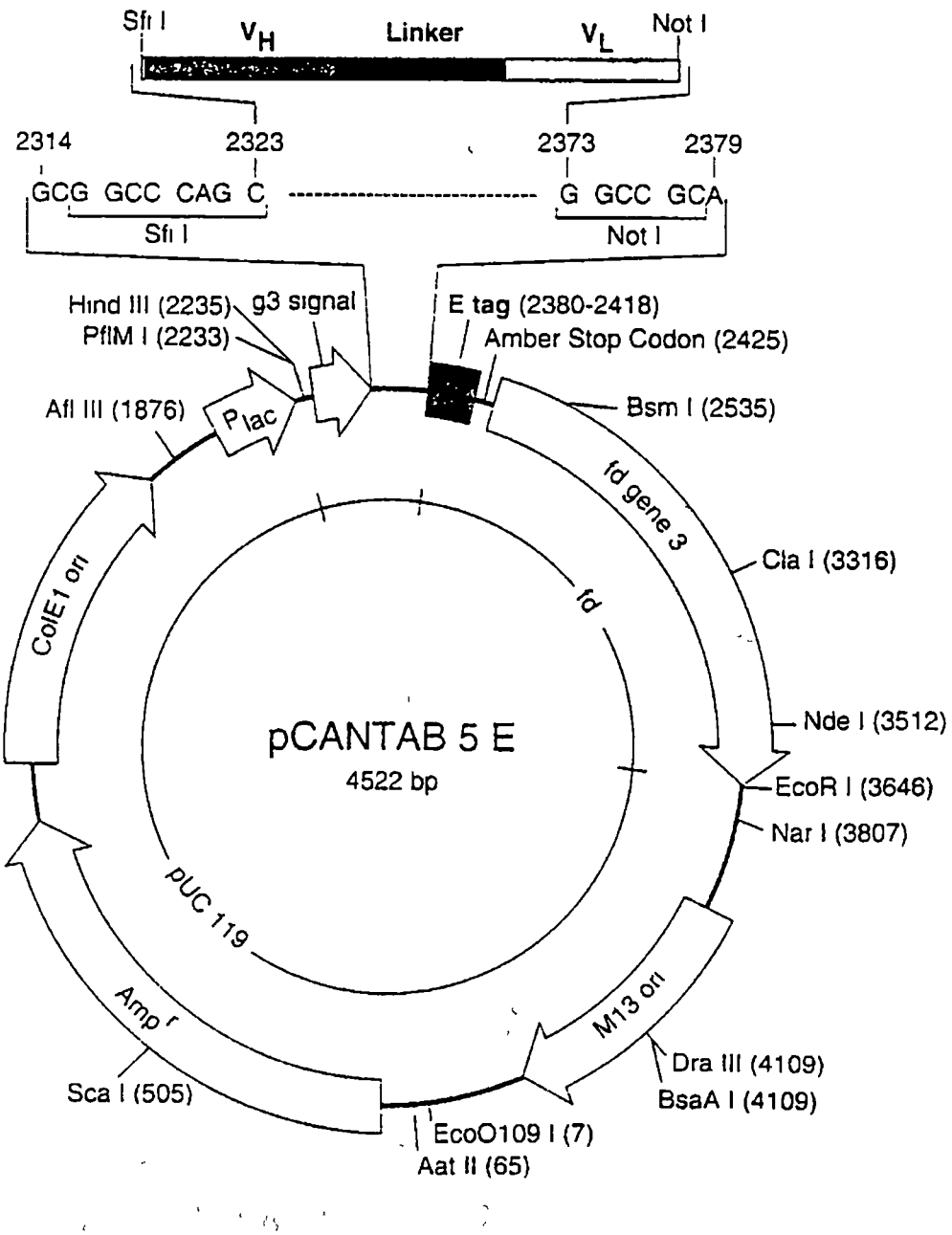


Figure 2.1. pCANTAB 5E phagemid vector map. This vector is used for the expression of recombinant ScFv. The section above the vector diagram depicts the orientation of the antibody fragments being cloned into pCANTAB 5 E

Competent *E coli* TG1 cells were prepared using the method outlined by Chung *et al* (46) Briefly, TG1 cells from glycerol freezer stock were aseptically streaked onto a minimal medium plate and grown overnight at 37° C One colony from this plate was used to inoculate 10ml of 2xYT broth, which was shaken overnight at 250rpm and 37° C Log phase TG1 cells were prepared as follows 100ml of fresh 2xYT medium were inoculated with 1ml of the overnight culture and shaken as before until the OD_{595} reached 0 5-0 6 The cells were pelleted at 2,500 x g for 15 minutes at 4° C and gently resuspended in 10ml of ice-cold transformation and storage buffer (TSS, 1g tryptone, 0 5g yeast extract, 0 5g NaCl, 10g polyethylene glycol (PEG, M W 3350, Sigma), 5ml dimethylsulfoxide (DMSO, Sigma), and 5ml 1M $MgCl_2$ (Sigma) / 100ml, pH 6 5, 0 22μm filter-sterilized) The competent cells were immediately transformed (within 2 hours)

Transformation was accomplished by the heat shock method (227) Briefly, the entire ScFv/pCANTAB 5 E ligation mix was mixed with 1ml of freshly prepared competent *E coli* TG1 cells and placed on ice for 30 minutes The mixture was then placed in a 42° C water bath for 2 minutes and then chilled on ice for 2 minutes 900μl of the transformation mix were used for phage rescue, the remaining 100μl were diluted and used for analysis of transformation efficiency and generation of freezer stock Briefly, 100μl of the transformation mix were combined with 900μl of LB-G (LB with 20mM glucose) and shaken for 1 hour at 37° C and 250rpm 100μl of this culture were plated on SOBAG medium (20g tryptone, 5g yeast extract, 0.5g NaCl; combined with 10ml sterile $MgCl_2$, 55 6ml sterile 2M glucose, 1ml of sterile 100mg/ml ampicillin (Sigma) / 1L) to

analyze transformation efficiency The remaining 900µl were combined with 250µl (20% v/v) of sterile 80% glucose as previously indicated and stored at –80° C

Rescue and Enrichment of Leukotoxin-Specific ScFv

In order to ensure the proper packaging of the recombinant ScFv/pCANTAB 5 E phagemid in TG1 cells and subsequent phage display, the construct must be rescued with the helper phage M13K07 (AP Biotech) as indicated in the RPAS™ Expression Module Briefly, 900µl of the undiluted transformation previously described were combined with 9 1ml of 2xYT-G medium (2xYT containing 2% glucose) in a 50ml Falcon™ screw-top polypropylene tube and incubated for 1hour at 37° C with shaking at 250rpm Subsequently, 10µl of 100mg/ml ampicillin and $4x10^{10}$ plaque forming units (pfu) of M13K07 were added to the cell suspension The culture was incubated under the same conditions for an additional 1 hour The cells were pelleted at 1,000 x g for 10 minutes at 25° C in a swinging bucket clinical centrifuge, the supernatant discarded, and the pellet resuspended in 10ml of 2xYT-AK (2xYT with 100µg/ml ampicillin and 50µg/ml kanamycin) to select for rescued cells and allow production of recombinant phage The culture was grown overnight at 37° C with shaking at 250rpm The recombinant phage were harvested by spinning the culture at 1,000 x g for 20 minutes at 25° C and filtering the phage-containing supernatant through a 0 45µm Acrodisc® filter (Pall Corp /Gelman Sciences, Ann Arbor, MI) into a sterile 50ml Falcon™ polypropylene tube Phage-displayed ScFvs were precipitated away from interfering soluble ScFvs by PEG precipitation as follows 2ml of PEG/NaCl (200g PEG, M W. 8000 (Fisher), 146 1g

NaCl / L of dH_2O) were added to the 10ml of phage-containing supernatant, incubated on ice for 1 hour, and centrifuged at 10,000 x g for 20 minutes at 4° C The supernatant was discarded and the pellet resuspended in 16ml of 2xYT medium

Enrichment for leukotoxin-specific phage-displayed ScFvs was carried out by panning according to the RPAS™ Expression Module Briefly, the pan was prepared by coating a $25cm^2$ tissue culture flask with 30ml of 20μg/ml ltx-35 (trapping antibody) in 1x PBS overnight at 25° C In order to block unoccupied protein sites, the pan was filled with 10% nonfat dry milk in 1x PBS(NFDM, Carnation Co ; Los Angeles, CA) for 1 5 hours at 37° C Concentrated leukotoxin was applied at a 1 50 dilution in 3% NFDM and incubated 1 5 hours at 37° C The pan was rinsed 3 times with 1x PBS-0 05% Tween 20 (PBS-Tween, Fisher), and 20ml of the 16ml phage resuspension, diluted with 14ml 10% NFDM / 0 01% thimerosal (Sigma)/0 1% Triton X-100 (Sigma), were applied to the pan and incubated for 2 hours at 37° C The flask was emptied and washed extensively with 1x PBS and PBS-Tween (20 times each) to remove clones not specific for leukotoxin In order to reinfect *E coli* with enriched leukotoxin-specific clones, 10ml of log phase TG1 cells (prepared as indicated previously to an OD_{595} of 0 3) were applied to the pan and incubated at 37° C for 1 hour with 250rpm shaking (In order to further enrich for leukotoxin-specific clones, the culture was transferred to a 50ml Falcon™ polypropylene tube, combined with 100μg/ml ampicillin, 2% glucose, and $4x10^{10}$ pfu of M13K07, incubated for 1 hour at 37° C with 250rpm shaking, and rescued and enriched as outlined above two more times) Subsequently, the reinfected culture was serially diluted 10-fold (1 10, 1 100, 1.1,00, etc), and 100μl of each dilution and the undiluted culture were

plated on SOBAG medium and incubated overnight at 30° C To prepare for leukotoxin-specific screening, individual colonies were sterilely transferred to each tube of a 96 cluster tube microtiter format (Corning Costar, Corning, NY) containing 400µl of 2xYT-AG (2xYT containing 100µg/ml ampicillin and 2% glucose) The cluster tubes were incubated in a humidified chamber overnight at 30° C with shaking at 250rpm This served as the stock plate for screening

Screening for Leukotoxin-Specific ScFv

Each of the 96 colonies obtained as previously indicated were assayed for the presence of both phage-displayed and soluble leukotoxin-specific ScFvs according to the protocol provided in the RPAS™ Expression Module Screening for phage-displayed ScFv involved transferring 400µl of a 50ml 2xYT-AG / 2 $5x10^{10}$ pfu of M13K07 stock to solution to each tube of a new set of 96 cluster tubes, which was subsequently inoculated with 40µl of respective saturated culture from the stock plate previously mentioned The cluster tubes were incubated in a humidified chamber at 37° C for 2 hours with shaking at 150rpm After incubation, the tubes were centrifuged at 1,500 x g for 20 minutes at 25° C in a refrigerated clinical centrifuge with microtiter plate adapters (Manhattan 21,000R, Fisher), the supernatant discarded, and each pellet resuspended in 400µl of 2xYT-AK medium The tubes were incubated overnight in a humidified chamber at 37° C with shaking at 250rpm The tubes were centrifuged as before, and 320µl of the phage-containing supernatant from each tube were transferred to a clean set of cluster tubes and diluted with 80µl of 10% NFDM

Leukotoxin specificity was determined by two-antibody sandwich (trap) enzyme linked immunosorbant assay (ELISA), a modification of that outlined in the RPAS™ Detection Module (AP Biotech) Briefly, each well a 96-well polyvinyl chloride plate (PVC plate, Dynatech Laboratories, Chantilly, VA) was coated with 100µl of 20µg/ml ltx-35 (trap) in 1x PBS overnight at 25° C Unoccupied protein sites were blocked with 200µl of 3% NFDM for 1 5 hours at 37° C and rinsed 3 times with PBS-Tween 100µl of concentrated leukotoxin were applied to each well of the plate at a 1 50 dilution in 3% NFDM, incubated, and rinsed as described 100µl of the NFDM-diluted phage-containing supernatant were applied to each respective well, incubated, and rinsed as described Bound phage displaying leukotoxin-specific ScFv were detected by adding 100µl of a 1 5,000 dilution of Anti M13/horseradish peroxidase (HRP) monoclonal conjugate (AP Biotech) in 3% NFDM to each well, incubating, and rinsing as described Development was by the addition of 100µl of a 2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS, Zymed) and hydrogen peroxide (H_2O_2, Zymed) mixture at 1 1 ratio in 0 1M citrate buffer, pH 4 1 (Zymed) Following a 30 minute incubation in the dark, the A_{405} was determined by a microtiter plate reader

Screening for soluble ScFv involved transferring 40µl of saturated culture from the stock plate previously described into respective tubes of a 96 cluster tube microtiter format containing 400µl of 2xYT-AG The tubes were incubated in a humidified chamber for 2 hours at 30° C with shaking at 250rpm. The tubes were centrifuged as previously described, the supernatant discarded, and each pellet resuspended in 400µl of 2xYT-AI (2xYT containing 100µg/ml ampicillin and 1mM isopropyl-β-D-

thiogalactoside (IPTG, Fisher)) The tubes were incubated in a humidified chamber for 5 hours at 30° C with shaking at 250rpm The tubes were centrifuged as before, and 320µl of the supernatant containing soluble ScFv were diluted with 80µl of 10% NFDM in a clean set of cluster tubes

Leukotoxin specificity was determined by trap ELISA, a modified version of that indicated in the Anti-E Tag Antibody Instruction Manual (AP Biotech) The protocol followed that previously described with the following modifications 100µl of the NFDM-diluted soluble ScFv-containing supernatant were applied instead of NFDM-diluted phage-containing supernatant, and detection was via 100µl of 1 8,000 Anti-E Tag/HRP monoclonal conjugate (AP Biotech). The remainder of the assay was unmodified

Transduction of *E. coli* HB2151 Cells

In order to achieve maximal expression of soluble leukotoxin-specific ScFv and avoid overexpression of toxic phage components, the phagemid construct must be transferred to the HB2151 nonsupressor strain of *E coli* via transduction as outlined in the RPAS™ Expression Module Briefly, HB2151 cells from glycerol freezer stock were aseptically streaked onto a minimal medium plate and grown overnight at 37° C One colony from this plate was used to inoculate 10ml of 2xYT broth, which were shaken overnight at 250rpm at 37° C Log phase TG1 cells were prepared as follows 50ml of fresh 2xYT medium were inoculated with 500µl of the overnight culture and shaken at 250rpm at 37° C until the OD_{595} reached 0 3-0 5 400µl of this log phase culture were

transferred to each tube in a 96 cluster tube microtiter format, and 5µl of recombinant phage from each respective tube of the phage-display screen previously described were added to the log phase cells The tubes were incubated at 37° C for 1 hour with gentle, intermittent shaking Individual SOBAG-N (SOBAG containing 100µg/ml nalidixic acid (Sigma)) plates were streaked to select for HB2151 transductants expressing leukotoxin-specific ScFv (as previously determined by ELISA) The plates were incubated overnight at 30° C

DNA Isolation and Sequencing

The HB2151 clone displaying the greatest affinity and neutralization capacity for leukotoxin was chosen for sequencing (clone #D9) The ScFv-containing phagemid from this clone was isolated using the Wizard™ Minipreps DNA Purification System (Promega) (9, 227) Briefly, two 1 5ml aliquots of overnight culture were placed in a 1 5ml eppendorf tube, centrifuged at 11,000 x g for 2 minutes at 25° C in a microcentrifuge, the supernatant discarded, and the pellet resuspended in 200µl of Resuspension Solution (Promega) 200µl of Lysis Solution (Promega) were added, vigorously mixed, and neutralized with 400µl of Neutralization Solution (Promega) for 10 minutes at 25° C The mix was centrifuged as described for 5 minutes, and the supernatant was aspirated and combined with 850µl DNA Purification Resin (Promega). The resin / DNA slurry was passed over a Miniprep Column (Promega) using a Vac-Man™ Laboratory Vacuum Manifold (Promega), washed with 2ml *endA*+ strain wash (40% isopropanol / 4 2M guanidine hydrochloride (Fisher)) and 2 ml column wash

(Promega) Residual wash was dried off by centrifugation for 2 minutes as indicated The isolated DNA was eluted from the resin by adding 50µl nuclease-free dH_2O, incubating for 1 minute at 25° C, and centrifuging as indicated for 30 seconds

The purified DNA was concentrated by ethanol precipitation(227) Briefly, four minipreps were pooled, treated with 1/10 volume 3M sodium acetate (Fisher), pH 5 2 and 2 volumes ice cold 100% ethanol, and incubated on dry ice for 30 minutes The precipitation was subsequently centrifuged at 14,000 x g for 30 minutes at 4° C in a refrigerated microcentrifuge, the supernatant discarded, the pellet rinsed with 75-100µl of ice cold 70% ethanol, and the rinse centrifuged as described for 15 minutes The rinse was discarded, and the pellet was vacuum-dried and resuspended in 50µl of nuclease-free dH_2O

DNA sequencing was performed at the University of Tennessee-Knoxville Molecular Biology Resource Facility using an ABI model 373A automated DNA sequencer (PE Applied Biosystems, Inc , Foster City, CA) and the ABI prism dye terminator cycle sequencing kit (PE Applied Biosystems, Inc) Cycle sequencing reactions were carried out using 400ng of phagemid DNA and 5 picomoles of each the pCANTAB 5-S1 forward (5'-dCAACGTGAAAAAATTATTATTCGC-3', AP Biotech) and pCANTAB 5-S6 reverse (5'-dGTAAATGAATTTTCTGTATGAGG-3', AP Biotech) primer in a GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer Corporation, Norwalk, CT), as directed in the ABI prism kit.

Analysis of *E. coli* HB2151 Cellular Fractions

In order to optimize protein expression, *E coli* HB2151 cells containing a neutralizing leukotoxin-specific ScFv, 2N-ScFv (clone #D9), were fractionated and assayed for presence of 2N-ScFv according to the RPAS™ Expression Module Briefly, an overnight culture of 2N-ScFv-containing cells was prepared by inoculation of 10ml of 2xYT-AG with a single colony from the appropriate SOBAG-N plate The culture was grown at 30° C with shaking at 250rpm 5ml of the overnight culture were added to 50ml of fresh 2xYT-AG and incubated as indicated for 1 hour The culture was centrifuged at 1,500 x g for 20 minutes at 25° C, the supernatant discarded, and the pellet resuspended in 50ml of 2xYT-AI The resuspension was incubated overnight at 30° C with shaking at 250rpm Subsequently, the induced culture was split into two 50ml Falcon™ polypropylene tubes and centrifuged as before The supernatant was filtered through a 0 22μm filter and stored at -20° C

The periplasmic fraction was isolated by the method described by Skerra *et al* (238) Briefly, one of the two pellets was resuspended in 0 5ml of ice cold 1x TES buffer (0 2M Tris-HCl, pH 8 0, 0 5mM EDTA, and 0 5M sucrose) and combined with 0 75ml of ice cold 1/5x TES The resuspension was incubated on ice for 30 minutes with intermittent agitation The mix was then transferred to a 1 5ml eppendorf tube and centrifuged at 14,000 x g for 20 minutes at 25° C in a microcentrifuge The supernatant was transferred to a clean tube and stored at -20° C

The whole cell extract was prepared by resuspending the remaining pellet in 0 5ml 1x PBS, transferring to a 1.5ml eppendorf tube, and incubating at 100° C for 5

minutes The boiled mix was centrifuged at 14,000 x g for 20 minutes at 25° C The supernatant was transferred to a clean tube and stored at -20° C

In order to discern the optimum induction time for the accumulation of 2N-ScFv in the either the supernatant or the periplasm, a time course study was performed Briefly, the induction previously described was scaled up to 500ml 25ml samples were drawn every 2 hours from 3 hours post-induction to 11 hours post-induction One 25ml sample was taken from the induced culture after overnight incubation as well The supernatant / periplasm from each sample was prepared and stored as before

Protein Purification

2N-ScFv was expressed in *E coli* HB2151 cells The protein was collected from the periplasmic extract 3-4 hours post-induction as previously described The protocol was scaled up to 4L of culture each time, with all volumes and materials being adjusted accordingly To reduce viscosity, the periplasmic extract was briefly sonicated (Fisher Model 60 Sonic Dismembrator, Fisher), and the sonicated extract was filtered through a 0 2μm filter Proteins present in the periplasmic extract were precipitated by slowly adding 65% v/v saturated ammonium sulfate (Mallinckrodt) The precipitation was carried out to completion by incubating the mix overnight at 4° C with gentle stirring The precipitated mix was then centrifuged at 25,000 x g and 4° C for 1 hour. The supernatant was discarded and the pellet containing the precipitated 2N-ScFv was stored at -80° C. Before purification, pellet masses representative of 1L of the original culture

were each resuspended in a total of 10ml of 1x PBS and dialyzed at 4° C against at least three changes of 4L 1x PBS

Purification of the 2N-ScFv was accomplished by affinity chromatography in accordance with the RPAS™ Purification Module (AP Biotech) Briefly, 25ml of resuspended periplasmic precipitate (representing 2.5L of culture) were passed over a HiTrap® Anti-E Tag affinity column (AP Biotech) at a rate of 2 5ml/minute, and the filtrate was passed over the column once more The column was washed with 25ml of 1x Washing/Binding Buffer (AP Biotech) at the same flow rate The bound 2N-ScFv was eluted via dropping the pH by passing a total of 10ml of 1x Elution Buffer (AP Biotech) over the column Only the last 5ml of eluate were kept and neutralized with a total of 500µl of Neutralization Buffer (AP Biotech) The column was immediately equilibrated with 25ml of Washing/Binding Buffer This purification was repeated at least two more times All of the retained eluate was pooled and dialyzed against 4L of 1x PBS overnight at 4°C to remove any residual elution/neutralization salts The dialyzed product was subsequently concentrated 10x using a 10ml Amicon stir-cell microconcentrator (Fisher) fitted with a low protein-binding regenerated cellulose10kDa molecular weight cutoff filter (Millipore) The final 2N-ScFv concentration was determined by the BCA method as previously indicated Purified 2N-ScFv was stored at 4° C for up to 3 weeks or at -20° C for longer storage times Figure 2 2 summarizes the major aspects of ScFv generation, expression, and isolation.

A panel of *lktA* gene deletional constructs was genetically engineered by Jackson McPherson of this laboratory (184) Briefly, all constructs were amplified from *P*

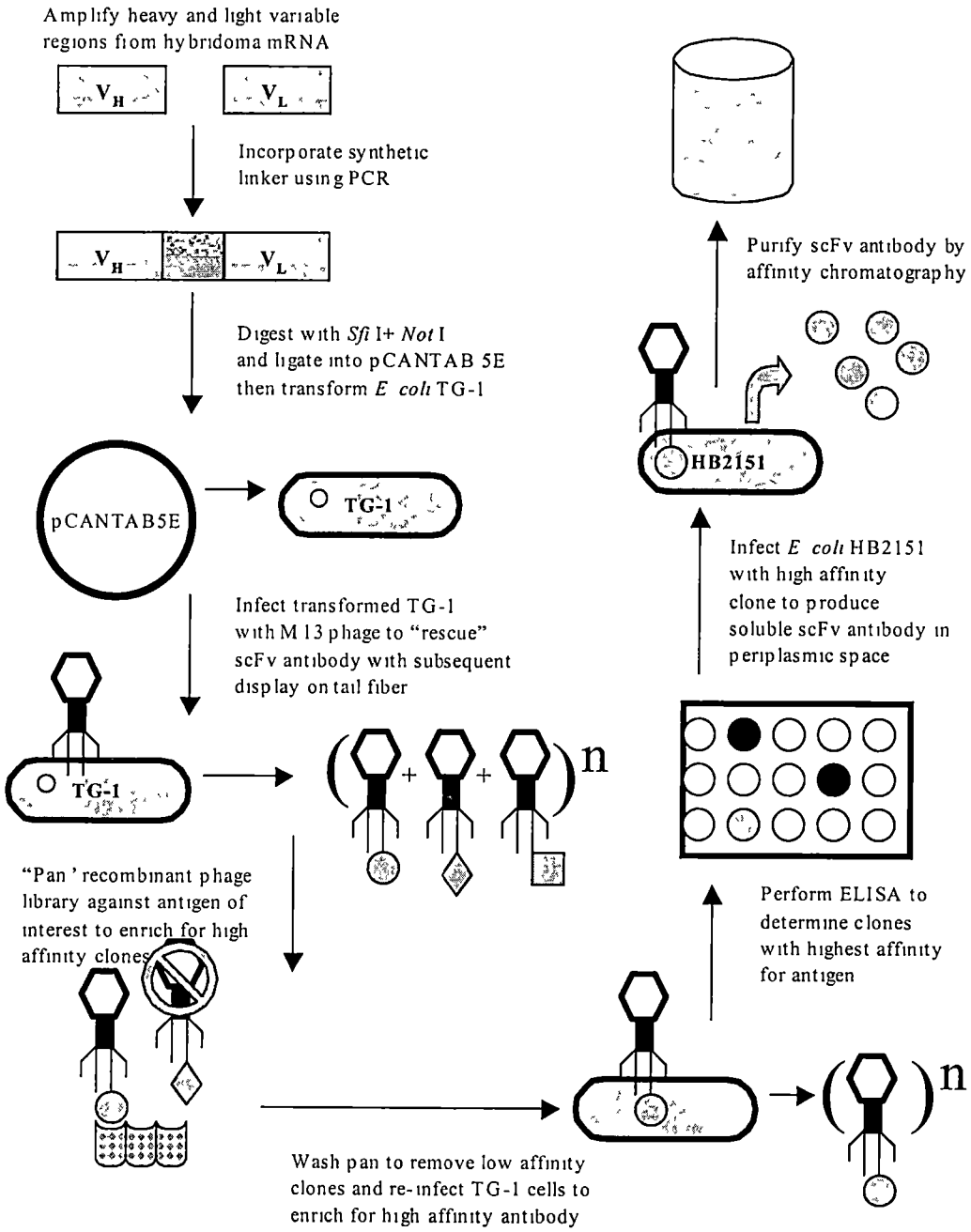


Figure 2.2. Schematic illustration of ScFv generation, selection, expression, and isolation.

haemolytica genomic DNA and recombined into the pGEX-4T-1 expression vector (AP Biotech, Figure 2.3) using restriction endonuclease digestion and ligation The pGEX-4T-1 expression vector contains a multiple cloning site immediately following the glutathione-S-transferase gene of *Schistosoma japonicum* such that the inserted *lktA* gene fragments were transcribed as carboxyl fusions with GST RNA These recombinant proteins were expressed in *E coli* JM-109 cells harboring the respective plasmid construct Cells were grown overnight in LB broth supplemented with 100μg/ml ampicillin at 37° C with 225rpm shaking Fresh, prewarmed LB/ampicillin^{100} medium was adjusted to 1% culture using the overnight culture, and then incubated as before to an OD_{595} of 0 6 The culture was then adjusted to 0 1mM IPTG and incubated as before for an additional 100 minutes The culture was centrifuged at 5,000 x g for 10 minutes at 4° C, the supernatant discarded, and the pellet resuspended in 30ml cold 1x PBS The cells were lysed by sonication Following sonication, the suspension was centrifuged for 30 minutes at 25,000 x g The supernatant was filtered through an Acrodisc® PF 0 8/0 2μm Supor filter (Gelman Sciences)

300-400μl Glutathione Sepharose® 4B matrix (AP Biotech) were added to the filtered supernatant and the suspension was incubated overnight at 4° C with gentle rocking Suspensions were then placed in a Poly-Prep chromatography column (BioRad Laboratories, Hercules, CA) and washed extensively with 1x PBS Fusion proteins were competitively eluted from the matrix by the addition of 4x the column bed volume of 10mM reduced glutathione (Sigma) The eluate containing the fusion protein was extensively dialyzed overnight in 4L 1x PBS at 4° C with at least two changes of 1x PBS

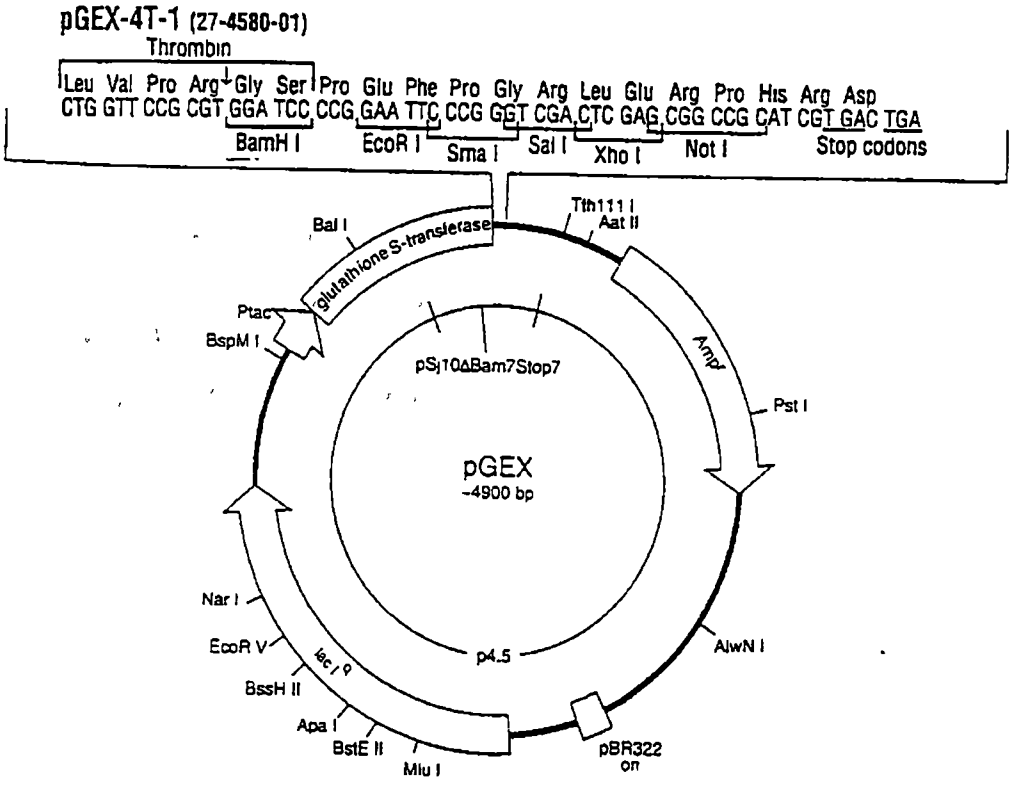


Figure 2.3. Diagram of pGEX-4T-1 vector. This expression vector was used to generate LktA-GST fusion proteins

