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I am submitting herewith a thesis written by Scott C. Fuller entitled "Modification and Heterologous Eukaryotic Expression of a Leukotoxin Reactive Monoclonal Antibody Derivative." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

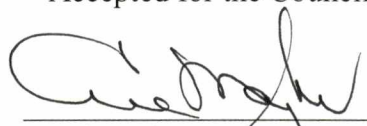

Robert N. Moore, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:


Interim Vice Provost and
Dean of the Graduate School

**MODIFICATION AND HETEROLOGOUS EUKARYOTIC
EXPRESSION OF A LEUKOTOXIN REACTIVE
MONOCLONAL ANTIBODY DERIVATIVE**

A Thesis

Presented for the

Master of Science

Degree

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Scott C Fuller

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DEDICATION

This thesis is dedicated to my wife Heather. I would not have chosen this path, nor been able to endure the rigors of graduate study without her encouragement and support. She not only believed in my potential and challenged me to pursue my passion, but also sacrificed her welfare in order to give me every opportunity to succeed. For this, I owe her an immeasurable amount of love and gratitude.

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ABSTRACT

Of the many illnesses that plague the stockyards and feedlots of the world, none is as refractory or costly as bovine pneumonic pasteurellosis, more commonly known as "shipping fever". A vast amount of evidence has implicated *Pasteurella haemolytica* serotype A1 as a major component in this respiratory disease complex. Included in the repertoire of virulence factors employed by this organism is a potent ruminant specific leukotoxin (LktA) that is suspected to contribute to the profound pulmonary lesions and fibrinous pleuritis that are characteristic of acute pneumonic pasteurellosis. The unfortunate result of this disease is manifested in staggering economic losses that include treatment costs, market delay, and animal mortality.

The importance of this toxin in the disease process has been punctuated by evidence suggesting that neutralizing antibodies against LktA are responsible for attenuating the severity of the disease. The fact that a potent humoral immune response against this critical bacterial epitope was noted in subjects recovering from the disease gave promise of developing an effective vaccine. However, despite some very creative efforts, a viable approach to vaccination has eluded scientific and veterinary personnel for years. These strategies have included live vaccines, bacterins, recombinant subunit vaccines, and DNA based approaches. While some of these approaches have shown promise in the laboratory, field trials have been disappointing, and at times inconclusive.

The focus of this work is to present a foundation for the concept of generating a transiently sustained form of passive immunity that will attenuate, but not completely abrogate the disease state. This mechanism of prophylaxis will allow the individual to

mount an active response, in the event that it becomes necessary, without suffering the extensive pathology associated with acute pneumonic pasteurellosis.

In sum, I propose a novel approach to passive immunity, whereby a recombinant single chain variable-fragment (scFv) derived from a defined LtxA neutralizing antibody is fused with an IgG1 signal peptide on the amino terminus and an Igk constant domain on the carboxy terminus. The novel DNA construct is placed under the influence of a viral promoter/enhancer element in a eukaryotic expression vector and transfected into SP2/0 and COS-7 cell lines to demonstrate the feasibility of heterologous gene expression in lymphoid and non-lymphoid cell lines.

The results of this study indicate that detectable expression levels of the fusion construct were appreciated in the COS-7 cell line but not the SP2/0 line. Furthermore, detectable levels of protein were not appreciated in the extracellular milieu, suggesting a possible defect in post-translational processing or folding of the molecule. Expression alone demonstrates the feasibility of this strategy, however the issue of secretion and functionality are central to the idea of DNA based passive immunity and remain to be resolved.

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LIST OF ABBREVIATIONS

A°	annealing temperature
A _n	absorbance at (n) wavelength
a.a	amino acid
ABTS	2,2'azino-di(ethyl-benzthiazoline-6-sulfonic acid)
ADCC	antibody dependent cell mediated cytotoxicity
AP	alkaline phosphatase
BCA	bicinchoninic acid
BHI	brain heart infusion
BHV-1	bovine herpesvirus-1
BL-3	bovine lymphoma cell line
BRSV	bovine respiratory syncytial virus
BVDV	bovine viral diarrhea virus
C	constant domain
°C	degrees centigrade
cAMP	cyclic adenine monophosphate
cDNA	copy DNA
CFU	colony forming units
cGMP	cyclic guanine monophosphate
CNBr	cyanogen bromide
CMV	cytomegalovirus

C-terminal	carboxy-terminal
CTL	cytotoxic T-lymphocyte
C5a	complement cascade chemotaxin
D	immunoglobulin diversity segment
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
D-PBS	Dulbecco's Phosphate Buffered Saline
EDTA	ethylene-diamine-tetracetic-acid
ELISA	enzyme linked immunosorbent assay
ErbB-2R	ErbB-2 receptor protein
EtBr	ethidium bromide
FBS	fetal bovine serum
Fc	complement/ phagocytic binding region
g	relative centrifugal force (gravity)
GST	glutathione-S-transferase
H	heavy chain
HIV-1	human immunodeficiency virus-1
HRP	horseradish peroxidase
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG1	immunoglobulin G subclass 1

IgG2a	immunoglobulin G subclass 2a
IgG2b	immunoglobulin G subclass 2b
IgM	immunoglobulin M
Ig κ	immunoglobulin light chain κ (kappa)
Ig λ	immunoglobulin light chain λ (lambda)
IL-2	interleukin-2
i.p	intraperitoneal
IPTG	isopropyl- β -D-thiogalactoside
J	immunoglobulin joining segment
kDa	kiloDalton
LB	Luria-Bertani medium
LFA-1	lymphocyte function associated antigen-1
LktA	leukotoxin structural gene A
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAC	complement membrane attack complex
mg	milligram
MHC	major histocompatibility antigen
ml	milliliter
M-MLV-RT	Moloney murine leukemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
ng	nanogram

nm	nanometer
N-terminal	amino-terminal
O antigen	antigenic capsular polysaccharide chain
O D.	optical density
OMP	outer membrane protein
ORF	open reading frame
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline-0.05% Tween 20
PCR	polymerase chain reaction
PI-3	parainfluenza 3 virus
PSG	penicillin, streptomycin, L-glutamine
PVDF	polyvinylidene difluoride
pmol	picomole
RER	rough endoplasmic reticulum
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal RNA
RTX	repeats in structural toxin
tRNA	transfer RNA
RPMI-1640	Roswell Park Memorial Institute-1640 medium
scFv	single-chain fragment (variable)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	scanning electron microscopy
TbpA/B	transferrin binding proteins A and B
TBS	Tris buffered saline
TBS-Tween	Tris buffered saline-0.05% Tween 20
TCR	T-cell receptor complex
TEM	transmission electron microscopy
Tris	Tris-HCl
U	unit
V _H	immunoglobulin variable domain, heavy chain
V _L	immunoglobulin variable domain, light chain
WC	whole cell
μg	microgram
μl	microliter
2X-YT	double strength yeast extract/tryptone medium

CHAPTER 1

INTRODUCTION

The Disease

Since the early 1900's bovine pneumonic pasteurellosis has been recognized as a major problem within the European and North American cattle industries (154). The severe economic impact associated with this disease has provided the impetus for investigating its causative agents. On a global scale, annual losses and expenditures for preventatives/treatments have been estimated at one billion and three billion dollars respectively (91). To date, a voluminous amount of empirical evidence has been compiled, illuminating the etiology of this affliction in addition to virulence factors and molecular mechanisms of pathogenesis observed in pneumonic pasteurellosis.

Acute bovine pneumonic pasteurellosis or "shipping fever" is presumed to manifest itself through a constellation of factors including stress, viral infection, and opportunistic bacterial infection (70). However, the common thread among these factors is the overwhelming prevalence of *Pasteurella haemolytica* serotype A1 in the lungs of infected calves (68, 70). The resultant infection is characterized by parenchymal lesions coupled with acute to subacute anterior ventral pleuropneumonia, fibrin exudation, profound neutrophil and macrophage infiltration, capillary thrombosis, and necrotic foci that are surrounded by bacteria and degenerate spindle-shaped leukocytes (212, 221).

Despite the current body of knowledge encompassing the etiology, epidemiology, and immunopathology of bovine pneumonic pasteurellosis, an effective approach to managing the disease has been difficult to achieve. Thus, shipping fever still remains one of the most important causes of sickness and mortality in cattle (175).

Etiology

The organisms that are primarily responsible for the bovine respiratory disease complex (BRDC) have a commensal relationship with normal, healthy feedlot age calves, colonizing the mucosal surfaces of the upper respiratory tract (66, 67). This evidence suggests that an environmental shift within the host to a state of impaired immunity is required for the opportunistic infection to occur. This shift is precisely why the term “complex” is applied to pneumonic pasteurellosis. Manifold factors contribute to the impairment of the immune system, providing an ideal situation for bacterial colonization within the lower respiratory tract of susceptible calves.

The first of these factors is physical stress, a result of the rigors that are inherent to the process of shipping. Practices such as bleeding, vaccination, rough handling, restraint, and herding are capable of eliciting a stress response in calves (129). Furthermore, it has been demonstrated that the process of shipping, acute exertion and restraint have resulted in altered immune function (22, 23, 117, 217).

The second factor in this complex is environmental stress which is manifested through irregular feeding and watering schedules, exposure to temperature extremes, crowded conditions, and unfamiliar individuals within the herd (66, 129). The result of

this composite stress, both physical and environmental, is a suspected depression in the host's immune response to opportunistic respiratory infection. Evidence for this suspicion resides in a decreased number of pulmonary macrophage numbers following transit, and the observation that stressed animals vaccinated with live "avirulent" *P haemolytica* may develop systemic infection (128, 233).

The third factor that is presumed to play a significant role in BRDC is viral infection. A number of viral agents have been implicated in compromising the host's nonspecific and specific immune defenses, resulting in a reduced ability to rapidly clear bacteria from the lower respiratory tract. In fact, it is estimated that individuals suffering from severe respiratory viral infection have a 40% chance of developing bacterial pneumonia (107). A host of viral agents are suspected to play a major role in enhancing the susceptibility of animals to pneumonic pasteurellosis. The most notable of these agents is parainfluenza-3 virus (PI-3) which is highly correlated with animals suffering from respiratory disease (28). Other agents of significance in this context include bovine respiratory syncytial virus (BRSV), bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) (12, 63, 108, 170).

The modality of viral-bacterial synergy in respiratory disease has been studied extensively and a number of mechanisms have been proposed (27, 28, 63). The most obvious of these mechanisms is the death or impairment of infected ciliated epithelium and immune effector cells as a result of the viral life cycle. Indeed, it has been shown that BHV-1, PI-3, and BVDV directly destroy ciliary activity, thereby reducing the efficacy of the mucociliary escalator (181). Furthermore, BHV-1 was shown to establish

a non-productive infection in bovine lymphocytes *in vitro* resulting in apoptotic cell death (90)

Another factor that is presumed to perpetuate the disease process as a result of virally induced cell death is the release of cellular proteases. These proteases may affect a variety of host defense mechanisms that are important in governing the rate of bacterial adherence or replication. One such host substance, fibronectin, is functionally important in enhancing macrophage and monocyte phagocytosis of particles both as a conventional opsonin, and also as a stimulant for macrophages to ingest opsonized particles (11). Not only has viral infection itself been shown to attenuate levels of fibronectin production, but a reduction of fibronectin on adjacent cells due to rogue cellular or viral proteases may pave the way for bacterial colonization (11, 223). In fact, it has been demonstrated that colonization by Gram-negative bacteria correlates with low fibronectin levels (223)

The final modality by which viruses may exert their influence in the context of pneumonic pasteurellosis is through induction of the inflammatory response with concomitant cytokine production. The specific cytokine profile that develops following viral infection may be directly or indirectly responsible for enhancing or reducing leukocyte activity, altering leukocyte trafficking, promoting localized tissue destruction, or facilitating an inappropriate immune response for bacterial clearance (11). Furthermore, Babiuk asserts that, "in respiratory disease complexes there may be an imbalance of cytokines because one pathogen overstimulates the production of a specific cytokine or inhibits cytokine production by another pathogen. This initial response can

then influence many subsequent events including leukocyte trafficking and cytokine production (11) ”

While several mechanisms have been proposed to explain the etiology of pneumonic pasteurellosis, one common denominator is required. The host must exhibit a degree of immunological impairment in order for bacterial colonization to occur in the lower respiratory tract. The most potent evidence for this requirement resides in the fact that healthy, unstressed calves exposed to an aerosol or intratracheal inoculation of *P haemolytica* remain subclinical or exhibit a one day febrile response (65). Conversely, a number of experimental models have successfully predisposed calves to disease using virally mediated or stress response approaches (65, 195, 230).

In cases of feedlot pneumonia, the exact causative agent of disease is often times obfuscated by the isolation of multiple bacterial species from pneumonic lesions (153). Indeed, concurrent infection with various combinations of *P haemolytica*, *P multocida*, *Haemophilus somnus*, *Actinomyces pyogenes*, and *Mycoplasma* species have been documented (40, 229, 231). However, an overwhelming number of cases presenting classical symptoms of shipping fever have yielded *Pasteurella haemolytica* serotype A1 isolates from pulmonary lesions (25, 40, 67, 68, 70, 153, 217). Although serotype A1 is considered to be a commensal organism colonizing the nasopharynx of healthy calves, it is generally outnumbered and cultured with less frequency in the nasopharyngeal secretions of normal individuals than serotype A2 (66). Curiously, there appears to be a drastic shift in serotypes from the farm, where A2 dominates, to the feedlot where A1 is the predominate isolate (68). While little evidence has been offered to illuminate the

mechanism of this serotype dynamic, it is clear that serotype A1 is the principal component of pneumonic pasteurellosis in cattle. Conventional wisdom suggests that viral infection or stress can lead to an explosive proliferation of serotype A1 in the nasopharynx, allowing for the domination of other normal flora. Subsequent to this proliferation, infected droplets may be aspirated into the lower respiratory tract, seeding in the lungs and resulting in development of pneumonic lesions (68). Horizontal transmission can occur to other stressed or virally infected calves via infected nasal secretions or sputum resulting in a profound outbreak of disease. Furthermore, it has been suggested that *P. haemolytica* A1 plays such a critical role in the genesis of respiratory infection, that if this single agent were eliminated, losses attributed to bovine respiratory disease would be dramatically reduced (70). Acknowledging the paramount importance of this organism in the disease process, much of the contemporary research and vaccination strategies have converged upon *P. haemolytica* A1 for an efficacious solution to the onerous problem of pneumonic pasteurellosis.

Characteristics of *Pasteurella haemolytica*

Organisms classified under the genus *Pasteurella* are described as pleomorphic, Gram-negative, non-motile rods that are facultatively anaerobic, catalase and oxidase positive, and able to ferment a variety of carbohydrates (2, 16, 19). *P. haemolytica*, arguably the most scrutinized species in this genus, is divided into two biotypes based upon carbohydrate fermentation patterns, colony morphology, antibiotic sensitivity, and growth characteristics (2). Between the two biotypes, seventeen distinct serotypes have

been identified by indirect hemagglutination techniques (2, 53) Biotype A is classified by the ability to ferment arabinose and encompasses serotypes 1,2,5,6,7,8,9,11,12,13,14,16, and 17 (2, 53). Biotype T is best characterized by the ability to ferment trehalose but not arabinose and consists of serotypes 3,4,10, and 15 (2, 18, 198). However, debate over this classification scheme has developed based upon recent serotype studies (8) and 16s rDNA sequencing/DNA-DNA hybridization results (7) Currently there is a movement to reallocate members of the trehalose negative *P haemolytica* serotypes 1-2, 5-9, 12-14, and 16 to an entirely new genus, *Mannheimia* Within this genus, species are defined according to host specificity, serologic reactivity, and phenotypic/genetic differences (7, 8). Furthermore, members classified under the *P haemolytica* biotype T umbrella have been proposed to be reclassified as a new species, *P trehalosi* (54, 199)

The degree to which an organism is able to exert its pathogenic effects correlates well with the ability to subvert or evade the host's immune system, avoid mechanical defenses, and satisfy essential metabolic needs in a hostile environment *P haemolytica* maintains a number of these suspected virulence factors, which have been characterized to varying degrees, enabling it to proliferate under adverse conditions These factors include endotoxin/lipopolysaccharide (LPS) (46, 115, 179), capsular polysaccharide (3), fimbriae (150, 151), a neutral sialoglycoprotease (166), a neuraminidase (205, 206), and a potent secreted leukotoxin (13, 114, 137, 191)

The LPS of *P haemolytica* A1 is homologous to that of other Gram-negative organisms, consisting of three parts, a glycopospholipid, called lipid A, a core

oligosaccharide, and the somatic or "O" antigen comprised of species specific sugar residues. LPS is known to exert a diverse array of immunological effects on the host depending on the dose. The result of LPS concentration ranges from general mobilization of the inflammatory response via thromboxane A₂, prostaglandins, serotonin, and cAMP/ cGMP (60, 61), to septic shock. The most common effect at low doses is fever, a result of IL-1 and TNF α production by mononuclear phagocytes stimulated with LPS. Interestingly, both IL-1 and TNF α have been shown to act in concert, enhancing *P. haemolytica* endotoxin induced endothelial cell damage (188). This endothelial cell damage can result in microvascular thrombi and initiation of the clotting cascade, interrupting normal vascular function and hemodynamics. Furthermore, pulmonary mast cells challenged with LPS engage in the production and release of histamine (4). This early inflammatory signal may result in the rapid influx of neutrophils, providing a target rich environment for the potent leukotoxin (24, 196). Similarly, LPS is responsible for activating the alternative pathway of the complement system, resulting in the production of the chemotaxin C5a, and subsequent extravasation of neutrophils along this gradient. Despite the undeniable role of LPS in the inflammatory response to pulmonary infection, circulating antibodies against endotoxin show little or no correlation with enhanced immunity to *P. haemolytica* (41, 45, 66).

An important virulence attribute of *P. haemolytica* A1 which provides a means to evade immune surveillance mechanisms is the polysaccharide capsule produced during the logarithmic growth phase (49, 68). This structure exerts its effect by impeding the processes of complement-mediated opsonization and phagocytosis by mononuclear cells.

in addition to inhibiting the intimate association of the pore-forming membrane attack complex (MAC) with the bacterial cell wall (32, 50). Evidence supporting the utility of the bacterial capsule resides in the observation that naturally occurring and experimentally derived rough (capsule deficient) strains of *P. haemolytica* show enhanced susceptibility to phagocytosis, opsonization, and complement mediated killing, compared to their smooth (capsule-bearing) counterparts (32). Furthermore, *P. haemolytica* recovered from the trachea of an infected calf had capsular material four to five times thicker than that seen on organisms grown in culture, suggesting a dependence on this structure for *in vivo* survival (151). While there are a number of benefits to encapsulation, the profound antigenicity of capsular material provides an ideal target for humoral effector molecules. Indeed, serum antibody titer to capsular antigen has been directly correlated with resistance to experimental bacterial challenge (47).

While the capsule may provide a nonspecific mechanism of adherence, many Gram-negative bacteria must rely upon filamentous bacterial structures known as fimbriae or pili for intimate attachment to mucosal surfaces within the host (86). Furthermore, given their exquisite specificity for eukaryotic cell surface ligands, fimbriated bacteria may possess a selective advantage for survival over non-fimbriated isolates with the capability to endure the innate cleansing mechanisms of the upper and lower respiratory tract (151). Fimbrial structures have been reported in *P. haemolytica* A1 recovered from the pulmonary lavage fluid of live infected calves as well as from *in vitro* cultures (150, 151). Using SEM, TEM, and negative staining techniques, two distinct fimbriae of approximately 10-12 nm and 5 nm in diameter were identified, suggesting a mechanism

of specific rather than nonspecific attachment to respiratory epithelium/mucosa (150) Currently there is no empirical evidence supporting a specific cellular ligand for these adhesive structures, making their importance in the colonization of the respiratory tract a matter of inference

In addition to the previously mentioned cell-associated virulence factors, *P. haemolytica* A1 also maintains a number of outer membrane proteins (OMP) that may play a supplementary role in terms of virulence (53, 106, 163) The most important of these membrane proteins is a 100 kDa bovine specific transferrin receptor (163, 171) It is a matter of fact that both the host and resident microbial flora require iron for normal cellular/ metabolic processes Therefore, as a mechanism of non-specific defense, the host produces substances that scavenge and chelate free iron, minimizing its availability to microbial invaders One such compound that executes this function is transferrin, which delivers its payload via receptor to host cells Bacteria have developed a number of different strategies to overcome the iron availability problem including siderophore production, molecular mimicry, or cytolysin synthesis Organisms that are capable of wresting iron from the host maintain a clear potential for increased virulence (219) *Pasteurella haemolytica* A1 exhibited the ability to acquire iron for growth from 30% saturated bovine transferrin in the presence of excess EDDA, but similar molecules from other species including pigs, humans, horses, and chickens could not serve as the iron source for growth under the same experimental conditions (163) Furthermore, it was asserted that the loss or drastic alteration of this transferrin receptor *in vivo* may reduce the ability of *P. haemolytica* to cause disease (163)

While *P. haemolytica* A1 possesses a number of cell-bound virulence factors, a vast amount of investigative effort has focused upon the soluble proteins that are known or suspected to play a role in the virulence of this organism. The first of these is neuraminidase, which is believed to exert its effect by cleaving fibronectin, facilitating the adherence of Gram-negative bacterium to respiratory epithelium (69, 209). Interestingly, neuraminidase activity was found to be 2.5 times greater in A1 isolates than A2 suggesting a possible relationship between enzyme activity and virulence (69). The second of these suspected virulence factors is a soluble neutral sialoglycoprotease that may be responsible for platelet activation and subsequent fibrin deposits that are observed in diseased cattle (160). Furthermore, this sialoglycoprotease has been implicated in IgG1-specific proteolysis in partially purified culture supernatant (ppCS) of *P. haemolytica* A1 (126). It was demonstrated that bovine IgG1 was hydrolysed into three distinct bands of 39, 12, 7 kDa following a six hour incubation in the ppCS with partial hydrolysis of IgG2 and no hydrolysis of IgA or IgM (126). Since IgG1 and IgG2 are major secretory immunoglobulins and suspected to play a major role in the defense against pneumonic pasteurellosis, proteases that target these effector molecules could directly abrogate an effective host response.

Without question, the most important and well-characterized virulence determinant of *P. haemolytica* A1 is a potent, chromosomally encoded leukotoxin (Lkt). This cytotoxin is exquisitely specific for ruminant leukocytes including alveolar macrophages, cultured blood monocytes, lymphocytes, and neutrophils (13, 17, 114, 191). While there is an obvious survival benefit to impairing phagocytic lung defenses

and the subsequent immune response necessary for bacterial clearance, the most striking result of leukotoxin activity is the exacerbated tissue destruction that is thought to be a consequence of leukocyte lysis (190). Acute lung injury that is consistent with pneumonic pasteurellosis is suspected to occur via cytoplasmic release of proteolytic enzymes such as elastase and collagenase and reactive oxygen intermediates from damaged leukocytes (196). Extracellular presence of these molecules may activate the alternative complement pathway or induce inflammation, resulting in extensive damage to lung parenchyma (196). Support for this assertion resides in the observation that calves depleted of neutrophils by prior administration of hydroxyurea were resistant to the effects of intratracheal challenge with live *P. haemolytica* A1, while normal calves developed severe exudative and necrotizing pneumonia (196, 197). Furthermore, mutant strains of *P. haemolytica* deficient only in the production of functional Lkt fail to establish clinical disease (73). The overall importance of this molecule in the disease process is punctuated by the fact that animals resistant to and recovering from the pneumonic pasteurellosis have detectable anti-leukotoxin antibody titers in lung secretions (66, 76).

Transcription of leukotoxin genes is tightly regulated and dependent upon many factors including pH, temperature, and iron availability (77). *In vitro* production of Lkt in the culture supernatant is appreciated during the logarithmic growth phase (13, 34, 191) with maximal concentrations achieved 6-8 hours following inoculation. Analysis of the genetic organization of this operon suggests that it is congruent with the typical RTX paradigm, exhibiting a polycistronic arrangement of C,A,B,D, with A corresponding to

the structural toxin unit (95, 130) Reports on the molecular weight of leukotoxin vary from >700 kDa to 150 kDa due to a tendency of this protein to aggregate (13, 96, 115, 155) Regardless of the disparate molecular weight reports for LktA, the gene encoding this protein has been cloned and sequenced, corresponding to a product of 953 amino acids and a molecular weight of 101.9 kDa (130, 132) While the core of the molecule is protein, carbohydrate moieties are presumed to play a role in its functionality, given that amylase treatment abolishes biological activity (34) Additional physiochemical toxin characteristics include oxygen stability and heat lability (34) Moreover, the toxin displays a short half-life at room temperature, exhibiting a complete decay of biological activity within two hours (13, 34)

Switching the objective to the molecular level, it is clear that calcium (Ca^{2+}) is absolutely essential for toxin function (38, 81, 191) Sequence analysis and homology studies of LktA indicate a domain in the carboxyl third of the protein exhibiting six repeating Leu-X-Gly-Gly-X-Gly-Asn-Asp-X arrays (204) These motifs function to bind extracellular calcium, which results in the conformational change that is necessary for target cell association (62, 134) Following this conformational shift, the toxin is poised to bind the $\beta 2$ integrin LFA-1 (CD18/ CD11a) cellular ligand, initiating leukocyte activation and cytolysis (111) Although the exact epitope involved in binding LFA-1 has yet to be completely resolved, it appears that a region within the carboxy terminal residues 840-939 is at least partially responsible for target cell association (123, 144)

When bound to the target cell, it is presumed that the amino terminal third of the protein is solely responsible for initiating cytolysis (52). Sequence analysis and

homology studies of related cytolysins indicate the presence of hydrophobic clusters within this region that are suspected to intimately associate with the target cell membrane (131). The result of this biological activity is the formation of small transmembrane pores of approximately 0.9-1.2 nm in diameter (37). Subsequent to this pore formation is the rapid efflux of potassium ions (K^+) and influx of calcium ions (Ca^{2+}), with the concomitant retention of high molecular weight biomolecules (37). The net effect of this deregulated ion traffic is a deranged colloid pressure that causes the cells to swell to twice their original volume, lose cytoplasmic organization and nuclear integrity and eventually rupture (13, 17). Furthermore, the rapid influx of Ca^{2+} is associated with activation in neutrophils, indicated by increased neutrophilic degranulation, superoxide generation, and leukotriene synthesis (93, 165). Moreover, reports indicate that low concentrations of *P. haemolytica* A1 toxin are capable of inducing apoptosis in bovine lymphocytes (207, 208). It is suggested that killing immune and phagocytic cells by apoptosis may allow bacteria that produce these toxins to escape host immune surveillance, prolonging their survival and allowing for colonization (35, 235).

Management Practices for Controlling Pneumonic Pasteurellosis

The severity of economic impact resulting from pneumonic pasteurellosis has compelled the beef industry to seek out viable strategies for mitigating this disease. The most obvious of these solutions is to prohibit overcrowding and intermingling of calves at the feedlot, thereby reducing the incidence of horizontal transmission. In theory, this would alleviate the risk of an epidemic initiated by one herd or a select group of

individuals. However, in practice this concept is economically unsound and would require a profound conversion of the current system. Another somewhat controversial management approach involves the wholesale administration of antimicrobial drugs at the feedlot. While this measure as a reactive approach has its merit, prophylactic administration not only raises the issue of facilitating bacterial resistance (33), but is costly and generally met with public disapproval. Aside from a comprehensive systemic change in the management of cattle, an economically acceptable alternative to controlling pneumonic pasteurellosis may be the development of a prophylactic vaccine against the infectious agents involved (154). The fact that a potent humoral immune response against these critical bacterial epitopes was noted in subjects recovering from the disease (66, 76, 78) gave promise of developing an effective vaccine. However, despite some very creative efforts, a viable approach to vaccination has eluded scientific and veterinary personnel for years. These strategies have included viral vaccines (75, 108, 110, 139, 140, 154, 201, 202), live bacterial vaccines (43, 71, 76, 121, 149, 156, 167), bacterins (43, 71, 76, 156), subunit vaccines (152, 189, 231), and DNA based approaches (125). While some of these preparations have shown promise in the laboratory, field trials have been disappointing, and at times inconclusive.

