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Induction of humoral immunity with plasmid DNA encoding an immunogenic fragment of Pasteurella haemolytica A1 leukotoxin

Kristin M. Lavender

To the Graduate Council:

I am submitting herewith a thesis written by Kristin M. Lavender entitled "Induction of humoral immunity with plasmid DNA encoding an immunogenic fragment of Pasteurella haemolytica A1 leukotoxin." I have examined the final electronic 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:

Habib Zaghouani, David A. Bemis

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Associate Vice Chancellor and
Dean of the Graduate School

INDUCTION OF HUMORAL IMMUNITY WITH PLASMID DNA ENCODING AN IMMUNOGENIC FRAGMENT OF *PASTEURELLA HAEMOLYTICA* A1 LEUKOTOXIN

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kristin M. Lavender

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DEDICATION

This thesis is dedicated to Jeffrey S. Price, whose patience and support enabled me to endure this process.

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ABSTRACT

Pneumonic pasteurellosis is an acute respiratory disorder that results from infection of the bovine lung by *Pasteurella haemolytica* A1. The primary virulence determinant is a secreted, ruminant-specific leukotoxin (LktA). The leukotoxin is a pore forming toxin that promotes inflammation and bacterial proliferation in the airways of infected animals. Studies continue to show that leukotoxin-neutralizing antibodies are required for significant levels of protective immunity. This laboratory has focused on characterizing this toxin in an attempt to define important immunogenic epitopes which could be incorporated into an effective vaccine. These studies were undertaken to explore the ability of a novel vaccination approach to elicit protective leukotoxin-specific antibodies in immunized mice. A plasmid encoding the immunogenic LktA fragment corresponding to amino acids 715-953 was used in a DNA-based vaccine and was examined for its efficacy in generating a leukotoxin-specific immune response in mice. In addition, the potential immunostimulatory peptides, human IL-1β 163-171 and human acidic isoferritin 172-185, were incorporated into DNA-based vaccines and into GST-fusion-protein vaccines, and monitored for the ability to augment the developing antibody response. Results from these studies indicated 1) the leukotoxin-encoding plasmid used in DNA-based vaccination is capable of generating protein-specific antibodies, however, these antibodies are not neutralizing; 2) the adjuvant peptides are capable of augmenting the immune response when incorporated in a DNA-based vaccine, although they do not

contribute to the development of neutralizing antibodies; and 3) the adjuvant peptides do not display immunostimulatory effects when incorporated between the GST moiety and the LktA fragment in recombinant protein vaccines.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di (ethyl-benzthiazoline-6-sulfonic acid)
APC	antigen presenting cell
BCA	bicinchoninic acid
BHI	brain heart infusion
BHV-1	bovine herpesvirus-1
BL-3	bovine lymphocyte 3 cell line
BRSV	bovine respiratory syncytial virus
BVDV	bovine viral diarrhea virus
C	degrees celcius
CFA	complete Freund's adjuvant
CFU	colony forming units
CNBr	cyanogen bromide
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
ELISA	enzyme linked immunosorbent assay

EtBr	ethidium bromide
FBS	fetal bovine serum
GST	glutathione-S-transferase
HBSS	hanks balanced salt solution
HLY	hemolysin
HRP	horseradish peroxidase
IBR	infectious bovine rhinotracheitis
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG1	immunoglobulin G subclass 1
IgG2a	immunoglobulin G subclass 2a
IL	interleukin
im	intramuscular
in	intranasal
INF-γ	interferon-γ
ip	intraperitoneal
IPTG	isopropyl-β-D-thiogalactoside
kDa	kilodalton
LB	luria-bertani medium
LKT	leukotoxin
LPS	lipopolysaccharide

mAb	monoclonal antibody
MHC	major histocompatability complex
mg	milligram
ml	milliliter
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
mRNA	messenger ribonucleic acid
N-terminal	amino-terminal
O antigen	antigenic polysaccharide chain
OD	optical density
ODN	oligodeoxyribonucleotide
OMP	outer membrane protein
OVA	ovalbumin
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline-0.05% Tween 20
PCR	polymerase chain reaction
PI-3	parainfluenza-3
PMSF	phenylmethylsulfonylfluoride
PSG	penicillin, streptomycin, L-glutamine
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
RNA	ribonucleic acid

RLF	rat liver ferritin
RPMI-1640	Roswell Park Memorial Institute-1640 Medium
RTX	repeats in structural toxin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNF-α	tumor necrosis factor-α
U	unit
μg	microgram
μl	microliter
2X-YT	double strength yeast extract tryptone broth

CHAPTER 1

INTRODUCTION

The Disease

Bovine respiratory diseases continue to be a challenging problem to the beef and dairy industry. Of particular concern is bovine pneumonic pasteurellosis, a fibrinous pleuropneumonia caused by *Pasteurella* species and often associated with cattle stockyards and shipping facilities (147). Also known as shipping fever, this pneumonia is one of the most prevalent causes of morbidity and mortality in cattle (208). The onset of the disease is influenced by a variety of factors and is characterized by fever, weakness, an influx of neutrophils and macrophages into the airways, accumulation of fibrinous edema fluid in lung tissue, vascular thrombosis, and parenchymal necrosis of the lung (227,266). Economic losses result from a delay in marketing animals, expensive treatments, and loss of animals. It is estimated that the loss to the North American beef cattle industry alone is approximately one billion dollars (101).

Etiology

The cause of shipping fever is multifactorial. Stresses involved in transportation and opportunistic viral and bacterial infections are key factors in the development of the disease (80,280). During transportation, cattle are exposed to dusts, overcrowding, extreme temperature changes, and irregular feeding schedules which when combined with

stresses resulting from the weaning transition may lower natural defense mechanisms (147,280). Viral infections also play a role in lowering the resistance of calves. Viruses such as bovine herpesvirus-1 (BHV-1), parainfluenza-3 virus (PI-3), infectious bovine rhinotracheitis virus (IBR), bovine viral diarrhea virus (BVDV), and bovine respiratory syncytial virus (BRSV) are able to damage the respiratory tract and reduce bacterial clearance mechanisms (9,25,75,117,204,280). Parainfluenza-3 virus, the most consistent viral isolate from respiratory diseased cattle, infects ciliated epithelium of the upper and lower respiratory tract as well as alveolar epithelium, interfering with normal clearance mechanisms (22,25,117,122). BHV-1 inhibits the antimicrobial actions of bovine leukocytes, also affecting the ability of the host to clear microbes (75). Infections with BVDV affect the gastrointestinal tract and lymphoid tissue and, in addition, may produce mild respiratory disease (203,204). BVDV is also thought to cause immunosuppression in cattle, impairing peripheral immunity and pulmonary resistance (203,204). BRSV and IBR infections also damage the ciliated epithelium of the respiratory tract and contribute to an overall suppression of the immune system. Viral infections in conjunction with physical stresses from shipping, alter the normal immune functions causing the animals to become more susceptible to infectious agents.

Many bacteria have also been associated with this type of bacterial pneumonia. *P. haemolytica*, *P. multocida*, *Haemophilus somnus*, *Corynebacterium* species, and *Mycoplasma* species have been isolated from lungs of diseased animals (39,106,279,280). The most common isolate from pneumonic calves, however, is *P. haemolytica* A1 (39,79) This bacterium is normally found in the nasopharynx and tonsils of healthy animals with

no adverse effects (80). Physical stresses and viral infections create an opportunity that allows for the proliferation in the upper respiratory tract followed by inhalation into the lower respiratory tract. The cause of death in animals diagnosed with pneumonic pasteurellosis is most often attributed to infection by *P. haemolytica* A1 (39). For this reason, current research has focused on this organism in an attempt to eliminate or reduce its consequences.

***Pasteurella haemolytica*-The Organism**

Pasteurella haemolytica is a gram negative, facultative anaerobe, non-motile coccobacillus which displays slight haemolysis when grown on 5% sheep blood agar (2,229) The species is divided into seventeen serotypes based on indirect hemagglutination techniques (2,283) and further divided into two biotypes based on carbohydrate fermentation, colony morphology, growth curves, and antibiotic sensitivity (224). Biotype A consists of serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16, and 17 which are arabinose-fermenting and appear as small grayish colonies (2,56). *P. haemolytica* A1 is the best characterized member of this group because of its role in the development of shipping fever. Biotype T consists of serotypes 3, 4, 10, and 15. These isolates are trehalose-fermenting and appear as larger brown colonies (2,229).