Protein concentrations were determined by BCA as previously indicated Fusion proteins were stored at 4° C for up to 2 weeks or at -80° C for up to a month

SDS/PAGE and Western Immunoblot

All cell fractions and protein preparations were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) using a Mini Protean II gel apparatus (BioRad, Hercules, CA)according to the method described by Laemmli (148) Stacking gels of 4% and separating gels of 10 to 15% (acrylamide/bisacrylamide 30 0 8, National Diagnostics, Atlanta, GA) were used Protein samples were diluted in 1x PBS, if necessary, and 2x sample buffer (2 5ml 4x Tris-HCl (J T Baker, Inc , Phillipsburg, NJ), pH 6 8, 2 0ml glycerol, 0 4g SDS (BioRad), 1mg bromophenol blue (BioRad), and 5 5ml dH_2O) and electrophoresed at a constant amperage of 14mA for approximately 2 5 hours

The SDS/PAGE gels were subsequently soaked in protein transfer buffer consisting of 0 025M Tris, pH 8 2, 0 19M glycine (Fisher), and 0 1M SDS (BioRad) for 20 minutes Proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (PVDF, Millipore), prewetted with methanol (Fisher) and soaked in protein transfer buffer, using a semi-dry protein transfer apparatus (Gelman) set at 176mA for 1 5 hours Unbound protein sites were blocked with 3% NFDM at 25° C overnight Proteins of interest were detected by adjusting the appropriate primary antibody to the correct molar concentration in 3% NFDM for purified mAbs (e.g ltx-2) or to a predetermined dilution for HRP-conjugated mAbs (e.g 2N-ScFv was detected using a 1 1,000 dilution

of Anti-E tag/HRP monoclonal conjugate) Primary antibodies were incubated with the membranes for 1 5 hours at 37° C with gentle agitation The immunoblots were washed two times with PBS-Tween Unconjugated primary antibodies were detected with the appropriate dilution of HRP-conjugated secondary (e g ltx-2 was detected with rat anti-mouse IgG_1/HRP conjugate, Zymed) at a dilution of 1.1,000 in 3% NFDM . The membranes were incubated at 37° C for 1.5 hours Following washing as before, the immunoblots were developed by the addition of 15mg 4-chloro-1-naphthol (BioRad) dissolved in 5ml of ice cold methanol and 15µl of ice cold 30% H_2O_2 in 25ml of 1x Tris buffered saline(TBS, 20mM Tris, 0 5M NaCl, pH 7 2) Development occurred at 25° C for no longer than 30 minutes and was terminated by washing the membranes extensively in water. Protein sizes were determined by comparison to a set of standards (Rainbow markers RPN 756, AP Biotech)

For coomassie blue visualization of proteins, SDS/PAGE was performed as previously described The gels were placed in 65° C coomassie staining solution (0 5% Coomassie Brilliant Blue R (BioRad) in 50% dH_2O, 40% methanol, and 9 5% glacial acetic acid) and incubated for 1 hour The dye was bound and retained by any proteins present All unbound dye was removed by 3 to 5 washes in 65° C destaining solution (50% dH_2O, 40% methanol, and 10% glacial acetic acid)

Enzyme Linked Immunosorbant Assays

ELISAs were performed to determine the presence of both the purified mAb, ltx-2, and the 2N-ScFv and to compare their relative binding capacities for native leukotoxin

and leukotoxin-derived fragments This was achieved using the antibody capture (direct) ELISA, the two antibody sandwich (trap) ELISA, and a ltx-2 / 2N-ScFv competition ELISA For the direct ELISA, concentrated leukotoxin was diluted to 20μg/ml in 1x PBS, and 50μl were added to each well of 96-well PVC plate and incubated overnight at 25° C in a humidified chamber The plates were then washed three times with PBS-Tween Any unoccupied protein sites were blocked with 150μl 3% NFDM for 1 5 hours at 37° C in a humidified chamber The plates were washed as before, and an equimolar concentration of ltx-2 and 2N-ScFv were then added to the appropriate wells and serially diluted two-fold, if titration was necessary, to a total volume of 50μl per well Following incubation and washing as before, 50μl of the appropriate HRP-conjugated secondary reagent at a normalized concentration were added to the appropriate wells for ltx-2 detection, a 1 2,000 dilution of rat anti-mouse IgG_1/HRP, for 2N-ScFv detection, a 1 8,000 dilution of Anti-E tag/HRP The plates were again incubated and washed as described Development was by the addition of 50μl of ABTS/H_2O_2 in citrate buffer as previously described Following a 30 minute incubation in the dark, the A_{405} was determined by a microtiter plate reader To assay the affinity of ltx-2 / 2N-ScFv for the *lktA*-GST fusion constructs and other proteins (both similar and dissimilar to LKT), the proteins were diluted to 20μg/ml and applied to the appropriate wells to a final volume of 50μl Ltx-2 and 2N-ScFv were applied to the appropriate wells at equimolar concentrations and serially diluted, if titration was necessary The remainder of the assay was unmodified All dilutions were performed in 3% NFDM and all washings were performed with PBS-Tween

The trap ELISA was carried out as previously described with minor modifications Briefly, each well of a 96-well PVC plate was coated with 50µl of 20µg/ml ltx-35 or a 1.100 dilution of LKT-specific F11 rabbit polyclonal serum (generated by Jackson McPherson of this laboratory (184)) in 1x PBS overnight at 25° C The plates were washed 3 times with PBS-Tween and blocked with 150µl of 3% NFDM as before Concentrated leukotoxin was diluted to 20µg/ml and 50µl were added to the appropriate wells The plate was incubated at 37° C for 1 5 hours in a humidified chamber The remainder of the assay was unmodified from the direct ELISA previously outlined The *lktA*-GST fusion constructs were trapped by applying 50µl of 20µg/ml GST-specific goat polyclonal antibody (AP Biotech) and incubating overnight at 25° C in a humidified chamber The plate was washed three times with PBS-Tween and blocked with 150µl of 3% NFDM as before 50µl of the fusion proteins were applied to the appropriate wells at 20µg/ml and incubated for 1 5 hours at 37° C in a humidified chamber The remainder of the assay was unmodified from the direct ELISA described for the fusion fragments All dilutions were performed in 3% NFDM and all washings were performed with PBS-Tween

In order to confirm that ltx-2 and 2N-ScFv bind to a common leukotoxin epitope, competition ELISAs were performed Briefly, 96-well PVC plates were prepared for direct ELISA analysis, blocked, and washed as before Either ltx-2 or 2N-ScFv, adjusted to the appropriate molar concentration, was applied to the first set of wells and serially diluted 2-fold such that each well had a final volume of 50µl The plates were incubated at 37° C for 1 5 hours in a humidified chamber Following incubation and washing, 50µl

of 2N-ScFv were consistently applied to the ltx-2 dilution series at 1/40 the starting molar concentration of ltx-2 50µl of ltx-2 were consistently applied to the 2N-ScFv dilution series in the same fashion The plates were incubated and washed as before Any 2N-ScFv not blocked by ltx-2 was detected using Anti-E tag/HRP conjugate as before, and any ltx-2 not blocked by 2N-ScFv was detected using rat anti-mouse IgG_1/HRP as before All dilutions were performed in 3% NFDM and all washings were performed with PBS-Tween Percent inhibition for each scenario was calculated by comparing the mean A_{405} of the ltx-2 / 2N-ScFv competed wells at each dilution to the mean A_{405} of the respective uncompeted control wells

Cytotoxicity and Neutralization Assays

The MTT cytotoxicity assay (198) was modified to determine the activity of the leukotoxin Briefly, active leukotoxin was adjusted to the desired concentration and serially diluted two-fold in RPMI-1640 in a volume of 25µl on a pre-chilled, sterile 96-well round-bottom polystyrene plate (Becton-Dickinson) BL-3 cells were centrifuged at approximately 500 x g in a swinging bucket clinical centrifuge, resuspended to 1 $6x10^6$ cells/ml in RPMI-1640, and added to each well in volumes of 25µl ($4x10^4$ cells/well) The plate was incubated at 37°C with 5% CO_2 for 1 hour Following incubation, 50µl of complete RPMI-1640 (RPMI-1640 + 10% FBS + 2% psg) and 10µl of 3-(4,5-Dimethylthiazol-2-ul)-2,5-diphenyltetrazolium bromide (MTT, Fisher/Acros Organics, Pittsburgh, PA) were added to each well. The plate was subsequently incubated as before for 4 hours To determine the long-term cytotoxicity of the leukotoxin, this step was

modified to the addition of 50µl complete RPMI-1640, followed by 20 hours of incubation as before, the addition of 10µl MTT, and a final 4 hour incubation as before Cells were then lysed and the tetrazolium dye solubilized by the addition of 100µl acid alcohol (0 04N hydrochloric acid in isopropanol, Mallinckrodt). The A_{562} was determined with a microtiter plate reader One unit (U) of leukotoxin was defined as the working dilution that kills 50% of the cells and was determined by linear regression

For leukotoxin neutralization assays, the relevant mAbs and purified 2N-ScFv were adjusted to equimolar starting concentrations and serially diluted two-fold in RPMI-1640 as before in 25µl volumes 1 5 U of active leukotoxin were then added to each well in a volume of 25µl and incubated on ice for 1 hour to 4 hours BL-3 cells were then added to the plate as before and the remainder of the assay was performed as previously described

For functionality testing of leukotoxin-specific ScFv clones, the assay was carried out as described with the following modifications 25 µl of crude ScFv-containing supernatant from each tube of the 96-cluster tube microtiter format was used for neutralization and there were no serial dilutions performed

Antibody Adsorption Assay

In order to reinforce the importance of the epitope(s) contained within the fusion proteins, an antibody absorption assay was performed (184) Fusion proteins were adjusted to 400µg/ml in RPMI-1640 and 50µl were added to the first wells of a sterile 96-well polystyrene plate The fusion proteins were then serially diluted 2-fold in RPMI-

1640 such that 25µl occupied each well 25µl of ltx-2 and 2N-ScFv in RPMI-1640 were added to the appropriate wells at a predetermined equimolar concentration The plate was incubated on ice for 1 hour 1 5 U of active leukotoxin in RPMI-1640 were then added in a volume of 25µl, and the plate was incubated for an additional hour on ice Subsequently, $4x10^4$ BL-3 cells in fresh RPMI-1640 were added to each well The plate was then incubated at 37° C and 5% CO_2 for 1 hour at which time a mix of 50µl complete RPMI-1640 and 10µl MTT were added to each well A final incubation at 37° C and 5% CO_2 for 4 hours was allowed The plate was developed with 100µl acid alcohol as before and the A_{562} was determined with a microtiter plate reader

Statistical Analysis

All linear regression and variability analysis was conducted using one-way ANOVA where applicable Results were considered significant if $P \leq 0\ 05$ All statistical analyses conducted on recognition (ELISA) and neutralization (MTT) titrations were based on $n = 3$ All non-titrated recognition ELISAs were based on $n = 6$

CHAPTER 3

RESULTS

Monoclonal Antibody Characterization

A panel of six monoclonal antibodies reactive with the 101 9 kDa leukotoxin of *Pasteurella haemolytica* was generated by Donald Gerbig and Mark Cameron of this laboratory (96, 97) The mAbs were named as follows ltx-2, ltx-4, ltx-35, ltx-37, ltx-58, and ltx-81 Preliminary studies based on eptitope mapping with cyanogen bromide cleavage fragments, mutant deletion proteins, and alkaline phosphatase/leukotoxin fusion proteins revealed that the region between LktA amino acids 768 and 939 was important for recognition by this panel of mAbs (96) Additional studies based on *Actinobacillus pleuropneumoniae* serotype 5 toxin/*P haemolytica* serotype A1 leukotoxin chimeric protein molecules created by Dr Douglas Struck (187)confirmed that the epitope for each mAb was located within this region (81) Using glutathione-S-transferase (GST) fused N-terminally deleted LktA constructs derived from the carboxy one-third of the leukotoxin molecule, the binding domains of ltx-2, ltx-4, and ltx-35 were narrowed down to amino acids 876-939, 876-939, and 846-862 respectively (184) No further epitope mapping has been conducted for ltx-37, ltx-58, or ltx-81 Finally, Pellet *et al* developed a mAb against the closely related RTX toxin, α-hemolysin (HlyA), of *E coli* which would only recognize the HlyC acyl-modified form of HlyA (211). However, it was demonstrated that none of the leukotoxin-specific mAbs required this form of acyl

modification of LktA to recognize the molecule (81) This study has shown that the region consisting of amino acids 768-939 contains the minimal elements necessary for ltx-2 recognition, that this region alone maintains many of the *in vitro* characteristics of native leukotoxin, and that the region 876-939 is essential, though not alone sufficient, for ltx-2 recogition

All of the derived leukotoxin-specific mAbs have been tested for their ability to neutralize the cytotoxic effects of LKT When purified IgG from each mAb-producing hybridoma was tested for neutralizing capabilities, it was found that ltx-2, ltx-4, ltx-35, and ltx-37 offered various degrees of protection from LKT in a target cell assay (81) Table 3 1 summarizes the characteristics of each mAb Because of the potent neutralization capacity of ltx-2 (96, 97), this mAb has been intensely investigated in hopes of understanding the region(s) involved in LKT-induced cytolysis Preliminary flow cytometric data revealed that ltx-2 prevents association with susceptible target cells (96, 97)

Molecular Cloning of a LKT-Specific Single Chain Fragment Variable (ScFv)

In order to further characterize the binding properties of ltx-2, elucidate the mechanism of ltx-2 neutralization, and create a novel LKT-specific reagent, a 29 5 kDa protein was generated This protein consists of the variable region of the light and heavy chains of ltx-2, linked together by a flexible polyglycine linker to allow for the formation of a stable LKT-specific paratope (binding pocket), and is dubbed a single chain fragment variable or ScFv Molecular techniques such as mRNA isolation, RT-PCR, PCR, and

Table 3.1. Characteristic profile for leukotoxin-specific mAbs

mAb	Isotype	Recognition Titer (ng/ml)[a]	Neutralization Titer (μg/ml)[b]
ltx-2	IgG_1	3,000	0 2
ltx-4	IgG_1	60	2
ltx-35	IgG_{2b}	< 25	120
ltx-37	IgG_{2b}	25	50
ltx-58	IgG_1	50	> 200
ltx-81	IgG_1	400	> 200

[a]Concentration of purified ascites IgG determined by direct ELISA on bound concentrated LKT culture filtrate (1μg protein/well) yielding A_{405} of 0 2 (96)
[b]Titer was equivalent to the mAb concentration at which 50% cytotoxicity was observed with 1U leukotoxin (81)

recombinant subcloning were of major importance in producing the ScFv First, in order to molecularly reduce the native structure of the 160 kDa ltx-2 down to a 29 5 kDa protein, it was necessary to isolate the total RNA and enrich the mRNA of the ltx-2-producing hybridoma Spectrophotometric quantitative analysis of the total RNA isolated from $5x10^6$ cells/ml showed that the RNA yield was approximately 0 11 μg/ml and gave an A_{260}/A_{280} ratio of 1 97 Both of these numbers were within the guidelines provided with the MicroPoly(A)Pure™ kit, indicating an adequate RNA yield relatively free of any protein contaminants Furthermore, electrophoretic qualitative analysis was performed on the RNA preparation to ensure the absence of any detrimental RNase contamination, which is often revealed as smeared rRNA bands and/or an abundance of EtBr-stained low molecular weight nucleic acid products The preparation was resolved on a 1% non-denaturing TBE agarose/EtBr gel As can be seen in Figure 3 1, the 28s and 18s rRNA bands were crisp with minimal smearing, and EtBr-stained low molecular weight nucleic acid products were absent (with the majority of stained nucleic acids being of high molecular weight) These observations indicated that the RNA preparation was adequate for downstream applications Finally, the mRNA within the preparation was isolated with a polyT column and immediately subjected to cDNA synthesis for stabilization

Subsequent to isolation, the ltx-2 mRNA was primed with random hexadeoxyribonuclotides in the presence of nucleoside triphosphates and cloned murine reverse transcriptase to yield cDNA via RT-PCR The resultant cDNA product was not directly analyzed, rather it was immediately subjected to PCR in order to amplify the

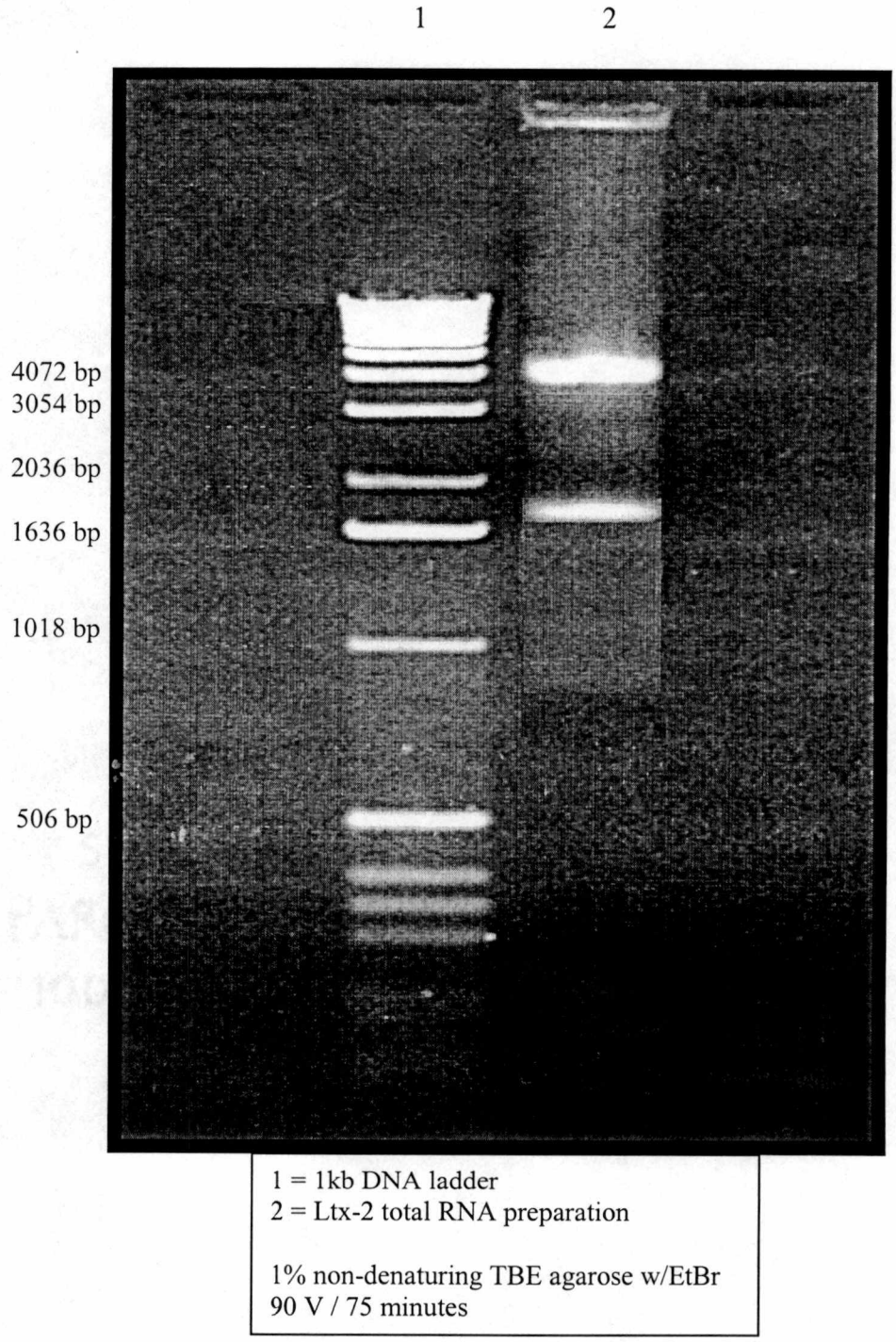
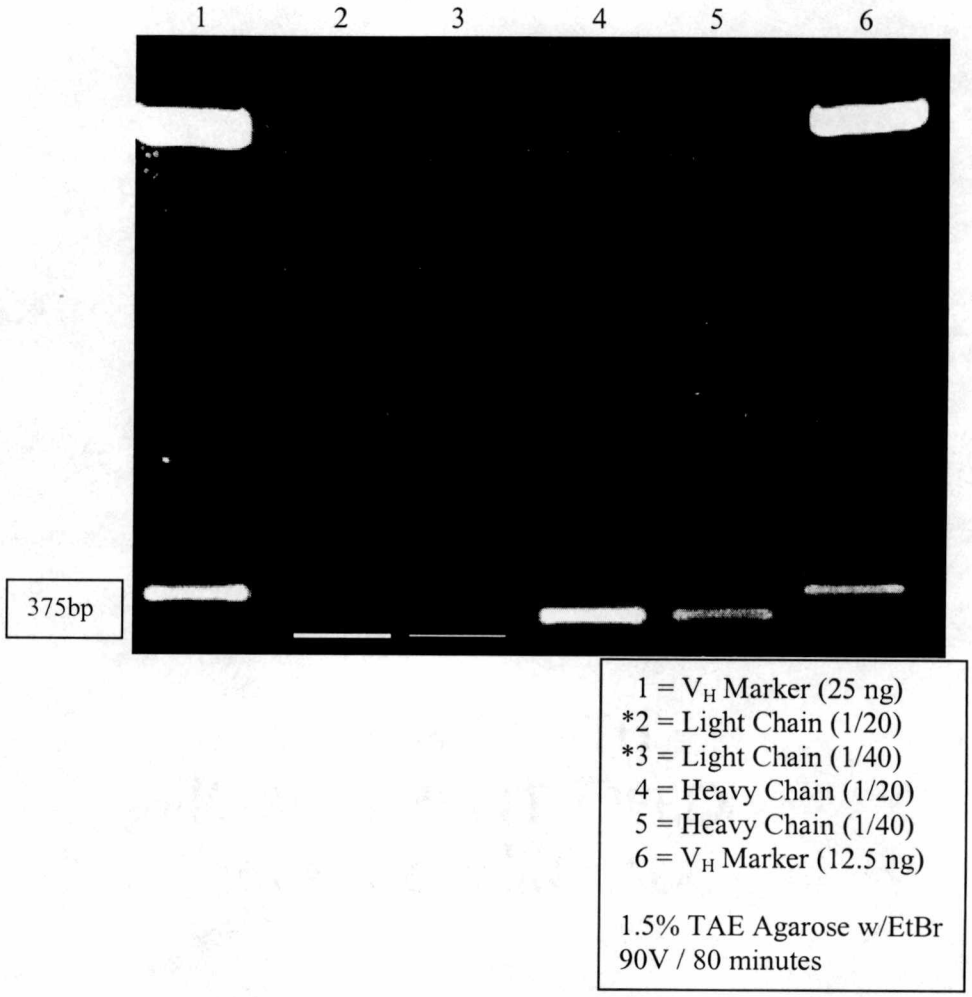


Figure 3.1. Gel photograph illustrating ltx-2 RNA. Total RNA was isolated from $5x10^6$ cells/ml of ltx-2-producing hybridoma.

variable region of both the heavy and light chain Using a degenerate mix of proprietary (AP Biotech) 5' and 3' primers, the light and heavy chain variable regions encoded by the cDNA were independently amplifed via PCR with *Taq* DNA polymerase This set of amplifications yielded a pool of heavy chain variable fragments, each having a molecular weight of approximately 350bp, and a pool of light chain variable fragments was also generated, each having a molecular weight of approximately 325bp Figure 3 2 shows the gel-purified amplified products of each chain, loaded as fractions of the total PCR mixture volume and resolved on a 1 5% TAE agarose/EtBr gel, and the relative size of each chain compared to a 375bp control marker As expected, the 350bp heavy chain fragment showed a slightly greater rate of migration than the control, and the corresponding band was clearly visible for both of the two volume fractions loaded Also, the DNA concentration, represented by band intensity, was in accordance with the volumes loaded Although the 325bp band representing the light chain fragment is not visible in the scanned image for either of the two volume fractions loaded, the band in each of lanes 2 and 3 were faintly visible on the original photo (inserted lines show the exact location and relative concentrations as determined by the original photo) The relative rate of migration for the slightly smaller light chain fragment was as expected, migrating further than both the control marker and heavy chain fragment in the same amount of time Using band intensity as an approximate measure of fragment concentration, the amplification of the heavy chain variable region was 2-3 times more efficient than the amplification of the light chain variable region This was most likely correlated to the priming efficiency for each chain; namely, the primer homolgy,



*Actual bands are not visible. The respective inserted lines show relative size and intensity.

Figure 3.2. DNA gel of ltx-2-derived V_H and V_L fragments. The gel photograph illustrates the relative sizes and concentrations of *Taq*-amplified heavy and light chain variable fragments derived from independently primed PCR mixtures.

annealing temperature conditions, etc were more conducive to successful amplification of the heavy chain than the light chain However, some loss may be attributable to differential recovery efficiency during gel purification of each fragment

To prevent aberrant pairing of mismatched or poorly associating heavy and light chain fragments that could arise by random interactions (which may severely diminish the specificity and function of the cloned paratope) and to promote correct association and proper folding, the heavy and light chain variable fragments were connected on an equimolar basis via a flexible $(Gly_4Ser)_3$ linker This was accomplished using a homology-based fill-in reaction with subsequent amplification of the full length ScFv During this amplification, 5' *Sfi*I and 3' *Not*I restriction sites were engineered The final product is a single chain typically consisting of 720-800bp as illustrated in Figure 3 3 This figure confirms that the final gel-purified single-chain product was approximately 760bp when resolved on a 1 5% TAE agarose/EtBr gel and compared to a 100bp DNA step ladder standard and ScFv positive control marker (molecular weight of 750bp), which is well within the expected range Again, the intensity of each ScFv band present was in accordance with the volume loaded The final full-length ScFv product was then digested with *Sfi*I/*Not*I and ligated into the pCANTAB 5E phagemid vector (see figure 2 1) for subsequent recombinant ScFv phage display and selection

Phage Display, Selection, and Preliminary Clone Screening

After ligation of the ScFv repertoire generated from PCR into a *Sfi*I/*Not*I pre-digested phagemid expression vector, pCANTAB 5E, competent TG-1 *E coli* cells were

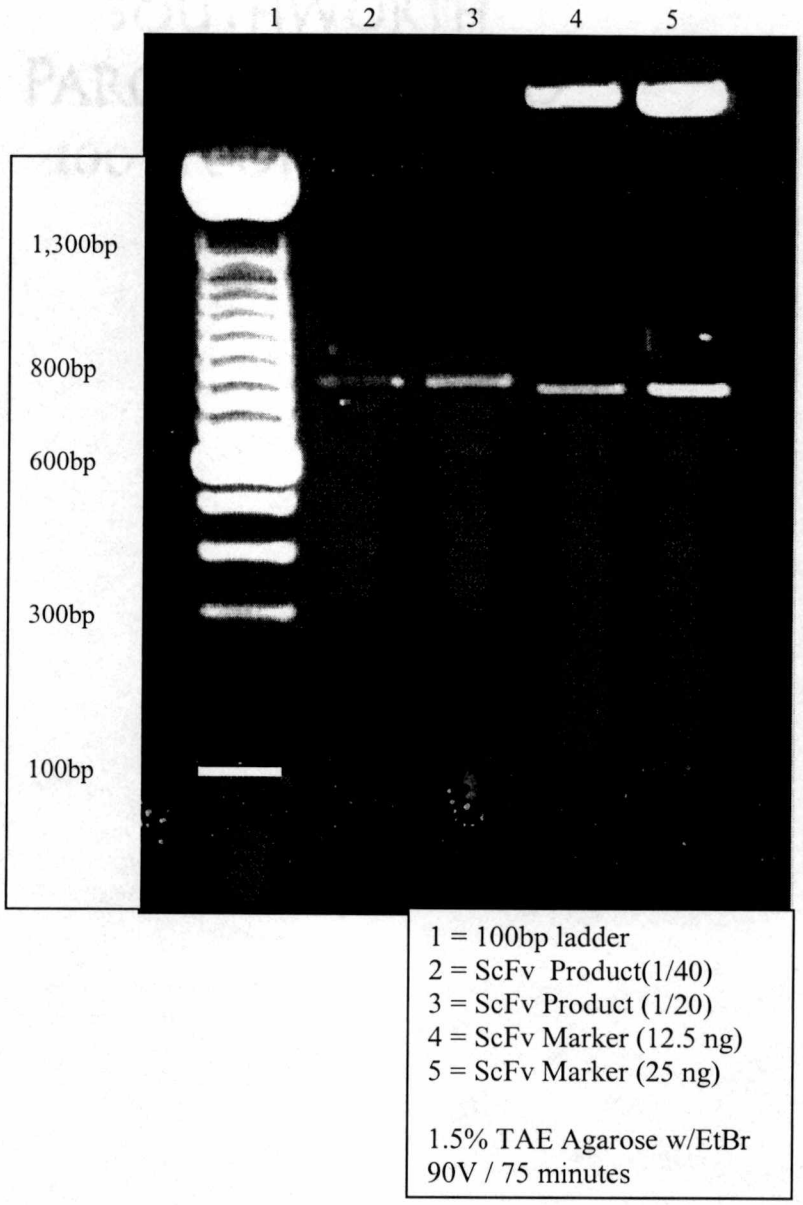
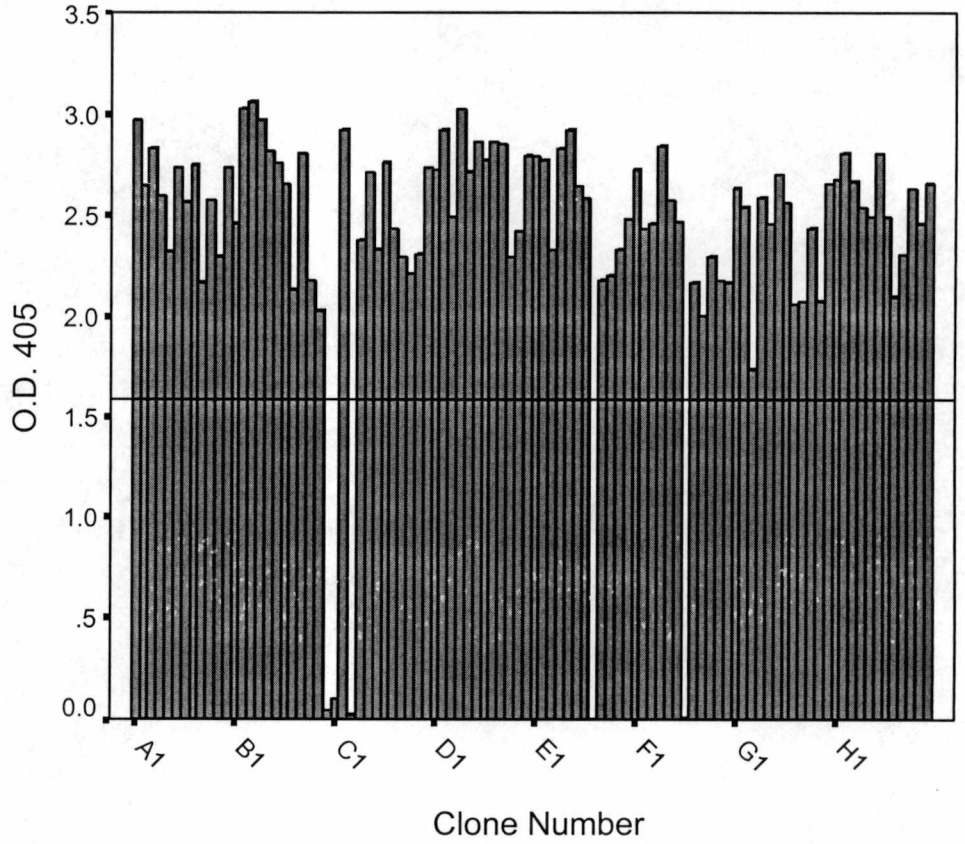