Because a large percentage of bovine bacterial pneumonias are suspected to be secondary to viral infection, attempts have been made to prevent shipping fever with viral vaccines (154). A number of experimental and commercial viral vaccines have been developed including such agents as PI-3, BHV-1, BRSV, and BVDV (75, 108, 110, 139, 140, 154, 201, 202). Although the justification for targeting viral agents implicated in the

genesis of pneumonic pasteurellosis appeared sound, the validity of this assumption was questionable following experimental and field trials of these vaccines (154). For instance, following vaccination with a preparation of PI-3 experimental subjects enjoyed no statistical difference in terms of protection when compared to unvaccinated controls (224-226). Furthermore, PI-3 vaccinated animals may be predisposed to disease (154). Alternatively, vaccination with BHV-1 preparations has yielded mixed results upon experimental challenge (109, 201, 202). While vaccination reduced disease following experimental challenge with aerosols of BHV-1 and *P. haemolytica*, protection was not complete, and was less evident in cattle that had been stressed by transport (201). At best, only a handful of viral preparations have shown limited success in field use under the conditions that commonly cause shipping fever (101, 203).

In light of the failure to mitigate disease using viral preparations, researchers set their sights on the principle agent of disease, *P. haemolytica* A1 as the source of an efficacious vaccine. Initial attempts to control shipping fever in this manner revolved around the use of bacterins. While this approach has the capacity to generate an immune response, some very discouraging observations were appreciated following administration and subsequent challenge with whole cell (WC) cultures (154). Most notably, formalin killed *P. haemolytica* bacterins administered by subcutaneous and aerosol routes resulted in more severe lesions in vaccinates than in controls following secondary challenge (71). It is hypothesized that the bacterins elicit a humoral response to somatic bacterial antigens, allowing for increased opsonization and phagocytosis of the live organism (162, 189). This facilitated association between phagocyte and bacteria

would enhance the kinetics of the toxin encountering its target, resulting in increased severity of disease. Another critical shortcoming of bacterin preparations is that they do not stimulate a humoral response to leukotoxin, given that they are metabolically inert and incapable of producing toxin. Since resistance to pneumonic pasteurellosis has been correlated with high antibody titers to this determinant (66, 76), its absence may impact the protective efficacy of the vaccine. Overall, bacterin preparations alone have consistently fallen short of inducing an effective prophylactic immune response (94, 154).

In contrast to bacterin preparations, live or live-attenuated *P. haemolytica* (WC) cultures have the capacity to elicit a protective response to the entire spectrum of immunogenic virulence determinants. Indeed these types of vaccines have shown merit in experimentally induced disease in an age, dose, and route dependent manner (42, 44, 108, 121). A study designed to assess the protective response as a function of culture age utilized vaccines prepared from either 6 or 22 hour cultures of *P. haemolytica*. Although enhanced resistance to experimental challenge infection was observed in both preparations, the six hour vaccine was associated with a decrease in lesion severity (42). In a investigation examining the dose response of *P. haemolytica* vaccines, it was found that 1×10^8 CFU/ml given subcutaneously significantly enhanced resistance to *P. haemolytica* challenge infection compared to the controls or calves vaccinated with 1×10^6 CFU/ml (44). In relation to immunization route, it was found that vaccination with live *P. haemolytica* administered by either intratracheal or subcutaneous routes caused increased resistance compared with the controls on the basis of the lung lesion severity

induced by experimental challenge (43, 159, 167) In contrast to these results, aerosol vaccination with live *P haemolytica* followed by a PI-3/ *P haemolytica* aerosol challenge infection did not provide enhanced protection compared with unvaccinated controls (108) Other notable vaccination strategies include the use of chemically altered or streptomycin dependent strains of *P haemolytica* Administration of these preparations with subsequent experimental challenge suggested a statistically significant level of protection in comparison to unvaccinated controls (31, 121) While it is clear that live or live attenuated vaccines elicit a better overall protective response than viral or bacterin preparations in experimentally induced disease, field trials of these preparations have failed to exhibit reliability (192). Since live vaccines are dependent on growth for antigen production, uncontrollable variables in the field such as concurrent administration of prophylactic or therapeutic antibiotics and improper handling or administration of the vaccines may retard their effectiveness (189).

In order to circumvent the limitations of live vaccines, much of the current research has focused on developing multivalent extract vaccines derived from *P haemolytica* culture supernatant (83, 152, 171, 189, 192, 231) These sub-cellular extracts harvested from log-phase cultures may include one or more of the following virulence factors leukotoxin, CPS, LPS, transferrin receptors TbpA/TbpB, and OMP involved in adhesion. While the clarity of the precise antigen or antigens responsible for maximal protection remains to be resolved, a degree of prophylaxis that is commensurate with live vaccines has been observed with combinations of LktA/ CPS (189, 192) and TbpA + TbpB (171) While LktA and CPS appear to be ideal candidates for inclusion in

a vaccine preparation, administration of recombinant leukotoxin alone was of little protective value (48). However, exogenous addition of recombinant LktA to culture filtrates offered a greater degree of protection than filtrate alone (189). Additionally, a novel approach utilizing an extract containing only *P. haemolytica* OMP/ carbohydrate-protein subunits was effective in generating partial protection upon experimental challenge (152). The sum of these experiments suggest that a threshold of key antigens must be present in order to elicit a protective immune response (48, 145, 154). In contrast to these results, Gilmour *et al* offers contradictory evidence, suggesting that immunization with a sodium salicylate extract of *P. haemolytica* failed to protect calves against experimental challenge (83). Interestingly, as seen in the case of live vaccines, the immunization route appears to play a role in the efficacy of protection, with intramuscular and subcutaneous delivery resulting in a reduced severity of disease in comparison to aerosol administration (154). In general, the use of specific antigen extracts has yielded promising results, obviating the intrinsic problems of live vaccines. However, the inability to identify the ideal antigenic formulation has limited experimental trials to a calculated protective efficacy of approximately 65% (189). It is generally agreed that the potential value of these preparations will grow as the antigens which provide protection against disease become more adequately characterized (154).

It is clear that the inconsistent and sometimes contradictory evidence derived from the various *P. haemolytica* vaccine field trials and experimental models may be attributed to the innumerable factors that can vary among individual trials. These factors can include challenge with viral pre-infection, challenge without viral infection,

challenge dose, route, mode of application, and observation times post-challenge. Other variables include the use and type of adjuvant, times of booster injections, sample sizes, and environmental factors. Given the extensive parameter variability of individual studies, it is not surprising that direct comparisons between independent investigations are difficult to evaluate. Fortunately, a statistical tool called meta-analysis has been developed to account for variability inherent to the numerous vaccine trials and summarize the accumulated knowledge from prior investigations. Each trial was categorized into 1 of 12 types based upon vaccine preparation/ content and following statistical normalization for individual variables, a mean protective index for each vaccine type was developed (200). A summary of this meta-analysis suggests that cattle vaccinated with live vaccines, culture supernatant, or potassium thiocyanate OMP extracts had the smallest lung-lesion scores which differed significantly from the controls (200). In contrast, bacterin preparations and streptomycin dependent live vaccines exhibited a meta-analysis protective index that was significantly weaker than the previously mentioned vaccine derivatives (200).

Despite the body of work aimed at preventing pneumonic pasteurellosis, prevalence of this disease in the feedlots of the world remains high. Subsequently, the economic burden associated with delays in market, treatment costs, and animal death attributed to shipping fever is still considered to be unsatisfactory. While it is clear that partial protection can be achieved through vaccination, an acceptable prophylactic strategy remains on the horizon.

Antibodies and Hybridoma Panel

In the context of infection, humoral immunity plays a critical role in neutralization and clearance of pathogens (1). The effectiveness of this aspect of acquired immunity is due primarily to antibodies, which execute different effector functions upon binding to a specific foreign antigen. The consequences of these functions may vary depending upon the structure, anatomic location, and isotype of the antibody (1). Although there are a number of antibody isotypes, the principle mediators of the humoral response to pneumonic pasteurellosis appear to be IgG and IgA (149). Whereas IgA is the major effector in the mucosal immune response and primarily involved in neutralization and antibody-dependent cell-mediated cytotoxicity (ADCC), IgG is the predominate class of soluble antibody executing such functions as neutralization, opsonization, ADCC, and complement activation (1).

In the context of this study, a brief discussion of IgG structure and production is necessary in order to appreciate the features of these molecules and the modifications attempted in their derivatives. Secreted IgG is a “Y” shaped heterodimeric protein of approximately 150 kDa consisting of two heavy and two light chains (1, 184). The heavy (H) chains are comprised of 450 to 575 amino acid residues while the light (L) chains consist of 220 amino acid residues (184). Both H and L chains can be divided into the variable (V) and constant (C) regions, each exhibiting discrete domains of approximately 100-120 residues (184). Although the amino terminal domains of both heavy and light chains exhibit extensive variability from antibody to antibody (184), moderate conservation can be appreciated in the four framework domains of the H and L variable

regions, allowing for classification of antibody families (113). The most significant aspect of the variable domain is the presence of three hypervariable complementarity determining regions (CDR) on the H and L chains which associate to form the exquisitely specific antigen binding pocket (1, 184). In addition to the variable domains, both H and L chains maintain constant regions that exhibit the same amino acid sequence for a given antibody chain of the same isotype except for single amino acid differences at a few positions (113, 184). Additional structural heterogeneity is supplied by the presence of diversity (D) and joining (J) segments within the heavy chain and a single J segment within the light chain (1). The segregation of the variable and constant domains confers a degree of bifunctionality to the molecule. While the V domain is solely responsible for the recognition and binding of antigens, the C domain of the heavy chain (Fc) mediates binding of the immunoglobulin to various cells of the immune system, some phagocytic cells, and components of the classical complement system (1, 184).

It is estimated that the antibody repertoire existing within unimmunized individuals is greater than 10^9 B-cell clones, each expressing individual specificity (1). This remarkably large pool of antibody specificity is supplied by somatic recombination of germline antibody DNA (186, 210) and RNA transcript splicing. Expression of these rearranged antibody genes is facilitated by specific transcriptional enhancer elements found between the J and C regions of the germline DNA (14, 147). It is hypothesized that VDJ recombination allows promoters upstream of the V region to come into close proximity with these enhancer elements, allowing for regulated tissue specific transcription of antibody genes (87, 147). Following transcription and splicing, the

mature mRNA is translated into a nascent polypeptide on the rough endoplasmic reticulum (RER), folded by chaperones (82) and delivered to the Golgi apparatus (116, 182) for constitutive secretion. After entry into the *cis*-Golgi, nascent proteins are subjected to removal of the leader peptide by a signal peptidase, glycosylated, and in the case of secreted Ig, packaged in vesicles for exocytosis.

A variety of substances can stimulate the process of antibody production, including proteins, polysaccharides, nucleic acids, and more complex particles such as pollens, viruses, bacteria, and tissue cells (184). Once stimulated, a particular clone will produce an antibody of defined specificity for the duration of its existence. Unfortunately, while these individual clones are effective at mounting an immune response to antigen challenge *in vivo*, they are exceptionally difficult to characterize in culture without immortalization. Recognizing the practical applications of specific antibodies in research and health-care, a method was developed in 1975 to generate immortalized cell lines that secrete a single species of antibody (monoclonal antibody) with the desired antigen specificity (118). The technique, known as "hybridoma technology" involved cell fusion between a normal antibody producing B-cell and a myeloma line followed by screening and selection of fused cells secreting specific antibody (1).

Our laboratory has generated a panel of murine-derived hybridomas secreting leukotoxin neutralizing monoclonal antibodies (mAbs) (79, 80). The degree to which these mAbs (ltx-2, ltx-4, and ltx-35) neutralize the leukotoxin is variable, however all recognize potentially protective epitopes within the leukotoxin of *P. haemolytica* A1 (79,

144) Of this panel, ltx-2 displays the greatest efficacy, requiring only 30 ng/ml of purified antibody to totally neutralize one unit (U) of leukotoxin (144). Furthermore, the epitope recognized by mAb ltx-2 appears to be conserved within the leukotoxins secreted by all *P. haemolytica* strains, with the exception of serotype A2 and biotype T strains (80). This protective epitope has been mapped to the carboxy third of the leukotoxin molecule between amino acids 768-939 (144), an observation that was corroborated by another independent panel of neutralizing mAbs (123). Additionally, neutralization appears to be independent of any effects mediated by the Fc portion of the antibody, suggesting a direct inhibition of binding or conformational change that is necessary for toxin activity (173).

The importance of leukotoxin in the disease process and the observation that significant anti-leukotoxin antibody titers are observed in cattle recovering from disease is well documented (66, 76, 78). This observation coupled with the inadequacy of current vaccination protocols has supplied the impetus for investigating a different approach to vaccine development based upon current protein engineering and recombinant DNA technologies.

Protein Engineering and scFv Technology

Recently, a new approach has been developed to bypass the laborious system of hybridoma generation, allowing for "immortalization" of rearranged immunoglobulin genes in bacteria (143, 164, 222). This technique relies on the ability to amplify variable domains from the heavy (V_H) and light (V_L) chains using the polymerase chain reaction

(PCR) Once amplified, these variable domains are linked by a synthetic oligonucleotide to form an open reading frame (ORF) corresponding to a single polypeptide or single chain variable fragment (scFv) (103) The synthetic linker is comprised of a repeating flexible, hydrophilic motif (Gly₄Ser)₃ that augments the stability of the molecule by facilitating close proximity of the V_H and V_L regions (103) Subsequently, this synthetic gene can be cloned into a commercially available phagemid vector (pCANTAB 5E) allowing for the expression of the antibody variable domain as a bacteriophage fusion protein Since the displayed single chain antibody fragment retains antigen binding capability, it is possible to enrich for recombinant phage expressing specific antibodies by affinity selection with the target antigen (143, 222) Following this enrichment procedure, soluble scFv molecules can be expressed, harvested, and partially purified from the periplasm of transfected *E coli* HB2151 cells

The benefits of this application in contrast to other reservoirs of monoclonal antibody production include an enhanced ease of cloning and screening from hybridoma or spleen cells, augmented genetic stability, reduction of experimental animal usage, economy, and ease of genetic manipulation (222) While this approach has obvious advantages in terms of immunological reagent development, affinity maturation studies, and diagnostic applications, it is hamstrung to some degree as a therapeutic/ clinical modality by virtue of the bacterial expression system. For clinical applications, a considerable amount of protein is needed, and the current approach of periplasmic export in *E coli* is unsatisfactory since the amount of exported protein is limited (10), and never exceeds 10% of the total soluble proteins (30) Conversely, cytoplasmic presence of scFv

is much higher, yet yields insoluble and inactive material forming aggregates and inclusion bodies (141) Current efforts have focused upon enhancing the stability of scFv molecules in this reducing environment, attempting to improve protein yields by manipulating cysteine residues (85) and other framework residues involved in the thermodynamic stability of the molecule (72, 84, 141) While these protein engineering approaches show promise, they are laborious and may partially or fully destroy the affinity for the antigen (184) An additional shortcoming of this system resides in the fact that glycosylation of the scFv cannot be executed in bacteria, obviating the functionality of proteins requiring this modification (184)

Although the current system of bacterial scFv expression is limited in terms of clinical applications, several characteristics of the scFv molecule make it an attractive candidate for therapeutic use. First, the scFv exhibits a reduced immunogenicity in comparison to native antibody by excluding the presence of the highly antigenic constant domains (124, 138, 184) Exclusion of these domains minimizes the probability of a xenoreactive immune response to the therapeutic molecules in addition to avoiding the difficult process of constant (C) domain reshaping/ grafting. Second, the scFv diffuses rapidly into the extravascular space, has increased volume distribution, and is thought to have improved renal elimination characteristics (39, 184) It is the latter characteristic that is most attractive in the context of cancer therapy, where scFv have been conjugated to toxic effector molecules (220) While rapid clearance is considered to be advantageous in some cases, in others it clearly is not A study examining the benefit of passive immunization through administration of coronavirus specific scFv revealed that

its half-life in the bloodstream was 55-fold shorter than Fab fragments, resulting in incomplete protection (124)

It is clear that antibodies and their derivatives are important in the clinical setting given the fact that they comprise over 30% of the proteins currently undergoing clinical trials (102). While bacterial expression of scFv can exhibit some clinical utility (124, 220), the therapeutic potential of these molecules is greatly enhanced by eukaryotic expression systems (178). By simply incorporating a modicum of regulatory DNA sequence information and an appropriate leader peptide, the successful ectopic expression of antibodies and their derivatives in mammalian cells was realized (55, 58, 178, 218). As a result of these advances, new vistas of antibody gene therapy and intracellular immunization have materialized.

Currently, some of the most fascinating research employing scFv technology is centered on the use of these molecules as "intrabodies" within the cell. Several groups have succeeded in targeting these molecules to different compartments of the cell such as the ER, Golgi, or nucleus by the addition of specific retention signals at the carboxy terminus (168, 234). These scFvs markedly inhibited the function of the intracellular proteins to which they were directed (234). A short list of these intracellular targets includes p21^{ras} (20), α subunit of the IL-2 receptor (177), and ErbB-2 (15, 56). However, the most extensive use of intracellular antibodies has been in the context of abrogating HIV-1 replication (59, 135, 136, 180, 187, 234). The net result of these studies demonstrates the clinical promise of intracellular immunization as a method to control viral replication and deranged cell cycle function.

In addition to the intracellular immunization approach, scFv chimeric genes have been utilized to effectively target tumors. Wels *et al* described the generation of transgenic cytotoxic T-cells expressing an ErbB-2R specific scFv fused with a short hinge region and the ζ -chain of the T-cell receptor (TCR) complex (220). Subsequent experiments demonstrated that target cells expressing the ErbB-2R gene were lysed with high specificity *in vitro* by the transgenic CTL. Additionally, the growth of ErbB-2R transformed cells in athymic mice was significantly retarded by adoptively transferred transgenic CTL's (220). These collective results enhanced the scope of scFv gene therapy by suggesting that selective tumor cell lysis can occur via a contrived MHC independent recognition molecule.

Aside from the remarkable therapeutic applications of mammalian scFv expression, this approach has produced a practical solution to the issue of insufficient yields and purification inherent to the bacterial system. Whereas high titers of scFv could be obtained from massive *E. coli* fermentations, the yields of soluble and active scFv from small scale cultures are mostly well below 1 mg/liter, with some scFv only recovered in trace amounts (178). In contrast, the transient transfection of a COS cell line with an scFv-Ig κ (C) domain construct was reported to generate 1 to 2 mg/liter of secreted protein in the culture supernatant (178). However, this production appears to be dependent upon the amino acid sequence of the V regions and glycosylation level of the scFv (112). Furthermore, the Ig κ (C) domain allows for the reliable purification of these molecules by affinity chromatography using anti-mouse Ig κ (C) sera. Most importantly,

the fusion protein had no appreciable difference in antigen affinity when compared to the *E. coli* derived scFv molecule (178)

The result of the literature survey suggests the power and potential of scFv molecules. While the bacterial expression system has merit in terms of developing immunological reagents and diagnostic applications, some limitations prevent its widespread use in the clinical/ therapeutic trials. In contrast, mammalian expression of these molecules has been able to overcome the intrinsic deficiencies of the bacterial system, thereby harnessing the full potential of the scFv as a conventional therapeutic modality.

Statement of Purpose

Using recombinant DNA techniques, I propose to generate a 2NscFv fusion protein that incorporates an amino terminal consensus murine IgG1 signal peptide and a murine Igk constant domain graft on the carboxy terminus. The former is a prerequisite for eukaryotic cellular trafficking and export, while the latter will serve as an immunohistochemical "tag" for purification and detection/localization of the protein within the cytosol or extracellular milieu following expression. Furthermore, previous reports (184) suggest that the addition of this entity confers stability to the scFv by enhancing its solubility, thus limiting aggregation. However, it has yet to be illuminated whether or not the fusion of this domain to the scFv inhibits the recognition and binding of the antigen. The second phase of this proposal involves the transient and stable transfection of lymphoid and non-lymphoid cell lines including SP2/0 and COS-7 to determine if protein

synthesis and export is possible. It is hypothesized that the 2NscFv construct, under the heterologous CMV promoter/enhancer element will result in the production and export of functional, detectable 2NscFv providing a reproducible protective function against *P. haemolytica* A1 Lkt that is commensurate with the native hybridoma derived antibody. If successful, this approach could provide the foundation for *in vivo* gene prophylaxis in the context of bovine pneumonic pasteurellosis.

CHAPTER 2

MATERIALS AND METHODS

Bacterium

Dr Robert D Walker (Michigan State University, East Lansing MI) generously donated *Pasteurella haemolytica* biotype A serotype 1 (# 853788) isolated from the lung of a pneumonic calf. The organism was passed twice in calves by intrapulmonic inoculation, re-isolated, and stored in skim milk at -80°C. *In vitro* propagation of the organism was achieved using BHI agar (GIBCO, Gaithersburg, MD) and 5% defibrinated sheep blood at 37°C (216).

Escherichia coli strain JM109 (Promega, Madison, WI) genotype (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* r_K^- m_K^+ , *relA1*, *supE44*, $\Delta lac-proAB$, F', *traD36*, *proAB*, *lacI^qZAM15*) (148) was used exclusively for transformation and propagation of plasmid DNA. Long-term cell stocks were prepared by growing cultures in LB broth (Fisher Scientific, Pittsburg, PA) to mid log phase (O D₅₉₅ of approximately 0.5). Cultures were pelleted by centrifugation (Beckman J-21 centrifuge, Beckman Instruments Inc, Palo Alto, CA) at 5000 x g for 10 minutes, 4°C. Cell pellets were resuspended in glycerol-based media (50 ml glycerol, 2 g tryptone, 1 g yeast extract, 1 g sodium chloride [Fisher Scientific, Pittsburg, PA], 150 ml deionized water) aliquoted and stored at -80°.

Escherichia coli strains TG1 and HB2151 (Amersham-Pharmacia Biotech, Piscataway, NJ) were used exclusively for the generation of phage displayed and soluble murine scFv constructs. TG1 cell line maintains the genotype (K12 $\Delta lac-pro$, *supE*, *thi*,

hsdΔ5/F', *traD36*, *proAB*, *lacI^q*, *lacZΔM15*) while HB2151 exhibits (*K12Δlac-pro*, *ara*, *nal^r*, *thi^r*/F', *lacI^q*, *lacZΔM15*) (193) Cell lines were received as lyophilized cultures and revived in 1 ml of double strength yeast extract/tryptone (2X-YT) medium (Fisher Scientific) overnight at 37°C Revived cultures were streaked for isolation on minimal medium plates (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 1 mM MgCl₂•6H₂O, 1mM CaCl₂•2H₂O, 1 mM thiamine hydrochloride, .001% glucose, 15 g granulated agar, pH 7.4) Long-term glycerol stocks were prepared by harvesting individual colonies from minimal medium plates and inoculating 5 ml of 2X-YT broth Following overnight incubation at 37°C, 200 μl of sterile 80% glycerol stock was added to 800 μl of stationary phase culture and stored at -80°C

Tissue Cultures

Leukotoxin neutralizing hybridoma clones ltx-2, ltx-4, and ltx-35 derived from murine splenocyte-SP2/0 myeloma fusion were developed and screened by Mark Cameron and Donald Gerbig of this laboratory (79, 80) Additionally, Dr Habib Zaghoulani (University of Tennessee, Knoxville) provided an SP2/0-Ag14 murine myeloma cell line (American Type Culture Collection # CRL-1581) used for DNA transfection studies. Cell lines were maintained in filter sterilized (Steritop™, Becton-Dickinson, Franklin Lakes, NJ) DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2000 U penicillin/ 20 mg streptomycin per liter of medium, 40 mM L-glutamine (PSG, Sigma), and 0.2%

sodium bicarbonate (Mallinckrodt, Paris, KY) Cultures were incubated at 37°C with a 5% CO₂ atmosphere and split at a 1:10 dilution every 2-4 days

A bovine leukemia derived B lymphocyte cell line BL-3 (American Type Culture Collection #CRL-8037) was used as the benchmark for leukotoxin neutralization assays. The cells were maintained in filter sterilized (Steritop™, Becton-Dickinson) RPMI-1640 medium (Sigma Chemical) supplemented with 10% FBS (Atlanta Biologicals), 2000 U penicillin/ 2 mg streptomycin per liter of medium, 40 mM L-glutamine (PSG, Sigma Chemical), and 0.2% sodium bicarbonate (Mallinckrodt). Cultures were incubated at 37°C with a 5% CO₂ atmosphere and split at a 1:10 dilution every 4-5 days.

An SV40 transformed african green monkey kidney cell line (COS-7, American Type Culture Collection # CRL-1651) was kindly provided by Dr Leon Potgieter (University of Tennessee, Knoxville). COS-7 cells were maintained in filter sterilized (Steritop™, Becton-Dickinson) DMEM (Sigma Chemical) supplemented with 10% FBS (Atlanta Biologicals), 2000 U penicillin/ 20 mg streptomycin per liter of medium, 40 mM L-glutamine (PSG, Sigma), and 0.2% sodium bicarbonate (Mallinckrodt). Cultures were incubated at 37°C with a 5% CO₂ atmosphere and split at a 1:10 dilution every 2-4 days. Briefly, the adherent cells were washed with sterile D-PBS (GIBCO) and treated with trypsin-EDTA (D-PBS, 0.5% trypsin, 0.2% EDTA•4Na, GIBCO) for 5 minutes at 37°C to dislodge the cells. Trypsin activity was arrested by addition of 5ml fresh medium containing 10% FBS. Cultures were passed by a 1:7 dilution into fresh medium.

Monoclonal Antibody Production

Leukotoxin neutralizing monoclonal antibodies (mAbs) were produced and collected from the ascitic fluid in mice. Four to six week old female BALB/c mice (Harlan-Sprague Dawley, Indianapolis, IN) were injected intraperitoneally (i.p) with 0.2 ml of 2,6,10,14-tetramethyldecanoic acid (Pristane, Sigma) on day 0 and day 14. On day 28, mice were administered an i.p injection of hybridoma cells at a concentration of 5×10^6 cells/ml in 0.5 ml PBS, pH 7.4. Within 7-10 days ascitic fluid developed. Subsequently, mice were anesthetized with methoxyflurane (Metaflane, Pitman-Moore, Inc., Mundelein, IL) and ascitic fluid was collected by peritoneal puncture using an 18-gauge needle. Harvested ascitic fluid was then mixed at a ratio of 1:5 to 1:10 with Seroclear[®] reagent (Calbiochem Corp., La Jolla, CA). The homogenate was centrifuged for 10 minutes at 3,000 rpm (International Instruments Co., Boston, MA). The top layer of lipid free ascitic fluid was removed, and the pH adjusted to 6.0-7.5 with wash buffer (pH 7.0, 0.01 M sodium phosphate, 0.15 M sodium chloride, Sigma Chemical).

For purification of IgG, the washed ascitic fluid was passed through a GammaBind[®] PLUS Prepack column (Genex Corp, Gaithersburg, MD). The column was continuously irrigated with wash buffer until the $O D_{280}$ fell below 0.075. The bound IgG was eluted from the column matrix with 0.05M ammonium acetate pH 3.0 (Mallinckrodt) and adjusted to pH 6.5-7.5 with 1M Tris base (Sigma Chemical). The IgG containing eluate was dialyzed against three changes of PBS, and the protein concentration was determined by BCA (Pierce, Rockford, IL) assay. Purified antibodies were aliquoted and stored at -80°C.