P. haemolytica A1 possesses many virulence factors. Lipopolysaccharide (LPS) (36,120,209), fimbriae (185), a polysaccharide capsule (3), a neuraminidase (81,243), a protease (197), and an exotoxin (leukotoxin) (10,120,121,221,222) are all potential virulence determinants.

In both gram-negative and gram-positive bacteria, the presence of a capsule correlates with virulence (46,82,161). Organisms that produce capsules are better protected from phagocytosis, antibody mediated opsonization, and complement-mediated bactericidal activity (27,49,256). The capsule of *P. haemolytica* is a complex polysaccharide that is primarily produced during log phase (2). The exact role of the capsule in infection is under investigation, but it is likely that the capsule is important in adhesion to alveolar and bronchiolar surfaces and in resistance to phagocytosis by neutrophils (23). Antibodies to capsular material are important in host resistance, and, therefore, the capsule may constitute an important vaccine component (39).

The LPS of *P. haemolytica* A1 is similar to the LPS of other gram-negative organisms. The LPS molecule is composed of the lipid A portion, a core oligosaccharide region, and the antigenic polysaccharide chain referred to as the O antigen. This molecule comprises up to 25% of the dry weight of the cell (124). Following colonization of the lung by *P. haemolytica*, LPS is responsible for initiating and enhancing a strong inflammatory response (36,265). This endotoxin has a wide array of biological activities including modification of metabolic activities, enhancement of phagocytic functions, alteration of migration of neutophils and macrophages, and mitogenesis of lymphocytes (36,40,66). LPS also binds to neutrophils, macrophages, endothelial cells, and the alveolar lining (40). Antibodies to the O antigen portion of LPS have been used to identify cross-reactivity among *P. haemolytica* serotypes; however, it is questionable whether antibodies to LPS enhance resistance (66).

Fimbriae are filamentous structures on the outer surface of some bacterial cells and are thought to be important virulence factors in many gram negative infections (98,126,174). These structures provide a mechanism of attachment to epithelial cells and interfere with the flushing and cleansing mechanisms of the host (15,40,174). Their role in the development of disease is speculative, but they may initiate colonization of the upper respiratory tract by acting as primary attachment factors to epithelial cells of the nasopharyngeal mucosa and tonsils (185). Another factor that enhances virulence through its role in mucosal adherence is a neuraminidase. It is postulated that this enzyme cleaves fibronectin which may enhance bacterial adherence to respiratory epithelium (81,265). Enzymatic activity has been monitored in culture fluid of bovine lungs and may serve as a marker for determining virulent *P. haemolytica* isolates (81). A neutral sialoglycoprotease is another enzyme believed to play a role in the pathogenesis of many bacterial diseases. Bacteria such as *Legionella pneumophilia* and *Pseudomonas aeruginosa* produce proteases and are associated with bacterial pneumonias which exhibit cytotoxic activity for alveolar macrophages (80,145). A recent study suggests that this protease may interact directly with bovine platelets to increase platelet activation and adhesion to induce platelet clumping. This platelet activation may be an important mediator of platelet and fibrin deposits which are observed in diseased cattle (195). The exact roles of fimbriae, neuraminidase, and the protease in the pathogenesis of shipping fever are unknown, but all are thought to contribute to the overall virulence of *P. haemolytica.*

The most important virulence factor produced by *P. haemolytica* is a potent cytotoxin. This toxin is specific for ruminant leukocytes and is referred to as a leukotoxin (10,121,221). It is believed that the leukotoxin contributes to the pathogenesis of shipping fever by impairing the primary lung defenses of the host (121,222,241). The leukotoxin is a calcium-dependent cytotoxin that damages target cells by its pore forming mechanism. The toxin must first bind calcium which enables it to then associate with the target cell (17,18). Transmembrane pores (0.9-3.0 nm in diameter) are quickly formed in the target cell. These pores act as channels for the rapid cytoplasmic efflux of potassium ions and the influx of calcium ions. The pores, however, are small enough to prevent leakage of cytoplasmic proteins (34). The cells are killed as they swell and round with loss of cytoplasmic organization and loss of nuclear integrity until they eventually rupture (10,13). The pathogenic effect of cell lysis instigated by the leukotoxin contribute to the pulmonary lesions characteristic of this disease (33,163).

The effects of the leukotoxin are multiple. The leukotoxin can kill mononuclear leukocytes as well as pulmonary alveolar macrophages and neutrophils, which function as the primary defense mechanism against bacteria that reach the lung (13). These damaged cells release proteolytic enzymes and reactive oxygen intermediates and may activate the alternate complement pathway causing damage to epithelial tissue contributing to the pathogenesis of pneumonic pasteurellosis (30,120,121,170,221,227). Increasing evidence shows that neutrophils are the primary effector cells of lung injury associated with this disease (184,227). Recent studies have indicated that IL-8 is a chemotatic factor that may be responsible for the influx of neutrophils into the alveolar milieu during the initial

stages of infection (142). IL-8 is known to be an important chemoattractant in human pneumonia (22,235) and may be a key player in neutrophil-mediated tissue injury in cattle (26). Interestingly, studies show that there is a reduction of lung damage in calves that have been depleted of neutrophils by hydroxyurea (227). The effects of the leukotoxin on leukocytes, macrophages, and neutrophils results in more than tissue damage. Impaired phagocytic function might permit bacterial colonization, and depressed lymphocyte activation could alter the induction of an antigen-specific immune response (163). Studies show that the production of leukotoxin-neutralizing antibodies correlate with resistance to experimental challenge with *P. haemolytica* and are, therefore, believed to be essential in effective vaccination approaches (91,184).

The leukotoxin is secreted into the supernatant fluid during logarithmic growth of *P. haemolytica* with the highest concentration at 6 hours post inoculation (10,29,222). The leukotoxin is encoded by chromosomal determinants with its expression modulated transcriptionally by temperature, pH, and iron concentration (241). Studies show that the leukotoxin is protein; however, amylase treatment of supernatant fluid indicates that carbohydrate moieties may be associated with the toxin (29). The leukotoxin is oxygen stable but heat labile, therefore, purification is difficult (10,29,109). Reports on sensitivity to pH extremes vary (10,29,89)

There are discrepancies concerning the molecular weight of the leukotoxin which are due to inadequate purification techniques and a strong tendency of the protein to aggregate. A number of molecular weight values have been reported ranging from 150

kDa to >700 kDa (10,109,124,188). However, the leukotoxin has been cloned and sequenced, with results indicating the molecule is a protein of 101.9 kDa (149,150).

Analysis of the leukotoxin sequence has revealed an extensive homology to toxins in the RTX, for Repeat in Structural ToXin, family (107,149), in particular the α-hemolysin of *E. coli* (149). RTX toxins are related through the presence of Gly/Asp-rich nine amino acid tandem repeats which, depending on the toxin, number from six to forty one (97). Similarities in these toxins are also due to similar genetic organization. RTX genes are arranged in polycistronic operons in the order C, A, B, D where A is the structural gene (107,149). The way in which the toxins are activated and the manner in which they are secreted are also similar (264). Other members of the RTX family include the leukotoxins of *Actinobacillis pleuropneumoniae* (28) and *A. actinomycetimcomitans* (133,143,144,148), the hemolysins of *Morganella morganii* (67,130), *Proteus mirabilis* (130,263), and *P. vulgaris* (240), and the adenylate cyclase toxin of *Bordetella pertussis* (96).

The RTX prototype and the most characterized member is the *E. coli* α-hemolysin (HLY) (240). There are differences between *E. coli* HlyA and *P. haemolytica* leukotoxin (LktA) such as target range and specificity; however, the toxin mechanisms appear to be similar (149). The Hly operon is composed of four genes designated *hlyC*, *hlyA*, *hlyB*, and *hlyD*. Similarly the LKT operon genes are designated *lktC*, *lktA*, *lktB*, and *lktD* to conform to the nomenclature.