Figure 3.3. DNA gel of full length ScFv. The gel photograph illustrates the relative size and concentration of the *Taq*-amplified $(Gly_4Ser)_3$-linked full length ScFv.

transformed Transformation efficiency was tested by plating the transformed cells on selective media and deemed adequate (results not shown) Those cells stably transformed expressed the ScFv as a fusion partner of the phagemid-encoded M13 phage tail fiber due to suppression of a phagemid amber stop codon in the TG-1 strain (additionally, some soluble ScFv was expressed independently of the tail fiber due to variability in adhering to the stop signal), making this a suitable system for antigen specificity screening via phage-display of the antigen-specific ScFv However, without the replication and packaging assistance of the K07 helper phage, this fusion product could not be packaged into a mature phage for phage-displayed ScFv screening Consequently, the transformed mix was "rescued" with the K07 helper phage To further eliminate those clones that did not recognize or had weak affinity for LKT, which are often present in the repertoire due to sequence variability and random linking of heavy and light chains, the phage were panned on a solid-phase surface against 20μg/ml of mAb 35-trapped LKT three times Finally, these enriched, high affinity clones were used to tranduce log-phase TG-1 cells which were subsequently serially diluted and plated on selective media Each plate in a 10^{-1} through 10^{-6} dilution series showed appreciable colony counts, varying in accordance to the dilution plated (data not shown) Subsequently, ninety-six well-isolated colonies were independently grown and either rescued with K07 helper phage to produce phage-displayed ScFv or induced with 1mM IPTG to augment soluble ScFv production. Both the phage-displayed and soluble ScFv derived from each of the 96 colonies were screened against 20μg/ml mAb 35-trapped LKT, and specificity was quantified via ELISA Using the convention that any O D.^{405} > 0.4 was indicative of LKT specificity, it

was found that only 5 of the 96 clones screened showed no appreciable LKT recognition· B12, C1, C3, E8, and F7 (clones were named in accordance with a 96 well microtiter format, *i e* , A1-A12, B1-B12, etc) This result was expected given the extensive enrichment of LKT-specific clones during the panning process The remainder of the clones gave very high O D ^{405} signals, often surpassing that given by the ltx-2-HRP conjugated positive control No signal was appreciated for the negative control (developing secondary against LKT) As expected, the results were consistent for both the phage-displayed and soluble ScFv screenings, illustrated in Figures 3 4 and 3 5 respectively, although soluble ScFv gave lower signals on the average than phage-displayed ScFv This was most likely due to differences in the secondary detection rather than LKT recognition variability

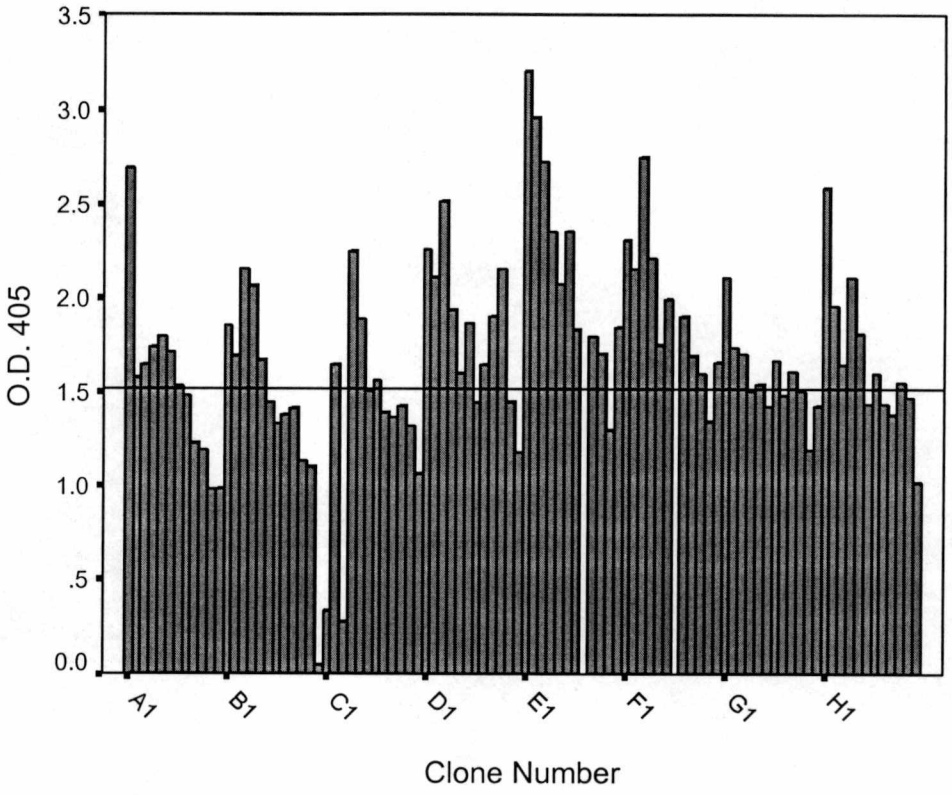
Even though the preliminary screening showed that 95% of the clones displayed LKT specificity, it was desirable to know which of these LKT-specific clones, if any, would neutralize the leukotoxin's cytotoxic effects In order to determine this a neutralization assay, based on MTT reduction, was carried out to quantify the ability of each clone to neutralize 1 U of LKT targeted at 4 x 10^4 bovine-derived BL-3 cells The results, seen in Figure 3 6, revealed that virtually all of the clones which displayed appreciable LKT recognition also protected the BL-3 cells as indicated by values representative of the cells only positive control (live value) and well above the cells plus LKT negative control (kill value) Only one exception was noted clone G3 showed positive LKT recognition but failed to protect the BL-3 cells However, this clone was later retested and shown to neutralize the LKT under the same set of conditions (data not



[a]Clone Population Mean = 2.4 ± 0.6
Positive Control (ltx-HRP conjugate) Mean = 1.6 ± 0.1 (indicated as a straight line in the graph above)
Negative Control (anti-M13 coat protein-HRP conjugate only) Mean = 0.0 ± 0.0

[a]Means are expressed as the average O.D.^{405} of the given population ± standard deviation.

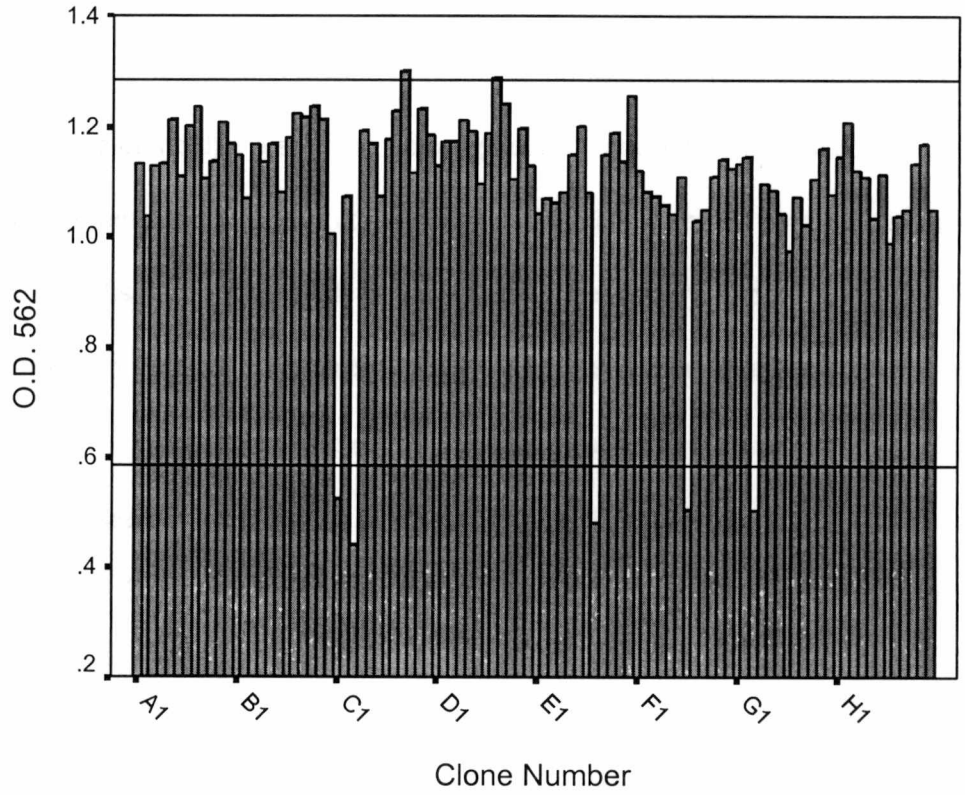
Figure 3.4. Quantitation of LKT specificity of phage-displayed ScFv clones A1-H12 as determined by trap ELISA. Each clone was tested against 20μg/ml of LKT trapped with 20μg/ml mAb 35. The bound phage was detected with a 1:8,000 dilution of anti-M13 coat protein-HRP conjugate. O.D.^{405} > 0.4 was considered LKT-specific.



Clone Population Mean = 1.7 ± 0.6
Positive Control (ltx-2-HRP conjugate) Mean = 1.5 ± 0.3 (indicated as a straight line in the graph above)
Negative Control (anti-E tag-HRP conjugate only) Mean = 0.0 ± 0.0

[a]Means are expressed as the average O.D.^{405} of the given population ± standard deviation.

Figure 3.5. Quantitation of LKT specificity of 1mM IPTG-induced soluble ScFv clones A1-H12 as determined by trap ELISA. Each clone was tested against 20μg/ml of LKT trapped with 20μg/ml mAb 35. The bound phage was detected with a 1:8,000 dilution of anti E tag-HRP conjugate. O.D.^{405} > 0.4 was considered LKT-specific.



[a]Clone Population Mean = 1.1 ± 0.2
Live Value (cells only) Mean = 1.3 ± 0.0 (upper line on the graph above)
Kill Value (cells plus LKT) Mean = 0.6 ± 0.0 (lower line on the graph above)

[a]Means are expressed as the average O.D.^{562} of the given population ± standard deviation.

Figure 3.6. Quantitative analysis measuring the neutralizing capability of each of clones A1-H12. Each clone was tested for its ability to neutralize 1 U of LKT targeted against 4 x 10^4 BL-3 target cells. Values reflect the amount of reduced MTT released upon lysis of the BL-3 cells with acid alcohol.

shown) Conversely, those clones which displayed no specificity for LKT did not neutralize the toxic effects, displaying values comparable to the kill value Again, one exception was noted clone B12 was originally found to be negative for LKT recognition but provided appreciable protection against LKT Subsequent sequence analysis (not shown) on this clone revealed drastic sequence abnormalities when compared to clone D9 (a LKT-specific clone that displayed a strong neutralization capacity, see below) Although an interesting exception to the expected behavior shown by all the other clones, this clone was disregarded since detection in subsequent assays would not be feasible Finally, based on the values obtained during the neutralization assay, thirteen clones giving an O D $^{562} \geq 1\ 2$ were selected at random for subsequent transduction of *E coli* HB2151 cells (see below) A5, A8, B8, B10, B11, C8, C9, D4, D8, D9, E6, E12, and H2 Tables 3 2 and 3 3 give the actual assay values found for each clone during the initial screening and characterization

Soluble Expression of ScFv Clones

Although phage-display of ScFv is an extremely effective, time efficient method of screening for antigen specificity, the fusion partner of the ScFv, the M13 tail fiber, actually accumulates within TG-1 cells and is cytotoxic This is especially true if the cells are induced to express proteins with an inducing agent such as IPTG However, in cell lines that adhere to the amber stop codon present on the phagemid, such as *E coli* HB2151 cells, readthrough is suppressed and no tail fiber protein is made Although the capacity for phage-display is lost, the production of soluble ScFv can be induced with

Table 3.2. Summary of clones A1-D12 These values were derived from the three initial assays run to characterize the LKT recognition and neutralization tendencies of clones A1-D12

Clone Number	O D ^{450}- Phage	O D ^{450}- Soluble	O D ^{562} -MTT	Clone Number	O D ^{450}- Phage	O D ^{450}- Soluble	O D ^{562}- MTT
[a]Positive Controls	1 6 ± 0 1	1 5 ± 0 3	1 3 ± 0 0	[a]Negative Controls	0 0 ± 0 0	0 0 ± 0 0	0 6 ± 0 0
A1	3 0	2 7	1 1	C1	0 1	0 3	0.5
A2	2 6	1.6	1 0	C2	2 9	1 7	1 1
A3	2 8	1 6	1 1	C3	0 0	0 3	0 4
A4	2 6	1 7	1 1	C4	2 4	2 3	1 2
[b]**A5**	2.3	1 8	1 2	C5	2 7	1.9	1 2
A6	2 7	1 7	1 1	C6	2 3	1 5	1 1
A7	2 6	1 5	1 2	C7	2 8	1 6	1 2
A8	2 8	1 5	1 2	**C8**	2 4	1 4	1 2
A9	2 2	1 2	1 1	**C9**	2 3	1 4	1 3
A10	2 6	1 2	1 1	C10	2 2	1 4	1 1
A11	2 3	1 0	1 2	**C11**	2 3	1 3	1 2
A12	2 7	1 0	1 2	C12	2 7	1 1	1 2
B1	2 5	1 9	1 1	D1	2 7	2 3	1 1
B2	3 0	1 7	1 1	D2	2 9	2 1	1 2
B3	3 1	2 2	1 2	D3	2 5	2 5	1 2
B4	3 0	2 1	1 1	**D4**	3 0	1 9	1 2
B5	2 8	1 7	1 1	D5	2 7	1 6	1 2
B6	2 8	1 4	1 1	D6	2 9	1 9	1 1
B7	2 7	1 3	1 2	D7	2.8	1.4	1 2
B8	2 1	1 4	1 2	**D8**	2 9	1 7	1 3
B9	2 8	1 4	1 2	**D9**	2 8	1 9	1 2
B10	2 2	1 1	1 2	D10	2 3	2 2	1 1
B11	2.0	1 1	1 2	D11	2.4	1 5	1 2
[c]*B12*	0 0	0 0	1 0	D12	2 8	1 2	1 1

[a]Control values are expressed as optical density means ± standard deviation.
[b]Bold-faced clones represent those that were transduced into *E coli* HB2151 cells for large-scale soluble ScFv production
[c]Italicized clones represent those with screening inconsistencies.

Table 3.3. Summary of clones E1-H12 These values were derived from the three initial assays run to characterize the LKT recognition and neutralization tendencies of clones E1-H12

Clone Number	O D ^{450}- Phage	O D ^{450}- Soluble	O D ^{562} -MTT	Clone Number	O D ^{450}- Phage	O D ^{450}- Soluble	O D ^{562}- MTT
[a]Positive Controls	1 6 ± 0 1	1 5 ± 0 3	1 3 ± 0 0	[a]Negative Controls	0 0 ± 0 0	0 0 ± 0 0	0 6 ± 0 0
E1	2 8	3 2	1.0	G1	2 6	2 1	1 1
E2	2 8	3 0	1 1	G2	2 5	1 7	1 1
E3	2 3	2 7	1 1	[c]*G3*	1 7	1 7	0 5
E4	2 8	2.3	1 1	G4	2 6	1 5	1 1
E5	2 9	2 1	1 2	G5	2 5	1 5	1 1
[b]**E6**	2 6	2 4	1 2	G6	2 7	1 4	1 0
E7	2 6	1 8	1 1	G7	2 6	1 7	1 0
E8	0 0	0 0	0 5	G8	2 1	1 5	1 1
E9	2 1	1 8	1 2	G9	2 1	1 6	1 0
E10	2 2	1 7	1 2	G10	2 4	1 5	1 1
E11	2 3	1 3	1 1	G11	2 1	1 2	1 2
E12	2 5	1 8	1 3	G12	2 7	1 4	1 1
F1	2 7	2 3	1 1	H1	2 7	2 6	1 1
F2	2 4	2 2	1 1	**H2**	2 8	2 0	1 2
F3	2 5	2.8	1 1	H3	2 7	1 6	1 1
F4	2 8	2 2	1 1	H4	2 5	2 1	1 1
F5	2 6	1 8	1 0	H5	2 5	1 2	1 0
F6	2 5	2 0	1 1	H6	2 8	1 4	1 1
F7	0 0	0 0	0 5	H7	2 5	1 6	1 0
F8	2 2	1 9	1 0	H8	2 1	1 4	1 0
F9	2 0	1 7	1 1	H9	2 3	1 4	1 0
F10	2 3	1 6	1 1	H10	2 6	1 6	1 1
F11	2 2	1 3	1 1	H11	2 5	1 5	1 1
F12	2 2	1 7	1 1	H12	2 7	1 0	1 1

[a]Control values are expressed as optical density means ± standard deviation

[b]Bold-faced clones represent those that were transduced into *E coli* HB2151 cells for large-scale soluble ScFv production

[c]Italicized clones represent those with screening inconsistencies.

IPTG without being deleterious to the host cell As a result, thirteen LKT-specific neutralizing phage-displayed clones (see above) were used to transduce *E coli* HB2151 cells, which were subsequently streaked on selective media containing nalidixic acid to eliminate any nonspecific susceptible TG-1 background Each of the thirteen transductants yielded appreciable growth and colony isolation At random, only the transductants of clones D8 and D9 were chosen for induction of ScFv production Both of these transductants were induced with 1mM IPTG overnight After induction, the cells were harvested and fractionated into supernatant, periplasmic, and cytoplasmic fractions Each fraction was analyzed for the presence of a 30kDa ScFv, based on the presence/absence of an inherent 13 amino acid E tag detectable marker, via Western immunoblot, illustrated in Figure 3 7 When probed with an anti-E tag-HRP conjugate, it was found that both clones showed appreciable levels of a 30kDa ScFv in the supernatant, minute levels in the periplasm, and no ScFv was detected in the cytoplasmic fraction Because clone D9 expressed greater levels of the ScFv in both the supernatant and periplasm as determined by visual inspection of band intensity and provided adequate protection in the initial neutralization screen, this clone was chosen for all future applications

In order to empirically determine the optimum induction strategy for soluble, D9-derived ScFv production, a time course assay was performed. In this assay, periplasmic and supernatant fractions were collected every 2 hours starting at 3 hours post-induction with 1mM IPTG Collection was terminated after overnight induction (24 hours) All of the fractions were analyzed by Western immunoblot as previously indicated The results

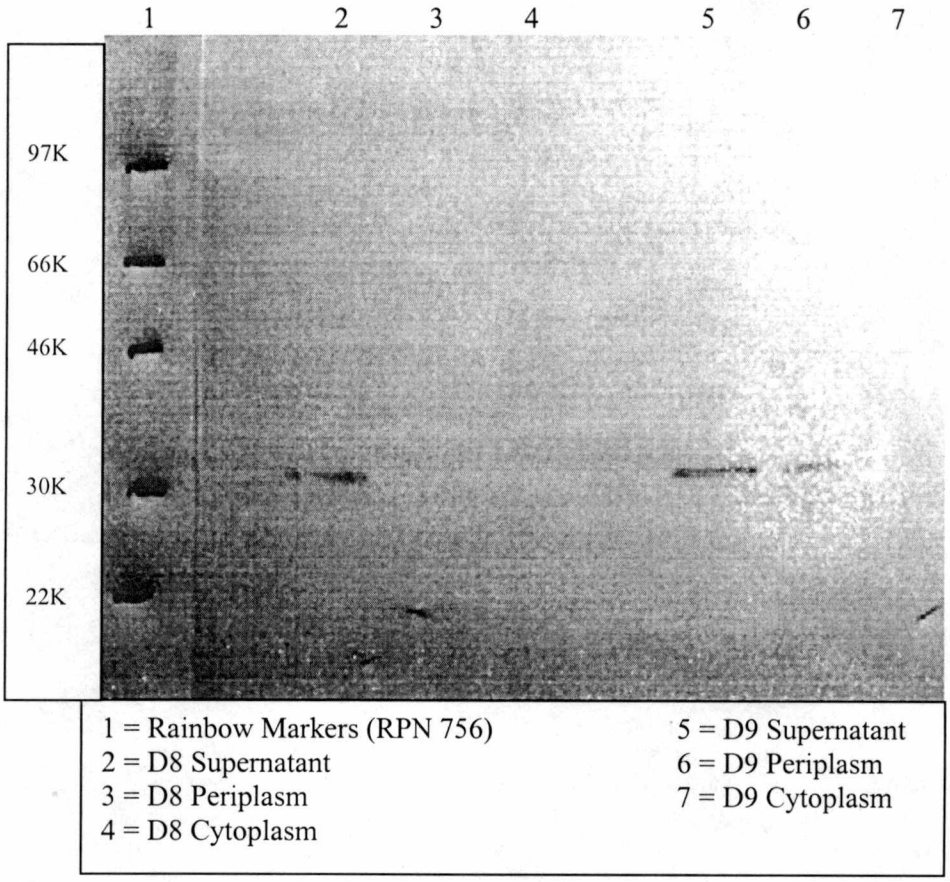


Figure 3.7. Western immunoblot illustrating the presence/absence of a 30 kDa ScFv. The supernatant, periplasmic, and cytoplasmic fractions of D8 and D9-transduced *E. coli* HB2151 cells were assayed for ScFv presence after overnight induction with 1mM IPTG. ScFv was detected with a 1:1,000 dilution of anti-E tag-HRP conjugate.

of this study are illustrated in Figure 3 8, which reveals an inverse relationship between periplasmic and supernatant-derived ScFv levels As periplasmic levels steadily decrease over time, supernatant levels steadily increase due to periplasmic leakage Based on the relative concentrations indicated by band intensities, optimal expression levels of soluble ScFv were determined to exist in the periplasmic fraction at 3 hours post-induction This finding was quantitatively reinforced when the 3 hour and overnight-induced supernatant and periplasmic fractions were titrated against LKT Figures 3 9 and 3 10 illustrate the titration curves and linear regression analyses of the titrations respectively as determined by direct ELISA As can be seen in Figure 3 9, only the 3 hour periplasmic fraction yielded O D ^{405} levels comparable to the ltx-2 positive control (20μg/ml ltx-2 against 20μg/ml LKT), which is labeled as 2N in this graph and all subsequent graphs Although parallel titration curves (as indicated by equivalent slopes) suggest comparable affinities between the differentially localized ScFvs, linear regression analysis on this set of titration curves revealed that the ScFv derived from the 3 hour periplasmic fraction has a titer (defined as the reciprocal dilution which gave an O D ^{405} of 0 8) of 235 9 This value is 2 3x greater than the titer for the peak concentration of supernatant-derived ScFv (overnight induction), indicating that there is 2 3x more functional LKT-specific ScFv in the periplasm at 3 hours post-induction than can be produced in the supernatant Also noteworthy in this set of figures is that the ScFv found in the periplasm after overnight induction titrated abnormally compared to the other species evaluated. This was probably due to protease destruction and/or aggregation of the ScFv within the periplasmic milieu, and suggested that periplasmic-localized ScFv loses a significant

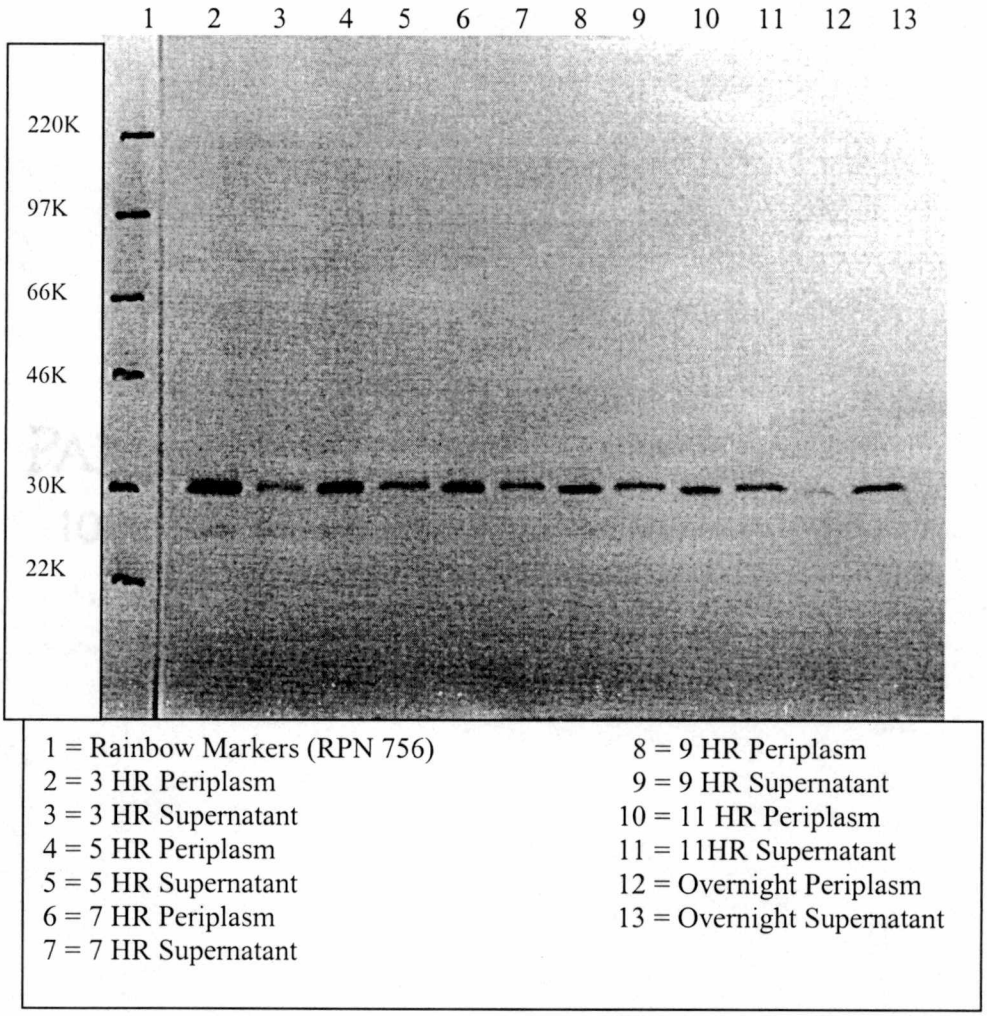


Figure 3.8. Western immunoblot illustrating the temporal induction of ScFv. The relative abundance of detectable D9-derived soluble ScFv in both the supernatant and periplasmic fractions of *E. coli* HB2151 cells was determined at varying time points. Time points began at 3 hours post-induction with 1mM IPTG and terminated after 18 hours (overnight) induction. ScFv was detected with a 1:1,000 dilution of anti-E tag-HRP conjugate.