Antibody Isotype Determination

Direct ELISA was employed to determine the heavy and light chain isotype of each neutralizing monoclonal antibody in the panel (ltx-2, ltx-4, ltx-35). Frozen aliquots from each purified mAb were thawed and diluted to 20 µg/ml. 96-well PVC plates (Dynatech Laboratories, Chantilly, VA) were divided into discrete sections corresponding to mAb and probe type. 50µl aliquots of diluted mAb suspension were administered to the appropriate sections and the plates incubated overnight at room temperature in a humid chamber. Following incubation, the plates were washed three times with PBS-0.05% Tween-20 (Fisher Scientific) and vacant binding sites were blocked with 100 µl of PBS-3% NFDM (Carnation Co., Los Angeles, CA) for 90 minutes at 37°C in a humid chamber. Subsequently, plates were washed as before in preparation for the administration of HRP conjugated antibody probes (Zymed, San Francisco, CA). The entire panel was probed with the following reagents, polyclonal rabbit α-mouse IgG1, monoclonal rat α-mouse IgG1, monoclonal rat α-mouse IgG2a, polyclonal rabbit α-mouse IgG2b, monoclonal rat α-mouse IgG2b, polyclonal goat α-mouse Igκ, and polyclonal goat α-mouse Igλ. Preparation of the probes included diluting polyclonal reagents 1:2000 in PBS-0.05% Tween and monoclonal reagents 1:5000 in a similar manner. 50µl of probe solution was added to the appropriate sections and plates were incubated for 90 minutes at 37°C in a humid chamber. Following this incubation, plates were washed as previously described and developed by the addition of ABTS (Zymed). Absorbance was characterized at 405 nm using a microtiter plate reader.

mRNA Enrichment Procedure

Tissue cultures were assessed for viability by mixing 20 μ l of cell suspension with an equal volume of 4% trypan blue solution (GIBCO). Cell density and culture viability values are obtained by charging a hemocytometer chamber with 10 μ l of this mixture and resolving under a 10X objective. Cultures were deemed suitable for mRNA extraction if their viability (live/ live+dead) exceeded 90%. Satisfactory cultures were transferred to a sterile 50 ml Falcon[®] screw top tube (Becton-Dickinson) and centrifuged at 2,500 rpm (International Instruments Co) for 8 minutes. The pellet was thoroughly resuspended in D-PBS so that the cell density was approximately 5×10^6 cells/ml. 1ml of suspended cells was transferred to a 1.5 ml eppendorf tube (Fisher Scientific) and centrifuged (Heraeus Instruments, South Plainfield, NJ) at $2,000 \times g$ for 5 minutes. The wash supernatant was aspirated and the final cell pellet was subjected to mRNA extraction using reagents contained in the MicroPoly(A)Pure™ kit (Ambion, Austin, TX). To the pellet, 250 μ l of lysis solution was added and the tube was vortexed (Baxter Diagnostics, Deerfield, IL) vigorously for 1 minute to ensure cell disruption. After cell lysis, 500 μ l of dilution buffer was added to the homogenate and mixed by inversion for 10 seconds. Homogenate was immediately centrifuged (Eppendorf, Westbury, NY) at $12,000 \times g$ (4°C) for 15 minutes. The supernatant was aspirated and transferred to a sterile RNase free 1.5 ml tube (Fisher Scientific). 1 pre-packaged vial of oligo dT cellulose resin was thoroughly resuspended in the supernatant and placed in a mechanical rotator (LabIndustries, Inc , Berkeley, CA) for 1 hour. Following this incubation, the resin with bound mRNA was pelleted by centrifugation (Heraeus Instruments) at $3,000 \times g$ (RT) for

3 minutes The supernatant was removed from the resin pellet and 1 ml of binding buffer was added with gentle pipetting to resuspend the resin The resin was subsequently pelleted as described before and this step was repeated twice more After the third addition and removal of binding buffer, three similar steps incorporating a more stringent wash buffer were employed Following the final wash step, the resin pellet was resuspended in 400 μ l of wash buffer and transferred to a sterile spin column assembly provided with the kit. The column was centrifuged (Eppendorf) at 5,000 x g (RT) for 20 seconds The eluate was removed from the catch tube and the resin was irrigated and centrifuged two times with 500 μ l of wash buffer to remove additional rRNA After the final wash, the column containing the resin was placed in a sterile 2 ml catch tube and 100 μ l of pre-warmed (65°) elution buffer was added to the resin The column assembly was immediately centrifuged (Eppendorf) at 5,000 x g (RT) for 30 seconds Without discarding the eluate, an additional 100 μ l of warm elution buffer was added and spun as described previously To precipitate the RNA, 20 μ l of 5M NH_4OAc , 1 μ l of glycogen, and 500 μ l of 100% ethanol were added to the final eluate, mixed briefly by inversion, and snap frozen on dry ice for 1 hour Following precipitation, the RNA was pelleted by centrifugation (Eppendorf) at 14,000 x g (4°C) for 20 minutes The supernatant was carefully aspirated and the pellet was dried under vacuum (Savant Instruments, Holbrook, NY) The dessicated pellet was resuspended in 15 μ l of RNase-free dH_2O / 0.1M EDTA and used immediately or stored at -80°C

Enriched mRNA yield was assessed by absorbance at 260nm using the GeneQuant DNA calculator (Amersham-Pharmacia) Integrity of the RNA preparation

was assessed by electrophoresis using a pH 7.8- 1% native agarose (Sigma)/ TBE gel, 0.5 μ g/ ml EtBr (Mallinckrodt) at 80 volts for 60-70 minutes

cDNA Synthesis

Synthesis of cDNA from enriched mRNA was accomplished by using reagents contained in the RETROSCRIPT™ kit (Ambion). In individual sterile, RNase free 0.5 ml thin wall PCR tubes (Fisher Scientific), 3-4 μ l of enriched mRNA template was combined with 4 μ l dNTP mix (2.5 mM each), 2 μ l first strand primer (Oligo dT₁₈ [50 μ M] or transcript specific primer [10 μ M]), and nuclease free dH₂O to 16 μ l. Individual tubes were mixed briefly and heated in a thermocycler (Perkin Elmer Cetus, Norwalk, CT) at 80°C for 3 minutes (Program 34) to abolish secondary structure. Following this step, tubes were chilled on ice, and pulsed in the centrifuge (Heraeus Instruments) to collect condensate, then returned to ice. Subsequently, the heat sensitive components of the reaction were added including 2 μ l of 10X RT-PCR buffer, 1 μ l placental RNase inhibitor (10 U/ μ l), and 1 μ l Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT, 100 U/ μ l) to a total reaction volume of 20 μ l. Individual tubes were gently mixed, pulsed as described previously, and incubated at 42°C for 1 hour (Program 32) in the thermocycler (Perkin Elmer Cetus). Following this incubation, Program 32 was automatically linked to a denaturation step of 92°C for 10 minutes (Program 31) to inactivate the reverse transcriptase and RNase inhibitor. Completed cDNA synthesis reactions were labeled and stored at -20°C or directly subjected to standard PCR amplification.

An internal positive control employing *rig/S15* primers was used to assess the efficacy of the reaction constituents. These primers amplify a highly conserved region of a housekeeping gene (*rig/S15*) that is constitutively overexpressed in murine tumor cell lines (105), resulting in a product of 361 bp.

PCR Amplification of Heavy and Light Chain DNA

Given the sequence variability in the 5' region of the heavy and light chain transcripts, a murine Ig-PRIME™ kit (Novagen, Madison, WI) containing a battery of degenerate oligonucleotides targeting the leader sequence of each molecule was employed. This system allows the user to vary the forward primer while keeping the reverse primer constant, resulting in the amplification of a wide range of IgG and Igκ variable regions. A list of these primers is supplied in Table 2.1. Additionally, a number of synthetic oligonucleotide primers (Genosys, The Woodlands, TX) were designed to amplify the entire transcript using published sequences for the heavy (98, 161, 211, 227, 228) and light (6, 142) chain constant domain and 3' UTR. Upon arrival, all primer stocks were diluted to 10 pmol/μl in nuclease free dH₂O unless otherwise stated.

For standard PCR amplification, 5 μl from the completed cDNA synthesis reaction was transferred to a sterile 0.2 ml thin wall PCR tube (Eppendorf) and combined with 2 μl each of forward and reverse primer, 5 μl 10X PCR buffer, 2.5 μl MgCl₂ (50mM), 0.5 μl recombinant *Taq* DNA polymerase (GIBCO, 5 U/μl), and nuclease free dH₂O to 48 μl. When possible, a reaction cocktail was prepared to minimize pipetting.

Table 2.1: Primers used to amplify variable regions and full length heavy and light chain DNA. Primers from the Novagen Murine Ig-Prime™ kit begin with the prefix **MuIg**. Synthetic oligonucleotides derived from published immunoglobulin constant region sequences are indicated with an asterisk. Sequence degeneracy's are underlined in **bold** type

<u>Primer Name</u>	<u>Sequence (5'→3')</u>	<u>AA Position</u>
MuIgV _H 5'-A	gggaattcatgrasttskgytmarctkgrttt <i>EcoR I</i>	-20 to -13
MuIgV _H 5'-B	gggaattcaggraatgsasctgggt ~~ywt~~ ctctt <i>EcoR I</i>	-20 to -13
MuIgMV _H 3'-1	cccaagcttacgagggggaagacatttgggaa <i>Hind III</i>	125 to 118
MuIgκV _L 5'-A	gggaattcatgragwacakwycaggtcttt <i>EcoR I</i>	-20 to -13
MuIgκV _L 5'-B	gggaattcatggagacagacacactcctgctat <i>EcoR I</i>	-20 to -13
MuIgκV _L 3'-1	cccaagcttactggatggtgggaagatgga <i>Hind III</i>	122 to 116
G1 C-reverse*	tgtgaccctagtaaattggtcctc	UTR
3'KapC-UTR*	tagctcgagtgtggggaagcctccaag <i>Xho I</i>	UTR

steps A general cycle profile for these reactions is characterized by an initial “hot-start” at 80°C with subsequent addition of 2 µl dNTP mix (GIBCO, 10 mM each) followed by denaturation (94°C, 30 seconds), annealing (50°C, 30 seconds), and extension (72°C, 1 minute) steps Some variance in the extension time was required to accommodate the polymerization rate of the enzyme and the size of the amplified products The denaturation, annealing, and extension steps were repeated for a total of 30 cycles with a final extension of 72°C for 5 minutes PCR products were assessed by gel electrophoresis on a 0.9-1.5% agarose (Sigma Chemical) TAE, 0.5 µg/ml EtBr (Mallinckrodt) matrix at 100V for 60-70 minutes Successfully amplified products were directly subjected to ligation with the pGEM[®]-T Vector System (Promega)

pGEM[®] T-Vector System

In a sterile 0.5 ml thin wall PCR tube (Fisher Scientific), 3 µl of PCR product was mixed with 5 µl of 2X rapid ligation buffer (Promega), 1 µl of pGEM[®]-T Vector (50 ng/µl), and 1 µl of T4 DNA ligase (2 U/µl) Reactions were incubated at 4°C overnight for maximum efficiency.

When *Pfu* polymerase (Promega) was used to amplify templates, an additional “A-tailing” protocol was employed before ligation into the T-overhang vector *Pfu* derived products were excised from the gel and purified by GENECLAN[®] protocol (BIO 101, Vista, CA) and resuspended in 15 µl of nuclease free dH₂O Subsequently, a reaction mix was prepared containing 6.5 µl of purified *Pfu* product, 1 µl 10X PCR buffer, 0.5 µl MgCl₂ (50mM), 1 µl dATP (2mM), and 1 µl *Taq* DNA polymerase

(GIBCO) Reactions were incubated for 25 minutes at 70°C then subjected to ligation with pGEM[®]-T as previously mentioned

Competent Cells and Transformation

Competent *E. coli* JM109 cells were prepared by the CaCl₂ method (183) Selected colonies were grown in LB broth (Fisher Scientific) to an O.D₅₉₅ of 0.5, chilled for 15 minutes, and centrifuged (Beckman) at 3,000 x g (4°C) for 10 minutes The cell pellet was gently resuspended in 33 ml of ice-cold RF-1 solution (100mM RbCl, 50mM MnCl₂•4H₂O, 30 mM potassium acetate, 10 mM CaCl₂•2 H₂O, 15% glycerol) and incubated on ice for 20 minutes. Following incubation, the solution was centrifuged as previously described and the cell pellet was resuspended in 5 ml of ice cold RF-2 solution (10mM MOPS, 10 mM RbCl, 75 mM CaCl₂•2 H₂O, 15% glycerol) Cells were immediately flash-frozen in dry ice and stored for long-term use at -80°C

The heat shock method of transformation (183) was used to introduce plasmid DNA into competent *E. coli* JM109 cells Briefly, 10 µl of ligation mixture was added to 90 µl of thawed competent cells in a sterile 3 ml Falcon[®] tube (Becton-Dickinson) and incubated on ice for 20 minutes Following incubation, the DNA/ cell suspension was exposed to heat shock (42°C, 90 seconds) and briefly returned to ice 900µl of 2X-YT broth was added to the suspension and incubated at 37°C for 1 hour to facilitate expression of resistance genes After incubation, cells were plated on LB agar supplemented with 100µg/ml of the appropriate selective antibiotic and incubated overnight at 37°C. It is important to note that the pGEM[®] vectors were qualified for

Blue/White insert screening, requiring the addition of X-GAL (50µl of 40mM stock) to the plate medium

Screening for Clones Harboring Insert DNA

Cells transformed with pGEM[®] vectors were screened using the Blue/White approach, with white colonies generally testing positive for insert DNA. However some ambiguity can be experienced with T-overhang vectors and inserts <1 Kb that do not possess any internal stop codons. Therefore a secondary PCR based approach was employed to verify the presence of recombinant insert DNA before any downstream applications were attempted. Additionally, this PCR approach served as the primary verification strategy for vectors that were not Blue/ White qualified.

The strategy involved the selection of isolated colonies from the growth medium with a sterile toothpick. The biomass was dislodged onto the wall of a 0.2 ml PCR tube (Eppendorf) and reaction cocktail (3 µl 10X PCR buffer, 1.5 µl MgCl₂, 1 µl dNTP mix, 1 µl each of forward and reverse primer, 0.25 µl *Taq* DNA polymerase, and dH₂O to 30 µl) was added. Forward and reverse primers were employed which flanked the multiple cloning site (MCS) in each case (*T7*, *SP6* [A°=48] pGEM[®]-T vector) (*T7*, *BGH-rev* [A°=50] pcDNA3.1+ vector) (*pGEX5'seq*, *pGEX3'seq* [A°=55] pGEX 4T-1 vector). 30 cycles were performed with variances in both annealing temperature and extension time depending upon the primer set used and length of the expected product. Products were assessed by gel electrophoresis on a 0.8% agarose (Sigma Chemical) TAE, 0.5µg/ml EtBr (Mallinckrodt) matrix at 100V for 60-70 minutes. Positive clones were subcultured.

individually onto LB plates containing 100µg/ml of selective antibiotic and incubated at 37° overnight

Enrichment and Purification of Plasmid DNA

Depending upon the amount of purified plasmid DNA that was required for downstream applications, Wizard™ mini or maxi-prep DNA Purification Systems (Promega) were used for plasmid isolation and purification. Typically, applications such as sequencing, fusion protein construction, and enzymatic cleavage of DNA required only µg amounts, ideal for the miniprep. Conversely, transfection studies required mg quantities of DNA, necessitating the large-scale purification system. Although the purity achieved by these methods was satisfactory for most applications, a phenol-chloroform (Sigma) extraction was performed on the large-scale preps to enhance the success of the transfection experiments. The quantity and quality of the plasmid preparations was assessed by spectrophotometry (A_{260} and A_{260}/A_{280} ratio) and by electrophoretic resolution on a 1% agarose/ TAE matrix.

DNA Sequencing

The University of Tennessee, Knoxville Molecular Biology Resource Facility performed the DNA sequencing using an ABI (PE Applied Biosystems, Inc., Foster City, CA) model 373A automated DNA sequencer and the ABI prism dye terminator cycle sequencing kit (PE Applied Biosystems, Inc.). DNA sequences were examined,

compared, and manipulated using the LaserGene99 software package (DNASTAR Inc , Madison, WI) All sequencing primers are listed in Table 2 2

Enzymes

Restriction endonucleases and *Pfu* DNA polymerase were purchased from Promega. *Taq* DNA polymerase and T4 DNA ligase were purchased from GIBCO Other enzymes with specific application are implied to be components of commercially available kits

Plasmid Vectors

The pGEM[®]T-Vector (Figure 2 1, Promega) is a high copy number vehicle designed for the rapid cloning and screening of PCR products It contains the ampicillin resistance marker in addition to a Multiple Cloning Site (MCS) interrupted *lacZ* gene for efficient α -complementation in Blue/White qualified cell lines Flanking T7 and SP6 RNA polymerase sites facilitate PCR screening across the MCS and DNA sequencing

The pGEX-4T-1 vector (Figure 2.2, Amersham Pharmacia Biotech) is a convenient system for regulated expression of recombinant fusion proteins within a bacterial system The MCS immediately follows the glutathione-S-transferase (GST) gene of *Schistosoma japonicum* which is in turn regulated by the *lac* operator sequence Recombinant DNA that is inserted in frame within the MCS will be expressed as a fusion protein with GST Additionally, pGEX 4T-1 confers ampicillin resistance and can be screened for insert DNA by targeting regions upstream and downstream of the MCS.

Table 2.2 List of primers used for DNA sequencing Note that G1FR3-SEQ was designed to target the Framework III region of each heavy chain to verify the cloned sequence Furthermore, the *rig/S15* primer set was not used in sequencing applications, but served as a quality control for cDNA synthesis reactions

PRIMER	SEQUENCE (5'→3')
SP6	TATTTAGGTGACACTATAG
T7	TAATACGACTCACTATAGGG
BGH-REVERSE	GCAACTAGAAGGCACAGTCGAGG
G1FR3-SEQ	GAGGACTCTGCGGGTCTATTACTGTGC
pGEX5'-SEQ	GGGCTGGCAAGCCACGTTTGGTG
pGEX3'-SEQ	CCGGGAGCTGCATGTGTCAGAGG
<i>rig/S15</i> forward	TTCCGCAAGTTCACCTACC
<i>rig/S15</i> reverse	CGGGCCGGCCATGCTTTACG

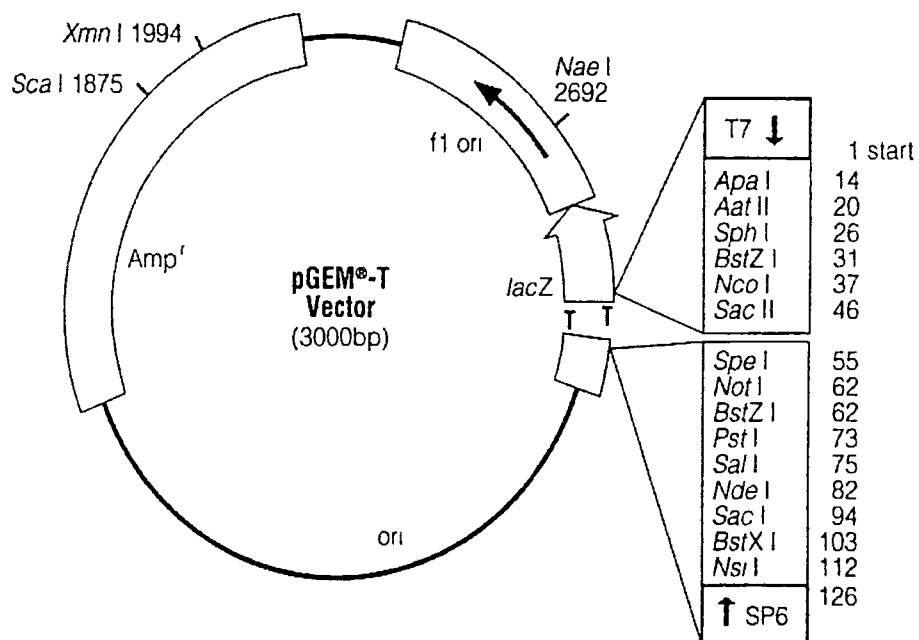


Figure 2.1: Diagram of pGEM®-T vector used for general cloning and DNA sequencing applications.

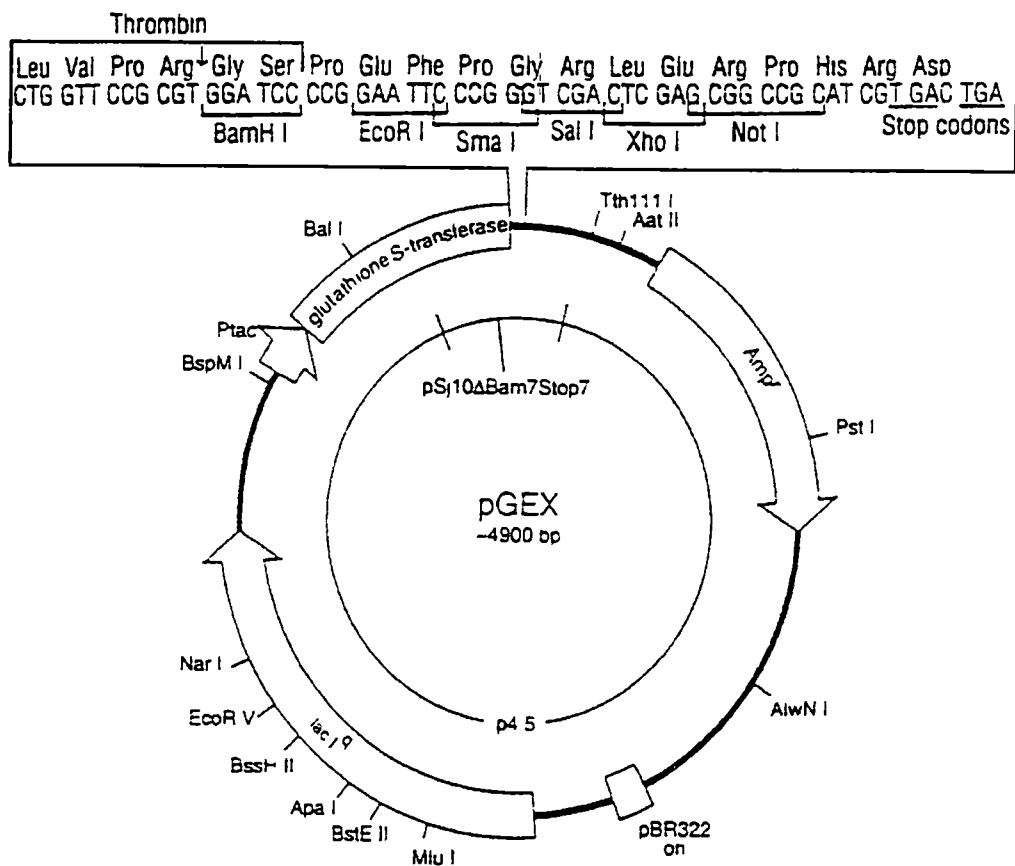


Figure 2.2: Diagram of pGEX 4T-1 vector used for bacterial expression of 2NscFXT, 2N Igk, and 2N IgG1 molecules as fusion proteins with GST

The mammalian expression plasmid pcDNA3.1(+) (Figure 2 3, Invitrogen, San Diego, CA) directs high level expression of recombinant genes using the cytomegalovirus (CMV) promoter. Furthermore, this vehicle employs two resistance markers, ampicillin and neomycin (G418) for positive selection in prokaryotic and eukaryotic systems

The phagemid pCANTAB 5E (Figure 2 4) supplied with the Murine Recombinant Phage Antibody Expression system (Amersham Pharmacia Biotech) is specifically designed to produce phage-displayed recombinant scFv antibodies through fusion with phage gene 3 protein. Expression in the *E. coli* TG-1 cell line with subsequent infection of M13KO7 Helper Phage results in phage display via suppression of the amber stop codon located between the scFv insert and phage gene 3. Phage displayed antibodies can be selected for functionality by “panning” against the antigen of interest. Subsequently, positive clones can be introduced into *E. coli* HB2151 for production of soluble antigen specific recombinant scFv. A brief diagram of this process is presented in Figure 2 5.

scFv Manipulation

A leukotoxin neutralizing scFv derived from ltx-2 was generated by Brian Ranger (173) using the Mouse scFv Module/ Recombinant Phage Antibody and Expression Systems (Amersham Pharmacia Biotech). With the neutralizing scFv DNA construct serving as the core element or “keystone”, a heavy chain signal peptide encoding DNA sequence was fused to the 5’ region and a Kappa constant domain encoding DNA sequence to the 3’ region by Overlap Extension (OE) PCR (99, 100). A succinct diagram of this process is provided in Figure 2.6

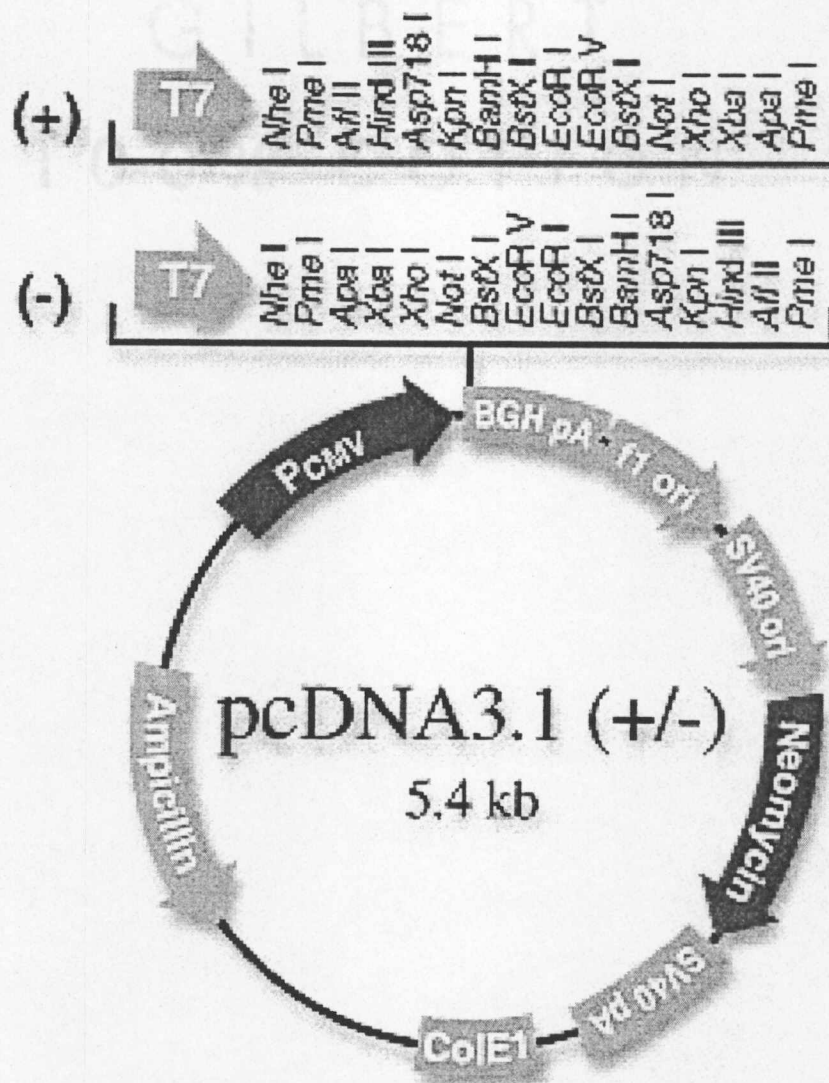


Figure 2.3: Diagram of the pcDNA3.1+ mammalian expression vector employed in the transfection of SP2/0 and COS-7 cell lines.