LktC and HlyC are proteins that exhibit 50.3% homology and are 19.8 and 20 kDa respectively (149). HlyC posttranslationally modifies HlyA by fatty acid acylation of lysine residues at positions 564 and 690 (111,114,236). This modification is thought to cause a conformational change of the toxin. The exact mechanism of LktC modification of LktA is not presently known, but it is possible that the mechanisms are similar (77).

HlyB (46 kDa) and HlyD (53 kDa) are proteins involved in transporting active hemolysin through the membrane and into the medium (67,100,105,131,158). This secretion pathway, designated type I, is not well understood. Unlike proteins secreted by type II mechanisms, the RTX toxins lack a cleavable signal sequence at the N-terminus (72,105). LktB (79.7 kDa) and LktD (54.7 kDa) share 80% and 60% homologies to their hemolysin counterparts respectively (107). A similar mechanism for both proteins is also assumed for these toxins. The export of the hemolysin across the cytoplasmic and the outer membranes requires the C-terminus of HlyA, the two specific translocator proteins HlyB and HlyD, and the outer membrane protein TolC (251). It is postulated that TolC and HlyD forms a pore in the outer membrane through which the hemolysin is released and that the C-terminal sequence of HlyD interacts with HlyB to form a closed channel spanning the periplasm (219). LktB and LktD are presumed to function in the same manner.

LktA is a protein of 953 amino acids with a molecular weight of 101.9 kDa (107,149). This protein is 36.4% homologous to HlyA, which is 1023 amino acids and has a molecular weight of 107 kDa (149,159,160). This homology is low compared to

that of the other proteins, however, LktA and HlyA are highly homologous over distinct regions of the two molecules (240). The first related regions are located in the N-terminal one third of each protein. These regions contain clusters of hydrophobic domains thought to be involved in the association of the toxin with the target cell membranes (148). Studies indicated that this region of HlyA acts by forming a single hydrophilic transmembrane pore about 3 nm in diameter in target cell membranes (14,52,148,155,156). It is also believed that the hydrophobic domains of LktA function to form pores in target membranes, however, these pores are much smaller, approximately 0.9 nm in diameter (34).

The carboxyl one-third of each protein makes up another functional domain. This region contains the tandemly repeating glycine-aspartate sequences (156,240) The sequence Leu-X-Gly-Gly-X-Gly-Asn-Asp-X is repeated eleven times in HlyA and six times in LktA (240). These repeats bind calcium strongly in such a way that changes the conformation of the toxin to allow for target cell binding (71,156). Studies with α-hemolysin show that deletion of a single repeat sequence causes an increase in calcium dependence (18,156) and multiple deletions eliminate hemolytic activity altogether (18,71,155,156,240). Recent studies also indicate a region within LktA between amino acids 768-939, in which at least two repeat sequences are located, contains an epitope recognized by a leukotoxin-neutralizing mAb. This mAb is believed to neutralize the leukotoxin by preventing association with target cell membranes (92,93).

Another functional domain of the leukotoxin and α-hemolysin is the terminal 70 amino acids. This region contains the secretion signal which is not cleaved during hemolysin export (130). This region shows little homology between the two proteins although circular dichroism analyses show that both molecules exhibit common biophysical properties (286). The precise function in secretion has not been clearly defined (108,193,286). The final domain is located in the central region of the proteins, just proximal to the glycine/aspartate repeats. In HlyA, this region contains the HlyC acylation site (236) It is presumed that LktA contains a similar LktC acylation site (181,214).

Control of Pneumonic Pasteurellosis

The ideal way to prevent cattle from contracting shipping fever is to protect them from the infectious agents that cause it. However, the economics of the industry do not allow for the measures necessary to control the overcrowding and intermingling of calves that occurs at feedlots. Therefore, the most effective control of this disease is a prophylactic vaccine (187). There have been numerous strategies examined with varying degrees of success.

The effectiveness of viral vaccines have been examined. Theoretically, prevention of viral infections should reduce or eliminate secondary bacterial pneumonias that result from increased susceptibility to bacterial colonization (147). Several viral agents have been tested such as PI-3, BHV-1, and IBR (117,171,172,173,187,238,239).

Studies have shown that a correlation between high PI-3 titers and protection from shipping fever does not exist. In fact, vaccines for PI-3 may enhance susceptibility to the disease (187). Vaccination with BHV-1 or IBR did provide some protection against experimental challenge, but protection varied widely among cattle (116,238,239). Multiple virus vaccines have provided some protection against experimental challenge, but these vaccine have not been effective in field vaccine trials (171,172,173).

The most common bacterial isolate from lungs of diseased cattle is *P.haemolytica* A1 (39,79). Because of the prevalence of this organism in shipping fever cases, research has focused on the most effective way to eliminate or neutralize this factor. Emphasis has been placed on determining the type of immune responses required to provide resistance as well as determining the potential antigens involved in stimulating immunity. Vaccines have been developed that combine *P. haemolytica* with various virus vaccines. These vaccines provided protection in experimental studies but were not consistently protective in field vaccine trials (39,171,172,173). This discrepancies may be attributed to the involvement of stress factors in the field that are not present in an experimental setting.

Vaccinations with bacterins have produced results that are variable and somewhat confusing (187). The type of adjuvant that is incorporated in the vaccine is important in generating a protective response (39,187). In one study, bacterins with aluminum hydroxide gel as an adjuvant did produce antibodies to *P. haemolytica*, however, protection was not induced after challenge with the bacterium (37). Other studies indicated increased susceptibility of animals to *P. haemolytica* infections after vaccination with formalin killed bacteria (83,184). These results could be attributed to

the generation of opsonizing antibodies to surface antigens of *P. haemolytica* enhancing phagocytosis by leukocytes. This would result in cell lysis and the production of lung lesions as phagocytosed organisms continue to produce leukotoxin (223,224). Studies also indicate that bacterins with Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (IFA), but not aluminum hydroxide gel or trehalose dimycolate, enhanced resistance to *P. haemolytica* challenge (41). Overall, vaccines containing bacterins failed to stimulate immunity and were ineffective in disease prevention (106).

Live P. haemolytica cells have also been tested in vaccination trials. This method attempts to expose animals to all antigens associated with the disease, however, conflicting data have resulted. The age of the bacterial culture is extremely important with log phase cultures more effective than stationary phase cultures (42). This can be attributed to the presence of virulence factors such as capsules and leukotoxin that are produced during log phase growth (10,46). Some studies have indicated increased resistance after challenge with BHV-1/*P. haemolytica* when immunized with live *P. haemolytica* (24,136,228). This increased protection may be due to stimulation of cell-mediated immunity as well as stimulation of antibodies to antigens produced by live organisms (200). However, conflicting results by one study showed no protection against similar challenge when animals were immunized with live organisms (116). This may reflect improper handling and storage of the live product or be due to the administration of antibiotics throughout the trial (223).

Perhaps the vaccination strategy with the greatest potential is to immunize animals with bacterial components or with bacterial culture supernatant fluid. Individual

studies evaluating several of the virulence components as vaccines have shown significant correlation between high antibody levels and protection against challenge with live *P. haemolytica*. These include leukotoxin and capsular polysaccharides (27,38,40,91,180). Outer membrane proteins (OMPs) have also been used in vaccine approaches and are capable of eliciting a potentially protective immune response to experimental challenge even though their role as a virulence factor is not well characterized (113,186). OMPs have been used as immunogens for other gram-negative bacteria, so it is likely that OMP-neutralizing antibodies play a role in protection against P. haemolytica infection (162,165,226,245).

Despite the increased number and usage of various vaccination approaches, respiratory disease in cattle remains astonishingly high. Financial losses continue to rise indicating the need to develop more effective ways to stimulate immunity. There is also a need to further evaluate the immunogens used in vaccination preparations as well as a need to develop more sensitive and reproducible assays to measure the level of antibodies induced by vaccines (232). The importance of the leukotoxin as a protective antigen has been well established and even though other components appear to be necessary to generate protective immunity, leukotoxin inclusion in future vaccines seems inevitable (231,233).