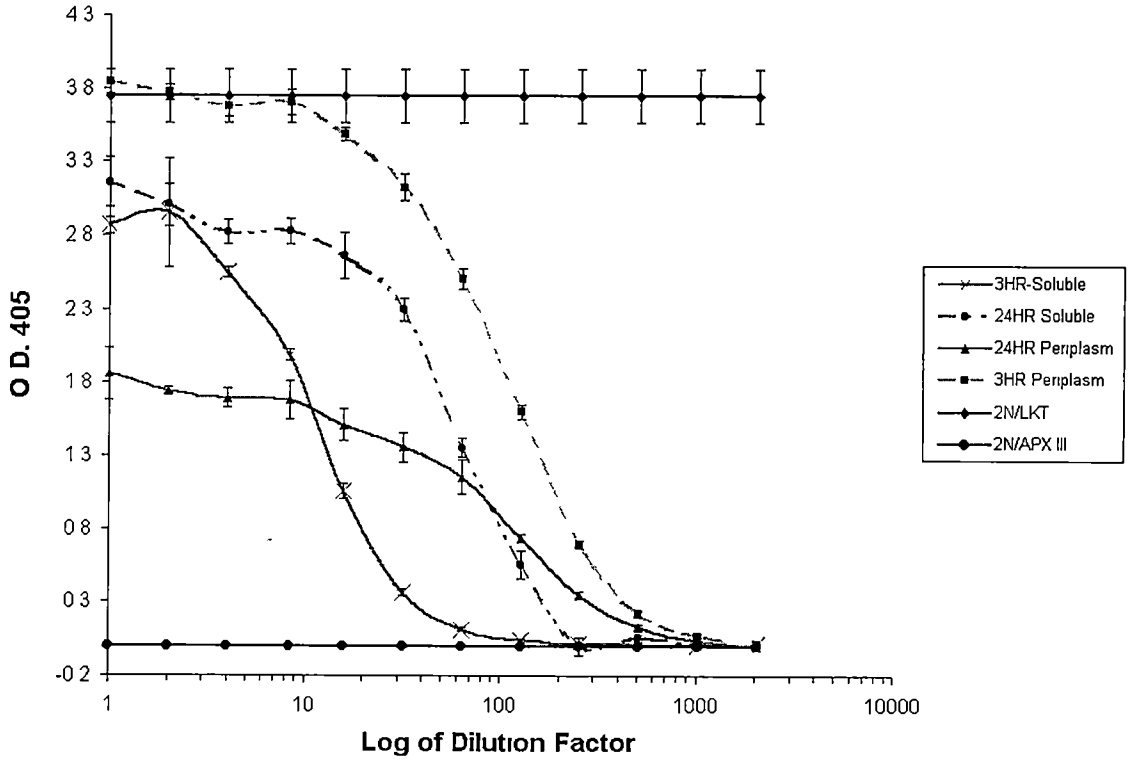
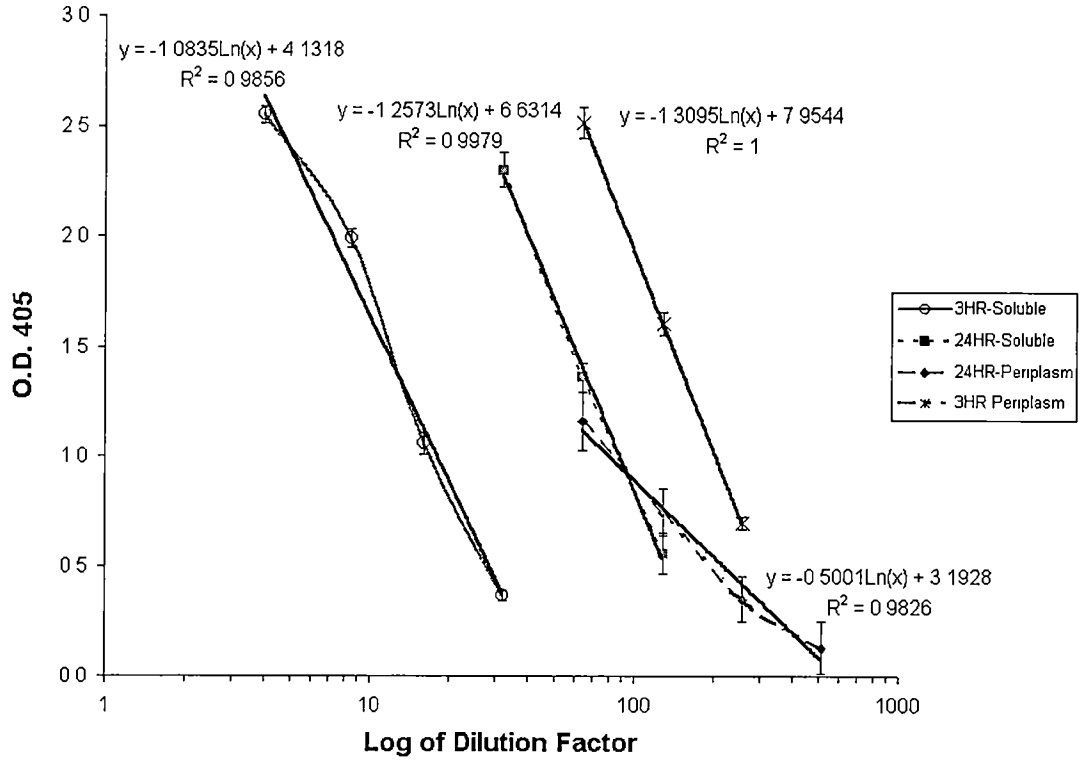


Figure 3.9. Titration of supernatant and periplasmic-derived ScFv against 20μg/ml LKT as determined by direct ELISA Each sample was directly loaded and serially diluted two-fold Error bars represent ± 1 standard deviation



[a]Titers		[b]Slope Interval	
3HR-Soluble	= 21 7	3HR-Soluble	= (-1 2, -1 0)
24HR-Soluble	= 103 3	24HR-Soluble	= (-1 4, -1 1)
3HR-Periplasm	= 235 9	3HR-Periplasm	= (-1 4, -1 2)
24HR-Periplasm	= 119 7	24HR-Periplasm	= (-0 6, -0 4)

[a]Titers are expressed as the reciprocal dilution of supernatant/periplasm that gives an O D ^{405} of 0 8

[b]Slope intervals are expressed at the 95% confidence level

Figure 3.10. Linear regression analyses on titration curves derived from a direct ELISA measuring the reactivity of periplasmic and supernatant-derived ScFv against 20µg/ml LKT Error bars represent ± 1 standard deviation

amount of function over time and must be harvested within a few hours after inducing In conclusion, the optimal levels of LKT-reactive soluble ScFv were found in the periplasm at 3 hours post-induction, and this population of ScFv recognized LKT in a comparable fashion to the ltx-2 control Consequently, this induction strategy was employed for all downstream applications

DNA Sequencing and Evaluation of Clone D9

Phagemid expressing the LKT-specific D9 clone were harvested from *E coli* HB2151 cells so that the respective ScFv could be sequenced The DNA sequence of the D9 ScFv is illustrated in Figure 3 11 This sequence was analyzed and a reading frame was established using LaserGene99 sequence alignment and translation software The primary amino acid sequence of the D9 ScFv clone is shown in Figure 3 12, which illustrates the framework regions and complementarity determining regions (CDRs) of both the heavy and light variable domains, g3 leader sequence, $(Gly_4Ser)_3$ linker, and the C-terminal, 13 amino acid E tag detectable marker The ScFv DNA sequence was compared to full-length kappa light chain and full length $gamma_1$ heavy chain cloned cDNA sequences derived from ltx-2 by Scott Fuller of this laboratory (92) Other than a minimal degree of diversity in the priming regions, the ScFv heavy chain variable region and light chain variable region sequences matched the respective regions of the full-length ltx-2 chains These same DNA coding sequences were compared to published sequences in the Kabat database (134) This analysis delineated that the heavy chain variable domain belongs to murine immunoglobulin heavy chain Family VIII (subgroup

63
GTG AAA AAA TTA TTA TTC GCA ATT CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG

GCC CAG GTC CAG CTG CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCT TCA GTG AAG
186
TTG TCC TGC AAG GCT TCT GGC TAC ACC TTT ACT AAC TTC TGG ATG CAG TGG GTA AAA CAG AGG

CCT GGA CAG GGT CTG GAA TGG ATT GGG GCT ATT TAT CCT GGA GAT GGT GAT ACT AGT TTC ACT
309
CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GCA GAT AAA TCC TCC AGC ACA GCC TAC ATG

CAA CTC ACC AGC TTG GCA TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA ACC CGG GTG GGG GAC
432
GTC TAC TGG GGC CAA GGC ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA

GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC CAG ATG ACA CAG TCT CCA AAA TTT CTG CTT GTC
555
TCA GCA GGA GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG GCT GAT GAT GTT

CTT TGG TAC CAA CAG AAG CCA GGA CAG TCT CCT AAA CTA CTG ATA TAC TAT GCA TCC AAT CGG
681
TTC ACT GGA GTC CCT GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC

AGC GCT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT ATT AGT TCT CCG TGG
804
ACG TTC GGT GGA GGG ACC AAG CTG GAA ATA AAA CGG GCG GCC GCA GGT GCG CCG GTG CCG
837
TAT CCG GAT CCG CTG GAA CCG CGT GCC GCA TAG

Figure 3.11. 2N-ScFv DNA sequence Sequence is presented in codon format with base numbers given in bold at the end of each line The g3 signal peptide is initiated with valine (GTG)

gene 3 leader sequence

Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met

V_H Framework I

Ala | Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys

V_H CDR I *V_H Framework II*

Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr | Asn Phe Trp Met Gln | Trp Val Lys Gln

V_H CDR II

Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly | Ala Ile Tyr Pro Gly Asp Gly Asp Thr Ser Phe

V_H Framework III

Thr Gln Lys Phe Lys Gly | Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr

V_H CDR III

Met Gln Leu Thr Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Thr | Arg Val Gly

V_H Framework IV

Asp Val Tyr | Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser | Gly Gly Gly Gly Ser Gly

$(gly_4 ser)_3$ linker *V_L Framework I*

Gly Gly Gly Ser Gly Gly Gly Gly Ser | Asp Ile Gln Met Thr Gln Ser Pro Lys Phe Leu Leu

V_L CDR I

Val Ser Ala Gly Asp Arg Val Thr Ile Thr Cys | Lys Ala Ser Gln Ser Val Ala Asp Asp Val

V_L Framework II *V_L CDR II*

Leu | Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr | Tyr Ala Ser Asn

V_L Framework III

Arg Phe Thr | Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe

V_L CDR III

Thr Ile Ser Ala Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys | Gln Gln Asp Asn Ser Ser

V_L Framework IV *spacer*

Pro Trp Thr | Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys | Arg Ala Ala Ala | Gly Ala Pro

E-TAG sequence

Val Pro Tyr Pro Asp Pro Leu Glu Pro Arg Ala Ala **stop**

Figure 3.12. 2N-ScFv primary amino acid sequence Amino acid residues correspond to codons presented in Figure 3 11 Vertical bars are inserted to denote regions of interest (134)

IIA), and the light chain variable domain may be classified in the murine kappa light chain Family XX (subgroup V) (92) To denote the confirmation that the D9 ScFv heavy and light chain sequences matched those of ltx-2 and that this clone contained all the expected structural elements in correct reading frame, this clone was renamed 2N-ScFv

Purification of 2N-ScFv and Ltx-2 (2N)

One of the major aspects of this project was to analyze the similarities and/or differences of LKT association and neutralization when the ltx-2 paratope (2N-ScFv) was removed from the rest of the molecule In order to assess the relative LKT association efficiencies of ltx-2 and 2N-ScFv, it was necessary to analyze them on a normalized equimolar basis To accomplish this, it was mandatory to purify both the 2N-ScFv and ltx-2 Ltx-2 was purified from hybridoma supernatant by affinity chromatography using a rat anti-mouse (kappa) column 2N-ScFv was purified from ammonium sulfate concentrated periplasmic extract via affinity chromatography using an anti-E tag column and subsequently concentrated 10x via ultrafiltration The results of these purifications are shown in Figures 3 13 and 3 14 Figure 3 13 illustrates a coomassie stained polyacrylamide gel As indicated, the purified ltx-2 was shown to be the correct 160kDa size and free from contaminating hybridoma proteins Since the sample was electrophoresed in the absence of a disulfide reducing agent such as 2-mercaptoethanol, the light and heavy chains did not dissociate well and weren't visible as 25kDa and 55kDa entities respectively Also, the purified 2N-ScFv concentrated product was the correct 30kDa size and free from other periplasmic proteins Although not appreciable

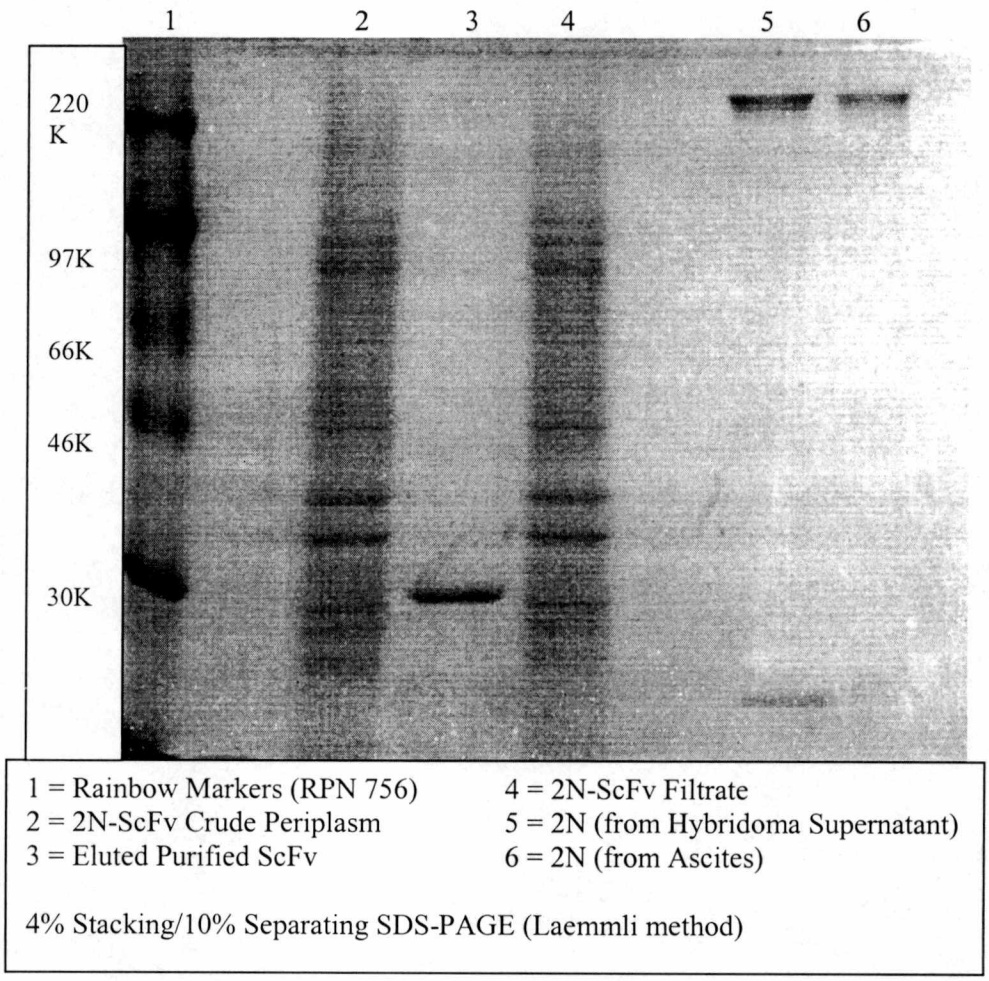


Figure 3.13. Coomassie stained polyacrylamide gel showing the efficiency of 2N-ScFv and 2N (ltx-2) purification.

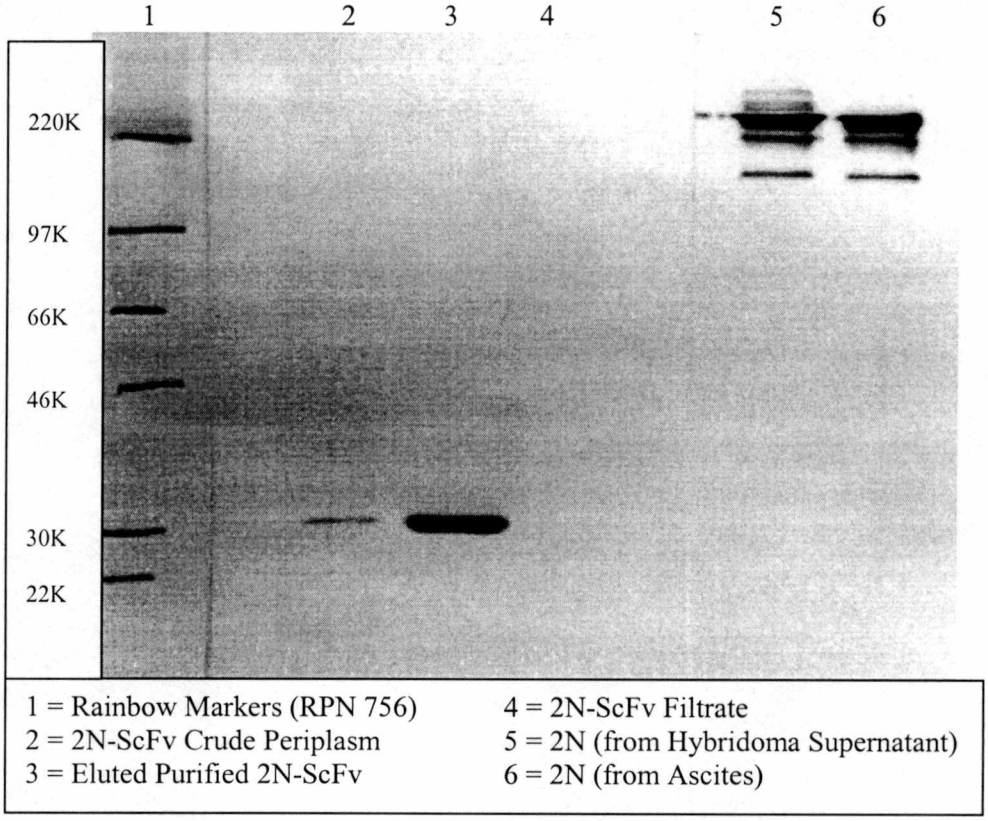


Figure 3.14. Western immunoblot illustrating detectable purified 2N-ScFv and 2N (ltx-2). 2N-ScFv was developed with a 1:1,000 dilution of anti-E tag-HRP conjugate. 2N was developed with a 1:1,000 of HRP-conjugated rat anti-mouse IgG_1.

on the coomassie stained gel photo, it can be seen in Figure 3 14, a Western immunoblot probed with anti-E tag-HRP conjugate, that all of the detectable 2N-ScFv was removed from the periplasm since none was detected in the column filtrate Purified ltx-2 was detected with HRP-conjugated rat anti-mouse IgG_1 and is shown in the same photo

To ensure that the purification and concentration did not significantly alter the 2N-ScFv's capacity to recognize native LKT, a titration was performed by direct ELISA against 20μg/ml LKT to compare the purified product to that found in the periplasmic extract at 3 hours post-induction Figure 3 15 illustrates that the purified 2N-ScFv recognized LKT and titrated in a parallel fashion to unpurified periplasmic 2N-ScFv This indicated that there was no significant loss of LKT recognition Furthermore, linear regression analysis, Figure 3 16, revealed that the titer (reciprocal dilution giving an $O D^{405}$ equivalent to 0 8) was 3x greater for the purified concentrate than for the crude periplasmic species No LKT recognition was detectable in the anti-E tag column filtrate, which is in agreement with the results of the Western immunoblot (Figure 3 14) Although no empirical studies were conducted, it was observed that there was no significant loss of LKT recognition by the purified 2N-ScFv after 3 weeks of storage at 4° C (data not shown) From these results, it was concluded that the 2N-ScFv purification and concentration strategies were acceptable

Reactivity of 2N-ScFv to Native Leukotoxin

Although reactivity of 2N-ScFv had been well-established, it was necessary to compare the LKT recognition efficacy of this molecule to that of an equimolar amount of

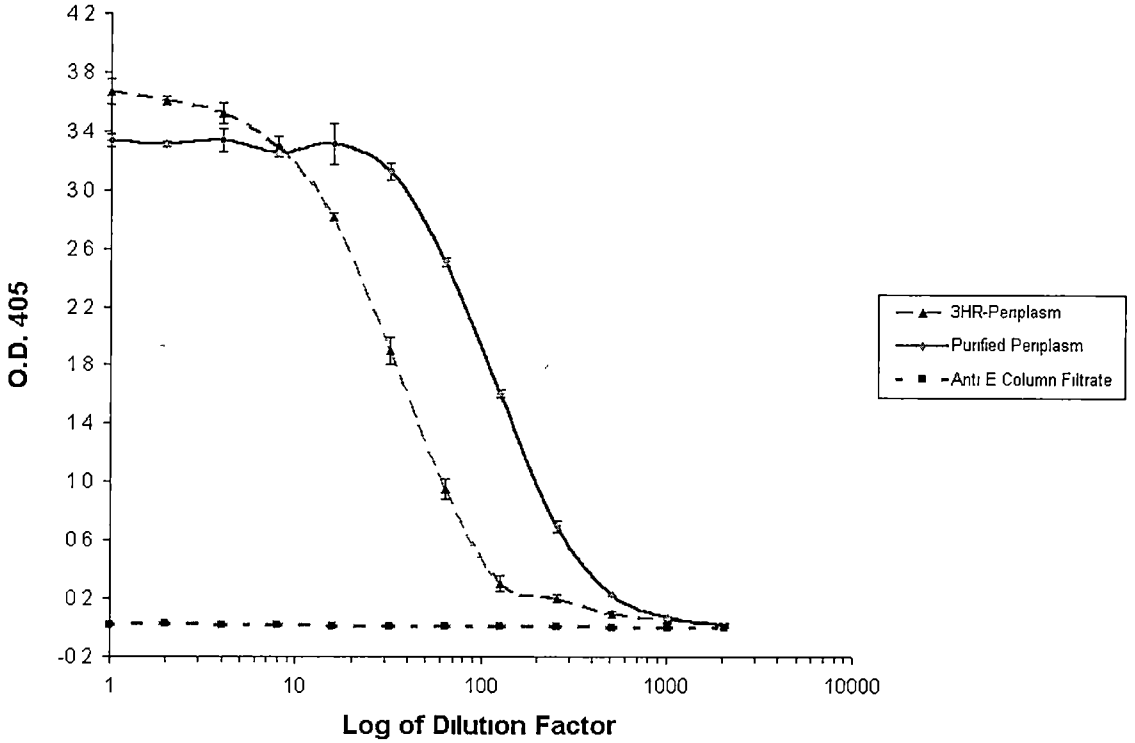
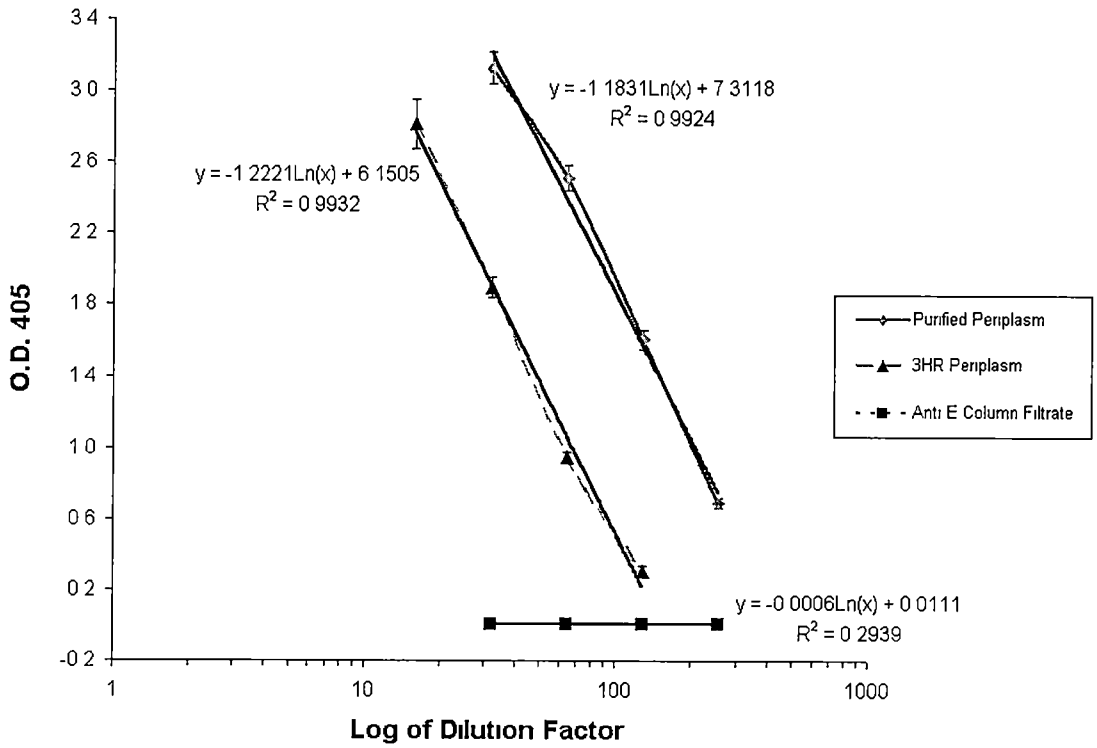


Figure 3.15. Titration showing the relative recognition of 20μg/ml LKT by purified 2N-ScFv concentrate in a direct ELISA Samples were loaded and serially diluted two-fold 2N-ScFv was detected with a 1 8,000 dilution of anti-E tag-HRP conjugate



[a]Titers	Purified Periplasm = 245 7	[b]Slope Interval	Purified Periplasm = (-1 3, -1 1)
	3HR-Periplasm = 79 7		3HR-Periplasm = (-1 3, -1 1)
	Anti-E Column Filtrate = N/A		Anti-E Column Filtrate = N/A

[a]Titers are expressed as the reciprocal dilution of supernatant/periplasm that gives an O D ^{405} of 0 8

[b]Slope intervals are expressed at the 95% confidence level

Figure 3.16. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of periplasmic and concentrated purified 2N-ScFv against 20µg/ml LKT. Error bars represent ± 1 standard deviation

native 2N (positive control) to ensure comparable LKT association characteristics This was quantitatively demonstrated using a standard two-fold titration series (starting molar concentration for 2N/2N-ScFv was 5,500nmol) in a direct ELISA against 20μg/ml of native LKT concentrate 2N-ScFv association was quantified by probing with a 1 8,000 dilution of HRP-conjugated anti-E tag secondary, and 2N was quantified by probing with a molar equivalent (1 2,000 dilution) of rat anti-mouse IgG_1 secondary Figure 3 17 shows this titration Linear regression analysis, Figure 3 18, revealed that the curve slopes were statistically identical, indicating comparable LKT association However, the titer (molar concentration giving an O D ^{405} of 0 8) for 2N was determined to be 2 4x lower than that found for 2N-ScFv, demonstrating that it takes 2 4x less 2N than 2N-ScFv to give the same signal This difference was a reflection of avidity differences inherent to the assay Because 2N had two recognition sites for secondary detection (each chain of each antibody molecule) and 2N-ScFv had only one (the E tag detectable marker), the expected result if an equal amount of molecules were bound (non-saturating conditions) was a two-fold shift in titration Although the observed value was slightly higher, this was probably due to a small degree of experimental error due to differences in association stability (solid phase avidity of bivalent 2N vs monovalent 2N-ScFv) and/or differences in the developing secondary reagents' respective affinities No reactivity of a LKT nonspecific antibody (negative control) was observed Given that all ELISA-determined titration curves against native LKT or LKT-derived fragments follow a similar protocol and have an appearance similar to that seen in Figure 3.17, only the linear analyses of the titrations will be presented henceforth.