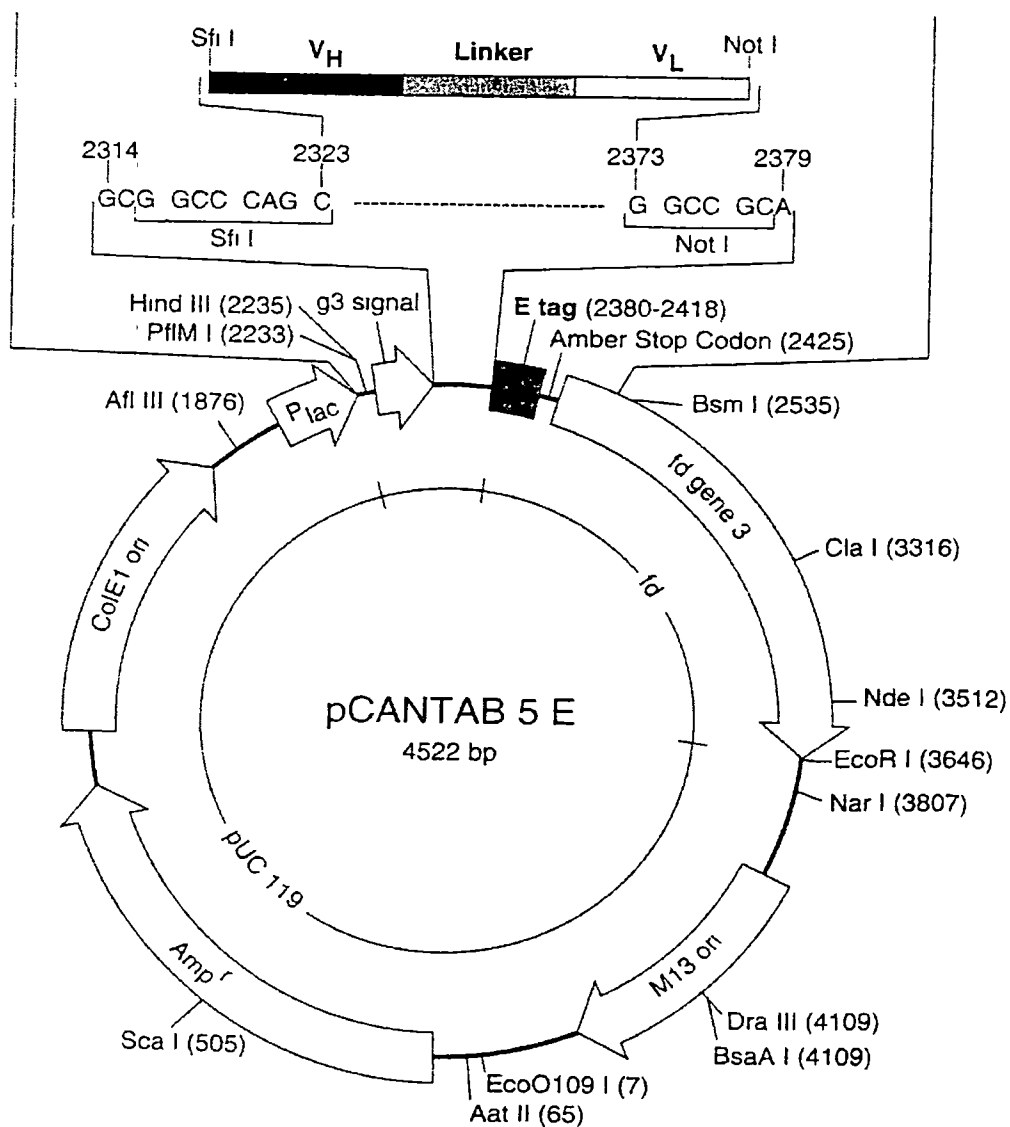


Figure 2.4: Diagram of the phagemid pCANTAB 5E used to generate soluble and phage-displayed recombinant murine scFv antibodies

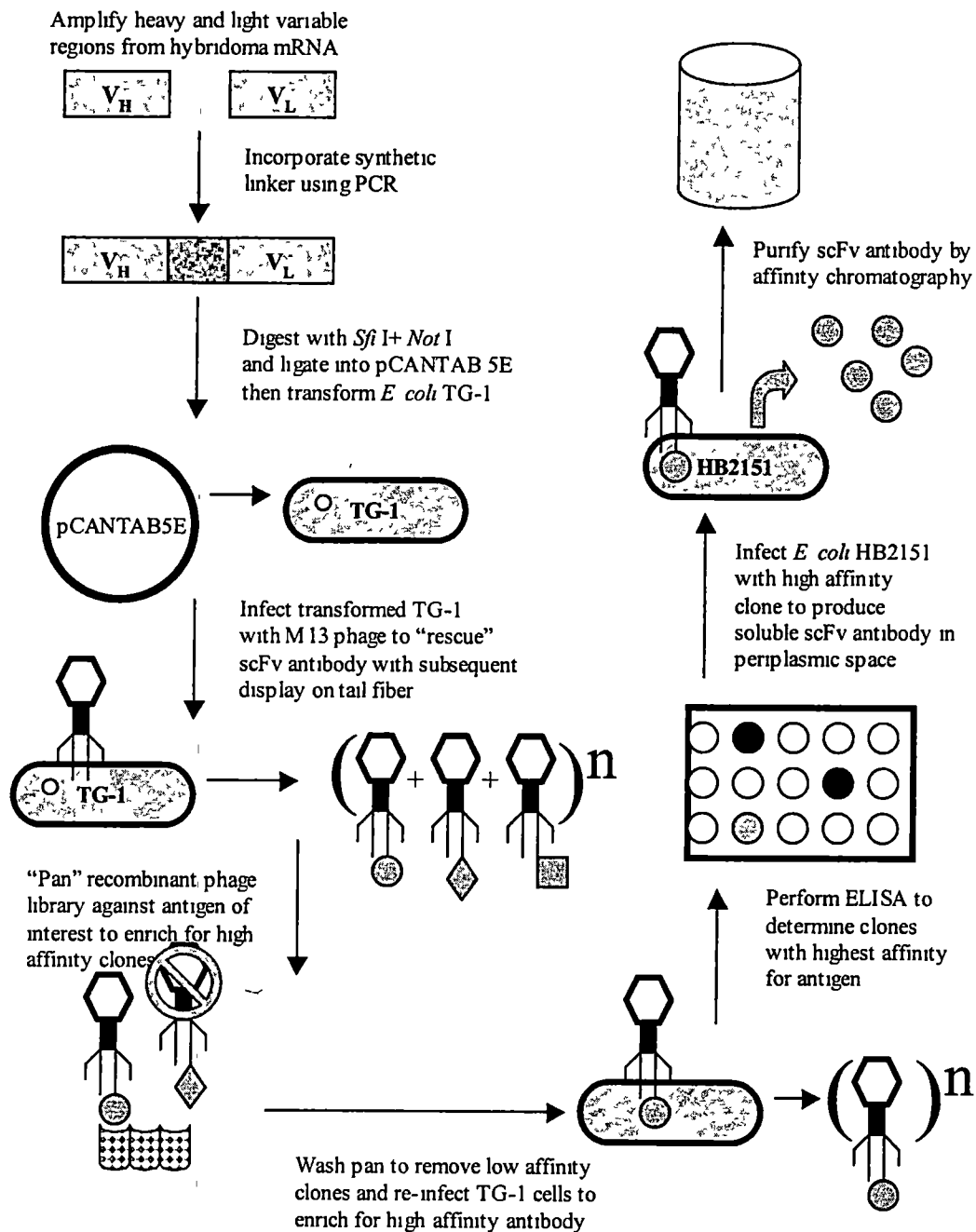


Figure 2.5: General scheme for generating, screening, and purifying recombinant scFv antibody.

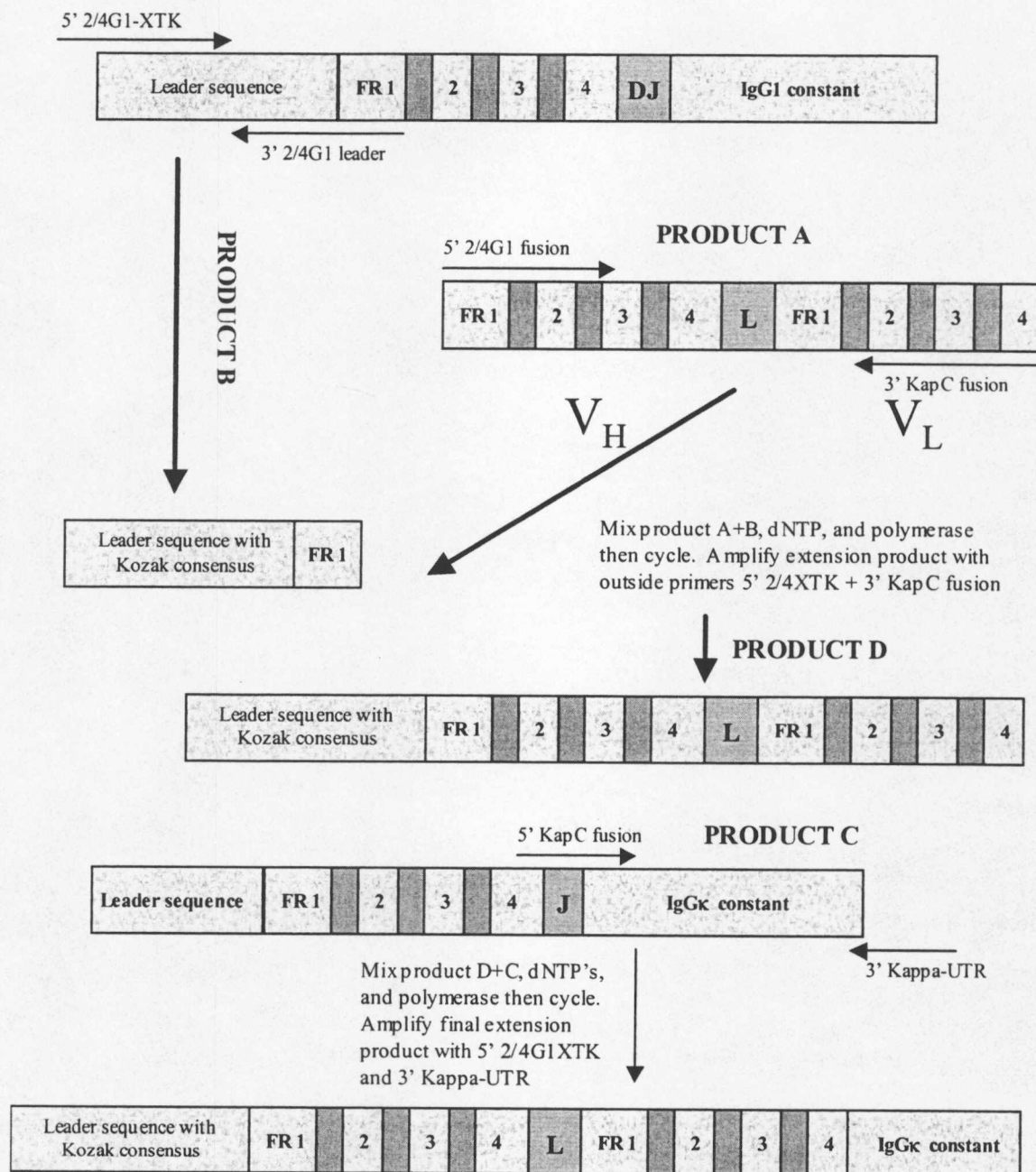


Figure 2.6: Diagram of the OE-PCR strategy used to generate the 2NscFXT fusion protein.

In a 0.2 ml PCR tube (Eppendorf) labeled tube A, 1 μ l of pCANTAB 5E/ 2NscFv was combined with 5 μ l 10X *Pfu* buffer, 2 μ l of forward primer (5' 2/4G1 fusion, Table 2.3), 2 μ l of reverse primer (3' KapC fusion), dH₂O to 47 μ l, and 1 μ l of *Pfu* polymerase (Promega 3U/ μ l). The reaction mix was incubated at 80°C and 2 μ l of dNTP's (GIBCO) were added with subsequent initiation of the amplification cycle. The cycle profile consisted of 94°C for 30 seconds denaturation step, 55°C for 30 seconds annealing step, and 72°C for 2 minutes extension step for a total of 30 cycles. Following amplification, the product was resolved on a 0.8% agarose TAE, 0.5 μ g/ml EtBr gel at 100V for 60 minutes. The product was excised from the gel, purified by the GENECLAN[®] protocol (BIO 101), and eluted in 15 μ l of dH₂O.

In a separate PCR tube labeled "B", 1 μ l of purified pGEM[®]-T/ ltx-4 IgG1 DNA was combined with 5 μ l 10X PCR buffer, 2.5 μ l MgCl₂ (50 mM), 2 μ l forward primer (5' 2/4G1-XTK), 2 μ l reverse primer (3' 2/4G1 leader), dH₂O to 47.5 μ l, and 0.5 μ l *Taq* polymerase. The reaction was incubated at 80°C and 2 μ l of dNTP's were added with subsequent initiation of the amplification cycle. The cycle profile consisted of a 30 second denaturation step at 94°C, a 30 second annealing step at 50°C, and a 30 second extension step at 72°C for a total of 30 cycles. Products were resolved on a 2% agarose/ 0.5X-TAE, 0.5 μ g/ml EtBr matrix at 100V for 60 minutes. Following resolution, the product band was excised from the gel and eluted into a sterile 1.5 ml eppendorf tube (Fisher Scientific) by centrifugation (3,500 x g (RT), 3 minutes) using a MicroSpin[™] column (Amersham Pharmacia Biotech).

Table 2.3: Synthetic oligonucleotide primers used to construct the 2NscFXT fusion protein. Restriction sites and degeneracy's are noted in **bold** type. Overlapping sequences required for OE-PCR are underlined.

<u>Primer name</u>	<u>Sequence (5'→3')</u>	<u>Base location</u>
5' 2/4G1-XTK	ggat ccgcccac <u>catg</u> gaa tgs acc tgg gty aty ctc ttc ctc c <i>Bam</i> HI met glu trp thr trp val ile leu phe leu	-12 to 31
3' 2/4G1-leader <i>sense strand code</i>	ggg gta gcc aga agc ctt gca gga yaa ctt cac aga gg <u>thr tyr gly ser ala lys cys ser leu lys</u> val ser	192-221
5'2/4G1 fusion	aag ttr tcc tgc aag gct tct ggc tac acc <u>lys leu ser cys lys ala ser gly tyr thr</u>	74-103
3' KapC fusion <i>sense strand code</i>	ccg ttt tat ttc cag ctt ggt ccc tcc acc gaa cg <u>arg lys ile glu leu lys thr gly gly gly phe</u>	699-733
5' KapC fusion	cg ttc ggt gga ggg acc aag ctg caa ata aaa cgg <u>phe gly gly gly thr lys leu glu ile lys arg</u>	352-387
3' KapC-UTR	tag ctcgag tgtggggaagcctccaag <i>Xho</i> I	786-812

An additional PCR tube labeled “C” was prepared containing 1 µl pGEM®-T/ ltx-2N Igk DNA, 5 µl 10X *Pfu* Buffer, 2 µl of forward primer (5' KapC fusion), 2 µl of reverse primer (3' Kappa-UTR), dH₂O to 47 µl, and 1 µl *Pfu* polymerase (Promega). The reaction was incubated at 80°C and 2 µl of dNTP's were added with subsequent initiation of the amplification cycle. The cycle profile was characterized by a 30 second denaturation step at 94°C, a 30 second annealing step at 55°C, and a 45 second extension step at 72°C for a total of 30 cycles. Following amplification, the product was resolved on a 0.8% agarose TAE, 0.5 µg/ml EtBr gel at 100V for 60 minutes. The product was excised from the gel, purified by the GENECLAN® protocol (BIO 101), and eluted in 15 µl of dH₂O.

To fuse the core scFv with the leader peptide, 5 µl of purified product “A” was mixed with 5 µl of product “B” in a sterile 0.2 ml PCR tube (Eppendorf). In addition to the templates, 2 µl of dNTP's, 2 µl of 10X *Pfu* buffer, dH₂O to 19 µl, and 1 µl of *Pfu* polymerase was added. The reaction was subjected to 10 cycles characterized by a 1 minute denaturation step at 94°C, a 1.5 minute annealing step at 60°C, and a 2.5 minute extension step at 72°C. This OE-PCR protocol allows complimentary regions within each product to overlap with free 3' OH ends accessible for primer extension. Following this step, 5 µl of OE product was placed in a sterile PCR tube labeled “D” and subjected to standard PCR amplification with the outside primers (5' 2/4G1-XTK and 3' KapC fusion) and *Pfu* polymerase (Promega). The amplified product was resolved on a 0.8%

agarose TAE gel, excised, then purified by the GENECLAN[®] method and eluted in 15 μ l of dH₂O

Finally, 5 μ l of product “D” was combined with 5 μ l of product “C” in addition to the previously mentioned OE-PCR reaction cocktail. Using the same cycle parameters as described before, the product was amplified then a 5 μ l aliquot was transferred to a standard 50 μ l PCR amplification mixture containing the primers 5’ 2/4G1-XTK and 3’ Kappa-UTR and *Pfu* polymerase. The final scFv derivative was resolved on a 0.8% agarose TAE gel, excised, then purified by the GENECLAN[®] method, and eluted in 15 μ l of dH₂O

The final scFv fusion (2NscFXT) was further subjected to a brief “A-tailing” procedure as previously described and ligated into pGEM[®]-T. Following ligation, the recombinant molecules were introduced into competent *E. coli* JM109 by the heat shock method and plated on LB agar containing 100 μ g/ml ampicillin (Sigma) and 50 μ l XGAL (40 mM stock, Sigma). Clones harboring insert DNA were assessed by both Blue/White screening and PCR amplification across the multiple cloning site using SP6 and T7 primers. Clones yielding amplified products of expected size were subjected to small-scale plasmid purification using the Wizard[™] miniprep (Promega) protocol and submitted for sequence analysis.

Plasmid Manipulation

In order to define the functionality of the Kappa constant domain “tag” fused with the scFv construct and conduct eukaryotic expression studies, inserts from pGEM[®] T

clones yielding satisfactory sequence data were liberated enzymatically and cloned into specialty vectors (pGEX 4T-1 or pcDNA3 1+) Briefly, 3 µg (5-10 µl) of purified scFv containing plasmid DNA or 1 µg (0.5-1 µl) expression vector were placed into separate 0.5 ml PCR tubes (Fisher Scientific). To the template DNA, 3 µl of 10X restriction buffer "B" (Promega), dH₂O to 29 µl, and 0.5 µl each of *Bam*H I and *Xho* I restriction enzyme (10 U/µl) were added. Reactions were incubated in the thermal cycler (Perkin Elmer Cetus) at 37°C for 90 minutes. Following incubation, the DNA bands were resolved on a 0.8% agarose TAE gel at 100V for 60 minutes. Bands corresponding to liberated insert and linearized target vector were excised from the gel and combined in a single GENECLAN™ DNA purification reaction. Purified vector and insert DNA was eluted from the silica matrix in 15 µl of nuclease free dH₂O. To the final eluate, 4 µl of 5X DNA ligase buffer and 1 µl of T4 DNA ligase (GIBCO) were added then incubated at 4°C overnight. Following incubation, the ligation mixtures were transformed into competent *E. coli* JM109 as previously outlined.

Expression of 2NscFXT/GST Fusion Proteins

Following transformation, colonies growing on LB agar spiked with 100 µg/ml ampicillin (Sigma) were briefly subjected to positive identification of insert presence using the MCS flanking primers (pGEX 5' seq / pGEX 3' seq.) and the PCR screening method previously described. Clones positive for growth and insert presence were enumerated in batch culture using 10 ml of Luria-Bertani (LB) broth at 100 µg/ml ampicillin (LB^{amp100}). Subsequently, 10 ml of prewarmed LB^{amp100} was inoculated with

100 μ l of overnight culture and incubated at 37°C in a shaking water bath. When the cells reached mid-log phase ($O.D_{595} = 0.5$) a 1 ml aliquot was extracted, pelleted by centrifugation, resuspended in 750 μ l of PBS, and lysed with 750 μ l of 2X sample buffer (2.5 ml 2M Tris-HCl pH 6.8, 2.0 ml glycerol, 2.0 ml 10% SDS, 0.25 mg bromophenol blue, 0.5 ml β -mercaptoethanol, and dH_2O to 10 ml). The remainder of the log-phase culture was induced for fusion protein expression by the addition of isopropyl- β -D-thiogalactoside (IPTG, Gold Biotechnology, Inc., St. Louis, MO) to a final concentration of 0.1 mM. The induced culture was allowed to incubate under the same conditions for an additional 75 minutes, then 1 ml was extracted and lysed as previously described.

2NscFXT Detection

To detect the presence of 2NscFXT in samples, Coomassie staining, Western immunoblot, and direct ELISA were employed. In regard to the Coomassie and Western protocols, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to separate the proteins according to the methods described by Laemmli (122). Stacking gels of 4% and separating gels of 10% (acrylamide/ bisacrylamide 30:0.8, National Diagnostics, Atlanta, GA) were used. Samples were electrophoresed in a Mini Protean II gel apparatus (BioRad) at 14mA through the stacking gel then at 28mA through the separating gel until the protein margin reached the bottom of the gel.

Separated proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a semi-dry protein transfer apparatus (Gelman Sciences, Ann Arbor, MI) at 176mA for 90 minutes. Following transfer, vacant

protein binding sites were blocked with 3% Nonfat Dry Milk (Carnation Co) in 30 ml of TBS with gentle agitation for 30 minutes at 37°C. Membranes were washed twice with TBS-0.05% Tween-20 (Sigma) then a 1:2000 dilution of polyclonal HRP goat anti murine IgG (Zymed) in 30 ml of TBS-Tween was added and incubated overnight at room temperature. After incubation, the membranes were washed twice for 5 minutes with TBS-Tween and developed with HRP Color Development Reagent (15mg, BioRad) suspended in 25 ml TBS, 5 ml methanol (Fisher Scientific), and 15 µl 30% H₂O₂ (Sigma).

ELISA detection of the 2NscFXT molecule followed the general protocol outlined in the isotype determination section. Briefly, 100 µl of sample was loaded into predefined wells and incubated as described previously. Plates were washed and probed with a 1:5000 dilution of polyclonal HRP goat anti murine IgG (Zymed) in PBS-Tween, then developed and read at O D₄₀₅ as outlined before.

Stable Transfection of SP2/0 Cells

In order to assess the efficacy of production and secretion within a eukaryotic cell line, the mammalian expression vector pcDNA3.1+ containing the 2NscFXT construct was introduced by electroporation into the SP2/0 cell line. Briefly, 60 µg of Wizard™ maxiprep/ phenol chloroform purified pcDNA3.1+/ 2NscFXT (25 µl) was linearized using 80 U of *Pvu* I enzyme (10U/µl) in a 100 µl reaction volume. The reaction was incubated at 37°C for 2.5 hours then 2 µl was subjected to qualitative analysis on a 0.8% agarose TAE gel. The remainder of the reaction was ethanol precipitated (10 µl 3M

sodium acetate, 250 μ l 100% ethanol), centrifuged at 14,000 (4°C) for 20 minutes to recover the pellet, and dried under vacuum in the SpeedVac (Savant Instruments) The final pellet was resuspended in 60 μ l of sterile D-PBS and stored at -20°C until use.

SP2/0 cells were grown in a total of 50 ml DMEM in two separate 75 cm² Falcon[®] Tissue Culture flasks (Becton-Dickinson) As previously described, health and cell density of the culture was assessed by Trypan Blue staining and subsequent microscopic analysis The culture was transferred to a sterile 50 ml Falcon[®] screw top tube (Becton-Dickinson) and pelleted at 1,000 rpm (RT) for 10 minutes The exhausted media was aspirated and the cells were gently resuspended in sterile, ice-cold D-PBS to a density of 1×10^7 cells/ml and placed on ice

Cells were prepared for electroporation by aliquoting 0.9 ml of cell suspension into a sterile 3 ml Falcon[®] snap-cap tube (Becton-Dickinson), then adding 10 μ l of linearized vector DNA while gently stirring the suspension with the pipette tip. The cell/DNA suspension was incubated on ice for 10 minutes prior to electroporation Following incubation, 0.8 ml of the suspension was transferred to a chilled 0.4 cm electroporation cuvette (BioRad), immediately capped and placed in the GenePulser[™] electroporation apparatus (BioRad) The cells were pulsed at 960 μ F and 220V (time constant of 14-16 msec) then immediately placed on ice for 10 minutes. Following recovery, the remaining intact cells were carefully aspirated from the cuvette and transferred to a sterile 15 ml Falcon[®] screw top tube (Becton-Dickinson) The cells were then thoroughly resuspended in ice-cold DMEM, 10% FBS, 2% PSG and distributed in 0.5 ml aliquots into 20 wells on a 24 well covered tissue culture plate (Corning/CoStar,

Cambridge, MA) and incubated at 37°C in a 6% CO₂ atmosphere. The remaining 4 wells were reserved for SP2/0 cells electroporated in the absence of vector and used to gauge the effectiveness of the resistance marker. In total, 5 plates were used for each individual construct to enhance the probability that the entire construct was fully incorporated and expressed within the cells.

24 hours following electroporation, 0.5 ml of selection medium (DMEM, 10% FBS, 2% PSG, 1.5 mg/ml G418) was aseptically added and the cells were incubated as previously described. 48 hours after transfection, the entire medium bathing the cells was aspirated and replaced with 1 ml of selection medium in each well. The plates were returned to the incubator and monitored daily for the appearance of foci. Within 5-8 days growth became apparent within individual wells and media was changed as needed. Focal growth was allowed to become nearly confluent, then cells were resuspended by gentle pipetting and aseptically transferred into 5 ml of fresh selection medium in a 25 cm² Falcon® Tissue Culture Flask (Becton-Dickinson) and incubated as previously described. Transfected cultures were split at 1:10 dilutions into fresh selection medium every 2-5 days.

Analysis of 2NscFXT Production in Transfected Cells

The supernatant from cells exhibiting growth in the selective medium was isolated by centrifugation at 2,500 rpm (RT) for 10 minutes. Briefly, 7 ml of cell free supernatant was concentrated into 1 ml using a stir-cell concentrator (Amicon) with a reconstituted cellulose 30 NMWL cutoff membrane (Millipore). The membrane was

washed briefly to dislodge any associated protein and the concentrate analyzed by Coomassie staining, Western immunoblot, and ELISA for extracellular presence of secreted 2NscFXT.

To assess the intracellular presence of 2NscFXT, cells growing in the selective medium were pelleted by centrifugation at 2,500 rpm (RT) for 10 minutes then resuspended to a density of 5×10^6 cells/ml in sterile ice-cold D-PBS. 1 ml of the suspension was then lysed by repetitive freeze/thaw cycles in dry ice. Following lysis, the suspension was centrifuged at $14,000 \times g$ (4°C) for 15 minutes, and the supernatant was filtered through an Acrodisc PF 0.8/0.2 μm Supor filter (Gelman Sciences).

The remaining cells suspended in D-PBS at 5×10^6 cells/ml were subjected to the mRNA extraction and cDNA synthesis procedures previously described in to ascertain the presence or absence of full-length polyadenylated transcripts corresponding to 2NscFXT. cDNA synthesis reactions were amplified by conventional PCR with the primers 5' 2/4G1 XTK and 3' KapC UTR and the products resolved on a 0.8% agarose TAE matrix.

In Vitro Transcription of 2NscFXT mRNA

Cultures exhibiting positive PCR results for full-length polyadenylated 2NscFXT transcripts were evaluated by *in vitro* transcription using treated rabbit reticulocyte lysate (Promega). Briefly, 9 μl (1.5 μg) of enriched mRNA from stably transfected positive cultures was heated at 65°C for 3 minutes and mixed with 21 μl rabbit reticulocyte lysate, 1 μl Placental RNase Inhibitor (10U/ μl , Ambion), 0.5 μl amino acid mix minus Met, 0.5

μl amino acid mix minus Leu, 1 μl Transcend™ biotinylated lysine tRNA (Promega), and nuclease free dH₂O to 30 μl in a sterile 0.2 ml PCR tube (Eppendorf). The reaction mix was incubated at 30°C for 90 minutes then analyzed for 2NscFXT presence by Western Immunoblot with streptavidin/Alkaline Phosphatase (AP, Promega) conjugated probe. Visualization of the separated translation products entailed blocking the PVDF membrane with 30 ml of TBS-0.05% Tween for 1 hour with gentle agitation at room temperature. Following this incubation, the blocking buffer was removed and probe applied (6 μl of streptavidin/AP probe in 15 ml of TBS-0.05% Tween). The probe was incubated in the presence of the membrane for 1 hour at room temperature with gentle agitation. The probe solution was removed and the membrane was washed twice for 1 minute with 30 ml TBS-0.05% Tween, then washed twice for 1 minute with 30 ml of TBS. After the final wash, the TBS was removed and the membrane was developed for approximately 20 minutes with 5 ml of Western Blue® stabilized development substrate (Promega).