This laboratory has generated several murine leukotoxin-neutralizing monoclonal antibodies, Ltx -2, -4, -35 (92,93). All of these mAbs recognized potential protective epitopes within the leukotoxin of *P. haemolytica*. Ltx-2 has proven to be a potent neutralizer. Neutralization assays show that only 30 ng/ml of Ltx-2 is necessary to

neutralize one unit (U) of leukotoxin, where one unit is the amount of leukotoxin required to kill 50% of the target cells. Flow cytometry has revealed that when leukotoxin is preincubated with Ltx-2 and then added to bovine leukocytes, no leukotoxin is detected on the target cell surface. Ltx -4 and -35 are also capable of neutralizing the leukotoxin but to a lesser extent than Ltx-2. Various methods of epitope mapping have been performed to localize the epitope of each mAb. These defined regions have proven to be important as potential candidates for vaccine incorporation.

DNA-based Vaccination Approach

Vaccination is the most effective means to control and prevent infectious diseases. To date, the majority of vaccines are based on either attenuated or inactivated pathogens (74,249). However, not all infectious agents can be safely attenuated, and inactivated vaccines often fail to induce a cytotoxic T lymphocyte (CTL) response as well as long term protection (262). Furthermore, live-attenuated vaccines may be immunosuppressive (8), cause disease if not attenuated properly (178), or provide limited immunity if severely attenuated (146). Killed vaccines are often unable to generate protective levels of immunity for reasons of antigen load and loss of important epitopes during inactivation (50). Vaccines consisting of synthetic peptides present an approach that could tailor the immune response based on the incorporation of the appropriate components. However, peptide vaccines often lack acceptable immunogenicity (78). A radical new approach to vaccinology has emerged within the past decade due to the advances in DNA-based technology. Vaccines composed of naked DNA, termed DNA-based vaccines or genetic

immunization, have been used to provide long lasting cellular and humoral immune responses to numerous antigens in a variety of animal models. The exact mechanism of immune induction is under investigation; however, this approach may address many of the shortcomings of present day vaccines.

History

The observation that purified DNA injected into mice could be expressed was made as early as 1962 (6). However, this observation remained relatively unnoticed until 1990 when Wolff *et al.* (270) demonstrated that prolonged expression could be achieved when RNA and DNA expression vectors encoding chloramphenicol acetyltransferase and β-galactosidase were separately injected into skeletal muscle of mice. Later in 1992, Tang *et al.* (248) also showed the ability to produce antibodies directed against a DNA encoded antigen, this time by the particle bombardment method using a gene gun to introduce the DNA into the skin of mice. In 1993, two studies involving plasmid DNA encoding proteins from influenza virus demonstrated for the first time the potential prophylatic use of DNA vaccines (183,211). These vaccines were able to elicit both humoral and cell-mediated immune responses as well as protect from subsequent lethal challenge. Since these initial reports, numerous studies have been reported to induce immunity to a growing number of infectious agents.

A major issue of recent studies has been to evaluate the different parameters of *in vivo* gene transfer and expression (201). The early stages of DNA vaccine development

focused mainly on testing the immunogenicity of the plasmid encoded antigen. However, the induced immune responses, both humoral and cellular, may vary greatly dependent upon the antigen. Modulation has now become the focus as researchers try to redirect or enhance the developing immune response. Various methods have been examined such as modifying the promoter, changing the route of injection, delivering DNA via vehicles, and co-administering cytokines (201).

Advantages-Risks

The use of plasmid DNA to induce protective immunity by genetic immunization has many advantages over traditional methods as well as accompanying risks. One advantage is the technical ease of generating expression vectors by the application of simple recombinant DNA methods (70). Many antigen epitopes can be cloned into available vectors in a reasonable amount of time compared to other approaches such as cloning into a vaccinia recombinant virus which could take months (70). Another benefit is the ability to produce DNA in large scale with a high level of purity and stability (201). Once purified, DNA can be lyophilized and stored at room temperature for an extended period of time which makes transport easy and inexpensive (168). In addition, there appears to be long-term antigen expression following DNA vaccination which may lead to the establishment of immunological memory as well as an enduring effector T cell response (168). Finally, the potential to encode multiple antigens in a particular vaccine is beneficial (168). Some infectious agents, such as *P. haemolytica* A1, may require several antigenic epitopes to achieve protective immunity.

Although DNA-based vaccines do not carry the risks associated with inactivated or attenuated pathogens, other safety considerations have arisen. One of the main concerns is the potential for the integration of plasmid DNA into the genome of the host cell. This integration could have potential mutagenic effects if there is a disruption of cellular genes or potential oncogenic effects if there is a disruption of genes that are involved in regulation of cell growth (64,70). Integration may occur randomly or by homologous recombination. However, this event does not appear to be likely because of the low sequence homology between the plasmids and the potentially transfected cells. Further, most plasmids do not contain an origin of replication that is functional in eukaryotic cells (194). Experiments at this point indicate that injected DNA remains episomal (54) and that integration into the host genome does not occur (70,194). In addition, some data has shown that mammalian cells can compartmentalize foreign DNA that penetrates the nucleus which may be a way a cell is able to prevent random integration of unwanted genetic material (176).

Another potential risk is the possibility that the injected DNA could elicit antibodies to itself and could therefore induce autoimmunity. However, this may prove to be a minor issue because studies have shown that bacterial DNA used in vaccinations actually suppresses autoimmune responses in lupus-prone mice (94). Further, double stranded DNA does not readily induce anti-DNA antibodies (95). Circulating nonpathogenic anti-bacterial DNA antibodies have been found in normal humans and animals, however, their specificity of reactivity is not cross reactive with mammalian DNA (210), Finally, studies involving animals immunized with plasmid DNA showed

little to no anti-DNA antibodies when measured by ELISA, immunoblot, or radioimmunoassay (118,123,273).

Vector

The level of antigen expression significantly affects the magnitude of the immune response. Therefore, the design and purification of the vector used for vaccination is critical. The basic structure of DNA vaccine plasmids consists of (1) an origin of replication suitable for high yield production in bacteria, (2) an antibiotic resistance gene suitable for selective growth in bacteria, (3) a strong eukaryotic promoter with broad tissue specificity, such as the promoter of cytomegalovirus (CMV), and (4) a mRNA transcript termination/polyadenylation sequence at the 3' end of the insert to ensure expression in mammalian cells, such as the poly A gene from bovine growth hormone (31,104).

Plasmid purification is also a critical step in vaccine preparation. Various purification methods exist. The first method is cesium chloride purification which results in a pure product, however, the procedure is time consuming and cesium chloride residue can be toxic to the animal. Another method is phenol-chloroform extraction which produces a less pure product, but is low-cost. Finally, plasmid DNA can be purified using silicon or anion exchange columns. These columns are available commercially and are much more time efficient that the other methods. Many researchers insist that the purity of the plasmid DNA effects the efficacy of gene expression. However, a recent study indicates no difference in transfection efficacy or in Ab responses to DNA-based

immunizations using plasmid DNA purified by the cesium chloride method or by anion-exchange chromatography (57).

The DNA backbone of the plasmid itself plays an important role in vaccine immunogenicity. Studies show that immunostimulatory sequences center on unmethylated CpG motifs which are more common in bacterial DNA (1/16 bases) than in mammalian DNA (1/50 bases) (16). These sequence motifs, which consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (135), have been shown to not only activate B cells (135), but also to stimulate cytokine production (103,127) and to induce specific antibodies (95). It is postulated that the host is able to discriminate between bacterial DNA and mammalian DNA based on methylation patterns and is stimulated to initiate an innate, antigen-nonspecific immune response at the site of vaccination (213,217).

Upon stimulation with the CpG motif in bacterial DNA, macrophages produce IL-12 and TNF-α (103). These cytokines regulate the production of IFN-γ, contributing to the activation of NK cells and T cells (19,285). In vitro experiments showed that CpG motifs in synthetic oligodeoxyribonucleotides (ODNs) and in plasmid used in DNA-based vaccines, induced the same cytokine profile as bacterial DNA (127). Furthermore, in vivo experiments demonstrated that the immunogenicity of a DNA-based vaccine was significantly reduced by methylating its CpG motifs and was significantly increased by coadministration of exogenous CpG containing DNA (128). CpG sequences also activate B cells and stimulate the production of IL-6 in vitro and in vivo, which contributes to the

further activation of B and T cells. (190,255). Although the molecular mechanism involved in the stimulatory effects of CpG motifs has not been clarified, they are appearing to be promising immune enhancers for vaccine applications.