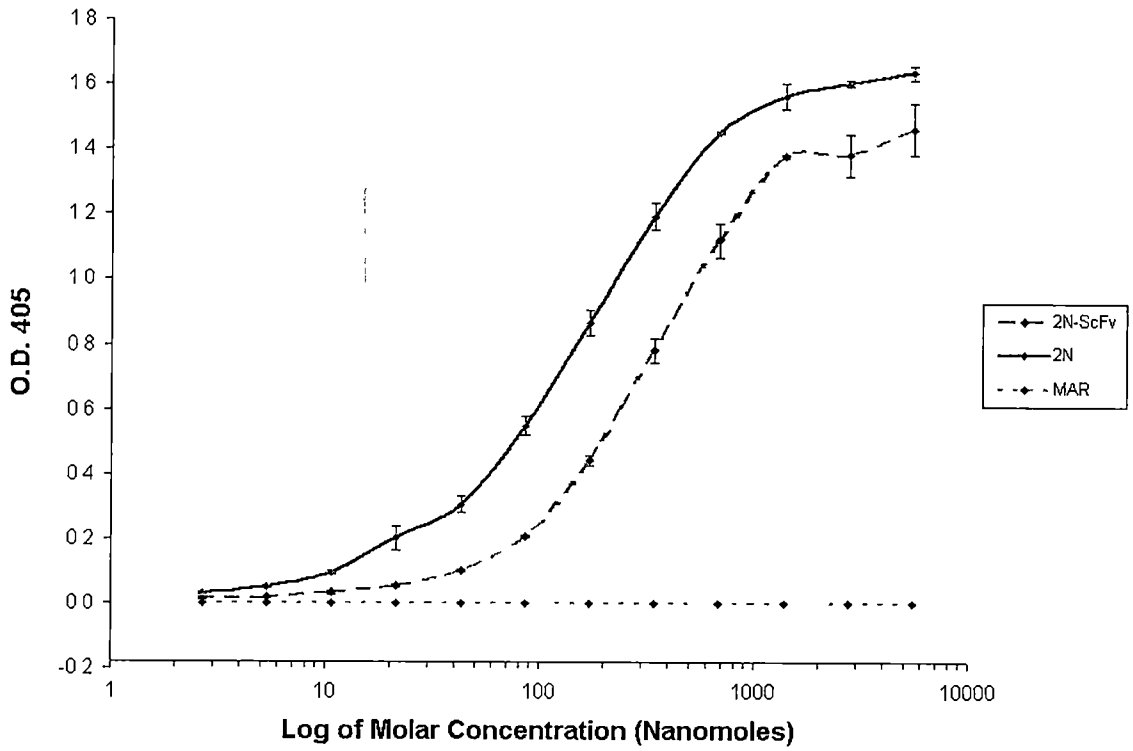
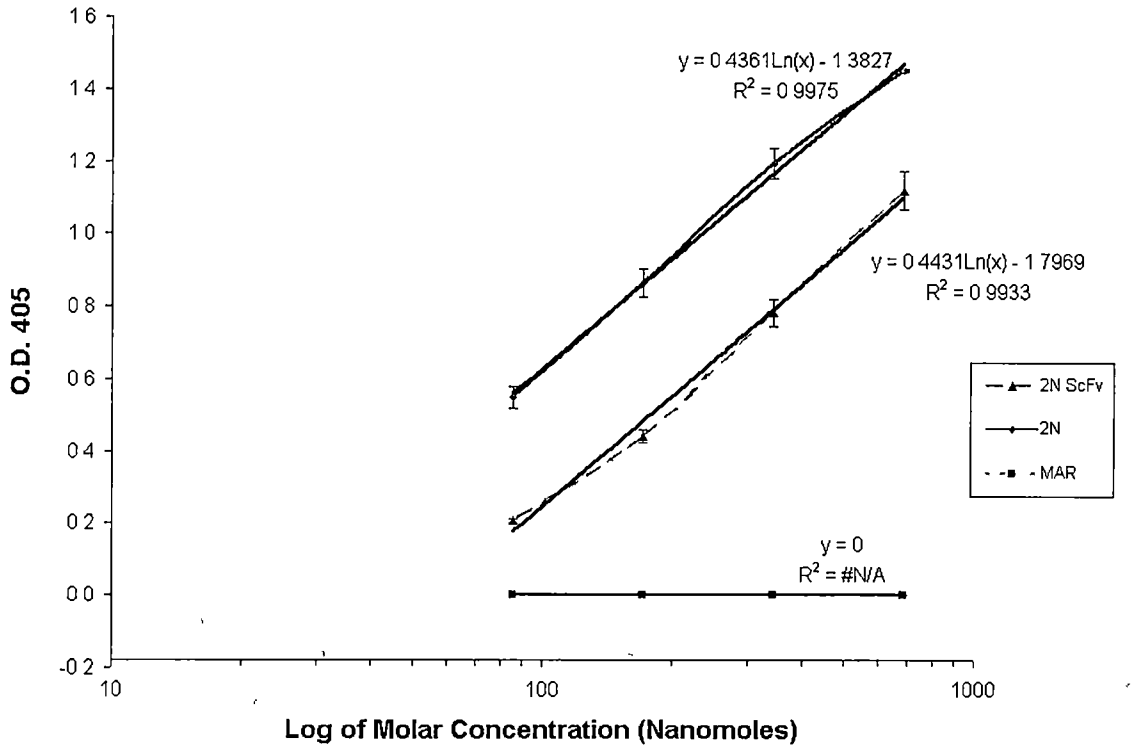


Figure 3.17. Titration showing the relative recognition of 20µg/ml LKT by 2N-ScFv and native 2N in a direct ELISA Samples were diluted to 5,500nmol, loaded, and serially diluted two-fold 2N-ScFv was detected with 1 8,000 diluted anti-E tag-HRP conjugate, and 2N was detected with 1 2,000 diluted rat anti-mouse IgG_1



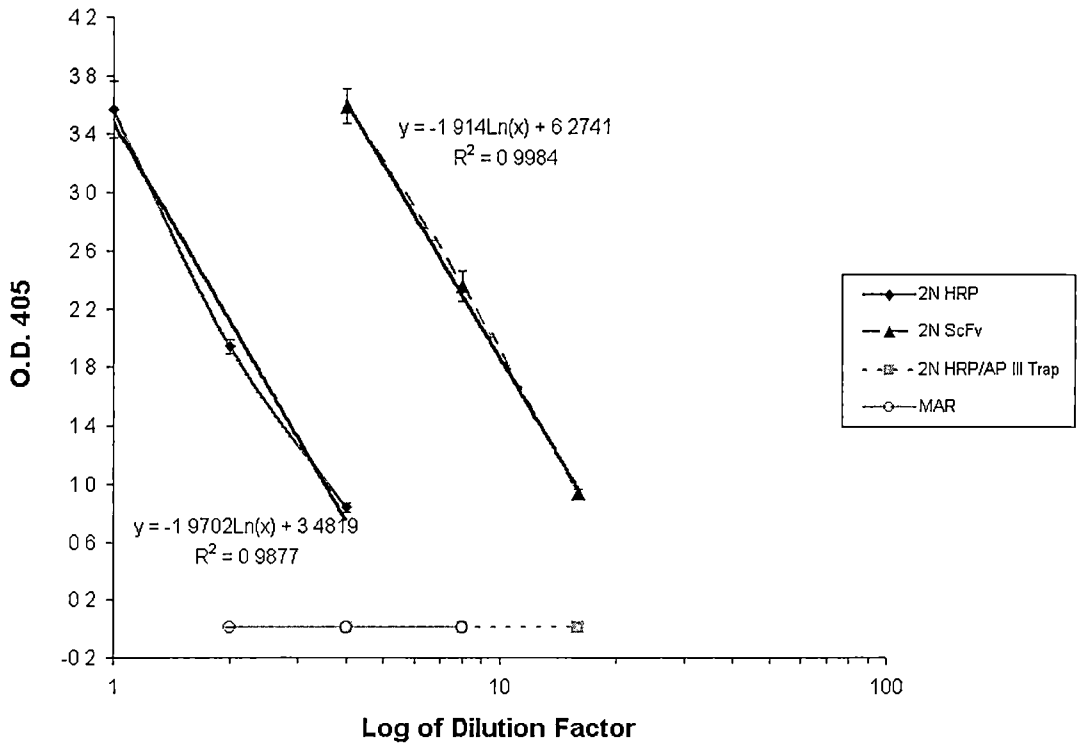
[a]Titers		[b]Slope Interval	
	2N-ScFv = 351 5		2N-ScFv = (0 4, 0 5)
	2N = 149 3		2N = (0 4, 0 5)
	MAR = N/A		MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8
[b]Slope intervals are expressed at the 95% confidence level

Figure 3.18. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20μg/ml LKT Error bars represent ± 1 standard deviation

In order to assess the practicality of 2N-ScFv as a LKT-specific reagent for future laboratory applications, this molecule was further compared to 2N in its ability to recognize LKT when presented by two-antibody sandwich (trap) ELISA Because trapping can often enhance epitope presentation and/or antibody recognition, this method is often important in characterizing serum samples which may contain LKT-specific antibodies that may have weak/no activity against LKT directly bound to a solid phase due to altered epitope presentation or epitope masking Although it had already been established during the initial screening of ScFv clones that 2N-ScFv would recognize LKT trapped by mAb 35, the relative abilities of 2N/2N-ScFv to recognize LKT in this mode were assessed However, given the limitations of detecting bound 2N due to cross-reactivity of secondary reagents with the trapping antibody, an impure, ascites-derived 2N-HRP conjugate was used Because of this, an equimolar comparison could not be made Therefore this assay only provided a qualitative assessment of 2N-HRP/2N-ScFv recognition of mAb 35-trapped LKT, illustrated in Figure 3 19 Because both species directed against 20μg/ml mAb 35-trapped LKT titrated in a parallel fashion as indicated by equivalent slopes, it was concluded that 2N-ScFv recognized trapped LKT in a manner comparable to that of 2N-HRP Negative controls showed no reactivity

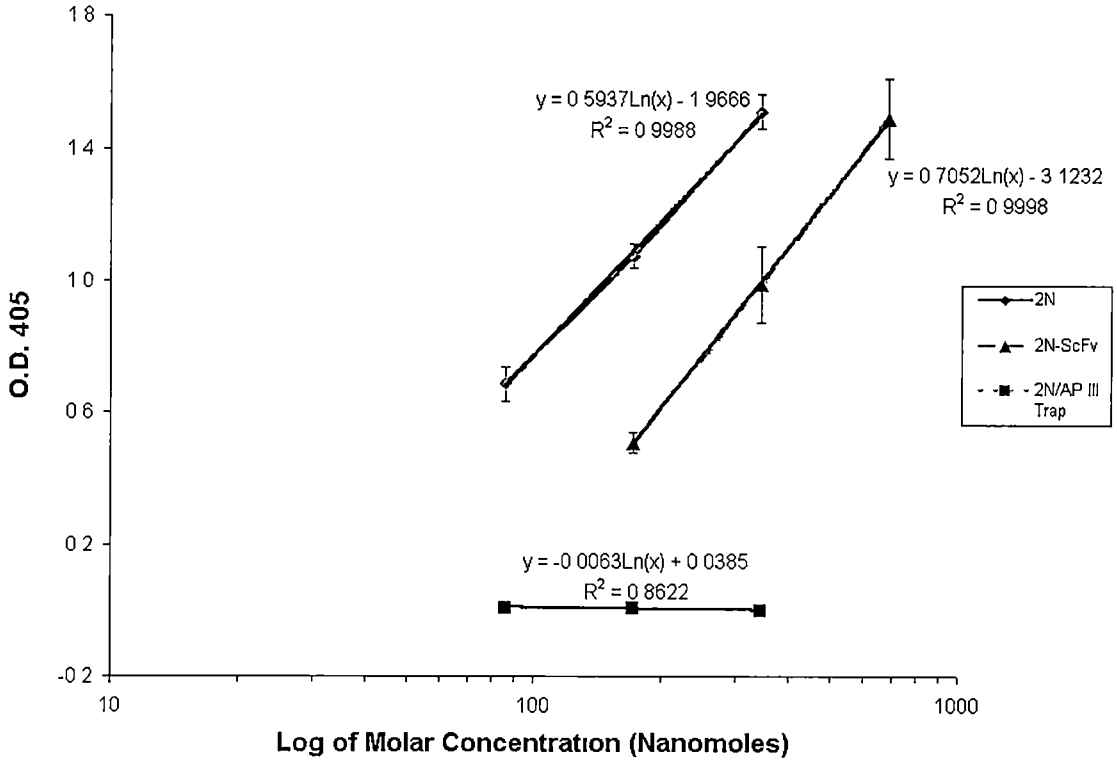
Because the mAb 35 trap assay was only qualitative, a more quantitative LKT trap assay using LKT-specific rabbit polyclonal antiserum as the trapping agent was conducted Figure 3 20 shows the results of this assay. As expected, both species showed appreciable recognition of 20μg/ml trapped LKT. Again, parallel titration curves (overlapping slopes) demonstrated that the LKT binding efficiencies of 2N/2N-ScFv



[a]Slope Interval 2N-ScFv = (-2 1, -1 8)
2N-HRP = (-2 2, -1 7)
MAR = N/A
2N-HRP/AP III Trap = N/A

[a]Slope intervals are expressed at the 95% confidence level

Figure 3.19. Qualitative analysis of 2N-HRP/2N-ScFv reactivity against 20μg/ml of mAb 35-trapped LKT Trapping antibody was at 20μg/ml Titrations represent serial two-fold dilutions of pre-diluted detecting antibody Error bars indicate ± 1 standard deviation



[a]Titers 2N-ScFv = 260 7	[b]Slope Interval 2N-ScFv = (0 6, 0 8)
2N = 105 6	2N = (0 5, 0 6)
MAR = N/A	MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8
[b]Slope intervals are expressed at the 95% confidence level

Figure 3.20. Linear regression analyses on the titration curves derived from the reactivity of 2N-ScFv and 2N to 20μg/ml LKT trapped with a 1:100 dilution of rabbit polyclonal anti-LKT antiserum 2N/2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold. Error bars represent ± 1 standard deviation.

were comparable For reasons already discussed, the same curve shift was noted (2 5x lower 2N titer based on molar concentration than that found for 2N-ScFv). Comparison of titers derived from this assay to those found against LKT by direct ELISA (Figure 3 18) confirm that trapping LKT does augment epitope presentation to both 2N and 2N-ScFv This assertion is supported by lower titer values for the trap assay 2N titer of 105 6 (T) vs 149 3 (D), and 2N-ScFv titer of 260 7 (T) vs 351 5 (D) Interestingly, quantification of this enhancement shows that 2N recognized trapped LKT 29% more efficiently, and 2N-ScFv recognized trapped LKT 26% more efficiently This reinforces that 2N and 2N-ScFv associate with LKT in almost the exact same fashion, as expected No LKT reactivity was noted for the negative control In conclusion, 2N-ScFv can be used as a detecting reagent for trapped LKT, and detection of LKT by 2N-ScFv is more efficient in a trap ELISA

Inhibition of 2N-ScFv Recognition of Leukotoxin Using Native 2N

Although 2N-ScFv displayed the same LKT association characteristics as its full-size counterpart, 2N, a competition ELISA was performed to confirm that they recognize the same LKT epitope(s) Starting with a molar excess (200μmol), 2N was serially diluted two-fold and incubated with 20μg/ml LKT for 1 5 hours prior to adding 2N-ScFv If both species share the same epitope(s), then 2N should block 2N-ScFv binding at saturating concentrations but not at non-saturating concentrations. Figure 3 21 shows that a molar excess of 2N was able to successfully block 2N-ScFv association as indicated by similarities to a 2N-ScFv negative control against an unrecognizable antigen

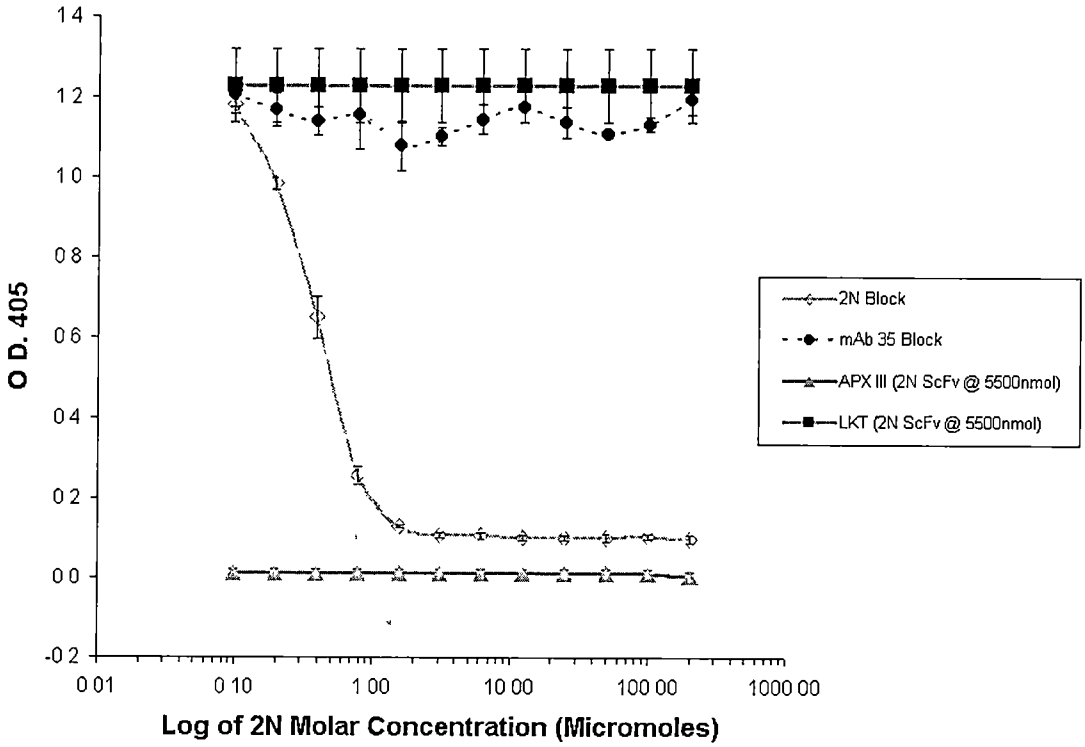


Figure 3.21. 2N inhibition of 2N-ScFv recognition of LKT as determined by direct ELISA Either 2N or mAb 35 was loaded at a molar excess of 200μmol, serially diluted two-fold, and preincubated with 20μg/ml LKT for 1 5 hours Any remaining LKT binding sites were detected with 5,500nmol of 2N-ScFv Error bars represent ± 1 standard deviation

(APX III), and that as 2N was diluted 2N-ScFv values were restored to positive control levels (uninhibited 2N-ScFv against 20μg/ml LKT) A negative control attempting to block 2N-ScFv with mAb 35 was unsuccessful, as expected since mAb 35 maps to a different epitope (see above) Linear regression analysis (r^2=0 9977) revealed that a significant level of 2N-ScFv association was restored (giving an O D ^{405} of 0 8) when the molar concentration of 2N was at 300nmol, or 1 2N molecule for every 18 2N-ScFv molecules This relatively low proportion of 2N 2N-ScFv indicated that steric hindrance was a likely factor However, because mAb 35, which maps to a region close to that determined to be the 2N epitope, did not significantly alter 2N-ScFv recognition, steric hindrance is probably not the sole cause of inhibition Therefore, the inhibition was also likely due to epitope masking, and the inference was that 2N and 2N-ScFv recognize the same eptiope(s)

2N-ScFv Antigen Cross-Reactivity

Although it was inferred that 2N-ScFv was only specific to LKT, a formal study on antigen cross-reactivity was conducted In this study 2N-ScFv was tested against 20μg/ml of several antigens LKT (positive control), structurally unrelated antigens, and closely related RTX toxins derived from different serotypes of *Actinobacillus pleuropneumoniae* Figure 3 22 summarizes the results As expected, LKT was the only antigen in the profile which was recognized when probed with saturating levels (100μmol) of both 2N and 2N-ScFv There was no statistical difference in LKT recognition at saturation between 2N and 2N-ScFv, as expected No other antigen, either

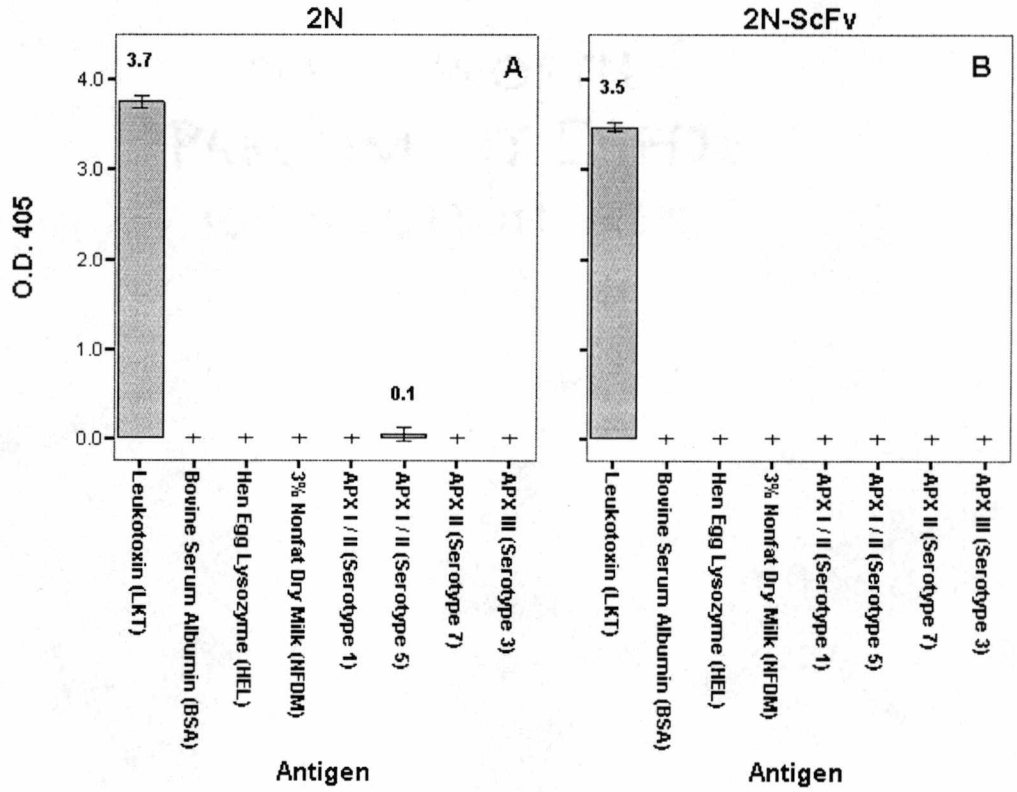


Figure 3.22. Profile of 100μmol 2N/2N-ScFv recognition of various antigens using direct ELISA. All antigens were at 20μg/ml. Bars represent the mean $O.D._{.}^{405}$. The mean value is in bold-face above the respective bar. Error bars represent the mean $O.D._{.}^{405} \pm$ 1 standard deviation.

structurally similar or dissimilar, was recognized by either species This demonstrated that 2N-ScFv, like its complete counterpart, is highly specific for LKT only

Reactivity of 2N-ScFv to LKT-Derived GST Fusion Proteins

A study by Jackson McPherson of this laboratory has implicated that the LKT region consisting of amino acids 876-939 comprises the ltx-2 (2N) binding domain (184) This definition of the potential epitope was derived using a panel of N-terminally deleted GST-fused LKT constructs, titled JM-1–JM-7, schematically illustrated in Figure 3 23 The nucleic acid sequences of all these constructs have been determined and compared to the *lktA* genetic sequence published by Lo *et al* (164) The sequence of each construct was found to be identical to the respective region of the published gene sequence (184) In order to ensure that 2N-ScFv also mapped to the aforementioned region, several recognition assays against these constructs were conducted All of the constructs, as well as unfused GST, were grown and purified via anti-GST affinity chromatography All constructs were analyzed for correct size and purity using SDS/PAGE and subsequent coomassie staining Figure 3 24 shows that all of the constructs were of the correct size and free from other contaminating proteins Western immunoblot analysis of these constructs, when probed with an equimolar concentration of either 2N or 2N-ScFv (Figure 3 25 and 3 26 respectively), revealed that both species only recognized LKT (positive control), JM-1, JM-2, and JM-4. The other fusions as well as the GST negative control showed no reactivity This finding was quantitatively reinforced using direct ELISA, probing 20µg/ml of each fusion protein with saturating concentrations of 2N or

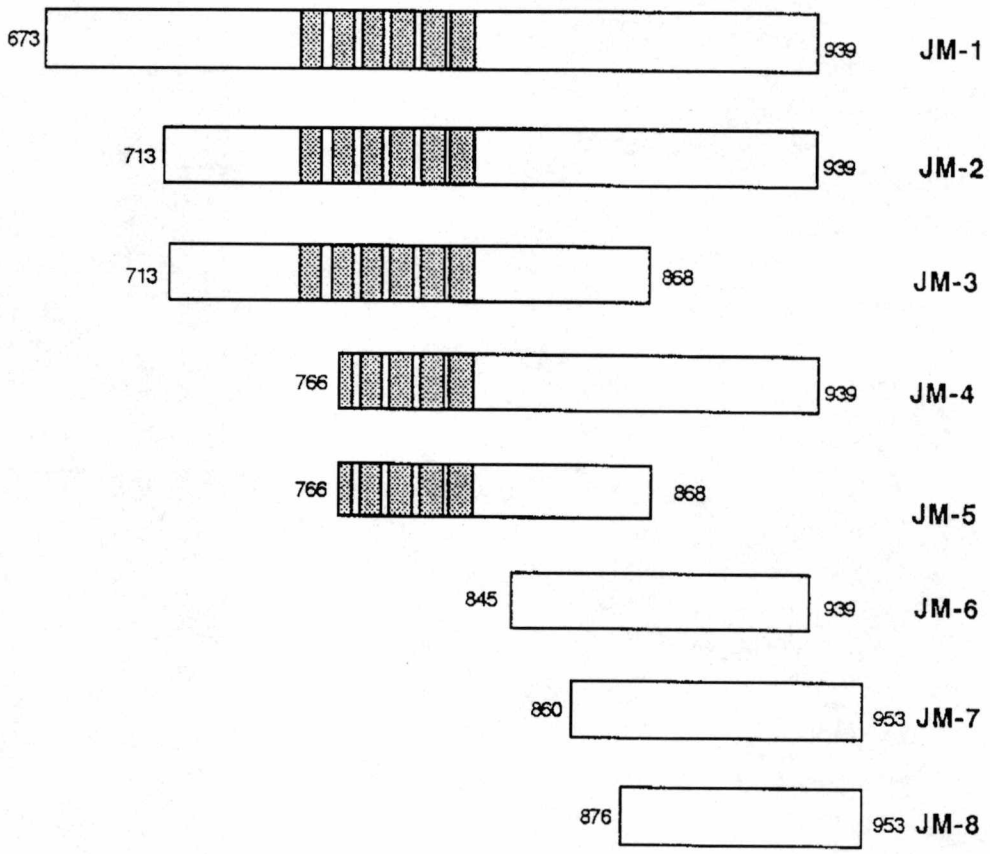


Figure 3.23. Schematic representation of LKT A peptides which are fused to GST at their amino termini. Numbers shown are amino acids. Shaded areas indicate tandem Gly-Asp repeat sequences.

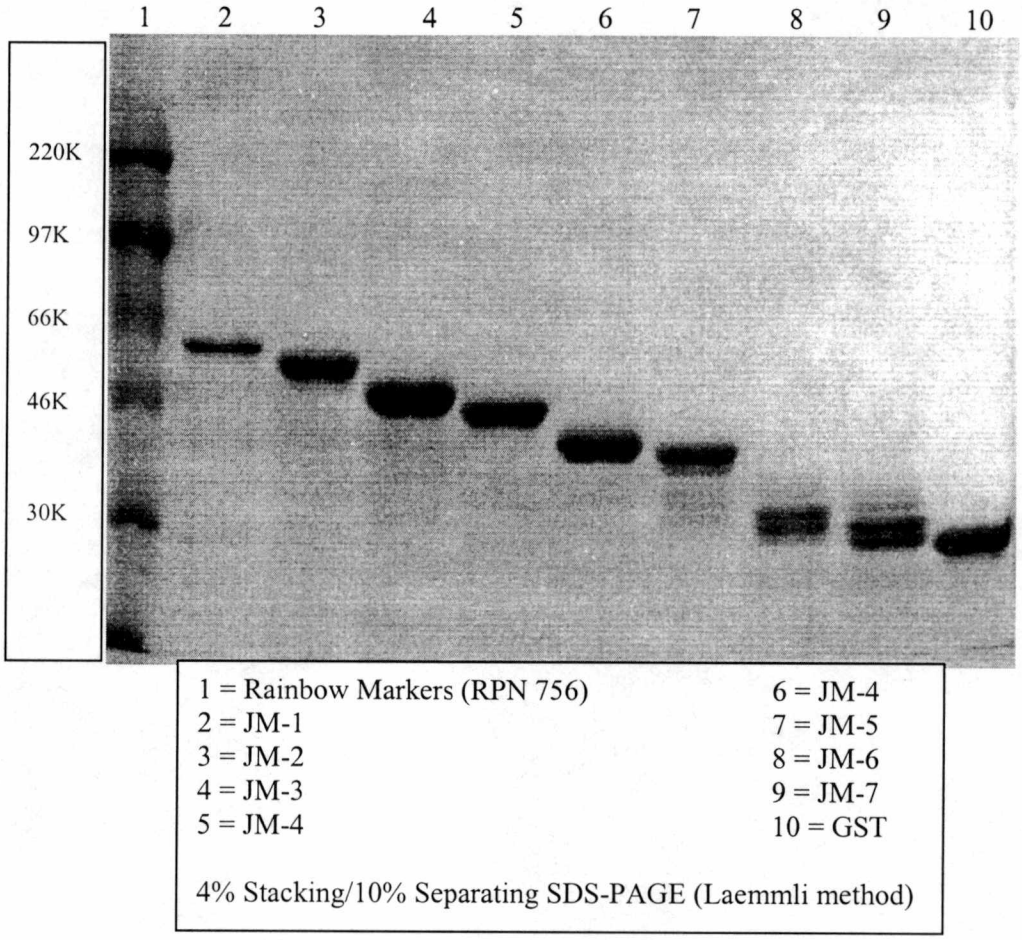


Figure 3.24. Coomassie stained polyacrylamide gel showing the relative size and purity of GST-fused N-terminally deleted LKT constructs. 3μg of each fusion protein was loaded and electrophoresed.

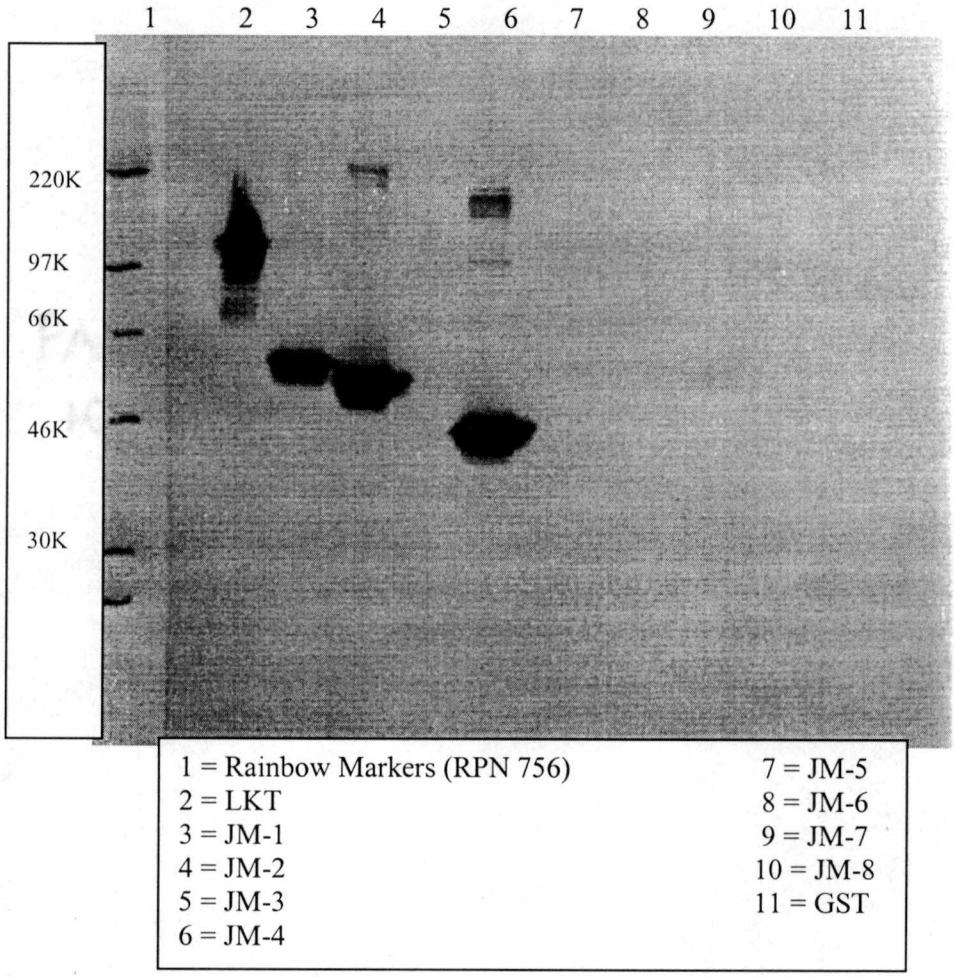


Figure 3.25. Western immunoblot of LKT and GST fusion proteins detected with 125μmol of 2N. The immunoblot was developed with a 1:1,000 dilution of rat anti-mouse IgG_1 HRP-conjugate.