Transient Transfection of COS-7 Cells

Transfection of the COS-7 cell line was accomplished using a modification of the calcium phosphate procedure established by Graham and van der Eb (88). The alternative protocol outlines the calcium phosphate transfection of cells in suspension, rather than a monolayer, resulting in a two-fold increase in transfection efficiency (36). The Profection® Calcium Phosphate Mammalian Transfection System (Promega) was purchased for the purpose of transient transfection. Briefly, COS-7 cells were grown to confluency in 75 cm² tissue culture flasks (Becton-Dickinson) as described previously.

4 hours prior to transfection, the growth medium was replenished, cells were returned to the incubator, and the transfection reagents were allowed to thaw to room temperature. In a sterile 1.5 ml eppendorf tube, 40 µg of purified plasmid DNA was added along with nuclease-free dH₂O to a total volume of 876 µl. The DNA suspension was vortexed briefly and 124 µl of 2M CaCl₂ solution was added to the suspension, then vortexed vigorously. In a separate 15 ml Falcon[®] screw top tube (Becton-Dickinson), 1 ml of 2X HEPES-Buffered Saline (2X HBS, Promega) was added. The calcium phosphate precipitate was prepared by slow, drop-wise addition of the entire 1 ml CaCl₂/DNA suspension into the 2X HBS solution with continual vortex agitation. Upon complete addition, the DNA transfection cocktail was allowed to incubate at room temperature for 30 minutes. Development of an opaque calcium phosphate/DNA coprecipitate devoid of particulate material in the bottom of the tube was indicative of successful cocktail preparation. While the transfection cocktail was incubating, COS-7 cells were trypsinized as previously described, aspirated and placed in a 50 ml Falcon[®] screw top tube (Becton-Dickinson) then pelleted by centrifugation (International Instruments, Co) at 1000 rpm (RT) for 10 minutes. The medium was aspirated from the tubes, leaving only the pellet, and the transfection cocktail was vortexed briefly prior to thorough resuspension of the pellet using the entire cocktail volume (2 ml). The COS-7 cells were incubated in the presence of the transfection cocktail at room temperature for 15 minutes then diluted 12-fold in 22 ml of DMEM, 10% FBS, 2% PSG, 6.25 mM CaCl₂, 0.05X HBS. The diluted cells were administered in 6 ml aliquots to four separate 25 cm² tissue culture flasks (Becton-Dickinson) to provide the appropriate cell density for adherence of

individual cells, then incubated for 5 hours at 37°C, 6% CO₂. Following this incubation, growth medium was aspirated and the adherent cells were overlaid with 1 ml of DMEM, 10% FBS, 2% PSG, 25% glycerol for 1 minute to enhance the transfection efficiency (74). Cells were then washed twice with 5 ml of fresh medium to remove the glycerol and a final 6 ml aliquot of fresh growth medium (either serum-free or 10%FBS containing DMEM) was added for extended incubation. Cells were allowed to grow for 60 hours before the growth medium was aspirated and concentrated as previously described for the assessment of secreted protein. Additionally, transfected cells were trypsinized as described, pelleted, resuspended in D-PBS to a density of 1×10^7 cells/ml, then lysed by three rounds of subsequent freeze-thaw cycles (dry ice→37°C x 3) to examine cytoplasmic contents via Coomassie staining, Western immunoblot, and ELISA.

Cytotoxicity Assay

A modification of the MTT cytotoxicity assay (157) was used to determine the activity of leukotoxin. Briefly, the leukotoxin was adjusted to the desired concentration and serially diluted two-fold in RPMI-1640. BL-3 cells were centrifuged, resuspended to 1.6×10^6 cells/ml in RPMI-1640 and added to each well in volumes of 25 µl. The plate was incubated at 37°C with 5% CO₂ for 1 hour. Following this incubation, 50 µl of complete RPMI-1640 (media + 10% FBS + 2% PSG) and 10 µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) were added to each well, and the plate was incubated as before for 4 hours. Cells were then lysed and the tetrazolium dye solubilized by the addition of 100 µl of acid alcohol (0.04 N HCl in

isopropanol, Mallinckrodt) The absorbance was read at 565nm on a microtiter plate reader

For leukotoxin neutralization assays, soluble scFv and 2NscFXT extracts were diluted in RPMI-1640 One unit of leukotoxin was then added to each well and incubated on ice for 45 minutes BL-3 cells were then added to the plate and the remainder of the assay was performed as previously described

CHAPTER 3

RESULTS

Monoclonal Antibody Characterization

The leukotoxin-neutralizing mAbs generated by Gerbig and Cameron of this laboratory (79, 80) have been examined to illuminate both neutralization efficacy (64) and epitope localization (80, 144). Flow cytometry results indicated that interaction of mAb ltx-2 with LktA prevents toxin association and concomitant lysis of BL-3 cells (64, 79, 80). Initial epitope mapping by CNBr cleavage, toxin deletion constructs, and alkaline phosphatase/ leukotoxin fusion proteins indicated that the region within LktA spanning amino acids 768 to 939 was chiefly responsible for binding mAb ltx-2 (80). Further deletion analysis narrowed the scope of the ltx-2 epitope to amino acids 876 through 939 in the carboxyl end of the toxin (144).

Before initiating any downstream molecular applications such as primer design, PCR amplification, and cloning of full-length heavy and light chain DNA it was necessary to verify the isotype of each neutralizing mAb within the panel. The results of a direct ELISA for each neutralizing mAb in our panel are presented in Figures 3 1-3 4. It is not surprising that some ambiguous reactivity is observed between mAbs exposed to polyclonal sera, given the modicum of conservation within the framework of the constant domain. However, reactivity with monoclonal probes resolves this issue, suggesting that mAbs ltx-2 and ltx-4 maintain the Igγ1 heavy chain isotype, while ltx-35 exhibits the

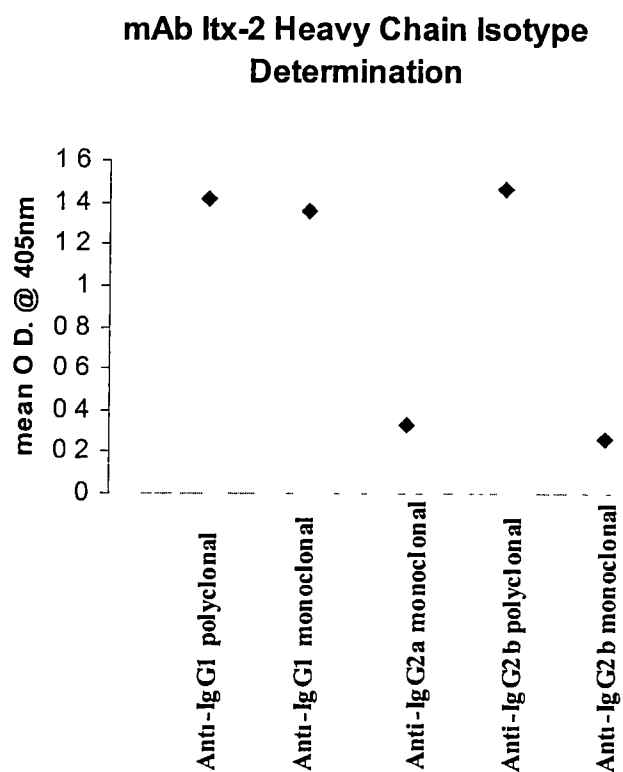


Figure 3.1: Itx-2 heavy chain isotype identified by direct ELISA. Data points represent the mean absorbance value for wells containing the probe indicated on the x-axis.

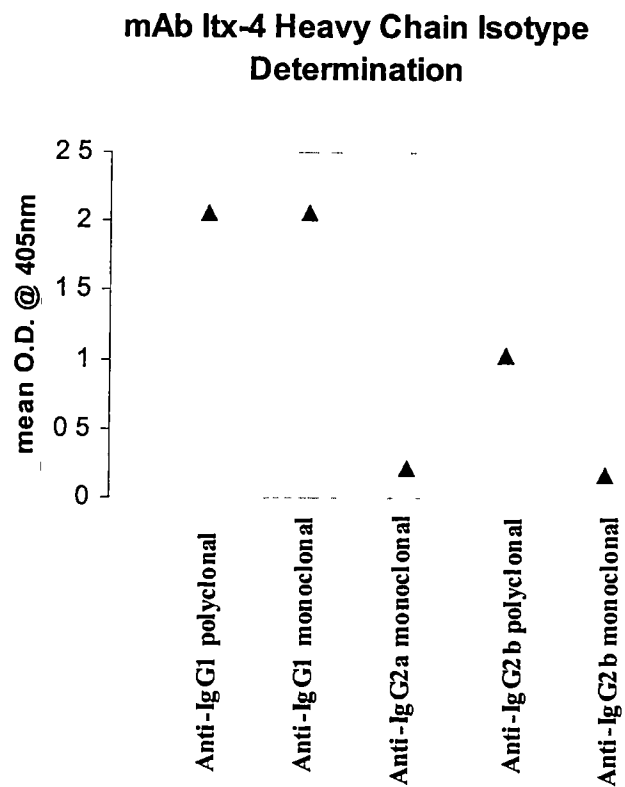


Figure 3.2: ltx-4 heavy chain isotype identified by direct ELISA. Data points represent the mean absorbance value for wells containing the probe indicated on the x-axis

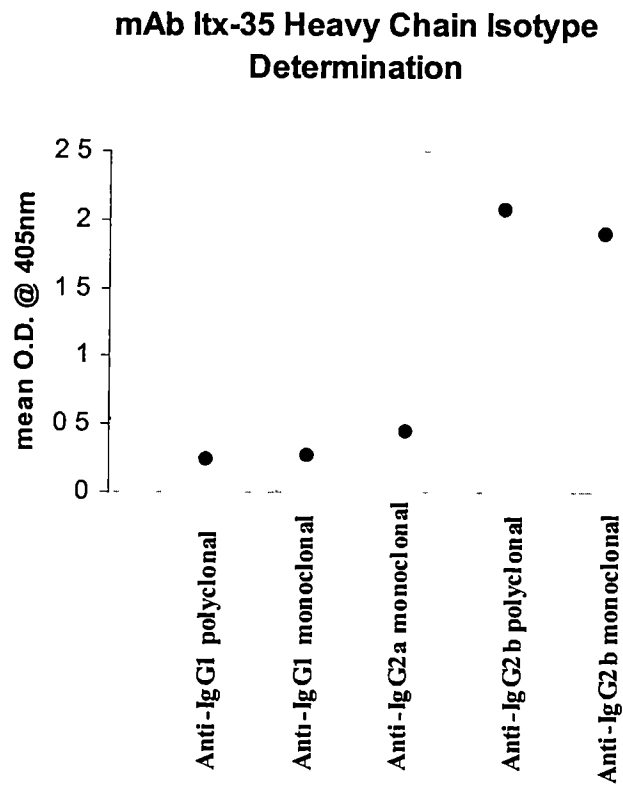


Figure 3.3: ltx-35 heavy chain isotype identified by direct ELISA Data points represent the mean absorbance value for wells containing the probe indicated on the x-axis

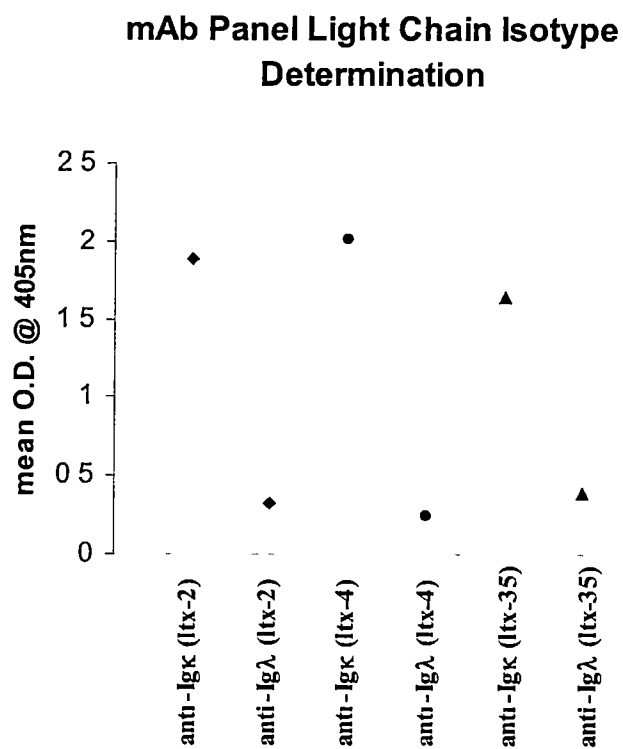


Figure 3.4: Light chain isotype for the entire mAb panel identified by direct ELISA. Data points represent the mean absorbance value for wells containing the probe indicated on the x-axis.

Ig γ 2b isotype. All neutralizing mAbs were consistent in light chain isotype, exhibiting profound reactivity with Ig κ antibodies, and little appreciable detection with an Ig λ probe.

RNA Preparation

To ensure the success of downstream applications, verification of the quantity, purity, and integrity of all RNA preparations performed during this work were required. Although variability in the quantity and purity of RNA was noted in individual preparations, typical RNA yields measured by spectrophotometry ranged from 0.05 to 0.2 $\mu\text{g}/\mu\text{l}$ with an A_{260}/A_{280} value within the range of 1.9-2.1. These numbers were well within the guidelines provided with the MicroPoly(A)Pure™ kit, suggesting satisfactory RNA yields devoid of significant protein contamination.

An additional qualitative assessment was performed to explore the presence or absence of RNase contamination within the preparations. Significant nuclease contamination can have a profoundly adverse effect on cDNA synthesis or *in vitro* translation, necessitating verification of transcript integrity. Preparations were resolved on a 1% non-denaturing agarose/ EtBr gel with subsequent appraisal of the 28s and 18s ribosomal RNA (rRNA) bands. Samples exhibiting negligible nuclease contamination present crisp rRNA bands with a majority of the RNA smear present in the higher molecular weight region. Samples tainted with endonuclease will exhibit nondescript, irregular, or smeared rRNA bands with the majority of EtBr stained nucleic acid in the low molecular weight vicinity. It can be concluded from Figure 3.5 that the integrity of

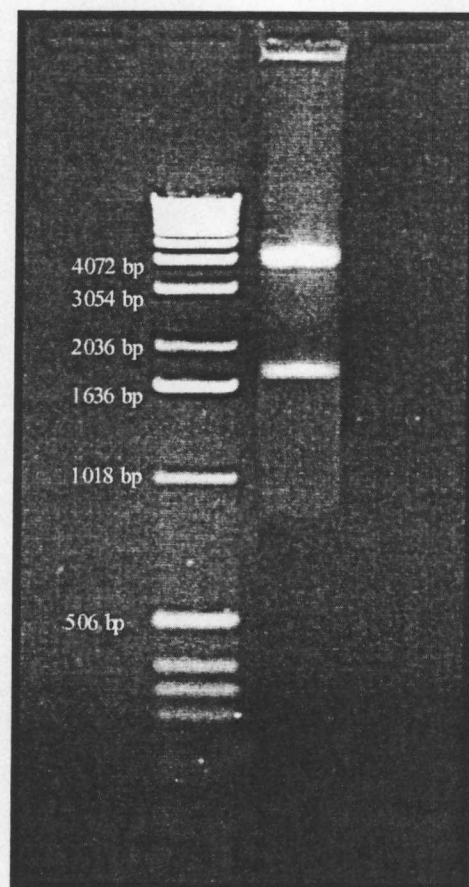


Figure 3.5: Characterization of enriched 2N mRNA integrity by 1% non-denaturing agarose/TBE/EtBr.

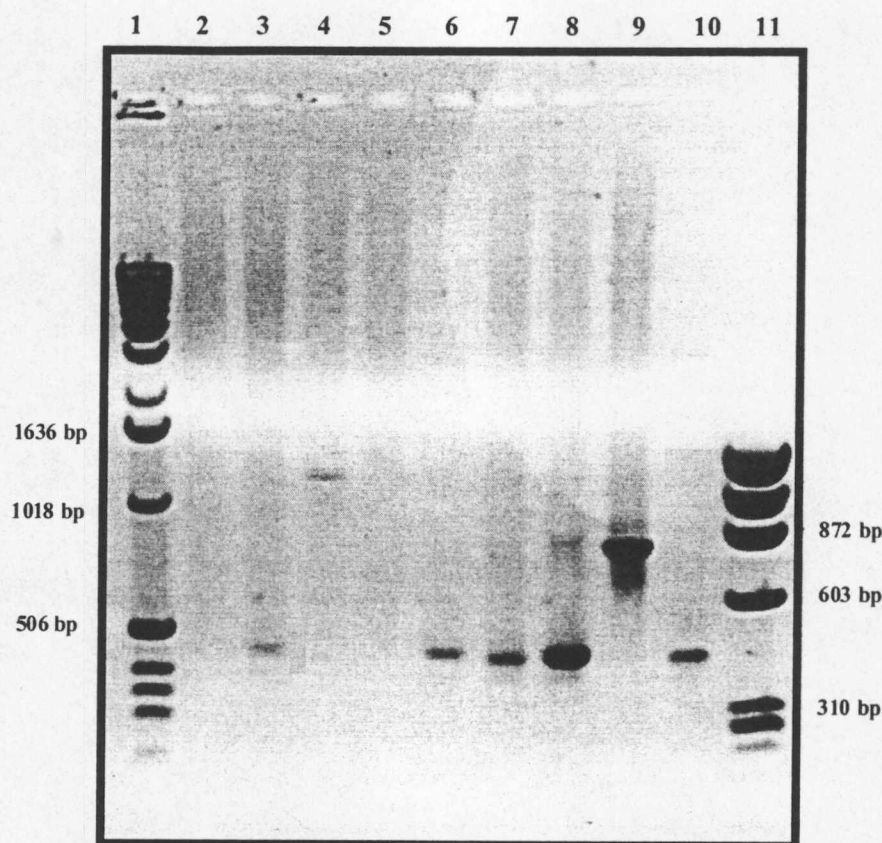
the ltx-2 enriched mRNA preparation is satisfactory considering the well-defined, sharp rRNA bands, and the absence of stained material in the extreme low molecular weight region

Amplification of Heavy and Light Chains from cDNA

Following verification of RNA integrity, enriched mRNA populations derived from each hybridoma were subjected to cDNA synthesis reactions employing poly(T) first strand primers. The cDNA synthesis reactions were then utilized as templates for PCR amplification with immunoglobulin specific primer sets designed to either amplify the variable region or the full-length sequence of the heavy and light chains. The results of this amplification scheme performed on ltx-2 derived cDNA can be seen in figure 3.6

In lanes 2 and 3, heavy chain variable region 5' IgG primers A and B from the Ig-Prime Kit[®] (Novagen) were employed, with an amplified product of the expected size (450 bp) appearing in lane 3, and no appreciable banding observed with the use of 5' IgG 5' primer A.

In lanes 4 and 5, an amplification of the entire heavy chain cDNA sequence was attempted with the same 5' Novagen primers and custom 3' primers targeting the UTR downstream of the constant domain sequence. Using this scheme, a faint band was observed in lane 5 with an apparent molecular weight of 1.4 kb relative to the dsDNA standard. This size is commensurate with the expected molecular weight of the full-length heavy chain molecule. Again, no appreciable banding was observed for the heavy chain with the use of 5' IgG primer A.



LEGEND

LN #	DNA SOURCE	PRIMERS
1	1 Kb ladder	
2	ltx-2 cDNA	Ig G5'A/Ig G3'
3	ltx-2 cDNA	Ig G5'B/Ig G3'
4	ltx-2 cDNA	Ig G5'A/G1C3'
5	ltx-2 cDNA	Ig G5'B/G1C3'
6	control IgG cDNA	Ig G5'A/Ig G3'
7	ltx-2 cDNA	Igκ5'A/Igκ3'
8	ltx-2 cDNA	Igκ5'B/Igκ3'
9	ltx-2 cDNA	Igκ5'B/3'KapC-UTR
10	control Igκ cDNA	Igκ5'B/Igκ3'
11	φX174/ <i>Hae</i> III ladder	

Figure 3.6: Amplification of ltx-2 full-length and variable regions from cDNA.

Lane 6 consisted of positive control heavy chain cDNA supplied with the Novagen kit. A band was observed with an apparent mobility of approximately 450 bp relative to the dsDNA standard.

Lanes 7 and 8 represented amplification of the light chain variable region using Novagen light chain 5' Igk primers A and B. It is readily apparent that both primers are equally capable of amplifying products from the cDNA synthesis reactions. The fact that both primers yielded products from the same cDNA was quite puzzling considering the disparity in primer sequence, particularly at the extreme 3' end. However, this issue was resolved upon discovering that some murine MOPC-21 myelomas (SP2/0-Ag14 included) produce an Igk transcript that is aberrantly rearranged in the J2-C region (29). The unfortunate result of this situation is that while one functional heavy chain transcript is produced in hybridoma lines generated via these fusion partners, two light chain transcripts are produced, only one being functional and attributed to the B-cell partner. The fact that 5' Igk primer B seemed to be more efficacious, given the enhanced intensity when compared to the 5' Igk primer A derived product, initially prompted the hypothesis that 5' Igk primer B yielded ltx-2 light chain DNA. However, sequence information from both products confirmed that 5' Igk primer A yielded the correct light chain variable region product, while 5' Igk primer B amplified a product identical to the published sequence for the aberrantly rearranged transcript (29).

Lane 9 consisted of ltx-2 cDNA amplified with 5' Igk primer B and a custom 3' oligonucleotide targeting the UTR of the Igk transcript. Although the product that is observed corresponds to the aberrantly rearranged transcript, subsequent amplification of

the same ltx-2 cDNA with 5' Igk primer A and 3' KapC-UTR produced a full-length product of similar apparent mobility (0.8 kb, not shown) in relation to the dsDNA standard with slightly less intensity.

Lane 10 consisted of positive control light chain cDNA supplied with the Novagen kit. A band was observed with an apparent mobility of approximately 470 bp relative to the dsDNA standard.

DNA Sequence, Translation, and Family Classification

Although ltx-2 was of principle interest in this investigation, the ease of RNA preparation and cDNA synthesis, availability of degenerate 5' primers, and published sequence information for the constant region(s) (6, 98, 161, 211) precipitated the decision to attempt cloning and sequencing of the entire mAb panel. Each consensus cDNA sequence was derived from no less than two independent clones to minimize the inherent errors in fidelity associated with a non-proofreading polymerase. The raw data from the DNA sequencing facility was examined for sequencing anomalies and entered into the LaserGene99 package for sequence alignment and comparison, delineation of reading frame, and restriction site analysis. Additionally, each consensus sequence for the variable heavy and light domains of ltx-2, and the variable heavy domains of ltx-4 and ltx-35 were analyzed to determine the immunoglobulin family and subgroup using the Kabat web database, *Proteins of Immunological Interest* maintained by Johnson and Wu (<http://immuno.bme.nwu.edu>).

The cDNA sequence of the ltx-2 heavy chain is given in figure 3 7 using a codon format. The translation of this sequence as determined by the LaserGene99 package is provided on the following page in figure 3 8. Additionally, regions of immunological interest including the leader peptide, framework domains, complementarity determining regions (CDR's), and constant domain are indicated. Comparison of the coding sequence DNA with the Kabat database suggests that ltx-2 can be classified as a member of the murine immunoglobulin heavy chain Family VIII (subgroup IIA). Additional analysis of this sequence indicated a total of 8 nucleotide deviations within the variable domain relative to closely matched sequences in the database. Three of these deviations occurred in the Framework I region at nucleotide positions 69 (C vs T or Ala vs Val), 73 (C vs T or His vs Gln), and 94 (A vs G- silent). Two nucleotide deviations were noted at positions 156 (A vs G or Asn vs Ser) and 159 (T vs A or (Phe vs Tyr) in the region corresponding to CDR I. Two more deviations were noted in the region corresponding to CDR II at nucleotides 241 (T vs G or Ser vs Arg) and 243 (T vs A or Phe vs Tyr). The final sequence deviation was noted in the Framework III region at nucleotide 325 (C vs G or Thr vs Ser). Furthermore, 3 of the 30 amino acid residues in the Framework I region varied from the consensus sequence established for Subgroup IIA. While this classification scheme is based upon the position of highly conserved amino acids within the immunoglobulin framework, it is noteworthy to mention variability may be observed in individual cases. The variations in this case included Ala vs. Val at amino acid (a.a) position 2, His vs Gln at a.a 3, and Thr vs Ser at a.a 30. Although variability was observed relative to the database entries, it does not undermine confidence in the ltx-2

63
 ATG GAG CTG GGT CTT TCT CTT ATT CTG TCA GTA ACT TCA GGT GTC TAC TCA | CAG GCT CAC CTC
 CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCT TCA GTG AAG TTG TCC TGC AAG GCT TCT 186
 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 CAG AAG TTC AAG GGC | 309
 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 CGA ACC | CGG GTG GGG GAC GTC TAC | TGG GGC 432
 CAA GGC ACC ACT CTC ACA GTC TCC TCA | GCC AAA ACG ACA CCC CCA TCT GTC TAT CCA CTG GCC
 CCT GGA TCT GCT GCC CAA ACT AAC TCC ATG GTG ACC CTG GGA TGC CTG GTC AAG GGC TAT TTC 558
 CCT GAG CCA GTG ACA GTG ACC TGG AAC TCT GGA TCC CTG TCC AGC GGT GTG CAC ACC TTC CCA
 GCT GTC CTG CAG TCT GAC CTC TAC ACT CTG AGC AGC TCA GTG ACT GTC CCC TCC AGC CCT CGG 681
 CCC AGC GAG ACC GTC ACC TGC AAC GTT GCC CAC CCG GCC AGC AGC ACC AAG GTG GAC AAG
 AAA ATT GTG CCC AGG GAT TGT GGT TGT AAG CCT TGC ATA TGT ACA GTC CCA GAA GTA TCA TCT 807
 GTC TTC ATC TTC CCC CCA AAG CCC AAG GAT GTG CTC ACC ATT ACT CTG ACT CCT AAG GTC ACG
 TGT GTT GTG GTA GAC ATC AGC AAG GAT GAT CCC GAG GTC CAC TTC ACC TGG TTT GTA GAT GAT 930
 GTG GAG GTG CAC ACA GCT CAG ACG CAA CCC CGG GAG GAG CAG TTC AAC AGC ACT TTC CGC
 TCA GTC AGT GAA CAT CCC ATC ATG CAC CAG GAC TGG CTC AAT GGC AAG GAG TTC AAA TGC 1050
 AGG GTC AAC AGT GCA GCT TTC CCT GCC CCC ATC GAG AAA ACC ATC TCC AAA ACC AAA GGC
 AGA CCG AAG GCT CCA CAG GTG TAC ACC ATT CCA CCT CCC AAG GAG CAG ATG GCC AAG GAT 1173
 AAA GTC AGT CTG ACC TGC ATG ATA ACA GAC TTC TTC CCT GAA GAC ATT ACT GAG GTG TGG CAG
 TGG AAT GGG CAG CCA GCG GAG AAC TAC AAG AAC ACT CAG CCC ATC ATG AAC ACG AAT GGC 1296
 TCT TAC TTC GTC TAC AGC AAC CTC AAT GTG CAG AAG AGC AAC TGG CAG GCA GGA AAT ACT TTC
 ACC TGC TCT GTG TTA CAT GAG GGC CTG CAC AAC CAC CAT ACT GAG AAC AGC CTC TCC CAC
 1380
 TGA *GGACCATTACTAGGGTCACA*

Figure 3.7: cDNA sequence of ltx-2 heavy chain is presented in codon format with base number given in bold type. 3' UTR is in *italics* and vertical bars provide reference for the protein translation.