Plasmid DNA Delivery

The observation that naked plasmid DNA could produce prolonged protein expression was made following intramuscular immunizations (270). Protein expression was readily detected in muscle for at least two months which led to further examination of this phenomena by the same infection route. Most early studies of DNA-based vaccines used intramuscular injections (i.m.).

Early in the development of DNA vaccines, various parameters of immunization were evaluated for their effect on the level of gene expression. Needle type, speed of injection, volume of injection fluid, type of muscle, and type of solute were examined (268). Results indicated that simple saline increased efficiency of expression (55,201,268), however, distilled water (274) and hypertonic solutions based on sucrose (58) were also effective. Drugs causing muscle damage such as buvocain (261) and cardiotoxin (55) were also shown by some investigators to improve the efficacy of genetic vaccination. Further, injection into smaller muscle groups such as the anterior tibial muscle tended to have better effects than larger muscle groups (281).

The fate of plasmid DNA after intramuscular immunizations in unclear. It is postulated based on *in vitro* studies that plasmid DNA may enter the nuclei through a nuclear pore by a process similar to other large karyophilic macromolecules (268). *In*

vivo studies show that intramuscular injections with plasmid DNA result in long term persistence of the plasmid and gene expression (267). It is thought that bacterial DNA does not replicate in muscle cells based on maintenance of its methylation pattern and that it remains extrachromosomal as indicated by electroporation of total cellular DNA from injected muscles into bacteria (267). Other cells besides muscle cells are believed to be involved in the immune induction following i.m. injections. Excision of an injected muscle bundle 10 minutes after injection does not affect the magnitude or the longevity of the antibody response (250). Infiltrating cells may play a critical role by taking up the DNA and traveling to the lymph nodes where antigen processing and presentation could initiate an immune response (70).

Particle bombardment is another method of introducing DNA into a host. This route involves the injection of DNA coated gold beads into the cytoplasm of a cell by a gene gun, a hand-held device (277). This method has several advantages over intramuscular immunizations. The first advantage is the amount of DNA required. For i.m. injections in mice, 50-100 μg are commonly used. Using the gene gun, this amount is reduced down to the nanogram range (69,248). This may be due to the direct involvement of dendritic cells which are efficient antigen presenters (250). Another advantage is the type of immune response that is induced. Intramuscular injections induce a predominantly Th1 response where as particle bombardment induces a predominantly Th2 response (73) which may be important in immunity to certain antigens. The nature of antigenic stimulation leads to the differentiation of naïve CD4+

helper T cells into the Th1 and Th2 subsets, which have relatively restricted cytokine production profiles and effector functions. Th1 cells secrete IL-2 and IFN-γ, which activates macrophages, and are the principal effectors of cell-mediated immunity and delayed type hypersensitivity reactions. The antibody isotypes stimulated by Th1 cells are effective at activating complement and opsonizing antigens for phagocytosis. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 which suppress cell-mediated immunity. The Th2 subset is mainly responsible for phagocyte-independent host defense. On the basis of potential use in man and the economy of the DNA dose, the gene gun approach is favored by some researchers (87).

More recently, investigators have begun to examine the possibility of mucosal DNA delivery. Although this method of delivery is not as efficient at eliciting a systemic immune response, the possibility of inducing a mucosal immune response is promising (164). This could prove to be critical in providing protection against pathogens which invade mucosal surfaces such as HIV, herpes simplex virus, and influenza virus (87,137,260). Secretory IgA, a hallmark of mucosal immune responses, has been induced in response to a number of antigens with the involvement of specialized inductive sites of the mucosal immune system such as the organized lymphoid tissue associated with the gut, rectum, bronchus, and nasopharyngeal/palatine tonsils (139,177).

The type of response elicited upon intranasal immunization has been shown by several groups to be predominantly a Th2 response as opposed to the predominance of Th1 responses following intramuscular immunizations (137,234). The Th2 type

responses generated in these studies may be a reflection of the action of cholera toxin B subunit (CTB) that was used as an adjuvant. Nasal or oral vaccination studies targeting mucosal immunity commonly employ CTB as a mucosal adjuvant, which is reported to induce a Th2-biased immune response (276). For this reason, CTB is not considered suitable for inducing systemic Th1-derived cell-mediated immunity. However, other adjuvants are being investigated that stimulate a Th1 type response. QS-21 saponin and plasmids expressing IL-2 and IL-12 have demonstrated the ability to enhance cell-mediated immunity (196,216,275).

Immune Response Generated by Plasmid DNA

Genetic immunization results in a full spectrum of immune responses to the expressed protein including antibodies, T helper cells, and cytolytic T cells. Administration of plasmid DNA has proven to be an effective means of generating a humoral immune response that is specific for a diverse array of proteins. The first account of an antibody response was demonstrated in mice following gene gun inoculation with DNA encoding human growth hormone and human α-1 antitrypsin (248). Antibodies against viral proteins were first generated after i.m. injections of plasmid DNA encoding influenza NP and HA (252). Other reports soon followed confirming the ability of DNA vaccination to generate humoral immune responses to a number of pathogens including viral, bacterial, parasitic, tumor, and eukaryotic proteins.

In some cases, the antibodies produced following immunization have been able to contribute to protection against challenge. This indicates that antigen expression in vivo is capable of assuming a native structure with intact epitopes able to induce neutralizing antibodies. DNA encoding a HIV envelope protein, herpes simplex glycoproteins, and influenza HA (21,119,154,166,254,258) have been able to generate neutralizing antibodies. Nonviral antigens have also protected against challenge. Plasmid DNA encoding circumsporozoite has generated protection against malaria (220) and a plasmid encoding an outer membrane protein elicited a protective response against *Borrelia burgdorferi* infection (157). In other instances, however, DNA plasmids carrying sequences from antigens of different pathogens failed to induce a measurable immune response and/or protection against a challenge (86,140,199,218).

The longevity of antibody responses induced by genetic immunization has been shown to be long-lived in mice, as exemplified by influenza NP (278), HA (119), and hepatitis B surface antigen (182). However, antibodies were more short-lived in some nonhuman primates (62,154). This is also true for protein subunit and inactivated whole-cell vaccines which may likely be a characteristic of the species in general (62). The antibody isotypes induced are generally IgG; however, serum IgM and IgA have also been detected and may be dependent on the route of immunization (59,119).

In many cases, DNA vaccines consisting of multiple discrete plasmids encoding several different antigens or one plasmid encoding several different antigens/epitopes may be desired to induce a broader spectrum of immune responses. This may be beneficial for viruses that undergo antigenic variation or to induce CTL responses against

some antigens and antibodies against others (62,283). Attempts to enhance the immune response have also included coinjection of plasmids encoding cytokines and co-stimulatory molecules. Plasmids encoding IL-2, IL-4, and granulocyte-macrophage colony stimulating factor (GM-CSF) have been used to enhance the humoral as well as cellular immune responses to various antigens that might otherwise be weakly immunogenic (32,88,125). These results suggest that this approach may be a way of modulating the magnitude and/or type of immune response induced by DNA vaccination.

An important finding following immunization is that DNA-based vaccines are capable of stimulating a cytotoxic T cell response. In early studies, MHC class-I restricted CD8+ T lymphocytes (CTL) could be detected when lymph node or spleen cells from mice injected i.m. with plasmid DNA encoding viral antigens were restimulated *in vitro* with antigen. CTL that were capable of recognizing and killing virus-infected target cells were first demonstrated by Ulmer *et al.* (252) against influenza virus and subsequently against targets infected with vaccinia-HIV gp160 (259), adenovirus-rabies virus glycoprotein recombinants (274), lymphocyte choriomeningitis virus (282), and herpes simples virus (167).