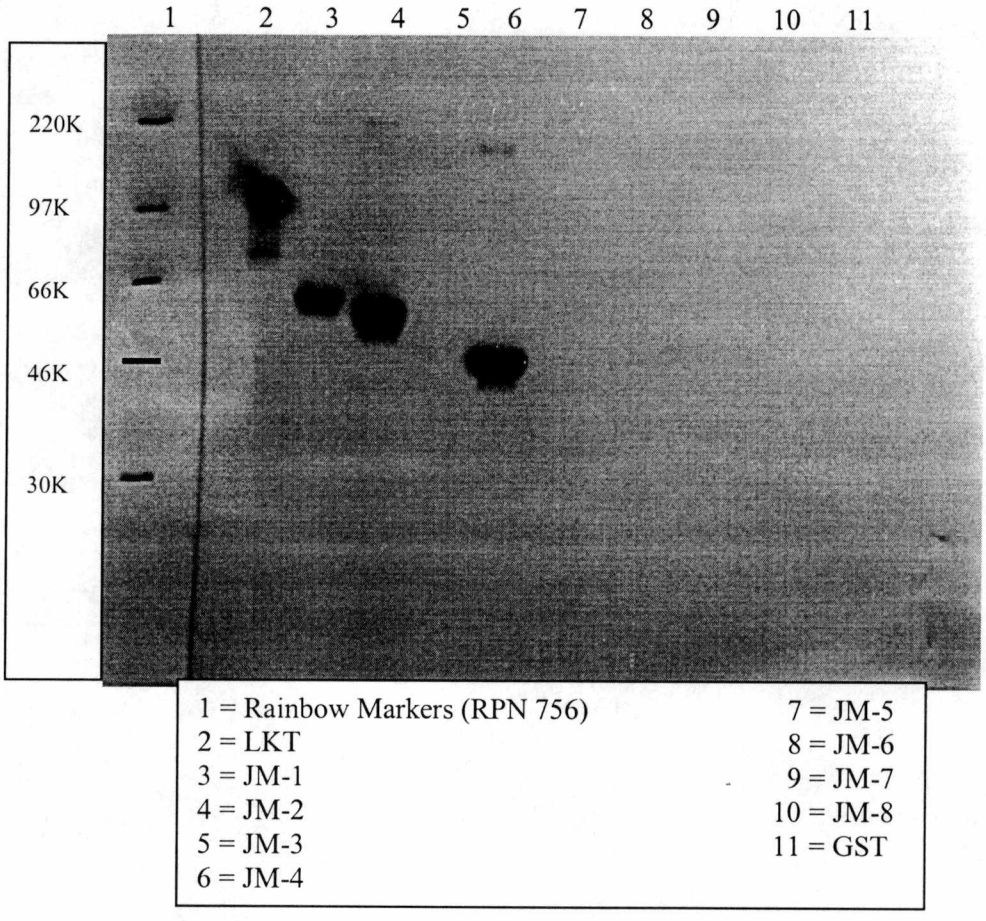


Figure 3.26. Western immunoblot of LKT and GST fusion proteins detected with 125μmol of 2N-ScFv. The immunoblot was developed with a 1:1,000 dilution of HRP-conjugated anti-E tag antibody.

2N-ScFv (100μmol) This study, Figure 3 27, confirmed the results found in the western blot both 2N and 2N-ScFv recognize LKT, JM-1, JM-2, and JM-4 Furthermore, 2N/2N-ScFv recognition of LKT and the indicated LKT fragments was statistically identical Again, no reactivity was detected against the remainder of the fusions as well as the GST negative control Because these results did not agree with previous findings (where both JM-7 and JM-8 were weakly recognized (184)), a polyclonal goat anti-GST trap ELISA was performed to see if this would change/enhance epitope presentation Also seen in figure 3 27, the results of this study were identical to those of the direct ELISA However, O D ^{405} values for the anti-GST trapped constructs were significantly reduced, which was attributed to experimental error

Finally, in an effort to demonstrate that both 2N and 2N-ScFv would react with those fusions recognized in a fashion similar to that of LKT, a titration series was performed on each This would hopefully reinforce that these fusions did indeed contain the elements necessary to form an epitope(s) representative of the one(s) found in native LKT The results are shown in Figures 3 28-3 32, which illustrate the linear regression analyses of the JM-1, JM-2, JM-4, JM-7, and GST respectively (the last two being negative controls) Table 3 4 summarizes the 2N/2N-ScFv titers for each fusion indicated as well as those previously indicated for native LKT It was observed that both JM-2 and JM-4 had similar 2N 2N-ScFv titer ratios, 2 7x and 2 5x respectively, to that of LKT (2 4, see above) This indicated that a 2 7x and 2 5x molar excess of 2N-ScFv was needed to produce the same signal as 2N for JM-2 and JM-4 recognition respectively Again, there was an expected 2x difference, and reasons for the slight excess have been discussed

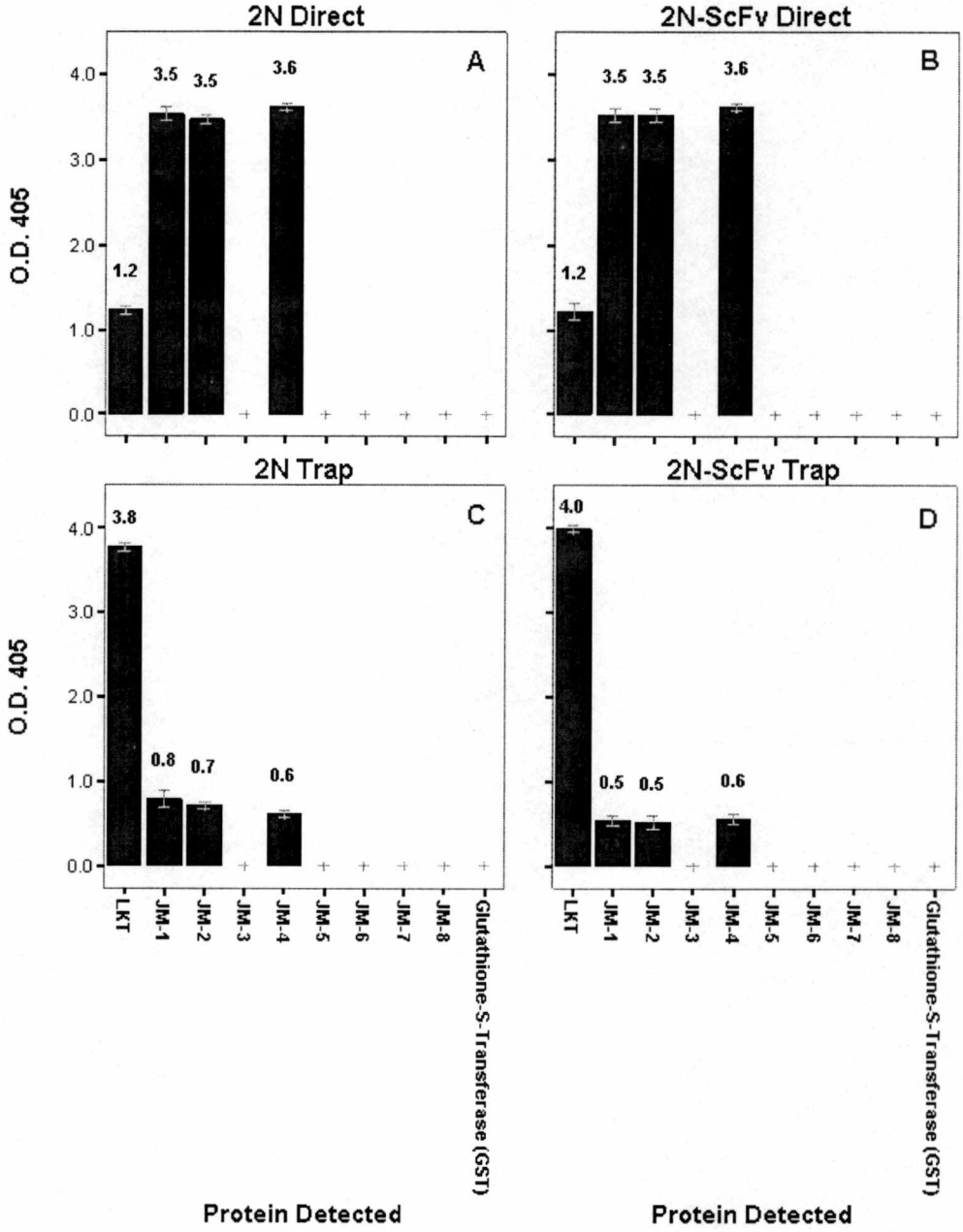
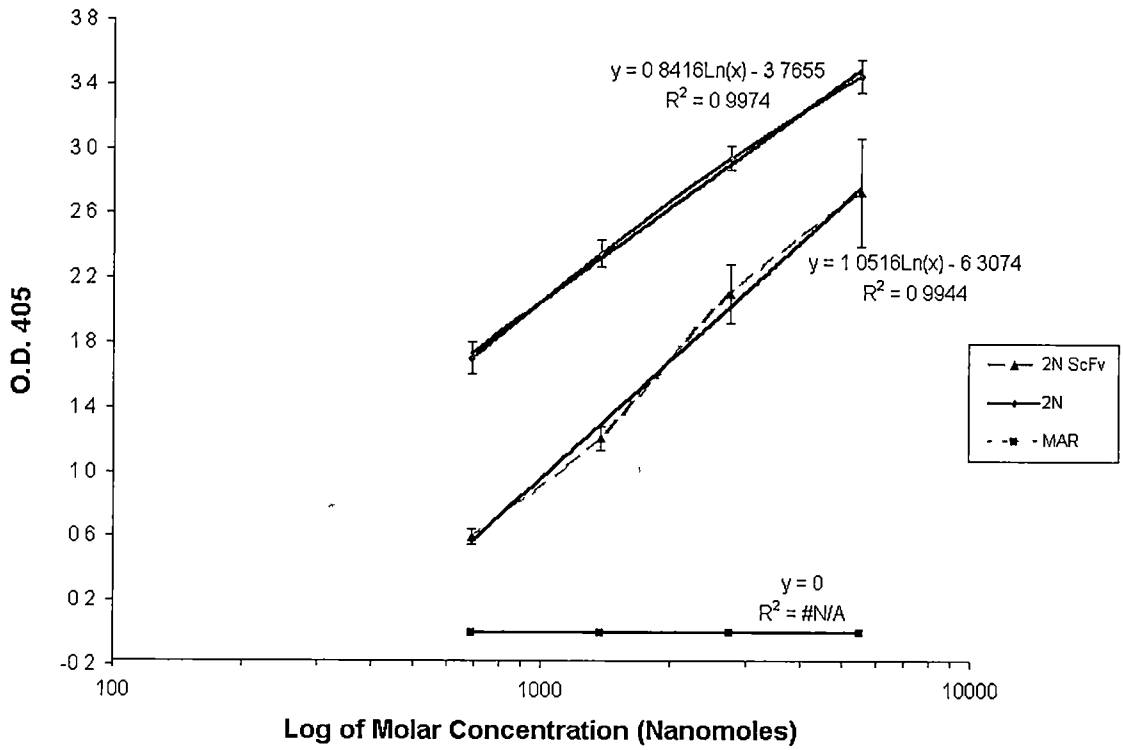


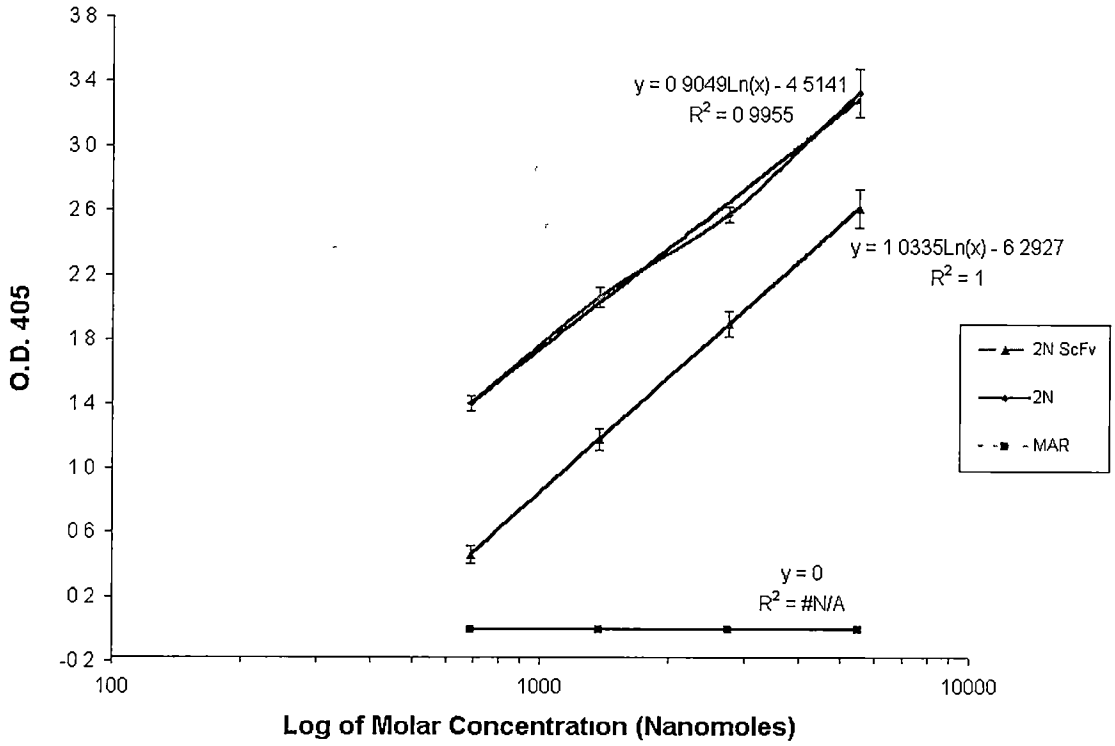
Figure 3.27. Profile of 100μmol 2N/2N-ScFv recognition of GST fusion proteins by both direct and trap ELISA. All antigens and trapping antibodies were at 20μg/ml. Bars represent the mean $O.D.^{405}$. The mean value is in bold-face above the respective bar. Error bars represent the mean $O.D.^{405} \pm$ 1 standard deviation.



[a]Titers		[b]Slope Interval	
	2N-ScFv = 861 5		2N-ScFv = (0 9, 1 2)
	2N = 227 0		2N = (0 8, 0 9)
	MAR = N/A		MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8
[b]Slope intervals are expressed at the 95% confidence level

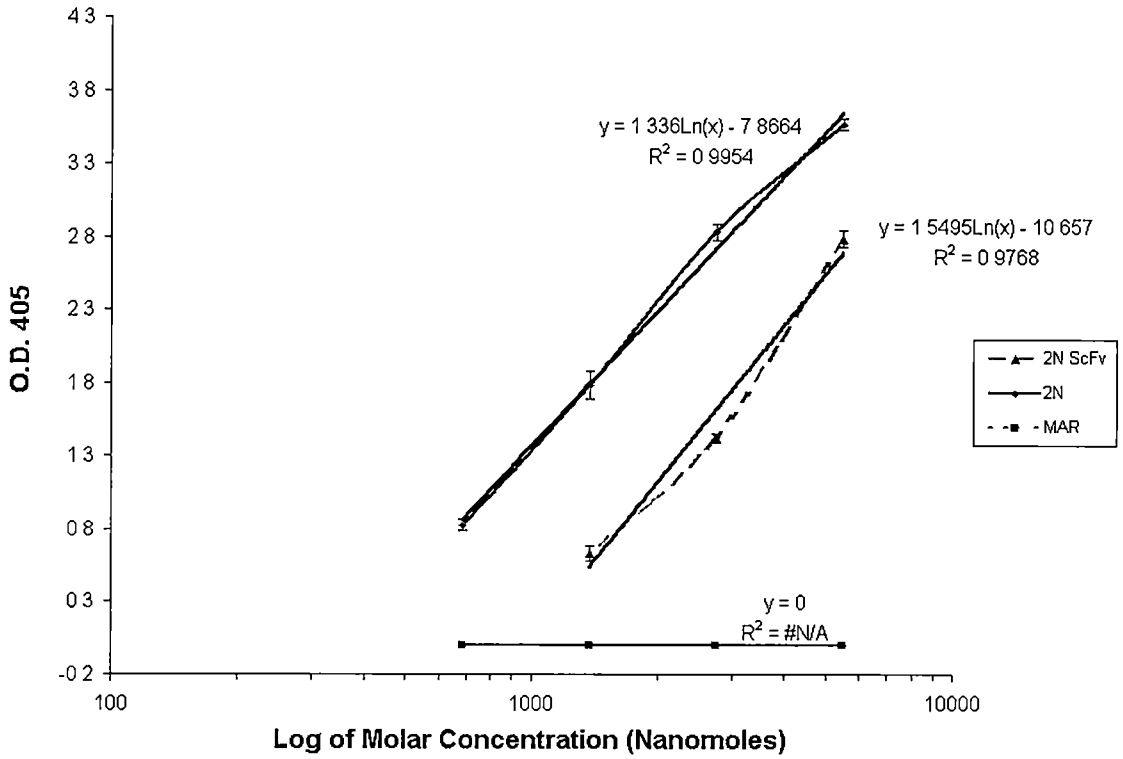
Figure 3.28. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20μg/ml JM-1 Both 2N and 2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold Error bars represent ± 1 standard deviation.



[a]Titers	2N-ScFv = 956 0 2N = 355 2 MAR = N/A	[b]Slope Interval	2N-ScFv = (1 0, 1 1) 2N = (0 8, 1 0) MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8
[b]Slope intervals are expressed at the 95% confidence level

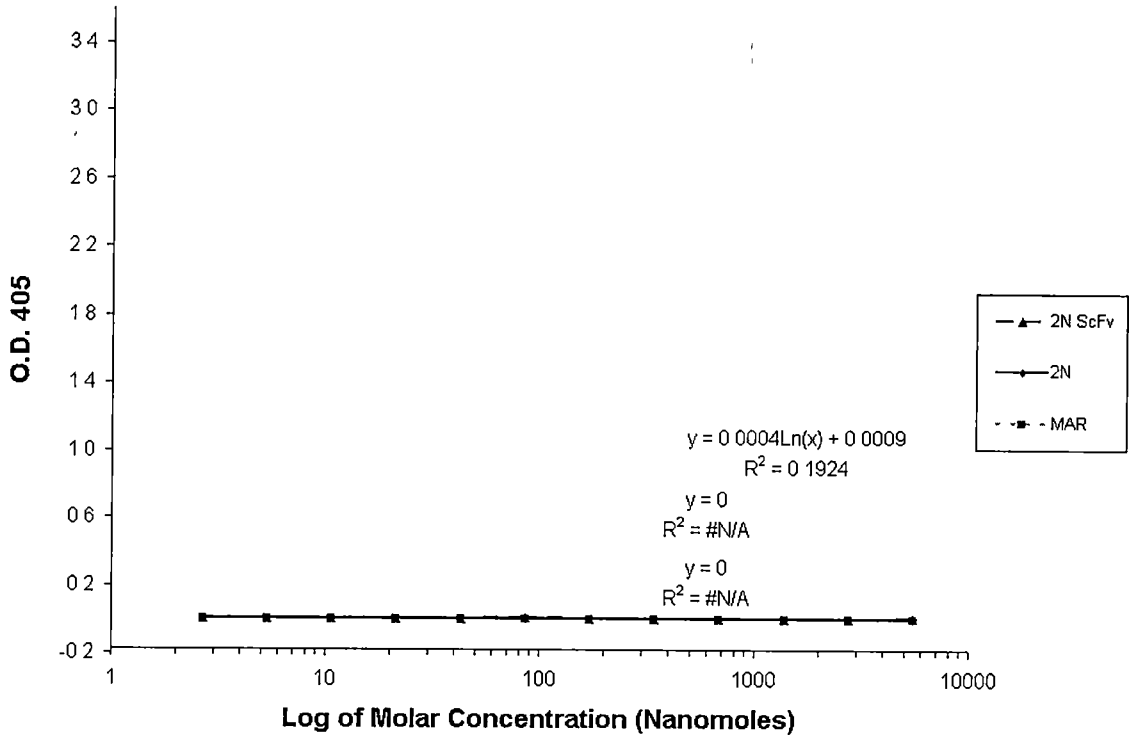
Figure 3.29. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20µg/ml JM-2 Both 2N and 2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold Error bars represent ± 1 standard deviation



[a]Titers	2N-ScFv = 1,626 2	[b]Slope Interval	2N-ScFv = (1 3, 1 8)
	2N = 656 4		2N = (1 2, 1 4)
	MAR = N/A		MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8
[b]Slope intervals are expressed at the 95% confidence level

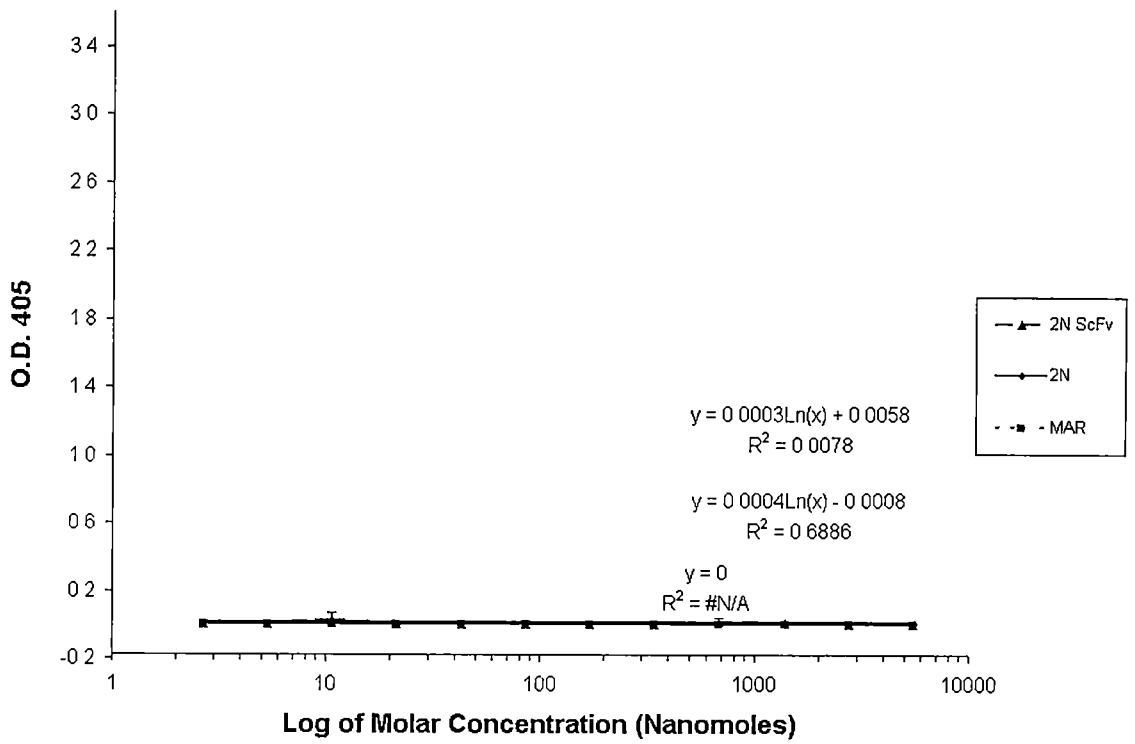
Figure 3.30. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20μg/ml JM-4 Both 2N and 2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold Error bars represent ± 1 standard deviation



[a]Titers 2N-ScFv = N/A
2N = N/A
MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D ^{405} of 0 8

Figure 3.31. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20μg/ml JM-7 Both 2N and 2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold Error bars represent ± 1 standard deviation



[a]Titers 2N-ScFv = N/A
2N = N/A
MAR = N/A

[a]Titers are expressed as the molar concentration that gives an O D [405] of 0 8

Figure 3.32. Linear regression analyses on the titration curves derived from a direct ELISA measuring the reactivity of 2N-ScFv and 2N against 20µg/ml GST. Both 2N and 2N-ScFv were prediluted to 5,500nmol and serially diluted two-fold Error bars represent ± 1 standard deviation.