Leader sequence *Framework I*

Met Glu Leu Gly Leu Ser Leu Ile Leu Ser Val Thr Ser Gly Val Tyr Ser | Gln Ala His Leu Gln Gln

Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe

CDR I *Framework II*

Thr | Asn Phe Trp Met Gln | Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly | Ala Ile

CDR II *Framework III*

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

Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Thr Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys

CDR III *Framework IV*

Ala Thr | Arg Val Gly Asp Val Tyr | Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser | Ala Lys Thr

Igγ1 constant region

Thr Pro Pro Ser Val try Pro Leu Ala Pro Gly Ser Ala Ala Gln Thr Asn Ser Met Val Thr Leu Gly

Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly

Val His Thr Phe Pro Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val Pro Ser

Ser Thr Trp Pro Ser Glu Thr Val Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys

Lys Ile Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val Ser Ser Val Phe

Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val

Asp Ile Ser Lys Asp Asp Pro Glu Val His Phe Thr Trp Phe Val Asp Asp Val Glu Val His Thr Ala

Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Ser Val Ser Glu His Pro Ile Met His

Gln Asp Trp Leu Asn Gly Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile

Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro Pro Lys

Glu Gln Met Ala Lys Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr

Glu Val Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile Met Asn Thr

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

Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His Thr Glu Asn Ser Leu Ser His stop

Figure 3.8: ltx-2 heavy chain translation Amino acid residues correspond to codons presented in figure 3.7 Vertical bars are inserted to denote regions of interest derived from the Kabat *et al* database

sequence information considering the range of immunoglobulin specificity encompassed by the comparison sequences

Due to the limited size of the light chain, I was able to conveniently present cDNA sequence in codon format and its translation within the same figure. Figure 3.9 outlines sequence information, translation, and regions of immunological interest for the ltx-2 light chain. Comparison of this sequence with the Kabat database suggests that it may be classified in the murine Kappa light chain Family XX (subgroup V). Additional analysis identified 18 deviations in nucleotide sequence relative to closely related comparison sequences. Of these deviations, 9 were silent, occurring in the wobble position at nucleotides 79, 103, 112, 172, 196, 211, 235, 352, and 358. Deviations in the Framework I region included positions 75 (A vs G or Asn vs. Ser) and 124 (C vs G or Asp vs Glu). Multiple deviation from the comparison sequences were appreciated in the CDR I region including nucleotides 162 (C vs G or Thr vs. Ser), 164 (G vs. A or Asp vs Asn), 173 (C vs G or Leu vs Val) and 174 (T vs G resulting in Ala or Pro depending on the nucleotide in the first position at 173). One deviation was noted in the region corresponding to CDR II at nucleotide position 237 (T vs A or Phe vs Tyr). One deviation was noted in the Framework III region at position 302 (G vs A or Ala vs Ser). Finally, one deviation was noted in the CDR III region at position 347 (A vs T or Asn vs. Tyr). Again, while some variability is expected when comparing the sequence to a database of variable domains with discrete and different specificity, a curious pattern appears to be present. It was noted that nucleotide deviations from the comparison sequences resulting in an amino acid change occurred twice in the Framework I region at

LEADER PEPTIDE

ATG GAG TCA CAT ACT CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT GGT GCT CAT
met glu ser his thr gln val phe val phe leu leu leu cys val ser gly ala his

FRAMEWORK REGION 1

GGG AAT ATC GTG ATG ACC CAG ACT CCC AAA TTT CTG CTT GTC TCA GCA GGA GAC AGG
gly asn ile val met thr gln thr pro lys phe leu leu val ser ala gly asp arg

CDR 1

GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG ACT GAT GAT GTT CTT TGG TAC CAA
val thr ile thr cys lys ala ser gln ser val thr asp asp val leu trp tyr gln

FRAMEWORK REGION 2

CAG AAG CCA GGA CAG TCT CCT AAA CTA CTG ATA TAC TAT GCA TCC AAT CGG TTC ACT
gln lys pro gly gln ser pro lys leu leu ile tyr tyr ala ser asn arg phe thr

CDR 2

GGA GTC CCT GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC
gly val pro asp arg phe thr gly ser gly tyr gly thr asp phe thr phe thr ile

FRAMEWORK REGION 3

AGC GCT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT AAT AGT TCT
ser ala val gln ala glu asp leu ala val tyr phe cys gln gln asp asn ser ser

CDR 3

FRAMEWORK REGION 4

CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT GAT GCT GCA CCA
pro trp thr phe gly gly gly thr lys leu glu ile lys arg ala asp ala ala pro

KAPPA CONSTANT DOMAIN

ACT GTA TCC ATC TTC CCA CCA TCC AGT GAG CAG TTA ACA TCT GGA GGT GCC TCA GTC
thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val

GTG TGC TTC TTG AAC AAC TTC TAC CCC AAA GAC ATC AAT GTC AAG TGG AAG AAT GAT
val cys phe leu asn asn phe tyr pro lys asp ile asn val lys trp lys ile asp

GGC AGT GAA CGA CAA AAT GGC GTC CTG AAC AGT TGG ACT GAT CAG GAC AGC AAA GAC
gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp

AGC ACC TAC AGC ATG AGC AGC ACC CTC ACG TTG ACC AAG GAC GAG TAT GAA CGA CAT
ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his

AAC AGC TAT ACC TGT CAG GCC ACT CAC AAG ACA TCA ACT TCA CCC ATT GTC AAG AGC
asn ser tyr thr cys glu ala thr his lys thr ser thr ser pro ile val lys ser

3' UTR

TTC AAC AGG AAT GAG TGT TAG AGA CAA AGG TCC TGA GAC GCC ACC ACC AGC TCC CCA
phe asn arg asn glu cys stop

GCT CCA TCC TAT CTT CCC TTC TAA GGT CTT GGA GGC TTC CCC ACA CTC GAG

Figure 3.9: Itx-2 Igk cDNA sequence and translation Sequence is presented in codon format with protein translation given in **bold type** Regions of immunological interest are separated by vertical bars and labeled

roughly the same position in the heavy and light chains. It was also observed that both the number and position of deviations in the CDR I region of both chains were similar with the obvious exceptions at positions 173 and 174 in the light chain. Furthermore, single deviations in the Framework III region were appreciated at roughly similar positions in both chains. Although the pattern of these changes may be coincidental, it is reasonable to consider the notion that they may contribute to the individual stability and discrete specificity of this neutralizing mAb via side chain or charge accommodation.

Due to considerable interest in ltx-2 downstream applications and lack of reproducible reactivity with primers other than murine Igk 5'B, the isolation of both ltx-4 and ltx-35 light chains was not pursued beyond a few preliminary failed cloning and sequencing attempts. The laborious process of isolating these transcripts from the preponderance of aberrantly rearranged Kappa transcripts produced by the myeloma fusion partner was deemed an unnecessary investment, given the original goal of this project. However, the heavy chains of both ltx-4 and ltx-35 were easily amplified, cloned, and sequenced from cDNA.

The cDNA sequence of ltx-4 is presented in figure 3.10 using a codon format. Immediately following this figure is a presentation of the reading frame translation and assignment of immunologically important domains within the molecule (Figure 3.11). Comparison of the ltx-4 nucleotide sequence with the Kabat database suggests a classification within the murine immunoglobulin heavy chain Family VII (subgroup IIA). Further analysis identified a total of twelve nucleotide deviations relative to the comparison database sequences. 5 of these deviation were silent occurring at nucleotide

ATG GAA TGG ACC TGG GTT ATT CTC TTC CTC CTG TCA GTA ACT GCA GGT GAC CAC TCC | CAG ATT **63**
 CAG CTG CAG CAG TCT GGA GCT GAG TTG ATG AAG CCT GGG GCC TCT GTG AAG ATA TCC TGC
 AAG GCT ACT GGC TAC ACA TTC AGT | AGT TAC TGG ATA AAG | TGG GTA AAG CAG AGG CCT GGA **183**
 CAT GGC CTT GAG TGG ATT GGA | GAG ATT TTA CCT GGA AGT GGA AAT TCT AAT TAC AAT GAG **303**
 AAC TTC AAG GGC | AAG GCC ACA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC CTG CAA
 CTC AGC CGC CTG ACA TTT GAG GAC TCT GCC GTC TAT TAC TGT GCA CTC | TAT GGG GAC TTC | TGG **429**
 GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA | GCC AAA ACG ACA CCC CCA TCT GTC TAT CCA CTG
 GCC CCT GGA TCT GCT GCC CAA ACT AAC TCC ATG GTG ACC CTG GGA TGC CTG GTC AAG GGC TAT **555**
 TTC CCT GAG CCA GTG ACA GTG ACC TGG AAC TCT GGA TCC CTG TCC AGC GGT GTG CAC ACC TTC
 CCA GCT GTC CTG CAG TCT GAC CTC TAC ACT CTG AGC AGC TCA GTG ACT GTC CCC TCC AGC CCT
 CGG CCC AGC GAG ACC GTC ACC TGC AAC GTT GCC CAC CCG GCC AGC AGC ACC AAG GTG GAC **678**
 AAG AAA ATT GTG CCC AGG GAT TGT GGT TGT AAG CCT TGC ATA TGT ACA GTC CCA GAA GTA TCA **804**
 TCT GTC TTC ATC TTC CCC CCA AAG CCC AAG GAT GTG CTC ACC ATT ACT CTG ACT CCT AAG GTC
 ACG TGT GTT GTG GTA GAC ATC AGC AAG GAT GAT CCC GAG GTC CAC TTC ACC TGG TTT GTA GAT **927**
 GAT GTG GAG GTG CAC ACA GCT CAG ACG CAA CCC CGG GAG GAG CAG TTC AAC AGC ACT TTC
 CGC TCA GTC AGT GAA CAT CCC ATC ATG CAC CAG GAC TGG CTC AAT GGC AAG GAG TTC AAA **1047**
 TGC AGG GTC AAC AGT GCA GCT TTC CCT GCC CCC ATC GAG AAA ACC ATC TCC AAA ACC AAA
 GGC AGA CCG AAG GCT CCA CAG GTG TAC ACC ATT CCA CCT CCC AAG GAG CAG ATG GCC AAG
 GAT AAA GTC AGT CTG ACC TGC ATG ATA ACA GAC TTC TTC CCT GAA GAC ATT ACT GAG GTG TGG **1170**
 CAG TGG AAT GGG CAG CCA GCG GAG AAC TAC AAG AAC ACT CAG CCC ATC ATG AAC ACG AAT **1290**
 GGC TCT TAC TTC GTC TAC AGC AAC CTC AAT GTG CAG AAG AGC AAC TGG CAG GCA GGA AAT
 ACT TTC ACC TGC TCT GTG TTA CAT GAG GGC CTG CAC AAC CAC CAT ACT GAG AAC AGC CTC TCC
 CAC TGA *GGACCATTTACTAGGGTCACA* **1380**

Figure 3.10: cDNA sequence of ltx-4 heavy chain is presented in codon format with base number given in bold type 3' UTR is in *italics* and vertical bars provide reference for the protein translation

Leader sequence *Framework I*

Met Glu Trp Thr Trp Val Ile Leu Phe Leu Leu Ser Val Thr Ala Gly Asp His Ser | Gln Ile Gln Leu

Gln Gln Ser Gly Ala Glu Leu Met Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Thr Gly Tyr

CDR I *Framework II*

Thr Phe Ser | Ser Tyr Trp Ile Lys | Trp Val Lys Gln Arg Pro Gly His Gly Leu Glu Trp Ile Gly | Glu

CDR II *Framework III*

Ile Leu Pro Gly Ser Gly Asn Ser Asn Tyr Asn Glu Asn Phe Lys Gly | Lys Ala Thr Phe Thr Ala

Asp Thr Ser Ser Asn Thr Ala Tyr Leu Gln Leu Ser Arg Leu Thr Phe Glu Asp Ser Ala Val Tyr

CDR III *Framework IV*

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

Igy1 constant region

Thr Pro Pro Ser Val try Pro Leu Ala Pro Gly Ser Ala Ala Gln Thr Asn Ser Met Val Thr Leu Gly

Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly

Val His Thr Phe Pro Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val Pro Ser

Ser Thr Trp Pro Ser Glu Thr Val Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys

Lys Ile Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val Ser Ser Val Phe

Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val

Asp Ile Ser Lys Asp Asp Pro Glu Val His Phe Thr Trp Phe Val Asp Asp Val Glu Val His Thr Ala

Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Ser Val Ser Glu His Pro Ile Met His

Gln Asp Trp Leu Asn Gly Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile

Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro Pro Lys

Glu Gln Met Ala Lys Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr

Glu Val Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile Met Asn Thr

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

Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His Thr Glu Asn Ser Leu Ser His stop

Figure 3.11: ltx-4 heavy chain translation Amino acid residues correspond to codons presented in figure 3 10 Vertical bars are inserted to denote regions of interest derived from the Kabat *et al* database

positions 94 and 114 in Framework I, position 156 in CDR I, positions 231 and 240 in the CDR II region relative to the related comparison database sequences. Other significant deviations within the sequence were noted in the Framework I region at nucleotide position 67 (A vs G or Ile vs Val), the CDR I region at position 166 (A vs G or Lys vs Glu), the CDR II region at positions 233 (A vs G or Asn vs Ser) and 235 (T vs A or Ser vs Thr), and the Framework III region at positions 304 (C vs A or Leu vs Met), 316 (C vs A or Arg vs Ser) and 326 (T vs C or Phe vs Ser). Furthermore, one instance of variability from the consensus Subgroup IIA sequence was noted in the Framework I region at a position 2 (Ile vs. Val). An additional striking characteristic of ltx-4 heavy chain variable region resides in the remarkably short CDR III. This motif consists of only 4 amino acids (Tyr-Gly-Asp-Phe) in ltx-4 whereas the others included 5 amino acids (ltx-2) and 10 amino acids (ltx-35). While curious, this observation does not lie outside the realm of possibility and may be classified as non-canonical according to the Kabat database.

The heavy chain cDNA sequence of ltx-35 is presented in figure 3.12. An adjunct figure delineates the reading frame translation and assignment of immunologically important domains within the molecule (Figure 3.13). Comparison of the ltx-35 nucleotide sequence with the Kabat database suggests a classification within the murine immunoglobulin heavy chain Family VII (subgroup IIB). Further analysis of this sequence suggests a total of 15 deviations in nucleotide sequence relative to closely related comparison sequences within the database. Of these, 4 deviations from the comparison sequences are silent occurring at nucleotide position 69 in the Framework I

ATG GAA TGG AGC TGG GTC ATT CTC TTC CTC CTG TCA GTA ACT GAA GGT GTC CAC TC**C**AG GTT **63**
 CAG CTG CAG CAG TCT GGG GCT GAG CTG GTG AGG CCT GGG TCC TCA GTG AAG ATT TCC TGC
 AAG GCT TCT GAC TAT GCA TTC ACT**A**GC TAC TGG ATG A**A**dTGG GTG AAG CAG AGG CCT GGA **183**
 CAG GGT CTT GAG TGG ATT GGA**A**AG ATT TTT CCT GGA GAT GAT GAT ATT AAT TAT AAT GGA AAA **306**
 TTC AAG GGT AAA GCC ACA TTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC
 AGC AGC CTA ACA TCT GAT GAC TCT GCG GTC TAC TTC TGT GCA AGA**A**GAG GGT AGT AGC TAC GAC **429**
 GTT GAC TA**A**dTGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA**A**GCC AAA ACA ACA CCC CCA
 TCA GTC TAT CCA CTG GCC CCT GGG TTT GGA GAT ACA ACT GGT TCC TCC GTG ACC TCT GGG TGC **555**
 CTG GTC AAG GGG TAC TTC CCT GAG CCA GTG ACT GTG ACT TGG AAC TCT GGA TCC CTG TCC AGC
 AGT GTG CAC ACC TTC CCA GCT CTC CTG CAG TCT GGA CTC TAC ACT ATG AGC AGC TCA GTG ACT
 GTC CCC TCC AGC ACC TGG CCA AGT CAG ACC GTC ACC TGC AGC GTT GCT CAC CCA GCC AGC AGC **681**
 ACC ACG GTG GAC AAA AAA GAG CCC AGC GGG CCC ATT TCA ACA ATC AAC CCC TGT CCT CCA **804**
 TGC AAG GAG TGT CAC AAA TGC CCA GCT CCT AAC CTC GAG GGT GGA CCA TCC GTC TTC ATC TTC
 CCT CCA AAT ATC AAG GAT GTA CTC ATG ATC TCC CTG ACA CCC AAG GTC ACG TGT GTG GTG GTG **927**
 GAT GTG AGC GAG GAT GAC CCA GAC GTC CAG ATC AGC TGG TTT GTG AAC AAC GTG GAA GTA
 CAC ACA GCT CAG ACA CAA ACC CAT AGA GAG GAT TAC AAC AGT ACT ATC CGG GTG GTC AGC **1047**
 ACC CTC CCC ATC CAG CAC CAG GAC TGG ATG AGT GGC AAG GAG TTC AAA TGC AAG GTG AAC
 AAC AAA GAC CTC CCA TCA CCC ATC GAG AGA ACC ATC TCA AAA ATT AAA GGG CTA GTC AGA **1167**
 GCT CCA CAA GTA TAC ACT TTG CCG CCA CCA GCA GAG CAG TTG TCC AGG AAA GAT GTC AGT
 CTC ACT TGC CTG GTC GTG GGC TTC AAC CCT GGA GAC ATC AGT GTG GAG TGG ACC AGC AAT **1290**
 GGG CAT ACA GAG GAG AAC TAC AAG GAC ACC GCC CCA GTT CTT GAC TCT GAC GGT TCT TAC TTC
 ATA TAT AGC AAG CTC AAT ATG AAA ACA AGC AAG TGG GAG AAA ACA GAT TCC TTC TCA TGC **1410**
 AAC GTG AGA CAC GAG GGT CTG AAA AAT TAC TAC CTG AAG AAG ACC ATC TCC CGG TCT CCG
1419
 GGT AAA TGA

Figure 3.12: cDNA sequence of ltx-35 heavy chain is presented in codon format with base number given in bold type. Vertical bars are inserted to provide reference for the protein translation

Leader sequence *Framework I*

Met Glu Trp Ser Trp Val Ile Leu Phe Leu Leu Ser Val Thr Glu Gly Val His Ser | Gln Val Gln Leu

Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Ser Ser Val Lys Ile Ser Cys Lys Ala Ser Asp Tyr

Ala Phe Thr | *CDR I* | Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly | *Framework II*

Gln Ile Phe Pro Gly Asp Asp Asp Ile Asn Tyr Asn Gly Lys Phe Lys Gly | *Framework III* | Lys Ala Thr Ala Asp

Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Asp Asp Ser Ala Val Tyr Phe Cys

Ala Arg | *CDR III* | Glu Gly Ser Ser Tyr Asp Val Asp Tyr | Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser | *Framework IV*

Igγ2b constant region

Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro Gly Phe Gly Asp Thr Thr Gly Ser Ser Val

Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Tyr Thr Val Thr Trp Asn Ser Gly Ser Ile

Ser Ser Ser Val His Thr Phe Pro Ala Leu Ile Gln Ser Gly Leu Tyr Thr Met Ser Ser Ser Val Thr

Val Pro Ser Ser Thr Trp Pro Ser Gln Thr Val Thr Cys Ser Val Ala His Pro Ala Ser Ser Thr Thr

Val Asp Lys Lys Glu Pro Ser Gly Pro Ile Ser Thr Ile Asn Pro Cys Pro Pro Cys Lys Glu Cys His

Lys Cys Pro Ala Pro Asn Leu Glu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Asn Ile Lys Asp Val

Leu Met Ile Ser Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Val Ser Glu Asp Asp Pro Asp Val

Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr

Asn Ser Thr Ile Arg Val Val Ser Thr Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe

Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ile Lys Gly Leu

Val Arg Ala Pro Gln Val Tyr Thr Leu Pro Pro Pro Ala Glu Gln Leu Ser Arg Lys Asp Val Ser Leu

Thr Cys Leu Val Val Gly Phe Asn Pro Gly Asp Ile Ser Val Glu Trp Thr Ser Asn Gly His Thr Phe

Glu Asn Tyr Lys Asp Thr Ala Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Ile Tyr Ser Lys Leu Asn

Met Lys Thr Ser Lys Trp Glu Lys Thr Asp Ser Phe Ser Cys Asn Val Arg His Glu Gly Leu Lys

Asn Tyr Tyr Leu Lys Lys Thr Ile Ser Arg Ser Pro Gly Lys **stop**

Figure 3.13: Itx-35 heavy chain translation Amino acid residues correspond to codons presented in figure 3 12. Vertical bars are inserted to denote regions of interest derived from the Kabat *et al* database.

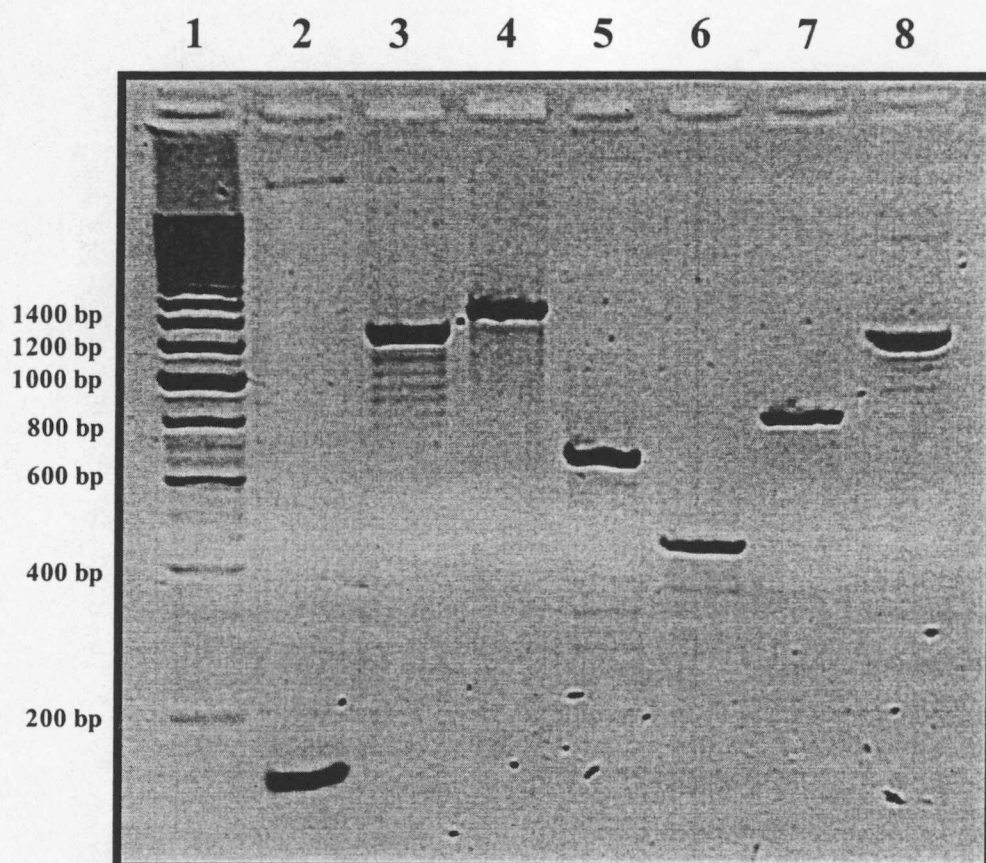
region, positions 240 and 252 in the CDR II region, and position 345 in the Framework III region. Other significant deviations were noted at positions 109-110 (TC vs GT or Ser vs. Val), 112-114 (TCA vs CTC or Ser vs Leu) and position 140 (A vs G or Asp vs Gly) in the Framework I region. The CDR II region maintained three significant deviations from the related comparison sequences at nucleotide positions 218 (T vs A or Phe vs Tyr), position 230 (A vs G or Asp vs Gly), and position 236 (T vs C or Ile vs Thr). Finally, two deviations were noted in the Framework III domain at nucleotide positions 271 (T vs C or Leu vs Arg) and 330 (T vs G or Asp vs Glu). Furthermore, 3 significant amino acid variances from the consensus Subgroup IIB sequence were noted in the Framework I region at a.a 16 (Ser vs Val), a.a 17 (Ser vs Leu), and a.a 26 (Asp vs Gly).

Engineering of the Eukaryotic Expression scFv Construct

The bacterially derived 2NscFv construct was graciously provided by Brian Ranger on the phagemid pCANTAB 5E. In order to achieve eukaryotic secretion of the engineered scFv protein, it was necessary to graft a signal peptide to the amino terminus for trafficking, processing, and export by the golgi apparatus (116, 182). While the leader sequence for ltx-2 would have been ideal for this purpose, the degeneracy introduced by the 5' amplification primers coupled with a complete lack of sequence homology in the ltx-2 clones relative to signal sequence database queries prompted the investigation of alternative means of accomplishing this goal. Fortunately, the ltx-4 signal sequence provided a viable alternative to this problem. A portion of the cloned ltx-

4 heavy chain was amplified with primers 5' 2/4G1-XTK and 3' 2/4G1-leader resulting in a DNA fragment of approximately 152 bp (lane 2, Figure 3.14). This fragment contained the entire ltx-4 leader sequence (-1 to -19) in addition to the first 28 amino acids encoded within the Framework I domain, and a Kozak motif (*CCGCCACCATGG*) required for proper translation initiation (119). While this strategy presented the best possible grafting alternative, ltx-4 harbored some possible downstream problems. First, the canon of signal peptide cleavage suggests that small, neutral residues must be present in the signal sequence at positions -1 and -3 (215). In the case of ltx-4, a charged residue (Asp) is present at position -3, suggesting that signal cleavage and subsequent packaging and export of the molecule may be compromised. This problem was remedied by using PCR primers and OE-PCR to mutate the (Asp) to the consensus (Val) in the final construct. Additional variations were appreciated in primary amino acid sequence relative to the scFv and ltx-2 Framework I region. It should be noted that grafting the ltx-4 signal sequence to the scFv molecule resulted in 3 amino acid substitutions within the Framework I region at positions 2 (Ile vs. Val), 12 (Met vs. Ala), and 13 (Lys vs. Arg). While the possible ramifications of these changes were considered during manipulation of the expression construct, a lack of reasonable alternatives precipitated the decision to maintain their presence until an assessment of molecule functionality could be made.

The scFv "keystone" sequence was amplified using primers 5' 2/4G1 fusion and 3' KapC fusion that targeted amino acids 19-28 in the V_H Framework I region and amino acids 1-10 in the V_L Framework IV region respectively. The product of this amplification scheme was approximately 659 bases (lane 5, Figure 3.14) and included the



LEGEND

- LANE 1: Promega 200 bp ladder
- LANE 2: amplified signal peptide (152 bp)
- LANE 3: ltx-4 heavy chain minus signal sequence
- LANE 4: ltx-4 full length sequence (1383bp)
- LANE 5: scFv "keystone" (659 bp)
- LANE 6: Igκ constant domain sequence (444 bp)
- LANE 7: scFv with leader fusion (784 bp)
- LANE 8: scFv/leader/kappaC fusion (1192 bp)

Figure 3.14: Amplification and OE-PCR assembly of 2NscFXT components resolved on a 1.7% agarose/TAE/EtBr gel.

entire functional region of the scFv minus the 18 amino acids of the Framework I region and the leader sequence provided by the ltx-4 graft. Note that the amino acid primary sequence is identical at residues 19-28 in the Framework I region of both ltx-4 and 2NscFv and provided a region of overlap in DNA sequence for OE-PCR.

The constant domain of ltx-2 Igk chain was amplified from a defined clone using the primers 5' KapC fusion and 3' KapC-UTR. This strategy generated a product of 445 bp (lane 6, Figure 3.14) and targeted the nucleotide sequence corresponding to amino acids 1-10 in the Framework IV region and a DNA sequence approximately 80 bp downstream of the stop codon in the 3' UTR of ltx-2 Kappa. Note that the sequence in the 5' end of this molecule aligns perfectly with the 3' end of the scFv keystone, allowing for an overlap of DNA sequence for OE-PCR.