CTL responses were also induced following gene gun immunizations. Hui *et al.* (112) first demonstrated this after injection with DNA encoding the MHC antigen H-2Kk. However, gene gun immunizations may induce a Th2 type response that may suppress the CTL response to a given antigen. This was initially shown by Fuller *et al.* (85). Mice immunized three times by gene gun injections with plasmids encoding gp120 and Rev had detectable CTL responses after two immunizations but not after the third. However,

antibody responses appeared only after the third dose. The authors suggest that the suppression of the CTL response was related to a switch from Th1 to Th2, with the administration of antibodies to IL-4 blocking the loss of CTL responsiveness (85). These observations differ from studies using i.m. immunizations where cell-mediated immune responses to influenza NP (199) and HIV env (218,258) were increased following repeated immunizations with the developing response of a Th1 phenotype.

Finally, the type of T helper cell response elicited by DNA vaccines is critical to the efficacy. These T cells provide "help" to other immune cells such as macrophages, B cells, and other T cells which aid in differentiation and development of effector and memory cells. Helper T cells are grouped into functional subsets characterized by the particular cytokines they produce (85). In mice, Th1 cells produce cytokines such as IL-2 and IFN-γ and support the development of cellular immune responses and the IgG2a immunoglobulin isotype; Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 and promote immunoglobulin class switching with a predominance of IgG1 immunoglobulin isotype (64). Similar types of T cells have been characterized in humans (212).

Mechanisms of Immunity

The precise mechanism of initiation of an immune response upon genetic immunization remains to be elucidated. Questions such as how the DNA is taken up by somatic cells, where the DNA is expressed, and how antigens are processed and presented to the immune system are all under investigation.

The mechanisms of DNA uptake are not yet clear, but several studies have investigated this event following intramuscular immunizations. Hgstrom *et al.* (102) examined nonnuclear membrane-associated DNA binding proteins from skeletal muscle revealing several sacroplasmic reticulum-restricted DNA binding species. This may indicate their possible role in the transport of DNA into the sacroplasm. Further *in vitro* experiments involving the injection of plasmid DNA into primary muscle cells or cell lines have shown that DNA can enter the nuclei through the nuclear pore (65).

Several early studies demonstrated expression of β-galactosidase (270) and hepatitis B virus surface antigen (55) in myoblasts and myotubules leading to the suggestion that muscle cells process and present the antigen and stimulate a primary immune response. Furthermore, Wolff *et al.* (268) showed that among several tissue cell types, myocytes are the most efficient at expressing foreign antigens encoded in plasmids. It is interesting to note, however, that the proportion of tissue cells expressing the antigen is relatively small, in some cases, less than 100 fibers or 1% per injection site (1,55).

Cooperative mechanisms of antigen processing and presentation may be of particular importance in immune responses induced by DNA immunizations. In the case of i.m. immunizations, muscle cells only express low levels of MHC class I antigens and lack MHC class II expression as well as costimulatory molecules (110). It is also unclear if myocytes act as the only source of antigen for the immune response, particularly for antigen presentation to achieve CTL induction (168). It therefore seems likely that

resident professional antigen presenting cells, such as macrophages and dendritic cells, act as the critical factor in the induction of an immune response (138,168,201).

The ability of secreted proteins or proteins released upon cell death to be endocytosed and to enter the pathway of presentation on MHC class-II molecules is well understood. In the case of DNA vaccination, it is postulated that infiltrating APCs capture antigens released by myocytes and carry them back to the lymph nodes where they can be presented to both B and T cells (138,201). The mechanisms by which viral antigens and DNA encoded antigens are presented by MHC class-I molecules appear to be more complex. Several researchers have postulated some means by which MHC class-I restricted CTL might by induced following genetic vaccination: (1) antigen presentation mediated directly by transfected myocytes, (2) professional antigen presenting cells becoming transfected and serving as the APC, and (3) transfer of antigen from transfected myocytes to professional APC (63,201).

Studies have shown that following DNA immunization, muscle cells can produce antigen that can be presented by professional APC in an MHC class-I restricted manner. Evidence for this mechanism includes the fact that muscle cells can be directly transfected with influenza NP DNA vaccines (253) and the intramuscular route of administration yielded the best CMI-mediated protection (64). Furthermore, antigen production by muscle cells is sufficient to induce CTL responses. This was demonstrated when C2C12 myoblasts were stably transfected with DNA encoding influenza NP and then transplanted into histocompatible C3H mice (253). An H-2KK restricted CTL response was induced even though synthesis of the antigen was limited to the myocytes

(253). In addition, IFN-γ was shown in vitro to upregulate MHC class I expression and to induce MHC class II expression on a number of cells including muscle cell lines which supports the possibility of antigen presentation by muscle cells (110).

It has also been postulated that the bone marrow derived APC become transfected with plasmid DNA. This would result in endogenous production of antigen within professional APCs and presentation in association with MHC class I molecules (35). This was demonstrated when parent into F1 bone marrow reconstituted mice were immunized with DNA encoding influenza NP by i.m. injection or injected with myoblasts stably transfected with a gene encoding NP. Detectable CTL responses were seen only to the peptide presented by the MHC class I molecules found on the donor bone marrow (84). In addition, it has been shown that gene gun immunizations result in the presence of bombarded projectiles in the cytoplasm of dendritic cells in the draining lymph nodes and that the introduced gene can be expressed as proteins by these particle-containing dendritic cells (35).

The mechanism of antigen transfer between somatic cells and professional APCs remains to be defined. It has been hypothesized that somatic cells such as muscle cells act primarily as factories for the production of antigen which then is transferred to a bone-marrow derived professional APC to be processed and presented in the context of MHC class I molecules (63). Support for this theory comes from evidence where exogenous proteins accessed presentation pathways necessary for CD8+ T cell induction. Rock *et al.* (131) demonstrated the existence of a pathway in cells to shunt proteins from

phagosomes to the cytosol where they can enter MHC class I associated pathways. In addition, peptides may be transported out of cells by chaperones such as HSP (5), and it has been suggested that certain APCs may express receptors for HSP-complexed peptides (246).

Potential Adjuvants

Successful vaccination against any potential pathogen is dependent on the induction of an appropriate immune response. Adjuvants have been used for the purpose of augmenting the immune response for the past 70 years (205). The term adjuvant defines a substance used in combination with a specific antigen that produces more immunity than the antigen alone (205). The number of molecules used for immunostimulatory purposes is enormous, and the substances are widely varied. Despite the number of available adjuvants, there is still a need for more effective immunomodulators due in part to the development of subunit vaccines which, although safer than inactivated or attenuated pathogens, are less immunogenic (68).

Cytokines are proving to play a critical role in augmenting the immune response to a given vaccine (151,247). Of particular interest is interleukin-1 (IL-1), a family of proteins involved in the development and maintenance of immunologic and inflammatory responses (61). IL-1 is a key player in regulating the immune response. It is involved in antigen-induced T cell activation and clonal expansion (230), as well as B cell differentiation and proliferation (202). *In vivo* administration of IL-1 enhances the immune response to various antigens (152,207,237) and also protects the host from lethal

challenge with microorganisms (54,198). However, IL-1 is also involved in the regulation of inflammatory effects such as pyrogenicity and acute phase responses (60,247). IL-1 is able to induce neutrophils and the release of prostaglandins and proteases (60) and is involved in the development of chronic inflammatory conditions such as rheumatoid arthritis (271). Although IL-1 possess immunostimulatory activities, these numerous side effects associated with proinflammatory actions makes IL-1 an unsafe adjuvant candidate.

After the cloning of IL-1, the possibility of exploiting IL-1's adjuvant effects was re-evaluated using recombinant material (7,152). Hydrophilicity and acrophilicity profiles of human IL-1β indicated two portions of the molecule likely to be found in well-exposed areas of the molecule (4). Corresponding synthetic peptides were examined for their ability to activate T lymphocytes in classic murine thymocyte proliferation assays. A nine amino acid peptide VQGEESNDK at positions 163-171 was identified as a potential immunostimulatory molecule. Furthermore, this peptide was shown to be devoid of the strong inflammatory activity associated with IL-1 due to its inability to stimulate prostaglandin E2 production (4). Additional experiments have demonstrated the stimulatory effect of this 9mer on antibody responses to both T helper-dependent and T helper-independent antigens, again with no pyrogenic and inflammatory effects (192).