Table 3.4. Summary of 2N/2N-ScFv recognition of 20μg/ml native LKT and GST-fused N-terminally deleted LKT constructs

GST Fusion	2N Titer[a]	2N-ScFv Titer[a]	Fold Difference
LKT	149 3 (998)[b]	351 5 (993)	2 4x
JM-1	227 0 (997)	861 5 (994)	3 8x
JM-2	355 2 (996)	956 0 (1 000)	2 7x
JM-4	656 4 (995)	1,626 2 (977)	2 5x
JM-7	N/A	N/A	N/A
GST	N/A	N/A	N/A

[a]Titers are expressed as the molar concentration (nanomoles) of 2N/2N-ScFv which gives an O D ^{405} of 0 8

[b]Numbers in parentheses indicate r^2 values as determined by linear regression

previously However, the 2N 2N-ScFv titer ratio for JM-1, 3 8x, was a little beyond this expected range of 2x This may be due to presentation differences inherent to JM-1 under these conditions, which may limit stable 2N-ScFv association If this assumption is correct, then the presented epitope may require the extra stabilization afforded by the bivalency of 2N (*i e* , there was a greater emphasis on avidity for JM-1 than for the JM-2 or JM-4) Also noteworthy in this set of experiments, as indicated by titer values, was that more of both 2N and 2N-ScFv was required for appreciable association with each of these constructs than that required to associate with an equivalent concentration of LKT This indicated that, although these fusions seem to have the elements required to mimic LKT under these conditions, recognition by either 2N or 2N-ScFv was not quite as efficient, perhaps due to weaker epitope formation and/or presentation or experimental error As expected, no appreciable titers were observed for the JM-7 or GST negative controls From these studies, it was concluded that the LKT-derived JM-1, JM-2, and JM-4 have the correct structural elements necessary for 2N/2N-ScFv association

Based on the recognition patterns observed for these fusions, two regions emerged as being of critical importance for LKT recognition First, from Figure 3 23 it can be seen that both JM-4 and JM-5 have the same elements, except that JM-5 is missing the carboxy 869-939 region Because JM-4 was recognizable to 2N/2N-ScFv and JM-5 was not, it was deduced that amino acids 869-939 play a critical role in epitope formation It can also be seen that the unrecognizable JM-3 construct is also missing this region Second, it was noted that none of the fusions JM-6-JM-8 were bound, but that all of these contain amino acids 869-939. However, these are missing the N-terminal amino acids

found on JM-4, namely amino acids 766-844 Therefore, it was deduced that this region is also important to epitope formation Examination of JM-1 and JM-2, the other two fusions recognized, show that they both contain these two stretches of amino acids which reinforces their importance Finally, because JM-4 is still recognized, it was assumed that the amino acids 673-765 were not of critical importance However, given the relatively large amounts needed to produce an appreciable titer (see Table 3.4), these amino acids may enhance or stabilize the epitope, thus increasing the efficiency of 2N/2N-ScFv association

2N-ScFv Neutralization of LKT-Induced Cytotoxicity

Although it had been extensively confirmed that 2N-ScFc specifically recognized LKT and LKT-derived fragmented proteins in a comparable fashion to its full-sized native counterpart, the functional significance of the molecule had to be determined Given that ltx-2 (2N) is such a potent neutralizer of leukotoxin's cytotoxic effects, it was hypothesized that the LKT-specific paratope (2N-ScFv) would prove to be a vital to neutralization The ability of 2N-ScFv to neutralize 1 5 U (1 U is defined as the reciprocal dilution of LKT which kills 50% of the target cells) of active LKT targeted at bovine-derived BL-3 cells was quantitatively assessed by a tetrazolium dye (MTT) reduction assay In this assay, the mitochondrial dehydrogenase of live cells reduces the MTT from yellow to purple by an oxidation/reduction reaction However, dead cells lose this ability, and the MTT remains yellow. As can be seen in Figure 3.33, both 2N and 2N-ScFv did offer 100% protection against 1.5 U of active LKT. This was concluded

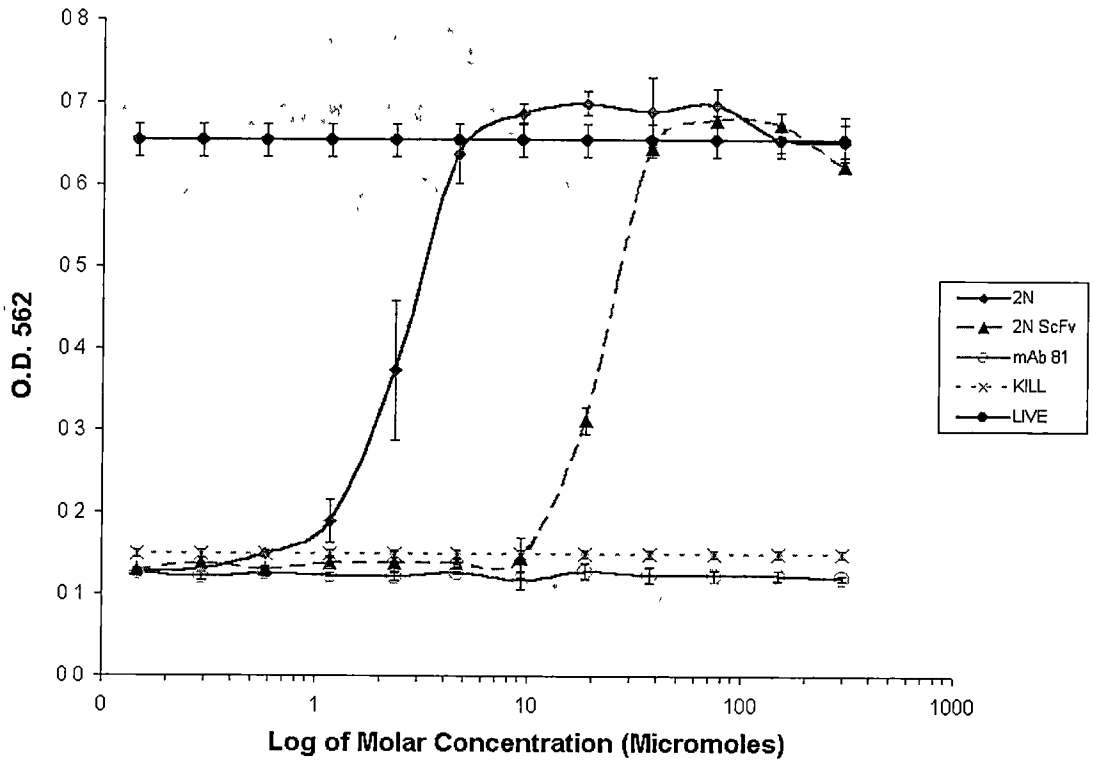
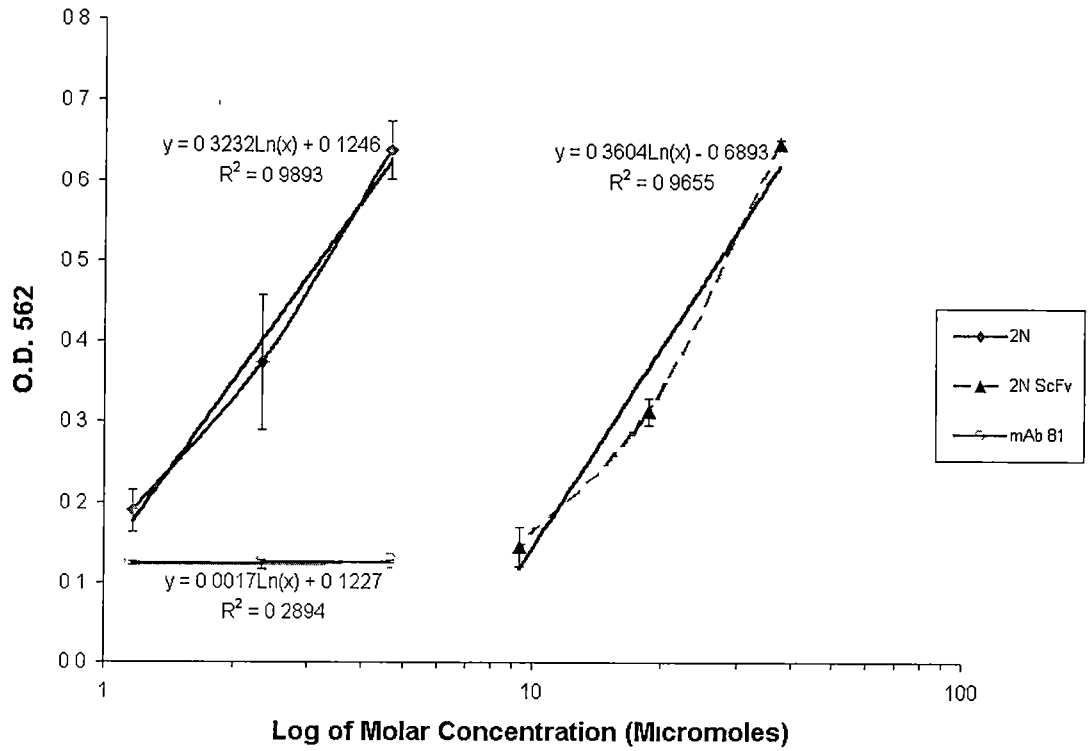


Figure 3.33. Quantitative analysis measuring the capability of 2N/2N-ScFv to neutralize 1.5 U of active LKT targeted against 4 x 10^4 BL-3 target cells Values reflect the amount of reduced MTT released upon lysis of the BL-3 cells with acid alcohol All antibodies were prediluted to 300μmoles and subsequently diluted two-fold

because high molar concentrations of both species yielded values at or above that of the cells only control (live value) A LKT-specific non-neutralizing mAb, mAb 81, served as the negative control, and, as expected, showed no protection against 1 5 U LKT This was reflected by values comparable to the cells + 1 5U LKT control (kill value) Linear regression analysis, Figure 3 34, revealed that 2N and 2N-ScFv had similar LKT association properties as evidenced by parallel titration curves However, from this figure, it can also be seen that the neutralizing titer (defined as the molar concentration needed to protect 75% of the BL-3 target cells) for 2N was 8 5x lower than that for 2N-ScFv (3 1 vs 26 4 respectively) This indicated that 75% protection could be provided by 8 5x less 2N than 2N-ScFv, and/or that 2N neutralized the LKT with 8 5x greater efficiency Because of valency differences not accounted for in the equimolar determinations, it was expected that 2N would neutralize 2x greater (*i e* , if there are the same number of molecules of 2N and 2N-ScFv, the bivalent 2N would show 2x greater neutralization because it can bind two molecules of LKT, while the monovalent 2N-ScFv can only bind one) This excessive difference between 2N and 2N-ScFv neutralization capacities was unexpected given the comparable LKT association tendencies previously determined by ELISA However, antigenic epitope presentation may drastically change when the antigen is in solution rather than bound to a solid phase (as with ELISA), and these alterations in the presentation of an antigenic domain are often difficult to predict. This presumable shift in epitope presentation may account for some of the observed difference. Other reasons for this discrepancy are most likely attributable to properties inherent only to the native antibody (e.g. the ability of the relatively large 160 kDa 2N to



[a]Titers		[b]Slope Interval	
	2N-ScFv = 26 4		2N-ScFv = (0 3, 0 4)
	2N = 3 1		2N = (0 2, 0 4)
	mAb 81 = N/A		mAb 81 = N/A

[a]Titers are expressed as the molar concentration that gives 75% protection against 1 5U of active leukotoxin

[b]Slope intervals are expressed at the 95% confidence level

Figure 3.34. Linear regression analyses on the titration curves derived from 2N/2N-ScFv neutralization of 1.5U LKT as determined by MTT reduction Error bars represent ± 1 standard deviation

cause significant conformational distortion of the smaller 101 9 kDa LKT), which are elaborated upon in the ensuing discussion Because 2N-ScFv was able to bestow 100% protection at high concentrations, it was concluded that the paratope alone was sufficient for neutralization However, given the large neutralization titer difference between 2N and 2N-ScFv, it was concluded that LKT recognition by the paratope alone does not constitute the entire neutralization capacity of 2N

During the initial investigation of the 2N-ScFv neutralization capacity, 1 5 U of active LKT was neutralized This was accomplished by preincubating the LKT with each dilution of a two-fold dilution series of either 2N or 2N-ScFv on ice for 1 hour Because the difference in neutralization titers between the two species was larger than expected, the experiment was repeated to empirically assess temporal effects on 2N-ScFv neutralization capacity The neutralization was carried out as indicated, but the time allowed for this step was varied 1 hour, 2 hour, and 4 hour preincubation increments No times beyond 4 hours were assessed due to the labile nature of LKT (which rapidly loses cytolytic activity at temperatures above 4° C) Figure 3 35 illustrates the results of this time course study As can be seen, extending the neutralization preincubation had no effect on the outcome Every time point gave results similar to those observed in the original neutralization assay neutralization by both 2N/2N-ScFv with titer differences of approximately 8x-10x Because of the similarity of each time point titration to the results indicated above, no linear regression analyses were performed The conclusion was that 2N-ScFv can neutralize but is not nearly as efficient as 2N native antibody (as stated above), regardless of the time allowed for neutralization

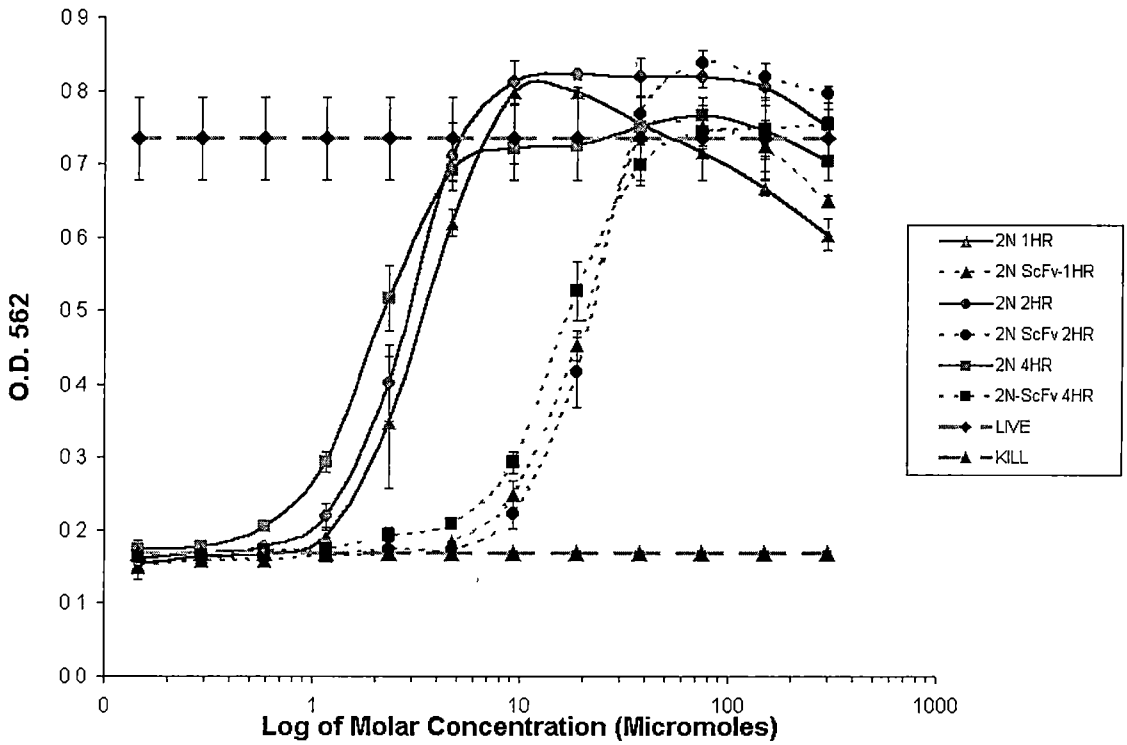


Figure 3.35. Quantitative analysis of temporal variability on the ability of 2N/2N-ScFv to neutralize 1.5 U of active LKT targeted against 4 x 10^4 BL-3 target cells
LKT was preincubated with each species on ice for either 1, 2, or 4 hours Values reflect the amount of reduced MTT released upon lysis of the BL-3 cells with acid alcohol All antibodies were prediluted to 300μmoles and subsequently diluted two-fold.

Adsorption of 2N/2N-ScFv by GST Fusion Proteins

Finally, in an effort to demonstrate that those GST fusion proteins already shown to be recognized maintain the ability to bind 2N and/or 2N-ScFv in a solution-based assay, antibody adsorption was performed In this quantitative MTT reduction-based assay, 100μmoles of 2N and 2N-ScFv (a molar concentration previously shown to provide 100% protection against 1 5 U LKT targeted at 4 x 10^4 BL-3 cells) were preincubated with varying concentrations of either JM-1, JM-2, JM-4, or the negative controls, JM-7 and GST 1 5 U of active LKT was then added and a 1 hour incubation on ice followed to allow for neutralization by any 2N/2N-ScFv not adsorbed in the initial preincubation Restoration of neutralization was indicative of reduced adsorption by each fusion protein The results of this assay are shown in Figures 3 36-3 39 As can be seen, each GST fusion protein was able to adsorb both 2N and 2N-ScFv with varying proficiency, except for the JM-7 and GST negative controls as expected This was indicated by values that represented the kill value at high fusion concentrations, showing that there was relatively little 2N and/or ScFv remaining to neutralize the LKT after the initial incubation As expected, this effect titrated as GST fusion concentration was reduced, restoring neutralization and values more representative of the live value

Even though each fusion shown to be recognized by 2N/2N-ScFv was able to adsorb these respective species with variable efficacy, some of these results were unexpected given the relative 2N/2N-ScFv association efficiencies previously found by direct ELISA The respective linear regression analyses are illustrated in Figures 3 40-3 42 (no linear regression analysis was conducted for the negative controls) and

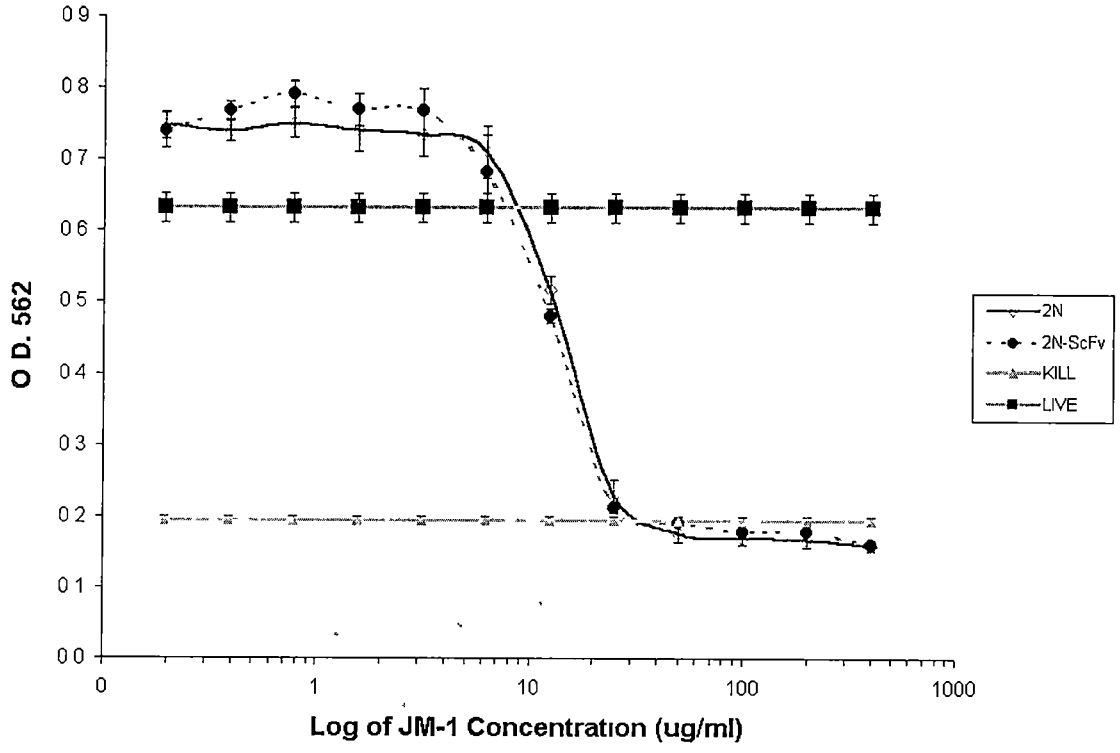


Figure 3.36. Quantitative analysis measuring the capacity of JM-1 to adsorb 100μmol of protective 2N/2N-ScFv JM-1 was prediluted to 400μg/ml and serially diluted two-fold Protection was assessed against 1.5 U of active LKT targeted at 4 x 10^4 bovine-derived BL-3 cells O D values are proportional to the amount of reduced MTT released upon lysis of cells with acid alcohol

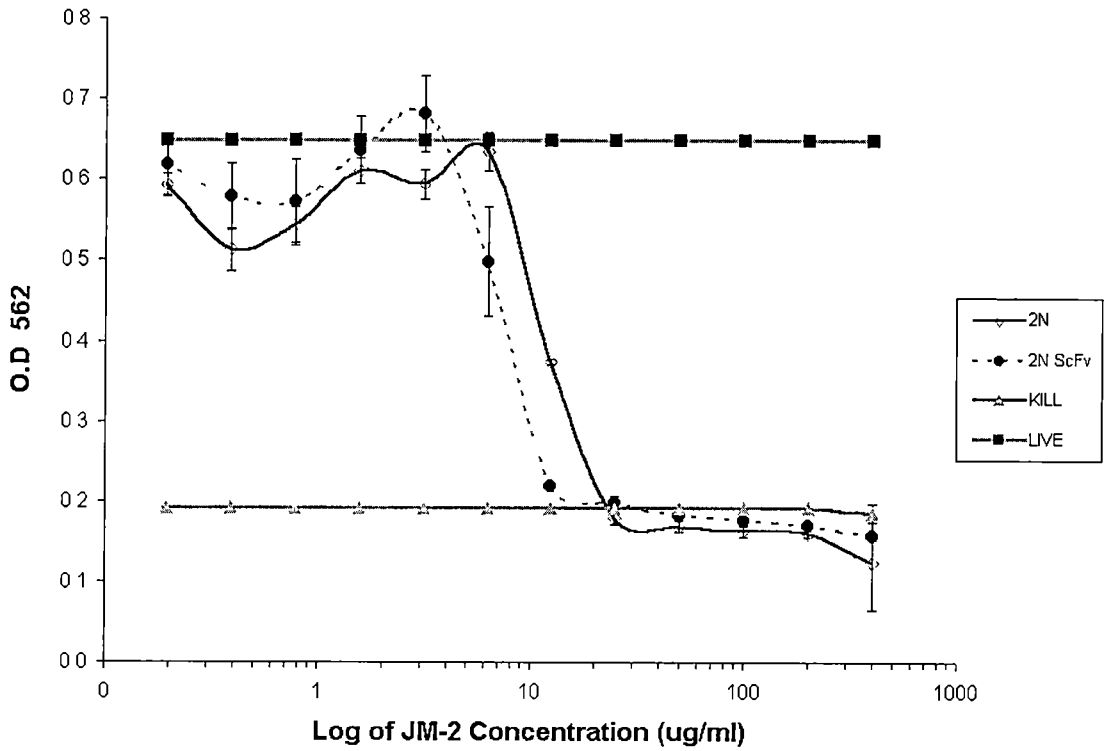


Figure 3.37. Quantitative analysis measuring the capacity of JM-2 to adsorb 100µmol of protective 2N/2N-ScFv JM-2 was prediluted to 400µg/ml and serially diluted two-fold Protection was assessed against 1 5 U of active LKT targeted at 4 x 10^4 bovine-derived BL-3 cells O D values are proportional to the amount of reduced MTT released upon lysis of cells with acid alcohol

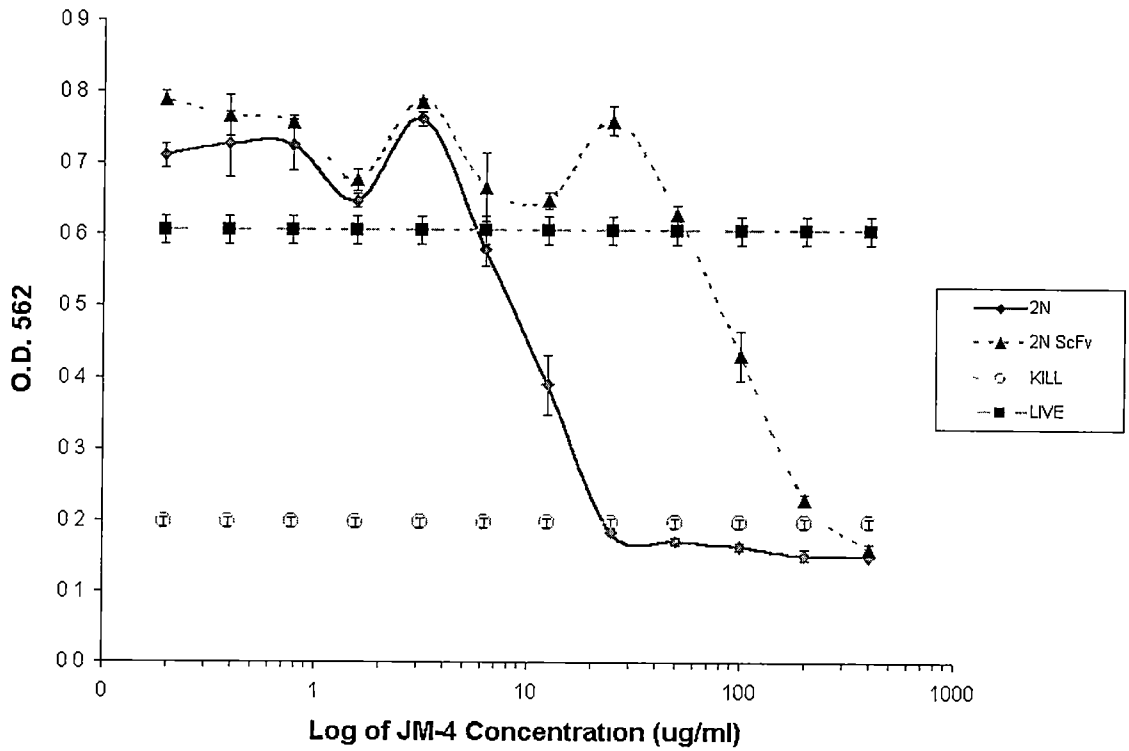


Figure 3.38. Quantitative analysis measuring the capacity of JM-4 to adsorb 100µmol of protective 2N/2N-ScFv JM-4 was prediluted to 400µg/ml and serially diluted two-fold Protection was assessed against 1 5 U of active LKT targeted at 4 x 10^4 bovine-derived BL-3 cells O D values are proportional to the amount of reduced MTT released upon lysis of cells with acid alcohol

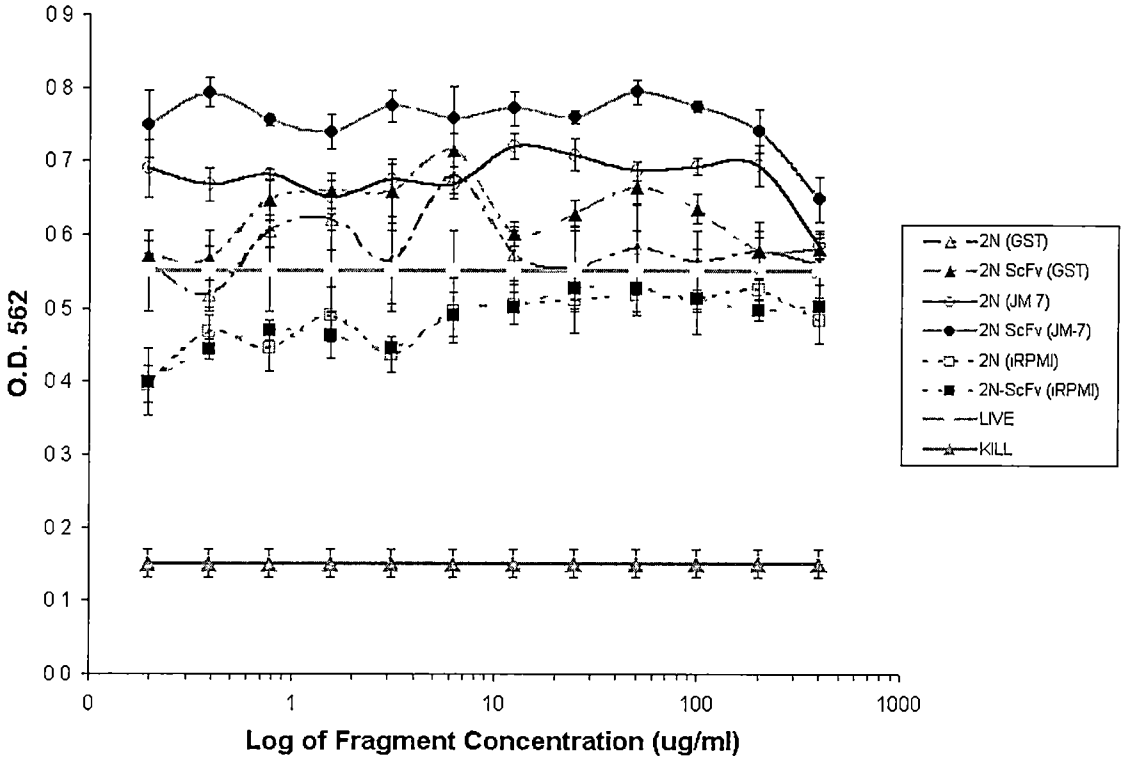
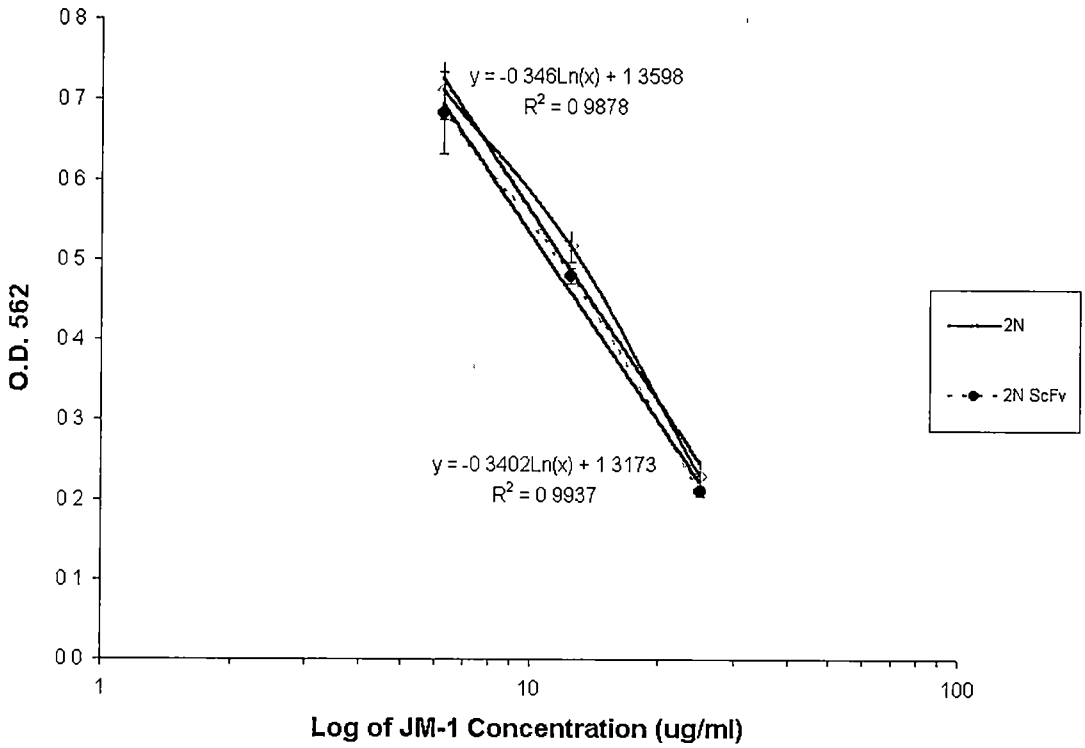


Figure 3.39. Quantitative analysis measuring the capacity of JM-7/GST to adsorb 100μmol of protective 2N/2N-ScFv Both JM-7 and GST were prediluted to 400μg/ml and serially diluted two-fold Protection was assessed against 1 5 U of active LKT targeted at 4 x 10^4 bovine-derived BL-3 cells O D values are proportional to the amount of reduced MTT released upon lysis of cells with acid alcohol

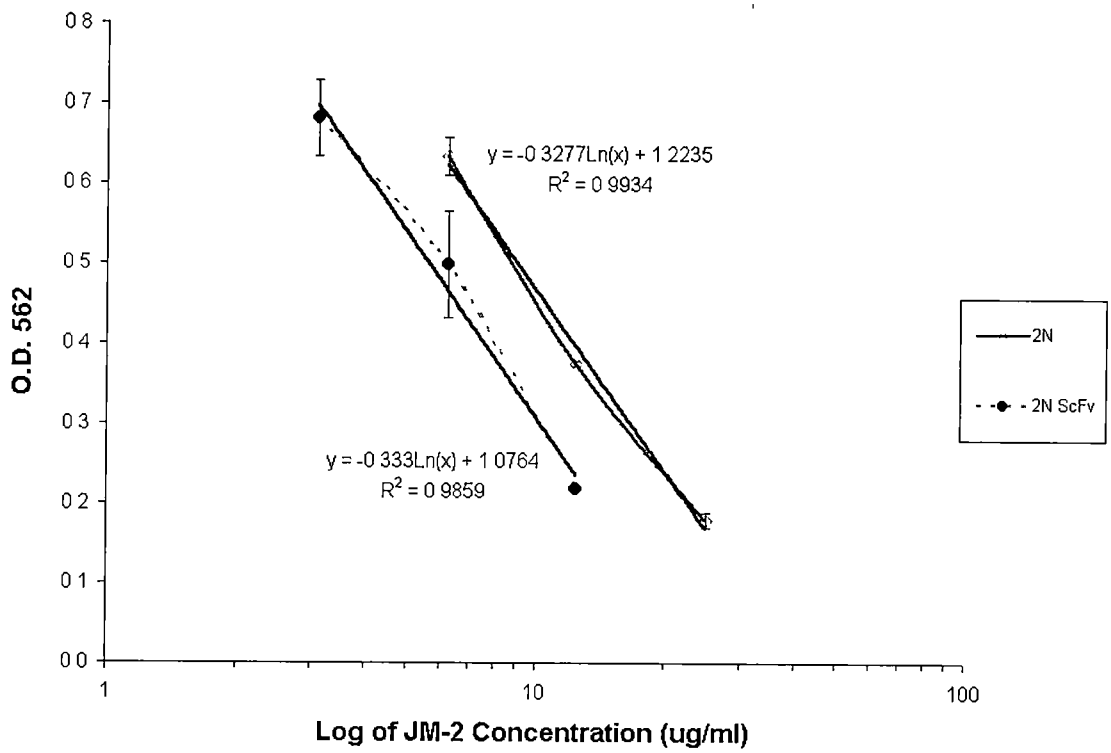


[a]Titers		[b]Slope Interval	
2N-ScFv = 12 1		2N-ScFv = (-0 4, -0 3)	
2N = 13 1		2N = (-0 4, -0 3)	