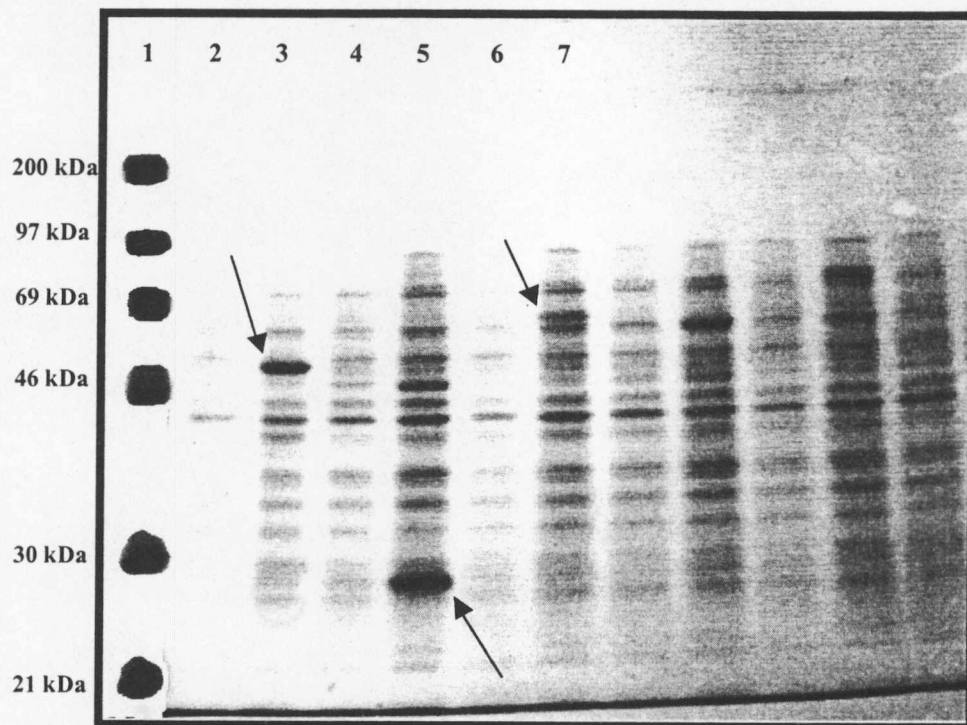
Lane 7 of Figure 3 14 depicts the resulting 794 bp product of ltx-4/ scFv keystone OE-PCR with subsequent amplification using outside primers 5'2/4G1-XTK and 3' KapC fusion. This product was then subjected to OE-PCR with the Kappa constant domain to provide a means for immunological detection of the molecule in addition to use in affinity chromatography purification. The resulting 1.2 kb 2NscFXT product can be seen in lane 8 of Figure 3 14.

It is worthwhile to note that the annealing temperatures of each primer set used was not optimized and occasionally led to multiple products of varied molecular weight following amplification (Figure 3 14). In order to minimize the carryover of these truncated or non-specific products, bands of the expected molecular weight for each product were excised from the gel and purified prior to OE-PCR.

Expression and Detection of 2NscFXT Using pGEX-4T-1

The forward (5' 2/4G1-XTK) and reverse (3' KapC-UTR) primers used to amplify the final 2NscFXT product contained the novel restriction sites *BamH* I and *Xho*I. These sites were employed exclusively in directional cloning of this construct into bacterial and eukaryotic expression vectors. In order to assess the efficacy of design and functionality of the immunological "tag", a number of individual 2NscFXT clones were excised from the shuttle vector pGEM[®]-T and ligated into the MCS of pGEX-4T-1. Additionally, full-length ltx-2 heavy and light chain clones were introduced into pGEX-4T-1 to assess their ability to react with anti-Igyl and anti-Igk HRP conjugated antibodies. Upon successful transformation of competent JM109 cells this strategy allows for the regulated bacterial expression of any construct as a fusion protein with GST, providing that the frame of the construct coincides with the reading frame of GST. Fusion protein synthesis is induced by the addition of IPTG, followed by a brief incubation period, cell lysis, and product resolution by SDS-PAGE with subsequent Coomassie staining or Western blot.

Figure 3 15 presents the results of a Coomassie stained gel containing protein extracts from induced and uninduced transformants harboring pGEX alone, pGEX/2NscFX (no tag), and 2NscFXT. Protein molecular weight calculations derived from the amino acid primary sequence and relative weight/ abundance of each amino acid were used to predict the expected size of each molecule. From these calculations, 2NscFX was predicted to have a molecular weight of approximately 31 kDa, 2NscFXT was predicted



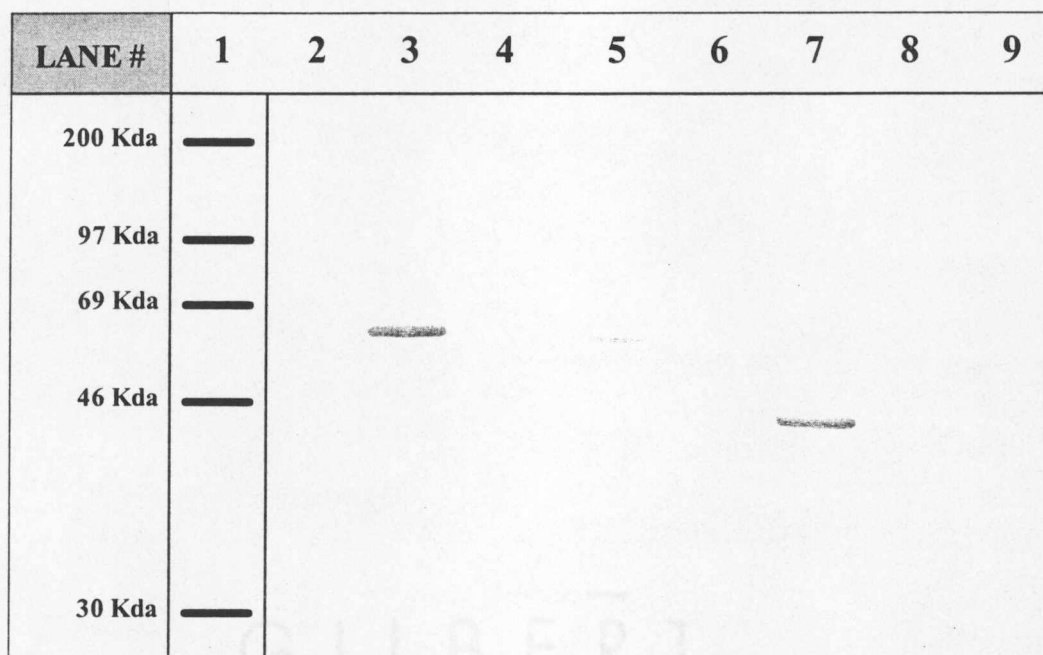
LEGEND

- LANE 1: Rainbow protein standard
- LANE 2: 2NscFv-GST (uninduced)
- LANE 3: 2NscFv-GST (induced)
- LANE 4: GST alone (uninduced)
- LANE 5: GST alone (induced)
- LANE 6: 2NscFXT-GST (uninduced)
- LANE 7: 2NscFXT-GST (induced)

Figure 3.15: Coomassie stained gel containing 2NscFv and 2NscFXT fusion proteins. Note that the protein standard has been enhanced to clarify the position of the molecular weight markers. Additionally, unlabeled lanes contain lysates unrelated to this work and should be disregarded. Bands of interest are clearly indicated with arrows.

to exhibit a molecular weight of 39.3 kDa, and GST is known to maintain a molecular weight of 29 kDa. Therefore, the GST/2NscFX fusion should exhibit a molecular weight of 60 kDa, and GST/2NscFXT should exhibit a molecular weight of 69 kDa relative to the molecular weight standard. From the photo, it is apparent that the bands of interest appear to have migrated further than predicted, with GST migrating at approximately 25 kDa, GST/2NscFX migrating at approximately 55 kDa, and GST/2NscFXT migrating at roughly 65 kDa. The consistency of this unexpected migration pattern suggests that the proteins were not sufficiently denatured, implying degradation of the β -mercaptoethanol in the loading buffer, or that the denaturation temperature/ time was not sub-optimal.

The results of a Western blot on two independent GST/2NscFXT clones and one GST/ltx-2 kappa chain clone are shown in Figure 3.16. As stated previously, the predicted molecular weight of the GST/2NscFXT fusion protein is approximately 69 kDa. The predicted molecular weight for the full-length Kappa chain is 25.9, suggesting the GST/ltx-2 Kappa fusion should exhibit a molecular weight of approximately 55 kDa relative to the protein molecular weight standard. Reactivity of the anti-Igk probe can be appreciated in lanes 3, 5 (faint), and 7 corresponding to 2NscFXT (A), 2NscFXT (B), and ltx-2 Igk respectively. Given the difference in band intensity between 2NscFXT clones A and B, it is reasonable to suspect that amino acid substitutions may have occurred in clone B that retard reactivity with the polyclonal probe. However, this difference is most likely attributed to a decreased amount of the clone B protein bound to the membrane relative to clone A. The fact that probe reactivity was only observed with both 2NscFXT clones and the Igk sequence suggests the structural integrity of the amplified sequences.



LEGEND

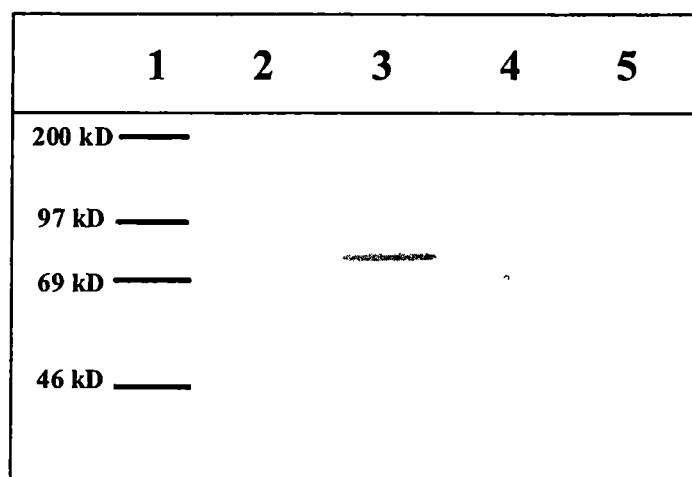
LANE1: Rainbow protein standard
 LANE2: 2NscFXT(A)-GST uninduced
 LANE3: 2NscFXT(A)-GST induced
 LANE4: 2NscFXT(B)-GST uninduced
 LANE5: 2NscFXT(B)-GST induced
 LANE6: Itx-2 Igκ full-GST uninduced
 LANE7: Itx-2 Igκ full-GST induced
 LANE8: GST alone uninduced
 LANE9: GST alone induced

Figure 3.16: Western blot of 2NscFXT and Igκ fusion proteins probed with HRP conjugated goat anti-murine Igκ polyclonal sera.

is sound and grafting of the Kappa constant domain to the scFv frame was indeed successful and cannot be attributed to cross-reactivity with the GST molecule. The fact that these bands exhibited an unexpected, but consistently smaller apparent molecular weight was addressed earlier and attributed to denaturation conditions rather than protein truncation. The pGEX-4T-1 plasmids containing the 2NscFXT constructs reactive with anti-Igk were purified and submitted for DNA sequencing.

Additionally, a Western blot was performed on the cloned Igyl ltx-2 chain to assess the integrity of the full-length cDNA sequence. The results of this experiment are presented in Figure 3.17. The calculated molecular weight for the ltx-2 heavy chain was 50 kDa, suggesting that the GST/ltx-2 IgG fusion should exhibit an apparent molecular weight of 79 kDa relative to the molecular weight standard. Indeed this fusion protein (lane 3) appeared to exhibit the expected pattern, with regression analysis suggesting an apparent molecular weight of 80 kDa. Furthermore, lane 5 corresponding to induced GST alone displayed no reactivity with the probe suggesting that the observed reactivity is a function of the ltx-2 heavy chain sequence and not attributed to GST artifact.

The observation that the GST/ltx-2 IgG1 protein exhibited the expected mobility while the others did not is puzzling, considering the consistency of sample treatment. A rational explanation resides in the fact that 2NscFv molecules are designed to form internal disulfide bonds for stability, which may account for an enhanced mobility if these bonds are not reduced. However, this argument has little merit when considering that both immunoglobulin light and heavy chains maintain this capacity (1), with the heavy chain being capable of forming intrachain disulfide bonds downstream of the hinge



LEGEND

- LANE 1: Rainbow protein standard
- LANE 2: ltx-2 IgG1 full-GST (uninduced)
- LANE 3: ltx-2 IgG1 full-GST (induced)
- LANE 4: GST alone (uninduced)
- LANE 5: GST alone (induced)

Figure 3.17: Western blot of ltx-2 IgG1/GST fusion probed with HRP conjugated rat anti-murine IgG1 monoclonal antibody.

region. If the disulfide bonds were not reduced effectively in the heavy chain, it is possible species of < 80 kDa or approximately 160 kDa could be observed corresponding to disulfide linked GST/ltx-2 IgG.

DNA sequence of 2NscFXT and Ligation into pcDNA 3.1+

Following receipt of the entire DNA sequence information for 2NscFXT, the raw data was examined for sequencing errors and 3 composite sequences were aligned using the LaserGene99 software package. The DNA sequence for 2NscFXT is presented using a codon format in Figure 3.18 with adjunct protein translation and delineation of regions of immunological interest given in Figure 3.19. Note that the Kozak sequence and 3' UTR of the Kappa constant region are present in the DNA sequence, but omitted for convenience in the final translation of 2NscFXT. Additionally, a Val residue has been substituted for an Asp at position -3 in the leader sequence to facilitate proper recognition and cleavage of the leader peptide. Furthermore, a Phe residue was substituted for an Ile at position -13 in the leader peptide to more closely match the consensus leader peptide sequences in the Kabat database.

Within the 2NscFXT Framework I it is important to note that amino acids 1-28 were contributed by the ltx-4 sequence. The result of this contribution is 3 amino acid substitutions relative to the original scFv sequence at position 2 (Ile vs Val), position 12 (Met vs Ala), and position 13 (Lys vs Arg). These substitutions also differ from the original ltx-2 heavy chain sequence with amino acid variance occurring at position 2 (Ala vs Ile), position 3 (His vs Gln), position 12 (Ala vs Met), and position 13 (Arg vs Lys).

CCG CCC ACC **ATG** GAA TGG ACC TGG GTT ATT CTC TTC CTC CTG TCA GTA ACT GCA GGT GAC CAC 63
 TCC CAG ATT CAG CTG CAG CAG TCT GGA GCT GAG TTG ATG AAG CCT GGG GCC TCT GTG AAG 186
 ATA TCC TGC AAG GCT ACT GGC TAC ACA 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 GCT GAT GCT GCA CCA ACT GTA TCC
 ATC TTC CCA CCA TCC AGT GAG CAG TTA ACA TCT GGA GGT GCC TCA GTC GTG TGC TTC TTG AAC 927
 AAC TTC TAC CCC AAA GAC ATC AAT GTC AAG TGG AAG ATT GAT GGC AGT GAA CGA CAA AAT
 GGC GTC CTG AAC AGT TGG ACT GAT CAG GAC AGC AAA GAC AGC ACC TAC AGC ATG AGC AGC 1047
 ACC CTC ACG TTG ACC AAG GAC GAG TAT GAA CGA CAT AAC AGC TAT ACC TGT GAG GCC ACT
 CAC AAG ACA TCA ACT TCA CCC ATT TGC AAG AGC TTC AAC AGG AAT GAG TGT TAG *AGA CAA AGG* 1176
 TCC TGA GAC GCC ACC ACC AGC TCC CCA GCT CCA TCC TAT CTT CCC TTC TAA GGT CTT GGA GGC TTC
 CCC ACA CTC GAG 1192

Figure 3.18: Complete DNA sequence of 2NscFXT presented in codon format with base numbers and start/stop codons given in **bold** type. 3' UTR is in italics

Leader sequence

Met Glu Trp Thr Trp Val Ile Leu Phe Leu Leu Ser Val Thr Ala Gly Val His Ser | Gln Ile
V_H Framework I
 Gln Leu Gln Gln Ser Gly Ala Glu Leu Met Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys
V_H CDR I *V_H Framework II*
 Ala Thr Gly Tyr Thr Phe Thr | Asn Phe Trp Met Gln | Trp Val Lys Gln Arg Pro Gly Gln
V_H CDR II
 Gly Leu Glu Trp Ile Gly | Ala Ile Tyr Pro Gly Asp Gly Asp Thr Ser Phe Thr Gln Lys Phe
V_H Framework III
 Lys Gly | Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Thr Ser
V_H CDR III
 Leu Ala Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Thr | Arg Val Gly Asp Val Tyr | Trp
V_H Framework IV *(gly₄ ser)₃ linker*
 Gly Gln Gly Thr Thr Val Thr Val Ser Ser | Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
V_L Framework I
 Gly Gly Gly Ser | Asp Ile Gln Met Thr Gln Ser Pro Lys Phe Leu Leu Val Ser Ala Gly Asp
V_L CDR I
 Arg Val Thr Ile Thr Cys | Lys Ala Ser Gln Ser Val Ala Asp Asp Val Leu | Trp Tyr Gln
V_L Framework II *V_L CDR II*
 Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr | Tyr Ala Ser Asn Arg Phe Thr | Gly Val
V_L Framework III
 Pro Asp Arg Phe Thr Gly Ser Gly Tyr Gly Thr Asp Phe Thr Phe Thr Ile Ser Ala Val Gln
V_L CDR III
 Ala Glu Asp Leu Ala Val Tyr Phe Cys | Gln Gln Asp Asn Ser Ser Pro Trp Thr | Phe Gly
V_L Framework IV *Igκ constant region*
 Gly Gly Thr Lys Leu Glu Ile Lys | Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro
 Ser Ser Glu Gln Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro
 Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn Gly Val Leu Asn Ser
 Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys
 Asp Glu Tyr Glu Arg His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro
 Ile Val Lys Ser Phe Asn Arg Asn Glu Cys STOP

Figure 3.19: 2NscFXT primary amino acid sequence. Amino acid residues correspond to codons presented in Figure 3.18. Vertical bars are inserted to denote regions of interest derived from the Kabat *et al* database

relative to the ltx-4 sequence. All other amino acids within 2NscFXT (V_H Framework I #29→ linker sequence) were congruent with the established sequence for the ltx-2 heavy chain

Deviations within the 2NscFXT V_L Framework I region from the established ltx-2 light chain sequence were also apparent and most likely attributed to the degeneracy of the primers that were used to amplify the original scFv construct and not a function of any manipulations during assembly of the 2NscFXT construct. These variances were identified at V_L Framework 1 positions 1 (Asp vs Asn), 3 (Gln vs. Val), and 7 (Ser vs Thr) relative to the accepted ltx-2 light chain sequence. As mentioned previously, the effect of these substitutions on the neutralization efficacy of the molecule is unknown, however the risk of altering the effectiveness of this molecule is implicit.

Satisfied with the sequence information, 2NscFXT constructs reactive with anti-Igk probe were excised from the pGEX-4T-1 in a directional manner using the restriction endonucleases *Bam*H I and *Xho* I. The eukaryotic expression plasmid pcDNA3.1+ was cut in a similar manner, and the gel purified fragment containing 2NscFXT was ligated into the linearized expression vector.

Stable Transfection of SP2/0 Cells with 2NscFXT

Following electroporation of SP2/0 cells with pcDNA3 1+/ 2NscFXT expression constructs, the cells were allowed to recover for 48 hours prior to addition of G418 (1.5 mg/ml). After addition of the selective marker, tissue culture plates were monitored daily for the appearance of resistant foci. Approximately 5-8 days following addition of

selective medium, foci began to appear in roughly 1/5 of the wells containing cells exposed to the expression vector, with no growth apparent in the untransfected control wells. Independent focal growth suggested that the linearized expression construct had undergone successful recombination/ integration into the cellular DNA and was at least expressing the resistance gene. While some transfected cells may originally harbor the vector, those failing to legitimately incorporate the resistance gene will eventually die. Furthermore, the rate of individual focal growth is directly proportional to expression level of the resistance gene, which in turn is a function of the local chromatin structure/ activity where the construct has integrated. Logic dictates that growth and cell division are intimately associated with the level of protein synthesis. Since G418 is a protein synthesis inhibitor affecting the small ribosomal subunit and chromatin activity plays a role in expression is key, it is not surprising that the foci appeared and flourished at different rates.

Analysis of 2NscFXT Expression in Stable SP2/0 Transfectants

Wells exhibiting focal growth were transferred to 25 cm² flask for enumeration. Following satisfactory growth, the cell density was determined and cells were pelleted by centrifugation. The cell free supernatant was concentrated 5-fold in a stir cell (30 NMWL cellulose membrane) and the remaining cell pellet was resuspended in a volume corresponding to 5×10^6 cells/ml and lysed to reveal the cellular contents.

The concentrated supernatant from each enumerated well yielded no reactivity with the anti-Igk HRP probe in repeated Western blot and ELISA experiments.

Furthermore, Coomassie stained gels containing concentrated supernatant from enumerated wells exhibited a complete absence of protein in the expected range. However, there were large amounts of protein in the vicinity of 25 kDa and 55-60 kDa attributed to the 10% FBS in the growth medium. It was hypothesized that the preponderance of FBS within the growth medium could inhibit the 2NscFXT protein from binding the ELISA plate. Selected cultures were grown in serum-free medium and concentrated as before, with a similar result being achieved.

Since no reactivity was observed in the culture supernatant, it was assumed that the problem existed at the level of processing and export. Cellular lysates exhibiting reactivity with the probe would indicate the possibility that the signal sequence was inadequately recognized by the signal peptidase or incapable of membrane transit. Unfortunately, the same phenomenon was observed with the lysed cell cultures that grew in the presence of selective medium. Both Western blot and ELISA failed to yield any reactivity with the anti-Igk probe. Furthermore, a Coomassie stained lysate gel suggested no appreciable difference in banding from the control lane. The sum of these results prompted investigation at the level of mRNA transcription within cells presumably containing the 2NscFXT construct.

2NscFXT Transcript Evaluation

In light of the previous failures to detect intracellular or extracellular 2NscFXT protein, it became necessary to evaluate the presence or absence of mRNA transcripts corresponding to this construct. Selected cultures exhibiting growth in selective medium

along with controls were exposed to mRNA enrichment and cDNA synthesis protocols. Following this series, each cDNA synthesis reaction was used for standard PCR amplification with primers specific for the 2NscFXT construct (5' 2/4G1-XTK, 3' KapC-UTR). The results of this experiment are presented in Figure 3.20 and suggest that cDNA derived from transfectant wells 1.3 and 3.3 yields a PCR amplified product commensurate with the expected molecular weight of 2NscFXT (1.2 Kb). Additionally, there is an apparent disparity in product concentration between the two positive samples given the profound intensity of well 3.3 vs. 1.3. However, these were not the only PCR positive samples, transfectant well 1.4, 2.3, 2.6, 3.4, and 4.1 (data not shown) yielded similar results following amplification from cDNA. It is also noteworthy to indicate that cells transfected with the expression vector alone (pcDNA3.1+CAT) grew quite well in the selection medium yet failed to yield any product using the 2NscFXT amplification scheme. Furthermore, 2NscFXT 1.1 (and 3.2, not shown) showed no appreciable amplification using the primers 5' 2/4G1-XTK and 3' KapC-UTR.

Success of the cDNA synthesis reactions and integrity of the mRNA preparations was verified by the successful amplification of the *rig/S15* housekeeping gene. In each sample preparation this product was present and displayed a uniform intensity and expected mobility (361 bp).

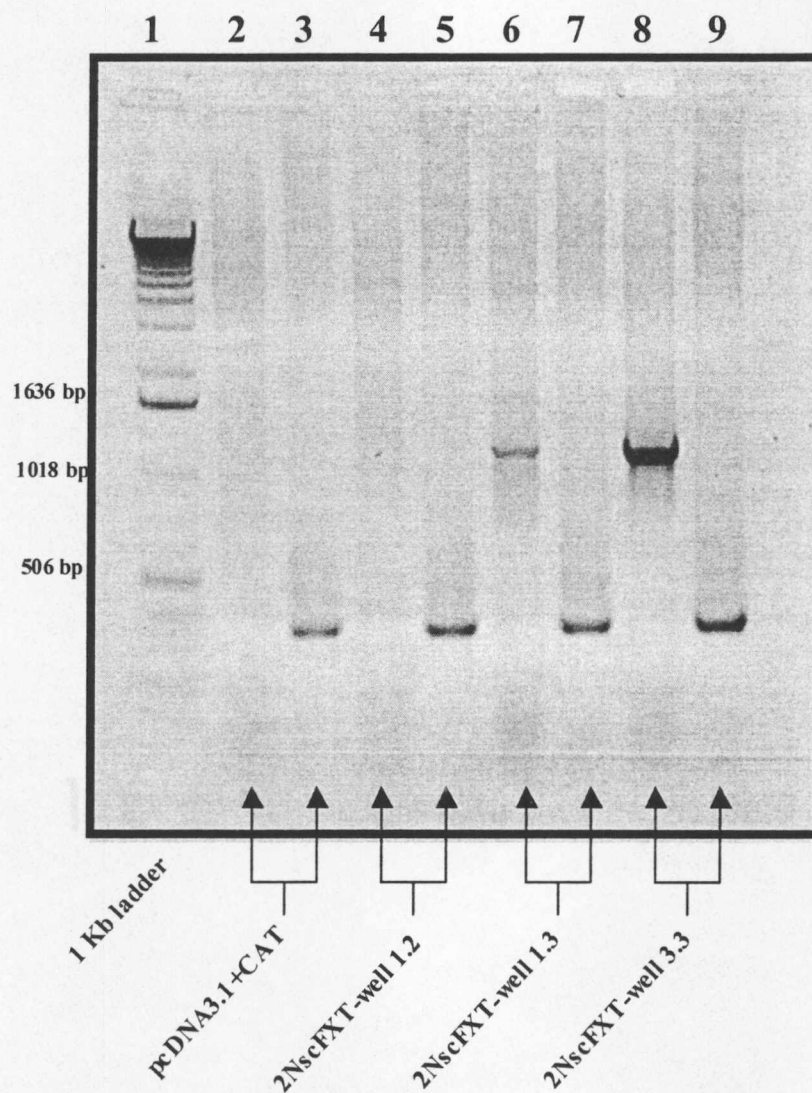
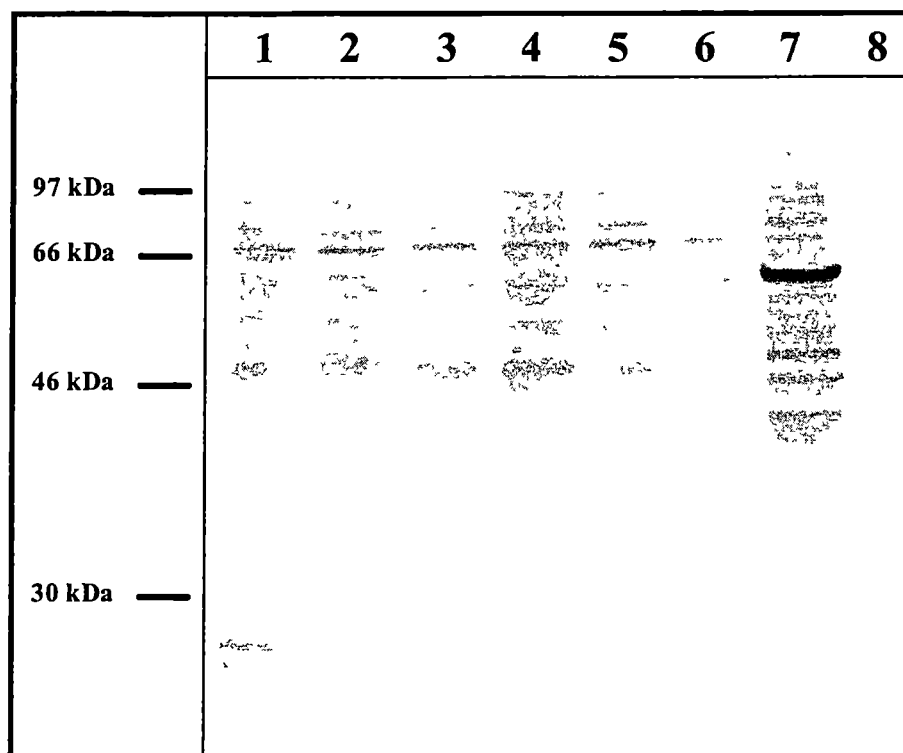


Figure 3.20: Amplification of 2NscFXT from stably transfected SP2/0 isolates. Lanes 2, 4, 6, and 8 consisted of cDNA synthesis reactions exposed to PCR amplification with 2NscFXT specific primers. Lanes 3, 5, 7, and 9 consist of the same cDNA synthesis reactions exposed to PCR amplification with the *rig/S15* primers.