Although hIL-1β 163-171 demonstrated effective immunomodulatory activity with no adverse side effects, the excessive amounts of the peptide required to elicit a significant enhancement in antibody response limits its applicability as an adjuvant. Rao

et al. (206) addressed this problem by direct coupling the adjuvant peptide and the antigenic peptide utilizing two glycine spacers to maintain the integrity of the two peptides. When presented as a composite peptide, the amount of IL-1 163-171 was 50 times lower than that of free peptide used by Necioni et al (192,206). Furthermore, Beckers *et al.* (12) employed the strategy of constructing a hybrid synthetic antigen in which immunogenic epitopes are convalently linked to the 9mer adjuvant. They inserted complementary oligonucleotides encoding IL-1 163-171 into antigen expressing plasmids and purified hybrid proteins. These approaches open the possibility of designing recombinant vaccines with "built in " adjuvanicity.

The molecular mechanisms underlying the activity of the IL-1 peptide are poorly understood. Circular dichroism studies indicate that the peptide possesses a β-turn secondary structure which is probably involved in T cell stimulation and antigenicity (225). *In vitro* experiments show that the 163-171 fragment is not an IL-1 receptor (IL-1R) domain and that it probably enters the cell through a receptor-independent mechanism, possibly by passive diffusion though the cell membrane (20). Once inside the cell, the peptide is localized in the cytosol where it can bind cytoplasmic proteins and initiate an intracellular pathway of cell activation which is independent of IL-1R triggering (20).

Another peptide sequence also has been described to have immunopotentiating properties. The 14mer peptide, ETVIMKAKPRANFP, inhibits the acquisition of M-CSF competence by bone marrow progenitors stimulated *in vitro* with bacterial LPS or TNF-α

(134). Similar observations have been made *in vivo* with peptide treatment of mice with no observable inflammatory side effects (Moore, unpublished data). Further experiments using rat lever ferritin (RLF) and ovalbumin (OVA) as antigens examined the potential use of this peptide for adjuvant purposes. Results indicate that the 14mer peptide significantly augmented IgG production in response to RLF as well as OVA, a less immunogenic protein, when injected with the antigen as free peptide. In addition, prior exposure to the peptide did not influence the primary response to the antigen with the peptide suggesting that the peptide does not act directly as a specific helper cell epitope (Moore, unpublished data).

The 14mer peptide sequence was taken from the H chain of human acidic isoferritin which was first cloned and sequenced by Constanzo *et al.* (47) in 1984 with the peptide corresponding to amino acids 172-185. In 1986, a reinvestigation of the original published sequence revealed mistakes at three positions which investigators conclude as artifacts (48). Ferritin itself is the major iron storage protein of eukaryotes and is found in most tissue (11). The protein consists of two subunit types, H and L, which share extensive homology but are derived from different gene families (115). This adjuvant peptide contains a four amino acid sequence Ala-Lys-Pro-Arg which is similar to the tufsin sequence Thr-Lys-Pro-Arg. Tufsin is a naturally occurring molecule with regulatory properties (191) and has been shown to stimulate phagocytes and their many functions. It is possible that this four amino acid sequence plays a role in the observed immunomodulatory effects of the entire peptide; however, *in vivo* experiments have shown that the intact 14mer peptide is necessary for augmentation of the immune

response and that the four amino acid peptide cannot substitute for the whole peptide (Moore, unpublished data).

Statement of Purpose

The purpose of this study has been twofold. The first goal of this study was to investigate the potential of generating humoral responses in mice following DNA-based vaccination with a leukotoxin fragment-encoding plasmid. This leukotoxin fragment contains the epitope believed to be recognized by the potent neutralizing monoclonal antibody, Ltx-2. Furthermore, this fragment is capable of inducing neutralizing antibodies in rabbits when injected as a fusion protein with glutathione-S-transferase (GST) (179).

The second goal of this study was to examine the potential immunostimulatory effects of human IL-1β 163-171 and human acidic isoferritin 172-185. These adjuvant sequences were incorporated into DNA-based vaccines as well as recombinant protein vaccines and were monitored for their ability to augment the antibody response in immunized mice.

CHAPTER 2

MATERIALS AND METHODS

Bacterium

Pasteurella haemolytica biotype A serotype 1 (isolate 853788), provided by Dr. Robert D. Walker (Michigan State University, East Lansing MI), was originally 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. The organism was grown on brain heart infusion agar (BHI, Grand Island Biological Company, GIBCO, Gaithersburg, MD) and 5% defibrinated sheep blood at 37°C (257).

Escherichia coli strains DH5α (GIBCO) and JM109 (Promega, St. Louis, MO) were used for transformations and plasmid propagation. Cell stocks were prepared from cultures grown in Luria-Bertani (LB) broth (Fisher Scientific, Pittsburgh, PA) to an OD_{595} of 0.5 by centrifugation (Beckman J-21 centrifuge, Beckman Instruments Inc., Palo Alto, CA) at 5,000 x g for 10 min. The cell pellet was resuspended in glycerol-based media (50 ml glycerol, 2 g tryptone, 1 g yeast extract, 1 g sodium chloride, 150 ml deionized water), aliquoted, and stored at -80°C.

Cell Culture

A bovine leukemia-derived B lymphocyte cell line BL-3 (American Type Culture Collection #CRL 8037) was used in this study. The cells were maintained in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2000 U penicillin/2.0 mg streptomycin per liter of medium, 40 mM L-glutamine (psg, Sigma), and 0.2% sodium bicarbonate (Mallinckrodt, Paris, KY). The cells were grown at 37°C in humid air of 5% CO_2 and were split at a 1:10 dilution every 3 to 4 days.

The hybridoma clones ltx-2, ltx-4, and ltx-35 were produced by Mark Cameron and Donald Gerbig of this laboratory (92,93). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% FBS, 2000 U penicillin/2.0 mg streptomycin per liter of medium, and 40 mM L-glutamine. The cells were grown at 37°C with 5% CO_2 and were split at a 1:10 dilution every 3 to 4 days.

Clone 929 Strain L (L929) of mouse connective tissue (American Type Culture Collection CCL1) was used in transfection studies. This adherent fibroblast cell line was maintained in DMEM (Sigma) supplemented with 10% FBS, 2% psg, and 0.2% sodium bicarbonate as before. The cells were grown at 37°C in 5% CO_2 and were split when cells were confluent, every 4 to 5 days. Briefly, cells were washed with Hanks Balanced Salt Solution (HBSS, GIBCO) and treated with trypsin-EDTA (0.05% trypsin, 0.53mM EDTA· 4Na, GIBCO) to dislodge the cells. Subcultures were prepared by shaking and added to fresh media at a dilution of 1:6.

Leukotoxin Production

Leukotoxin contained in the supernatant of log phase cultures was prepared using a modification of the protocol described by Shewen *et al.* (222). Briefly, 500 ml BHI broth (DIFCO Laboratories, Detroit, MI) was inoculated with several colonies from an overnight culture of *P.haemolytica* A1 grown on a blood agar plate and incubated for approximately 4.5 h at 37°C in a shaking water bath (Orbit Environ-Shaker, Lab-Line Instruments Inc., Melrose Park, IL). The culture was then centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was discarded and the remaining pellet was resuspended in sterile phosphate buffered saline, pH 7.0 (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.3 mM sodium phosphate, and 1.8 mM potassium phosphate monobasic). The bacteria were centrifuged again for 20 min at 10,000 x g at 4°C, and the supernatant was discarded. The remaining bacterial pellet was resuspended in RPMI-1640 (Sigma) at a concentration of $4x10^8$ CFU/ml according to a spectrophotometric determination. This concentration was determined based on a linear regression line produced by Donald Gerbig in which an OD_{595} of 1.2 is equivalent to $4x10^8$ CFU/ml. This suspension was incubated in a 37°C shaking water bath for an additional 1.5 h and then centrifuged as before. The supernatant fluid was filtered through a 0.2 μm filter (Nalge Company, Rochester, NY) and stored at -80°C. Cultured filtrates were then concentrated 100-fold using a Pellicon concentrator equipped with four 30 kDa exclusion membranes (Millipore, Bedford MA), aliquoted, and stored at -80°C.