[a]Titers are expressed as the fragment concentration at which 75% protection against 1 5U of active leukotoxin is restored

[b]Slope intervals are expressed at the 95% confidence level

Figure 3.40. Linear regression analyses on the titration curves derived from 2N/2N-ScFv adsorption by JM-1 as determined by MTT reduction Error bars represent ± 1 standard deviation



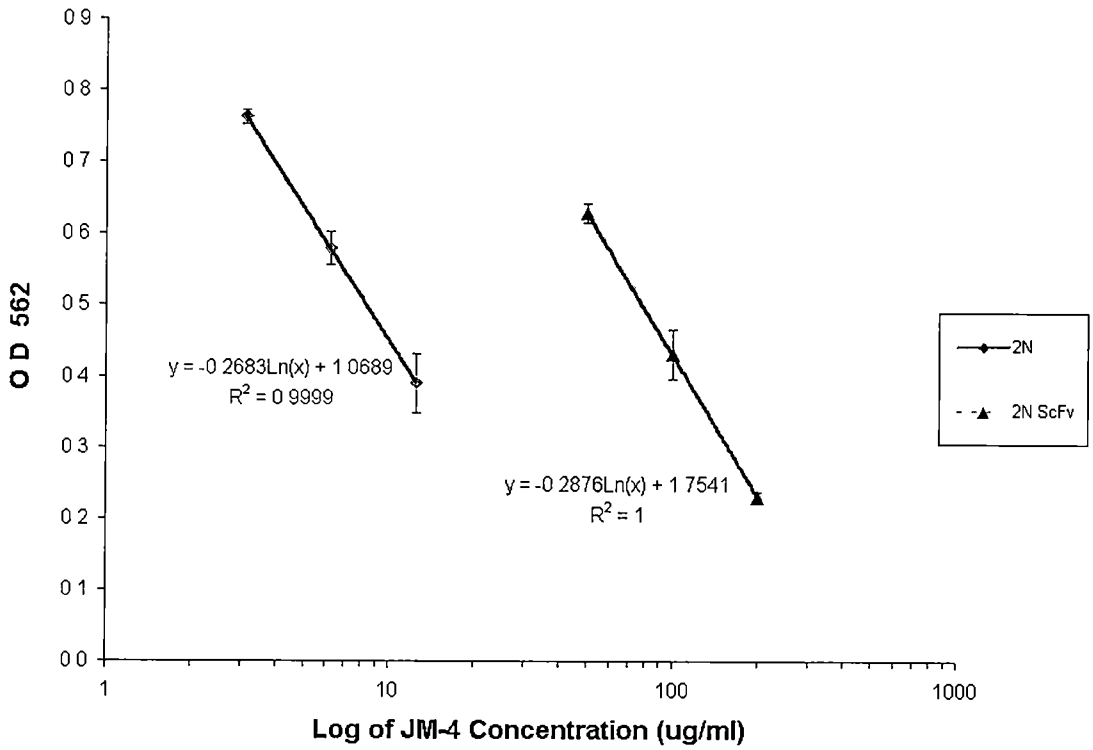
[a]Titers 2N-ScFv = 5 8
2N = 9 4

[b]Slope Interval 2N-ScFv = (-0 4, -0 3)
2N = (-0 4, -0 3)

[a]Titers are expressed as the fragment concentration at which 75% protection against 1 5U of active leukotoxin is restored

[b]Slope intervals are expressed at the 95% confidence level

Figure 3.41. Linear regression analyses on the titration curves derived from 2N/2N-ScFv adsorption by JM-2 as determined by MTT reduction Error bars represent ± 1 standard deviation



[a]Tıters 2N-ScFv = 93 2
2N = 10 0

[b]Slope Interval 2N-ScFv = (-0 3, -0 2)
2N = (-0 3, -0 2)

[a]Tıters are expressed as the fragment concentratıon at whıch 75% protectıon agaınst 1 5U of actıve leukotoxın ıs restored

[b]Slope ıntervals are expressed at the 95% confidence level

Figure 3.42. Linear regression analyses on the titration curves derived from 2N/2N-ScFv adsorption by JM-4 as determined by MTT reduction Error bars represent ± 1 standard deviatıon

summarized in Table 3.5 Again, it was noted that the association of 2N/2N-ScFv with each respective fusion construct was comparable as evidenced by parallel titration curves. However, the efficiency of this association varied The predicted outcome was that, for reasons already mentioned, there would be a 2x shift in adsorption· taking 2x more fusion protein to adsorb an equimolar amount of 2N due to valency differences (if 2N can bind two fusion molecules, then it would take 2x more fusion to adsorb the same number of bivalent 2N molecules as that required for the adsorption of the monovalent 2N-ScFv) As can be seen in Table 3 5, only the JM-2 adsorption approximated this expected result, showing that at 75% neutralization 1 6x more JM-2 was required for 2N adsorption This was in agreement with the results found for the direct ELISA measuring recognition of JM-2 by 2N/2N-ScFv, which indicated adherence to this valency difference The adsorption of 2N/2N-ScFv by JM-1 was found to be almost equivalent, giving neutralization restoration titers of 13 1/12 1 respectively Although this did not adhere to the valency restrictions discussed, it did reiterate the results found during the JM-1 recognition ELISA Recall that JM-1 gave an abnormal fold difference in 2N/2N-ScFv titers at 3 8x (see Table 3 4) Because this was nearly twice that expected, it suggested that JM-1 was recognized twice as efficiently by 2N. This held true in the adsorption assay, which revealed that 2N did not require the expected 2x increase in the amount of fusion at 75% neutralization This was interpreted as a 2x increase in 2N recognition efficiency compared to 2N-ScFv, results similar to that found in the recognition ELISA The possible reasons for this difference were presumed to be comparable to those given for the recognition differences observed in the ELISA

Table 3.5. Summary of 2N/2N-ScFv adsorption by GST fusion proteins Values were determined by MTT reduction assays

GST Fusion	2N Titer[a]	2N-ScFv Titer[a]	Fold Difference
JM-1	13 1 (988)	12 1 (994)	1 1x
JM-2	9 4 (993)	5 8 (986)	1 6x
JM-4	10 (1 000)	93 2 (1 000)	9 3x
JM-7	N/A	N/A	N/A
GST	N/A	N/A	N/A

[a]Titers are expressed as the fragment concentration (μg/ml) at which 75% protection against 1 5U of active leukotoxin is restored
[b]Numbers in parentheses indicate r^2 values as determined by linear regression

(discussed above) Finally, the results seen for JM-4 significantly deviated from the those expected In this assay, rather than showing that a 2x higher concentration of JM-4 was needed for 2N adsorption relative to 2N-ScFv adsorption at 75% neutralization, it was demonstrated that a 9 3x higher concentration was needed for 2N-ScFv adsorption This showed that 2N was 9 3x more efficient in recognizing JM-4 (and subsequently adsorbed 9 3x more efficiently), results not in agreement with the ELISA results previously conducted which indicated that there were no drastic differences between 2N/2N-ScFv association with JM-4 However, interestingly, this change in behavior from solid phase analysis to solution-based analysis closely resembled that seen for native LKT and was likely attributed to the reasons previously discussed for LKT If 2N/2N-ScFv association is adequate for LKT neutralization (shown to be the case in this study), then it was previously indicated that 2N was 8 5x more efficacious in LKT binding as determined by this assay Likewise, adsorption of 2N/2N-ScFv was a measure of the relative association efficiencies with each fusion construct within the confines of this assay In the case of JM-4, 2N was 9 3x more proficient in JM-4 binding, a fold difference comparable to that found for LKT Because 2N/2N-ScFv association values/titer differences determined by both this assay and the previously discussed ELISA were extremely similar, some credence was given to JM-4 (amino acids 766-939) as being the most representative of the true epitope found on the native active form of LKT

CHAPTER 4

DISCUSSION

Although there have been several recent investigations which have helped to elucidate the etiological mechanisms that contribute to pneumonic pasteurellosis, this disease remains the biggest economic burden of the North American beef cattle industry (11, 84, 107) Certainly, many questions about the intricacies of the disease processes associated with pneumonic pasteurellosis still exist The relative inability to recreate the field conditions which elicit this disease in a laboratory setting and the necessity of ruminant animal models are two factors which continue to limit the efficiency of contemporary research Furthermore, the lack of a suitable, widely accepted prophylactic treatment strategy, despite numerous time and resource-consuming laboratory and field studies, has heightened the frustrations of the beef industry Despite these shortcomings, current and past investigative endeavors have consistently shown that *Pasteurella haemolytica* serotype A1 plays a primary role in the genesis of pneumonic pasteurellosis and significantly contributes to the observed pathology As such, it is no surprise that this bacterium is the most frequent isolate from the lungs of pneumonic cattle (51, 59, 85, 86, 88, 194, 285)

Given the primary involvement of *P haemolytica* A1 in pneumonic pasteurellosis, many investigations have focused on the virulence mechanisms of this microbe as well as potential ways to diminish their detrimental effects in host animals. Considering *P haemolytica's* relevance to the bovine respiratory disease complex, many

researchers maintain that controlling this one etiological agent and its associated virulence factors could lead to a drastic reduction is the incidence of disease (88) However, preventative measures via various vaccination strategies have given contradictory and disappointing results These strategies have included live and live-attenuated *P haemolytica* vaccines (56, 89, 94, 147, 190, 197, 208, 285), bacterins (56, 89, 94, 147, 190, 197, 208, 285), and DNA-based vaccines (155) Despite the poor success of these strategies, vaccines prepared from cell-free culture supernatant containing appreciable levels of capsular polysaccharide, outer membrane proteins, and leukotoxin have shown some promise (53, 64, 215, 233, 236) In fact, many studies have indicated that enhanced protection correlates with high circulating titers of LKT-neutralizing antibodies (94, 185, 190, 233, 236) Furthermore, LKT has been well-established as an important virulence factor involved in both colonization and tissue pathology (13, 17, 48, 135, 174, 234) Considering leukotoxin's importance as a virulence factor and immunogen, further elucidating its mechanism(s) has been a primary goal of contemporary investigators

Our laboratory has focused on defining and characterizing important immunoreactive epitopes within the LKT molecule (96, 97, 184) This approach has been facilitated by a panel of monoclonal antibodies generated by Donald Gerbig of this laboratory (96, 97) Given their ability to neutralize active LKT, three of these mAbs (ltx-2, ltx-4, and ltx-35) presumably recognize protective epitopes within the LKT molecule Of these, ltx-2 shows the greatest capacity to neutralize LKT (96, 97) Initial epitope mapping with cyanogen bromide cleavage fragments, mutant deletion proteins,

alkaline phosphatase/leukotoxin fusion proteins, and *P haemolytica/A pleuropneumoniae* serotype 5 RTX chimeras suggested that the LKT epitope recognized by ltx-2 is in the region between amino acids 768-939 (96, 97, 187) A more contemporary investigation has narrowed down the ltx-2-specific epitope to the region between amino acids 876-939 (184) Furthermore, a preliminary characterization of ltx-2 has revealed that this neutralizer prevents toxin association with BL-3 target cells (96) This study has sought to further characterize the underlying mechanisms of ltx-2 neutralization in an effort to implicate the aforementioned LKT region as a putative cell association domain

In order to evaluate the LKT association properties of ltx-2, it was decided to construct a single-chain fragment variable This novel approach was chosen for three reasons First, ScFv technology employs a number of time-efficient recombinant strategies which ultimately pare down native, full-length antibody molecules to antigen binding pockets, or paratopes, independent of any constant region effector domains In this case, this provided a suitable way to analyze the ltx-2 paratope and its contribution to LKT binding/neutralization Second, if successful, this strategy ensures a stable, antigen-specific molecule which may be applicable as an immunochemical and/or a therapeutic reagent This is in contrast to enzymatic cleavage of native antibody in order to generate Fab or $F(ab')_2$ fragments, an approach that may be unpredictable or completely ineffective for some IgG subclasses (209) In fact, initial attempts at cleaving ltx-2 with pepsin and papain failed due to inadequate cleavage sites and/or fragment instability (Amanda Royer, personal communication) Finally, due to its versatile molecular basis,

ScFv technology facilitates DNA sequencing, genetic analysis, homology studies, site-directed mutagenesis, etc

Using ltx-2 mRNA, V_H and V_L cDNA was generated by RT-PCR and subsequently amplified and linked using conventional PCR techniques. After cloning the ScFv construct into a phage-display fusion vector (pCANTAB 5E) and transforming *E coli* TG-1 cells, 96 phage displaying LKT-specific ScFvs were enriched and screened against the native antigen (LKT) Once LKT recognition of these clones had been established, each one was preliminarily screened for its capacity to neutralize LKT Clone D9, a clone which gave a strong ELISA signal indicative of LKT specificity as well as a strong MTT reduction signal indicative of LKT neutralization, was transduced into a non-phage generating *E coli* cell line for large-scale soluble expression Stable LKT-specific ScFv was successfully isolated from both the cellular periplasm and the cell culture supernatant of this HB2151 cell line upon IPTG induction As a result, the ScFv-encoding DNA from clone D9 was isolated and sequenced Sequence analysis of this ScFv (2N-ScFv) yielded an open reading frame encoding the proper 29 5 kDa V_H-$(Gly_4Ser)_3$ linker-V_L-E tag polypeptide Based on amino acid sequence homology, ltx-2 was identified as a member of the murine Ig heavy chain Family VIII (subgroup IIA) and the murine kappa light chain Family XX (subgroup V) (92, 134) Also noteworthy was the fact that all six CDRs represented in this polypeptide, which form a putative six-loop ligand binding motif (1, 29), had at least one charged amino acid and/or polar, uncharged amino acid, with V_H CDR II and V_L CDR III having the most (9 and 8 respectively) This is important because these classes of amino acids lend themselves to forming

electrostatic interactions and hydrogen bonds respectively, both very important in ligand binding (29) Although this sequence analysis does little in illuminating the precise mechanism(s) of ltx-2 binding/neutralization of LKT or the immunoreactive epitope, it may provide the impetus for site-directed mutagenesis/affinity maturation studies For example, amino acid residues and/or CDR composition could be systematically manipulated in an effort to decipher which amino acids are crucial in LKT binding Subsequently, the LKT association properties of 2N-ScFv could be enhanced for future applications

Given that 2N-ScFv represents the ltx-2 paratope, both molecules are expected to react with LKT in a similar fashion In order to confirm this assertion, several *in vitro* experiments were performed Initially, purified 2N-ScFv was tested for its antigenic specificity, ability to bind native LKT, and reactivity to a panel of GST-fused LKT fragments. Using a comparative approach, 2N-ScFv characterizations were performed alongside an equimolar amount of its full-length ltx-2 counterpart First, it was demonstrated that both ltx-2 and 2N-ScFv are extremely specific for LKT This was evidenced by the lack of cross-reactivity with a panel of closely related *A pleuropneumoniae* RTX toxins, suggesting that the ltx-2 epitope may be unique to LKT However, the *A pleuropneumoniae* toxins tend to show markedly reduced homology to LKT in the region of the putative ltx-2-specific epitope Current efforts are being made to extend this investigation to other RTX toxins including the more closely related α-hemolysin of *E coli*. Hopefully, this study will help to illuminate LKT-specific sequence elements which contribute to its effector function(s).

Next, it was demonstrated that the 2N-ScFv affinity for native LKT was comparable to that of ltx-2 as indicated by parallel titration curve slopes However, due to inherent avidity differences between the two molecules, ltx-2 titers, or the amount required to produce an O D.^{405} signal of 0 8 against 20μg/ml LKT, in both direct and trap ELISAs were consistently 2x less than those of 2N-ScFv This effect was expected for solid-phase *in vitro* assays due to differences in secondary detection of ltx-2/2N-ScFv (2 detectable ligands on ltx-2 versus only one on 2N-ScFv) and/or subtle differences in the stability of binding, with bivalent ltx-2 association expected to be more stable (avid) than monovalent 2N-ScFv association (1, 108)

Finally, in an effort to confirm that the ltx-2-specific epitope resides between amino acids 768-939, 2N-ScFv was tested for its ability to recognize a panel of GST-fused LKT fragments (see Figure 3 23) derived from the respective region of the LKT molecule (184) It was found by Western immunoblot, direct ELISA, and trap ELISA that JM-1, JM-2, and JM-4 all possess the ltx-2/2N-ScFv-specific epitope Furthermore, as determined by direct ELISA, each of these fragments yielded similar ltx-2/2N-ScFv titration patterns to those found for native LKT. This lends further credence to the assertion that these constructs contain the immunoreactive epitope Based on the recognition patterns observed for these fusions, two regions emerged as being of critical importance for epitope formation First, from Figure 3 23 it can be seen that both JM-4 and JM-5 have the same elements, except that JM-5 is missing the carboxy 838-969 region Because JM-4 was recognizable to ltx-2/2N-ScFv and JM-5 was not, it was indirectly demonstrated that amino acids 869-939 play a critical role in epitope

formation The fact that the unrecognizable JM-3 construct is also missing this region reinforces this finding Unfortunately, the relevance of this region was not directly established by either ltx-2 or 2N-ScFv since neither molecule was able to associate with the smaller JM-6, JM-7, and JM-8 fragments, all of which contain this region However, it has been previously reported that ltx-2 weakly recognizes this region solely (184) This discrepancy may be due to fragment instability and/or variability in experimental protocol Again, fusions JM-6-JM-8 were not bound despite the presence of amino acids 869-939 However, these are missing the N-terminal amino acids found on JM-4, namely amino acids 766-844 Therefore, it was deduced that this region, which primarily consists of the carboxyl 4 5 tandem repeats, is also important to epitope formation Given this scenario, the ltx-2/2N-ScFv-specific epitope is most likely conformational, its formation relying on at least two different stretches of primary sequence Examination of JM-1 and JM-2, the other two fusions recognized, show that they both contain these two stretches of amino acids which reinforces their importance Finally, because JM-4 was recognized, it was assumed that amino acids 673-765 present in JM-1/JM-2 were not of critical importance, though they may help stabilize the ltx-2/2N-ScFv-specific epitope in these constructs It should be noted here that other potent LKT neutralizing antibodies have been mapped to the carboxyl one-third of the molecule, thus ascribing some functional significance to this region (96, 97, 150, 184) However, some findings have shown that not all antibodies which map to the carboxyl region are neutralizing (150) Although this seems contradictory, it actually reinforces the importance of specific domains and motifs within this part of the molecule On a related note, recent studies

have shown that the leukocyte-specific adhesin LFA-1 is a potential LKT receptor (131, 160, 268) The normal ligand for this receptor is ICAM-1 on endothelial cells In an effort to justify the functional significance of the carboxyl region of LKT in cell association, both DNA and protein homology studies were performed Unfortunately, there is no appreciable homology between the carboxyl one-third of LKT and bovine-derived ICAM-1 However, this does not eliminate the possibility of similar motifs due to comparable protein folding Hopefully, future molecular studies on LKT will probe this possibility

In addition to LKT binding, the effector function of 2N-ScFv, namely LKT neutralization, was also assessed In this context, antibody-mediated neutralization may occur in one of four ways, though it should be noted that these are not mutually exclusive steric hindrance at or near the site of target cell association motifs, antibody-induced structural distortion, which has the potential to disrupt both proximal and distal elements important for cell association; Fc-stabilization of bivalent crosslinking of antigen, which may facilitate molecular "clumping", and Fc-mediated formation of immune complexes (1) In terms of mAbs, the latter is possible only if there are overlapping or repeating epitopes within the same antigen which are cross-reactive with the same mAb, thus facilitating the formation of large, stable lattices or aggregates Within the confines of ltx-2 LKT association there is some evidence to support this First, it has been noted on several occasions that ltx-2 can trap for itself, indicating that there may be more than one epitope within the 766-939 region (unpublished observation) However, the tendency of LKT to form multimolecular aggregates, either spontaneously

or via Fc-stabilized bivalent clumping, may contribute to this unlikely scenario of a mAb trapping for itself Second, this study found that ltx-2 blocked 2N-ScFv LKT association much more effectively than 2N-ScFv inhibited ltx-2 LKT association Potentially, this could be due to the ability of the bulkier ltx-2 to shield other putative adjacent epitopes whereas the small 2N-ScFv does not have the same steric hindrance capacity However, this is speculation and has not been directly demonstrated Nevertheless, prior to this investigation it remained that any of these four mechanisms of neutralization were feasible

Because previous studies have indicated that neutralization of LKT by ltx-2 is due to inhibition of target cell association (96, 97), neutralization by 2N-ScFv would provide some insight into the effector mechanism(s) of ltx-2 Furthermore, this approach could indirectly implicate the aforementioned carboxyl regions as important cell association domain constituents Using a BL-3 target cell cytotoxicity assay to measure the neutralization capacity of ltx-2/2N-ScFv, it was found that the native ltx-2 was a much more effective neutralizer than its paratopic counterpart However, it was clearly demonstrated that 2N-ScFv has the capacity to neutralize active LKT Furthermore, at high molar concentrations there was no appreciable difference in the two molecules' neutralization capacity The disparity was only appreciated by titration, which revealed that 75% protection could be afforded by 8 5x fewer ltx-2 molecules than 2N-ScFv molecules This was approximately a 4 25x deviation from the expected 2x valency difference Furthermore, this effect was not abrogated by temporal variability or assay repetition, suggesting that this difference in neutralization capacity is real.

The results obtained from these neutralization studies suggested that epitope masking (steric hindrance) is certainly important in preventing LKT association with the target cell, but it may not be the entirely responsible given the ltx-2/2N-ScFv neutralization capacity disparity. This is based on the fact that the 2N-ScFv neutralization mechanism is limited to steric hindrance or structural distortion Though the latter cannot be ruled out, the 29.5 kDa 2N-ScFv is substantially smaller than the 101 9 kDa LKT diminishing this possibility Furthermore, 2N-ScFv neutralization cannot be attributed to immune complex formation or Fc-mediated clumping as there is no associated Fc region on this monovalent molecule Conversely, these phenomena may account for some of the noted titer difference in that it is feasible for ltx-2 to neutralize by these mechanisms If, in fact, the bivalency of ltx-2 (clumping) contributes to its enhanced neutralizing capability, then this would be in agreement with the observation that increased LKT aggregation correlates with diminished activity (13, 115, 196) However, neither of these mechanism can be solely responsible due to the observed 2N-ScFv neutralization Certainly, structural distortion is a distinct possibility for the much larger native 160 kDa ltx-2 As a result, some of the observed difference may be attributed to differing levels of structural distortion brought about by ltx-2/2N-ScFv association Finally, as argued above for 2N-ScFv, ltx-2 association probably inhibits target cell binding by virtue of steric hindrance. Again, the noted disparity may be explained by molecular size differences For example, if the putative cell association domain was not entirely represented by the aforementioned regions but also relied on adjacent motifs, ltx-2 would be much more efficient in shielding these as well based on

its bulky size. Conversely, 2N-ScFv would not be efficient at blocking unbound adjacent areas due to its small molecular size Other *in vitro* factors such as altered epitope presentation in a soluble assay, avidity, and molecular stabilization/half-life may play minor roles in the observed ltx-2.2N-ScFv neutralization difference Nevertheless, the ability of 2N-ScFv to neutralize the effects of active LKT showed that steric hindrance (and perhaps to a lesser extent structural distortion) is an important facet of ltx-2 neutralization

Because of the strong likelihood that steric hindrance plays a significant role in LKT neutralization, those amino acid sequences involved in ltx-2 epitope formation (discussed above) may be vital in forming a potential cell association domain or the ligand for leukocyte-specific LFA-1 In order to emphasize this point, antibody adsorption assays were performed using those fragments recognized by ltx-2/2N-ScFv, JM-1, JM-2, and JM-4 All three fragments were successful to varying degrees in adsorbing both ltx-2 and 2N-ScFv in a soluble assay (thus preventing neutralization), results which suggest that all three fusion proteins contain an epitope(s) similar to the cell association epitope(s) of native LKT Interestingly, the difference in ltx-2/2N-ScFv adsorption by JM-4 gave disparate results very reflective of those found for LKT neutralization (*i e*, 2N-ScFv being approximately 5x less efficient in binding JM-4 in a soluble environment) Although this may lend some support for JM-4 having the most similar epitope presentation to that of native LKT within the confines of this assay, it must be noted that this assay was only a measure of fragment recognition and not a measure of ltx-2/2N-ScFv function Therefore, the disparity betweeen ltx-2/2N-ScFv

adsorption with JM-4 in this assay may merely be coincidence and not at all related to the neutralization capacity difference observed using the same type of assay In this case, the difference is most likely attributed to altered epitope presentation in a solution-based assay, an effect which could have a greater impact on Fc-deficient monovalent molecules due to the lack of avid stabilization

In this study, every effort was made to maintain suitable positive and negative controls However, two issues must be addressed First, although the results of this study indicate that 2N-ScFv has a comparable character to that of its native ltx-2 counterpart, many of the assays used in this project employed different means of secondary detection of ltx-2/2N-ScFv Because ScFv molecules are devoid of any conserved constant domains, there are no suitable animal-derived anti-IgG reagents An initial attempt at grafting a kappa-specific constant domain onto 2N-ScFv was unsuccessful, resulting in the abrogation of LKT recognition (92) Moreover, ScFv molecules do not adhere well to plastics, thus limiting assay normalization via a two-antibody sandwich strategy For these reasons, the 2N-ScFv design employs a small 13 amino acid tag, the E tag, for detection Therefore, ltx-2 was detected with rat anti-mouse IgG_1-HRP conjugate and 2N-ScFv was detected with recombinant anti-E tag-HRP conjugate However, detecting secondary reagents were normalized on an optimal, equimolar basis Nevertheless, some experimental error is likely attributable to subtle differences in secondary affinities for the respective primary molecule and/or experimental conditions Second, although GST was included as a pertinent control in all fusion protein experiments, the effect of a GST moiety on the LKT deletional constructs is unpredictable. GST could potentially alter the

conformation of these peptides and/or the formation/presentation of LKT-specific epitopes Countless efforts have been made to enzymatically cleave off the GST adduct or use a different purification tag (184) However, these attempts have not been lucrative, usually resulting in the destabilization of these constructs and/or poor protein yield Because of the fused GST, caution must be exercised when comparing the LKT deletional fragments to native LKT since they are not precise reflections of the in tact molecule

The objectives of this study were realized in that a leukotoxin-specific ScFv with neutralization capacity was generated, characterized, and used to explore the underlying mechanisms of ltx-2 epitope recognition and neutralization Undoubtedly, 2N-ScFv has already proven to be a valuable laboratory reagent given the relative ease of its production, its stable nature in both periplasmic extract and purified forms, its reliability as a LKT-detecting molecule, and its capacity to neutralize Hopefully, future endeavors will enhance this molecule, making more versatile and applicable Future experiments might include the following affinity maturation studies/site-directed mutagenesis targeted at defining key amino residues involved in LKT recognition/neutralization; utilization of 2N-ScFv to probe other RTX toxins in hopes of defining conserved antigenic regions important in eliciting effector function (e g the cell association domain of LKT), suitable strategies to enhance the production, purification, and stabilization of 2N-ScFv, and placing 2N-ScFv encoding DNA into eukaryotic expression systems as a preliminary step toward DNA-based passive immunity (72, 74, 133, 221, 228, 273). The latter has already been attempted by Scott Fuller of this laboratory (92) However,

preliminary results were disappointing. Currently, other methods to improve the success of this strategy are being explored Hopefully, this idea will come to fruition since the efficacy of 2N-ScFv as a form of passive immunity might have unlimited potential.

Despite many years of research and the laborious efforts of several investigators, there are still many questions about *P haemolytica* A1, its virulence mechanisms, and ways to control it during cattle transport and at feedlots Suitable vaccination strategies and other methods of control and prophylactic prevention remain atop the research priority list As yet, there is still not a widely-accepted, efficacious vaccine Another pressing question includes finding the underlying factors that contribute to LKT's incredible specificity for ruminant leukocytes If LFA-1, a highly conserved mammalian leukocyte receptor, is indeed the LKT receptor, then 1) how does LKT mimic ICAM-1 such that it can bind this receptor? and 2) what feature(s) of ruminant leukocyte LFA-1 and/or its associated signal transduction pathways is/are accountable for the remarkable specificity of LKT-induced cytolysis? Questions such as these should elicit an explosion of future research efforts since the respective answers may lead to a quantum leap in our understanding of *P haemolytica's* mode of virulence and in designing suitable preventative strategies Finally, efforts to obtain a crystallographic structure of LKT should continue Although homology studies and algorithmic modeling are certainly useful, understanding this potent virulence factor's three-dimensional conformation could potentially answer several other pressing questions about the molecular mechanism(s) of this toxin. Hopefully, ScFv technology will play a substantial role in future LKT

research, eventually contributing to the answers to questions like these and enhancing our understanding of bacterial pathogenesis

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VITA

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