In Vitro Translation of 2NscFXT Enriched mRNA

The results of the cDNA amplification scheme made it clear that some of the failure encountered in the immunological detection of 2NscFXT could be ascribed to an absence of mRNA. However, the ability to amplify product from a majority of the wells exhibiting growth precipitated the hypotheses that in these cases the protein was either unstable and rapidly degraded or problems inherent to the transcript itself were inhibiting effective translation. Since the mRNA was apparently stable and present, it seemed reasonable to investigate whether or not detectable levels of protein could be produced in a rabbit reticulocyte lysate system using enriched mRNA derived from positive wells. If 2NscFXT protein could be produced and detected in this fashion, it would be logical to assume that the SP2/0 cell line is not amenable to production of this contrived molecule.

The results from this line of experimentation were nebulous at best (Figure 3.21). The positive control reaction using luciferase mRNA was successful, yielding a pronounced band at roughly 61 kDa. The success of this reaction was tempered by the fact that the experimental lanes mirrored the control reaction containing no mRNA to some degree. While some bands are apparent in these lanes, those at approximately 90, 66, and 48 kDa can be attributed to biotinylated proteins or tRNA that are inherent to the reticulocyte lysate. Attempts at optimization of the experimental mRNA concentration yielded some smearing that differed from the negative control, yet no discrete bands were observed at the expected molecular weight of 2NscFXT and no disparity in banding was appreciated between mock control and experimental transfectants. While it is possible



LEGEND

<u>LANE #</u>	<u>mRNA CONTENT</u>
1	enriched MOCK control
2	enriched 2NscFXT well # 1.3
3	enriched 2NscFXT well # 1.4
4	enriched 2NscFXT well # 3.3
5	enriched 2NscFXT well # 2.3
6	enriched 2NscFXT well # 2.6
7	positive control
8	negative control (no mRNA)

Figure 3.21: Western blot of *in vitro* transcription products. Biotin-containing molecules (protein and tRNA) are recognized by the addition of a streptavidin-AP probe in the presence of development substrate.

that the experimental wells may contain the 2NscFXT protein, it cannot be distinguished from the background cellular mRNA's that are coordinately transcribed

Transient Transfection of COS-7 Cells

The apparent failure of the SP2/0 line to yield any detectable protein necessitated the decision to transfect an alternative cell line with the 2NscFXT construct. A number of reports (21, 55, 178, 218, 234) suggest that the COS cell line is amenable to transient transfection with subsequent production and export of reconstituted lymphoid molecules and their derivatives. In light of this information, COS-7 cells were transiently transfected with the 2NscFXT construct using a modified calcium phosphate approach and grown in standard as well as serum-free DMEM. Following a 60 hour incubation, the medium was removed and centrifuged to remove non-adherent cells. The medium was concentrated 10-fold and analyzed via Western blot, Coomassie stain, and ELISA. Non-adherent as well as adherent cells were harvested separately and lysed by successive freeze-thaw rounds.

The results of the Western blot and Coomassie staining procedures indicated no reactivity with the anti-Ig κ probe and no appreciable difference in protein banding between control and experimental samples. However, a direct ELISA yielded a significant and detectable signal in wells corresponding to 2NscFXT transfected non-adherent cells grown in serum-free and standard DMEM (Figure 3.22). Curiously, both the 2NscFXT transfected adherent cells and the concentrated medium failed to yield a

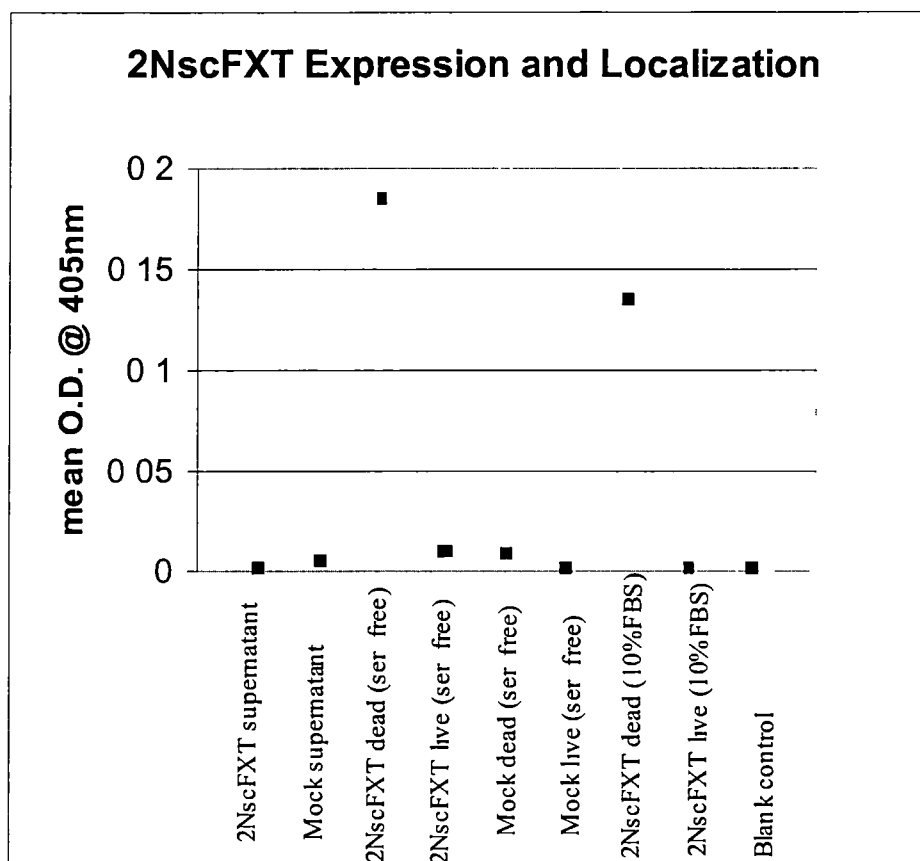


Figure 3.22: Direct ELISA results from COS cell transfection with 2NscFXT. Data points represent mean absorbance value from wells containing samples indicated on the x-axis. Note that “dead” cells refer to those floating in the medium after 60 hours, while “live” cells are indicative of those still adherent to the flask. Mock transfectants received vector alone.

significant signal relative to the experimental and negative controls when probed with the Igk antibody.

The fact that the Western and Coomassie procedures failed to detect any protein while the more sensitive ELISA did implies that the level of protein production falls below their limits of detection. Positive reactivity with the probe and a lack of significant signal in the ELISA controls suggests that 2NscFXT is being produced within the COS cell line and is not an experimental artifact.

CHAPTER 4

DISCUSSION

The current body of knowledge relating to pneumonic pasteurellosis continues to increase and may ultimately lead to a viable vaccination strategy capable of complete protection. To date, researchers and clinicians have illuminated the etiology, causative agent, and a host of virulence factors that are central to the progression of this disease. While it is clear that our understanding of pneumonic pasteurellosis is greater than ever, translation of this information into universally applicable therapeutic strategy remains elusive. An enormous amount of time, effort, and resources have been invested in the development of an effective vaccination protocol, targeting both viral and bacterial epitopes. Although some of these efforts have shown promise in artificially induced disease models, field trials have yielded spurious results. Given the complex etiology of pneumonic pasteurellosis, these failures are not difficult to understand. The many factors involved in genesis of the disease make it difficult to elucidate the proper immunological target or antigenic combination.

Contemporary understanding of the disease process suggests that *Pasteurella* (*Mannheimia*) *haemolytica* A1 is the principle agent of disease, garnering much of the current investigative attention. Its role in pneumonic pasteurellosis has prompted individuals within the vanguard of vaccine development to assert that elimination of this organism alone would drastically reduce the incidence of disease (70). The central

importance of this organism suggests that an appropriate vaccine should precipitate an immunological response to crucial virulence factors such as LktA, CPS, and TbpA/TbpB (171, 189, 192). In fact, a number of successful vaccine trials have definitively proven that enhanced protection correlates with circulating titers of neutralizing antibody against LktA (145, 149, 189, 190), a bacterial cytolysin known to exacerbate disease pathology. The shortcomings of prior vaccination protocols using live *P. haemolytica* vaccines (43, 71, 76, 121, 149, 156, 167), bacterins (43, 71, 76, 156), subunit vaccines (152, 189, 231), and DNA based approaches (125) have prompted the investigation of alternative vaccination modalities.

Our laboratory has focused on characterizing a panel of LktA neutralizing monoclonal antibodies to further illuminate the immunoreactive epitopes within the leukotoxin molecule (79, 80, 144). This characterization revealed the neutralization capacity of ltx-2 (64) and localized the binding region of this antibody to the carboxy-third of the LktA molecule encompassing amino acids 876-939 (144). Furthermore, the current study has illuminated the complete heavy and light chain cDNA sequence information for ltx-2 as well as the complete heavy chain cDNA sequence information for ltx-4 and ltx-35.

Current advances in recombinant DNA technology now allow researchers the possibility to explore new therapeutic vistas through antibody encoding DNA. Antibodies that have been empirically proven to bind and/ or neutralize their respective targets can now be manipulated and improved, giving rise to the term "designer antibodies". The technology currently exists to alter murine mAb heavy chains making

them less immunogenic (51, 174), retrofit antibodies and their derivatives with effector molecules such as toxins (39, 176, 220), improve binding efficacy through artificial antibody maturation (214), and execute heavy chain “swapping” for different immunological effector functions (9, 194). Furthermore, the development of scFv technology has enhanced the velocity of this field by eliminating the laborious process of hybridoma generation in addition to providing alternative therapeutic Fv molecules with a reduced immunogenicity and high rate of clearance.

The molecular engineering bonanza has broadened the scope of clinical antibody therapy in addition to providing the foundation for novel approaches to passive immunity through delivery of antibody encoding DNA. Unfortunately, the rate-limiting steps for the latter prospect reside in the delivery mode of therapeutic DNA and the necessity for *in vivo* administration. Currently, several methods are employed to deliver therapeutic DNA including; naked DNA injection (26, 57, 133, 213), cationic liposome mediated DNA delivery (89), and a host of viral strategies (97, 127, 158, 169, 172, 185). These strategies have exhibited varying degrees of success in terms of stimulating an immune response to DNA-encoded antigens or successful expression of a deficient gene in target cells, however no mention of *in vivo* heterologous antibody expression as an alternative to passive immunity was found. This lack of antibody gene therapy in the context of passive immunity is probably due in part to the ability to generate and purify prodigious amounts of antigen specific mAb's in the laboratory setting and administer them on demand. While this approach is suitable for most passive immunity applications such as

anti-venom therapy, it becomes problematic if a sustained presence of the antibody is required or a specific parenchymal target rather than systemic dissemination is desired

In this study the foundation for a proactive rather than a reactive approach to passive immunity is explored. The therapeutic scFv would potentially serve as an immunological “crutch”, attenuating the severity of the disease while an active immune response to LktA and other critical bacterial epitopes could be mounted. Unfortunately, the reported half-life for scFv molecules is approximately 6 minutes (124, 194), precluding conventional administration in the field. Furthermore, native immunoglobulins maintain a half-life of approximately 2-16 hours (194) depending upon their intrinsic level of stability, complicating their exogenous administration in this context as well. In an effort to compensate for this rapid clearance Lamarre and colleagues used a small peristaltic pump to achieve sustained intravenous delivery of anti-coronavirus scFv in a murine model (124). While this apparatus performed well in the laboratory, it is wholly unacceptable for field applications. It seems that the only reasonable means to achieve sustained presence of a heterologous recombinant antibody is to transfect host cells with antibody-encoding DNA and capitalize upon the promiscuity of vector-encoded promoter/enhancer elements and cellular pathways of protein synthesis and secretion.

If the capacity to express a leukotoxin neutralizing scFv in a eukaryotic system were established, the issue surrounding sustained presence of the molecule by reasonable means would be addressed. Furthermore, demonstration of this phenomenon suggests that it is not outside the realm of possibility to consider the delivery and expression of

this molecule as a potential prophylactic measure in the case of pneumonic pasteurellosis and other toxin mediated diseases. The only prerequisites for this strategy are demonstrably neutralizing antibody cDNA, a suitable expression vector, a reliable signal sequence, and a target cell type amenable to secretion of heterologous protein.

In order to satisfy the first prerequisite, it was necessary to clone and sequence the cDNA encoding both heavy and light antibody chains of ltx-2. This sequence data was invaluable during the generation of OE-PCR primers and verification of the functional scFv molecule. While these sequences may have questionable value in further defining the LktA epitope, they may present some utility in affinity maturation studies or perhaps be useful in heavy chain swapping experiments. Since the CDR's have been defined, future studies may employ this information in the systematic manipulation of the binding pocket to enhance the affinity of mAb ltx-2 for LktA. Furthermore, this sequence information provides essential information for the prospect of grafting a bovine heavy chain constant domain onto the murine ltx-2, 4, or 35 variable regions to reduce xenoreactivity in the context of passive immunization.

A cursory comparison of the disparate CDR's in the individual heavy chains lends support to the previous observations that each mAb within the panel has a discrete epitope within the carboxy third of LktA (80, 144). Between the two IgY1 isotypes (ltx-2 and ltx-4) only 20% (1/5) amino acid homology by position was appreciated in CDR I, 41% homology (7/17 aa) in CDR II, and there was no basis for comparison between the two mAb's in CDR III due to size disparity. A comparison of ltx-2 to ltx-35 (IgY2b) yielded similar results. A 40% (2/5) amino acid homology by position was appreciated in

CDR I, a 53% homology (9/17 a a) for CDR II, and again there was no basis for comparison between the two mAb's in CDR III due to a size disparity. Additionally, the heavy chain cDNA sequence information for ltx-2 and ltx-4 suggests a difference of 60 bases or 20 amino acids in comparison to the ltx-35 sequence (1359 bp vs 1419 bp). This difference is not surprising considering the extended CDR III loop in ltx-35 and the fact that it is of Igγ2b isotype, which may have a larger hinge region than Igγ1 antibodies.

(1) Furthermore, the aberrant migration patterns of ltx-2 heavy and light chain GST fusion proteins in the SDS-PAGE experiment opened the possibility that the entire gene was not cloned. However, verification of the cDNA sequence information assuaged these concerns and the migration anomalies were attributed to protocol rather than incomplete sequence.

The successful assembly of the tagged eukaryotic expression construct 2NscFXT was verified by DNA sequence analysis. This data suggested a reading frame consisting of, (1) a Kozak sequence in the context of the start codon allowing for proper translation initiation, (2) an appropriate leader peptide capable of intracellular targeting and signal peptidase recognition/ cleavage, (3) a LktA neutralizing scFv, (4) an Igκ constant domain for immunological detection, and (5) a stop codon to terminate protein translation. Furthermore, the functionality of the 2NscFXT Igκ tag was demonstrated in a bacterial system through GST fusion proteins probed with polyclonal goat anti-Igκ. The ability of the probe to recognize only 2NscFXT fusions coupled with the DNA sequence information provided preliminary encouragement regarding the integrity of the engineered protein.

This encouragement was short-lived once the expression phase of this project was initiated. Initially, stable transfection of SP2/0 cells by electroporation seemed to present a viable model for the production and secretion of 2NscFXT, considering the sequence information, fusion protein reactivity, and the reported mRNA transcription levels (89, 146, 158, 232) of the vector encoded CMV promoter/enhancer. The appearance of foci in the experimental wells and the lack of growth in the negative control wells suggested successful integration of the construct and production of the selective marker. In light of this observation, it seemed logical that at least some of these foci would be producing and secreting detectable amounts of 2NscFXT. Unfortunately, this assumption proved to be invalid. Repeated attempts to detect extracellular 2NscFXT by Western blot and ELISA failed to yield any signal. It was suggested that this problem was a result of the decision to directly probe concentrated supernatant rather than affinity purified material. There is no doubt that the concentration of FBS within the supernatant could complicate detection, particularly in an ELISA by monopolizing limited binding space. However, this issue was weighed against the concern that the immunological tag may assume a conformation in 2NscFXT that is not amenable to column recognition, resulting in partial or total protein loss.

Another facet of the detection issue involves the ability of 2NscFXT to bind PVC plates. Soluble 2NscFv weakly associates with PVC plates in direct ELISA experiments (B. Ranger, personal communication). If the protein was present in amounts only detectable by ELISA and was unable to bind the plates, then the following assumptions can be made; (1) the protein is not being secreted from the cytoplasm, (2) the protein may

be present and requires an alternative detection approach. The former assumption was tested by lysing 2NscFXT transfected SP2/0 cells while the latter was explored by coating the plate with antigen then subsequently adding the concentrated supernatant. The LtxA coated wells would theoretically eliminate the problems encountered with 2NscFXT protein binding to the wells as well as FBS inhibition. However, this strategy was complicated by two major concerns, (1) changes in the Framework I domain supplied by ltx-4 may compromise the binding efficacy of the molecule, and (2) the behavior of the scFv with the constant region attached was completely unknown, and this region may partially or completely obfuscate the ability to recognize the LktA epitope. Therefore, the failure of this experiment supplied little information regarding the extracellular presence or absence of 2NscFXT produced by SP2/0 cells.

Following the failure to detect extracellular presence of 2NscFXT in the supernatant of transfected SP2/0 cells, the investigation focused on the intracellular milieu. If an intracellular presence of 2NscFXT could be detected, it would be reasonable to assume that the protein was improperly targeted for export, suggesting a defect in the leader sequence, or had experienced a folding dysfunction that resulted in aggregates incapable of membrane transit. Again, experimental detection followed the protocol described for the concentrated supernatant, with no appreciable detection of 2NscFXT in the cytoplasm. While the same issues of protein/ PVC plate binding and epitope recognition were in play, this curious observation broached suspicions that perhaps little or no 2NscFXT transcript was being produced in the transfected cells.

Investigation at the transcript level suggested that there was some validity in the hypothesis that 2NscFXT mRNA was completely absent. Indeed some of the wells exhibiting positive growth in the selection medium failed to yield any 2NscFXT product from the cDNA synthesis reactions. However, these nonproductive transfectants were the minority, with only 3 of the 10 examined positive wells failing to yield a product of expected size using the PCR primers specific for 2NscFXT. The observation that these cells were able to grow in the presence of selective medium and untransfected cells were not precipitated the notion that in these cases an illegitimate recombination event had occurred, eliminating all or part of the 2NscFXT sequence while maintaining the resistance gene.

The observation that 7 of the 10 examined wells exhibited a product commensurate with the expected size for 2NscFXT suggested that stable transcripts encoding this molecule were being produced. The nature of the RNA enrichment protocol virtually eliminates the probability that false positives from genomic DNA could occur. Evidence for this assertion resides in the ability to amplify control products from cDNA that were of the expected size. Appearance of unexpectedly large PCR products in these reactions would suggest DNA contamination and amplification of germline products rather than a derivative of mature mRNA. Furthermore, the primer combination employed is specific for the 2NscFXT target, negating any background amplification of intrinsic SP2/0 proteins. The fact that the PCR positive experimental wells failed to yield detectable 2NscFXT protein precipitated a number of questions about the nature of the

message, its thermodynamic properties, its ability to be successfully translated, and the positional insertion within the genome

Given that the Kozak sequence appeared to be sound and the reading frame had been verified by sequence analysis and fusion protein detection, only a handful of explanations remained which adequately described the experimental data. First, the protein may be secreted at a very low level and is incapable of binding PVC or recognizing the LktA epitope. Second, the message maintained internal secondary structure at or close to the ribosome entry site that was too stable to be unwound by the 40S ribosome ($\Delta G \geq -60$ kcal/mol) (120), resulting in inhibition of translation. Third, it was possible that since SP2/0 cells overexpress an aberrant Kappa transcript, this locus was predisposed to recombination with the 2NscFXT construct, yielding a product that is truncated shortly after the V_L Framework IV region (29), eliminating the possibility of detection. Finally, it seemed reasonable to consider the possibility that SP2/0 cells are simply not amenable to the production of such a contrived molecule, maintaining the exquisite capacity to recognize and degrade stable or unstable 2NscFXT protein shortly after it is synthesized.

In order to address the thermodynamic properties of the 2NscFXT message, enriched mRNA from PCR positive transfectants was used for *in vitro* translation experiments in the presence of rabbit reticulocyte lysate. In light of the results, this was not the most suitable course of action for determining the translation capacity of this message. While the technical literature suggests that as little as 5ng of protein can be detected using the Transcend™ kit, the presence or absence of protein corresponding to

2NscFXT cannot be ascertained from the background cellular products on the Western blot. Furthermore, incorporation of the biotin adduct on lysine residues would no doubt inhibit molecule functionality and immunological detection. In retrospect, a coupled transcription and translation system using the pcDNA3 1+/2NscFXT DNA construct as a template would have provided a more reliable and definitive picture of the translation capacity by eliminating the cellular background. Therefore, the results obtained from the *in vitro* translation experiments using enriched mRNA from 2NscFXT transfectants were of little value in addressing thermodynamic issues.

Based upon the serious impediments encountered in the SP2/0 system, an expression protocol using the COS cell line was adopted. The benefits of using this cell line reside in the constitutive cellular expression of the SV40 T-antigen along with a relative ease of transfection. Delivery of plasmid vectors containing the SV40*ori* results in high levels of expressed heterologous protein (104) due to SV40 T-antigen/*ori* interaction. The SV40 T-antigen induces DNA replication starting at the SV40*ori*, autoregulates transcription of the early genes and stimulates synthesis of late mRNA both by increasing the gene copy number through its role in viral DNA synthesis and by augmenting the efficiency of the late promoter (92). Simply put, the plasmid vector will be replicated in transformed cells resulting in a phenomenon that is analogous to gene amplification. Multiple copies of the plasmid in each transfected cell coupled with the enhanced efficiency of the early and late genes will result in augmented protein synthesis of heterologous plasmid encoded genes in COS cells.

The direct ELISA results from transfected COS cell lysates suggest that 2NscFXT is being synthesized within the cells and is capable of binding the PVC plates directly. A significant signal was detected in experimental lysates from floating (dead) cells after 60 hours of incubation. This signal was 40X stronger than the controls, both blank well and mock transfected cells, suggesting that it could not be attributed to probe cross-reactivity or cellular peroxidase activity. Furthermore, no reactivity was appreciated in adherent cell lysates or concentrated supernatant. The fact that only dead cells exhibited reactivity with the probe after 60 hours is reasonable, considering that the massive accumulation of plasmid DNA and protein within the cytoplasm of these cells would lead to cell stress or even death. Additionally, the calcium phosphate approach boasts a modest efficiency of 15% of the total cells transfected, suggesting that the cells remaining in the monolayer may not have been transfected initially. The fact that no detectable protein was present in the culture supernatant suggests one of two possibilities; (1) the signal sequence is defective, hindering proper targeting and export of the protein, or (2) the protein is folding improperly, resulting in cytoplasmic aggregates incapable of secretion. The latter assertion would add validity to the observation that only dead cells exhibited reactivity to the probe. If protein were aggregating in the cytoplasm rather than being secreted, cell stress or death would be dramatically increased. Moreover, the consensus signal sequence was confirmed by the Kabat database (113) and the repair procedure should have allowed the signal to be recognized and cleaved from the mature peptide by signal peptidase (215). Thus, the most probable explanation for the exclusive cytoplasmic

presence of 2NscFXT may reside within an intrinsic instability of the molecule, resulting in attenuated secretion

The COS cell evidence illuminates some of the curious observations derived from the SP2/0 transfection series. It is now clear that 2NscFXT is capable of directly binding PVC, suggesting that if there was sufficient presence in the SP2/0 samples it could have been detected. Additionally, it is obvious that the message is capable of being translated, refuting the hypothesis that transcript anomalies may have resulted in translation inhibition. The most attractive hypothesis regarding the SP2/0 transfection series is that this cell line is simply not amenable to production and export of the current 2NscFXT construct. Considering the collective body of information, it is reasonable to assume that the minimal amount of protein produced by the SP2/0 cell line relative to COS cells could easily be degraded, particularly if it was forming aggregates in the cytoplasm. Skowronek and colleagues indicate that partially folded Igk light chains in the absence of a heavy chain partner bind to the chaperone protein BiP. This ER chaperone protein recognizes the V_L domain and based upon the stability of this interaction determines the half-life (1-4 hours) of the molecule (194). Additionally, it is known that aggregate or misfolded proteins may be directly targeted for rapid degradation (5), explaining the absence of detectable signal in SP2/0 cell lysates. Furthermore, while the amount of expressed intracellular 2NscFXT was not calculated, it was suspected to be within the ng/ml range, commensurate with some of the observations in other heterologous scFv expression studies employing COS cell lines (55, 178). However, the estimated yields from this work suggest that eukaryotic expression of this

scFv antibody is no more efficacious than the bacterial system. This unexpected yield may be due in part to the variable region sequence (178) or rapid degradation of the 2NscFXT protein rather than secretion. The fact that COS cells exhibit a detectable signal in the presence of similar regulatory machinery utilized by the SP2/0 line suggests the possibility that heterologous protein synthesis overwhelms the rate of protein degradation in these cells.

While the addition of an immunological detection entity is necessary to assess the presence of and localize the construct in this study, it remains unclear as to whether or not this adduct inhibited the functionality of the engineered molecule. It is possible that the size of the “tag” relative to the scFv or inter-chain interactions affected the stability of the neutralizing scFv, resulting in an inability to recognize the LktA epitope or a folding anomaly that prevents secretion. Therefore, while it is known that the molecule can be expressed, it would be advisable to assess the efficacy of secretion and neutralization by replacing the large Igk constant domain present in the 2NscFXT construct with the smaller (14 a.a.) E-Tag. The bacterially derived soluble scFv-E-Tag is stable and has demonstrated the ability to bind and neutralize LktA (173), presenting a proven strategy for neutralization and detection. Once the extracellular presence of the molecule has been established experimentally and neutralization capacity verified, the adduct may be removed for *in vivo* application if desired.

This study provides evidence that an scFv derivative can be expressed in a non-lymphoid eukaryotic system. This successful demonstration may enhance the exploration of DNA based antibody prophylaxis, particularly in the case of toxin-involved diseases.

such as pneumonic pasteurellosis and intracellular immunization. Additionally, this technology may have significance in the military arena, considering the current shortage of bio-weapons vaccines and the velocity and adaptability of this technique. However, while this approach may yield some potential prophylactic benefit in the future, some vital issues must be addressed. First, the fact that production was only observed in COS cells poses serious questions about *in vivo* application. Since target cells will not be constitutively expressing the SV40 T-antigen, it may be necessary to fit this construct with a tissue specific promoter or include the T-antigen sequence in the delivery vehicle. The former suggestion becomes problematic when considering that gene delivery will have to be targeted. The only current strategy capable of targeted delivery employs replication deficient viruses, which may pose ethical and/or health concerns. The latter suggestion may result in augmented heterologous protein production, however there is no reason to suspect that target cells would have a greater longevity than the experimental cells in this case.

Therefore, future investigations must assess the activity level of this therapeutic molecule including how much prophylactic protein is required to reduce disease severity. From this information, it may be determined which tissues are amenable to supporting the production of therapeutic amounts of 2NscFXT. After these questions have been addressed, the construct can be manipulated to suit the delivery and production prerequisites. A realistic example of this strategy could involve targeting surfactant secreting alveolar type II cells with an expression construct containing the surfactant

leader peptide and a tissue specific promoter, localizing expression of the therapeutic molecule to the area where it is needed most

In conclusion, this technology offers an exciting new approach to conventional vaccination as well as intracellular therapy. The development of viable approaches to DNA-based antibody prophylaxis may contribute to the control and management of diseases ranging from bacterial and viral infections to cancer.

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VITA

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