Monoclonal Antibody Production

Leukotoxin monoclonal antibodies (mAbs) were generated by the production of ascitic fluid in mice. Four to six week old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were injected with 0.2 ml of 2,6,10,14-tetramethydecanoic acid (Pristane, Sigma) intraperitoneal (i.p.) on day 0 and day 14. On day 28, mice were injected i.p. with hybridoma cells at a concentration of $5x10^6$ cells/ml in 0.5 ml PBS, pH 7.4. Within 7-10 days, ascitic fluid developed and was collected by peritoneal puncture using a 18 gauge needle while animals were anesthetized with methoxyflurane (Metafane, Pitman-Moore, Inc., Mundelein, IL). The obtained ascitic fluid was then mixed at a ratio of 1.5 to 1.0 with Seroclear® Reagant (Calbiochem Corp., La Jolla, CA). The mixture was centrifuged for 10 min at 3,000 x g (International Instruments Co., Boston, MA). The top layer of lipid free ascitic fluid was then removed, and the pH was adjusted to 6.0-7.5 with wash buffer, pH 7.0 (0.01 M sodium phosphate, 0.15 M sodium chloride).

For purification of IgG, the collected ascitic fluid was passed through a Gamma Bind® PLUS Prepack column (Genex Corp., Gaithersburg, MD). The column was washed with wash buffer until the OD_{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). The collected IgG was dialyzed against three changes of PBS, and the protein concentration was determined by a

colormetric method using bicinchoninc acid (BCA, Pierce, Rockford, IL) method. Purified antibodies were aliquoted and stored at -80°C.

Enzymes

Restriction endonucleases, DNA *Taq* polymerase, and DNA modifying enzymes were purchased from GIBCO.

Synthetic Oligonucleotides

Synthetic oligonucleotides used as primers were purchased from Genosys Biotechnologies (The Woodlands, TX). Primer sequences are listed in Table 1. Synthetic oligonucleotides encoding the adjuvant sequences to be incorporated into plasmid constructs were also purchased from Genosys Biotechnologies and were purified by reverse phase cartridge. Oligonucleotide sequences are listed in Table 2.

Polymerase Chain Reaction

A portion of the leukotoxin A gene (*lkt A*) of *P. haemolytica* A1 was amplified by the polymerase chain reaction (pcr). The reaction mixture contained 1 µl genomic DNA (1 µg/µl), 5 µl 5' primer (5 ng/µl), 5 µl 3' primer (5 ng/µl), 5 µl 10X pcr buffer (GIBCO), 1.5 µl magnesium chloride (50 mM, GIBCO), 4 µl deoxynucleoside-triphosphate mixture (dNTPs, 10 mM each, GIBCO), 0.5 µl *Taq* DNA polymerase (GIBCO), and 28 µl deionized water. The above were mixed in a 0.5 ml eppendorf tube (Fisher Scientific)

Table 1. Primers used to amplify gene fragments from *P. haemolytica* leukotoxin.

	Primer	Direction	Location	Sequence
pcDNA3.1 construct (pclkt)	HindIII 707-719	Forward	2120-2155	CCTTGAAAGCTTTTGAA GCAACTATGGGTACATC AC
	XhoI InterAB	Reverse	2857-Inter-genic region	GAAACAATCTCGAGTTG CCAATCATTCAAAATTA AGC
pGEX constructs (pGlkt.9, pGlkt.14, pGlkt.14scr)	EcoRI Pinsert	Forward	pcDNA mcs-lktA 2155	GCGTTTAAACTTAAGCT GTTGAAGAATTCATGGG TAC
	XhoI Pinsert	Reverse	lktA 2875-pcDNA mcs	GGCAACTCGAGTCTACA GGG

Underlined sequences denote restriction sites.

Table 2. Oligonucleotides encoding adjuvant peptide sequences.

Adjuvant	Direction	Sequence
9mer peptide	Forward	atcGGGGGAGTACAAGGAGAAGAAA GTAATGACAAAGGAGGG
	Reverse	CCCTCCTTTGTCATTACTTTCTTCTCC TTGTACTCCCCCgat
14mer peptide	Forward	atcGGGGGAGAGACAGTGATAATGAA AGCTAAGCCTCGGGCTAATTTCCCA GGAGGG
	Reverse	CCCTCCTGGGAAATTAGCCCGAGGCT TAGCTTTCATTATCACTGTCTCTCCCC Cgat
14mer peptide scrambled (control)	Forward	atcGGGGGAAAGTTCGAGCGGACAGCT CCAATAGCTATGGTGCCTAATAAAGG AGGG
	Reverse	CCCTCCTTTATTAGGCACCATAGCTAT TGGAGCTGTCCGCTCGAACTTTCCCC Cgat

Lower case sequences denote oligos that recreate the EcoRV restriction site.
Underlined sequences denote oligos that encode for glycine residues used as spacers.

and overlaid with sterile mineral oil (Sigma). The reaction was carried out in a DNA Thermocyler (Perkin Elmer Cetus, Norwalk, CT) under the following conditions: one cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min, followed by one cycle of 72°C for 10 min. Following amplification, the resulting products were electrophoresed on a 1% agarose gel containing ethidium bromide (Mallinckrodt). The visualized band was excised from the gel and purified using the Geneclean II kit (Bio 101, Inc., Vista, CA) and stored at -20°C.

Plasmids

The mammalian expression plasmid pcDNA3.1(+) (Invitrogen, San Diego, CA) was used in the DNA-based vaccine studies with the gene of interest under the control of the cytomegalvirus (CMV) promoter. Figure 1.

The vector pGEX 4T-1 (Pharmacia Biotech) was used to generate recombinant proteins by expression in a bacterial system. The multiple cloning site immediately follows the glutathione-S-transferase gene of *Schistosoma japonicium* with the resulting product being expressed as a fusion with GST. Figure 2.

Plasmid Manipulation

The pcr amplified *lkt A* fragment and the plasmid pcDNA3.1 were digested with the restriction enzymes *HindIII* and *XhoI* at 37°C for 2 h. To ensure complete cleavage, enzymes were added a second time and digested for an additional 2 h. An aliquot of each

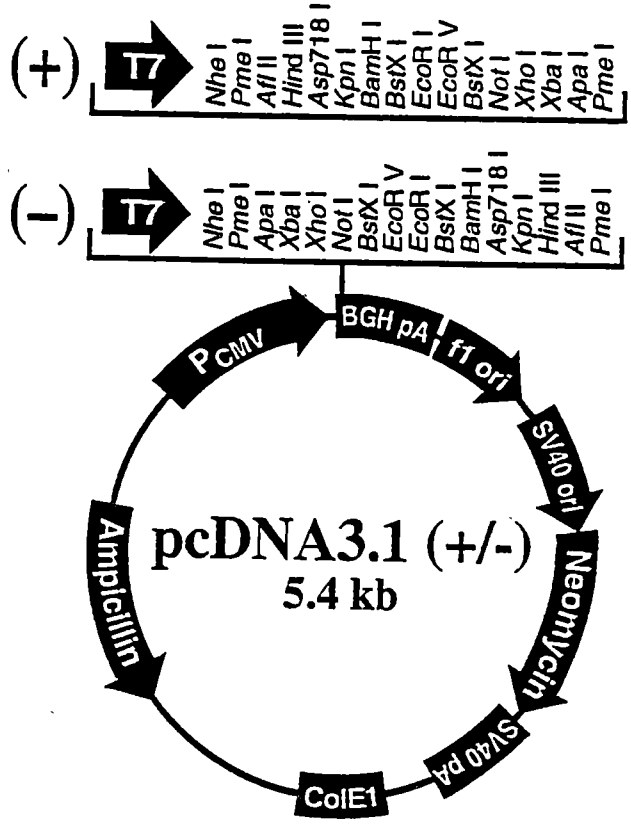

Figure 1. Diagram of pcDNA3.1 vector used in DNA-based vaccination.

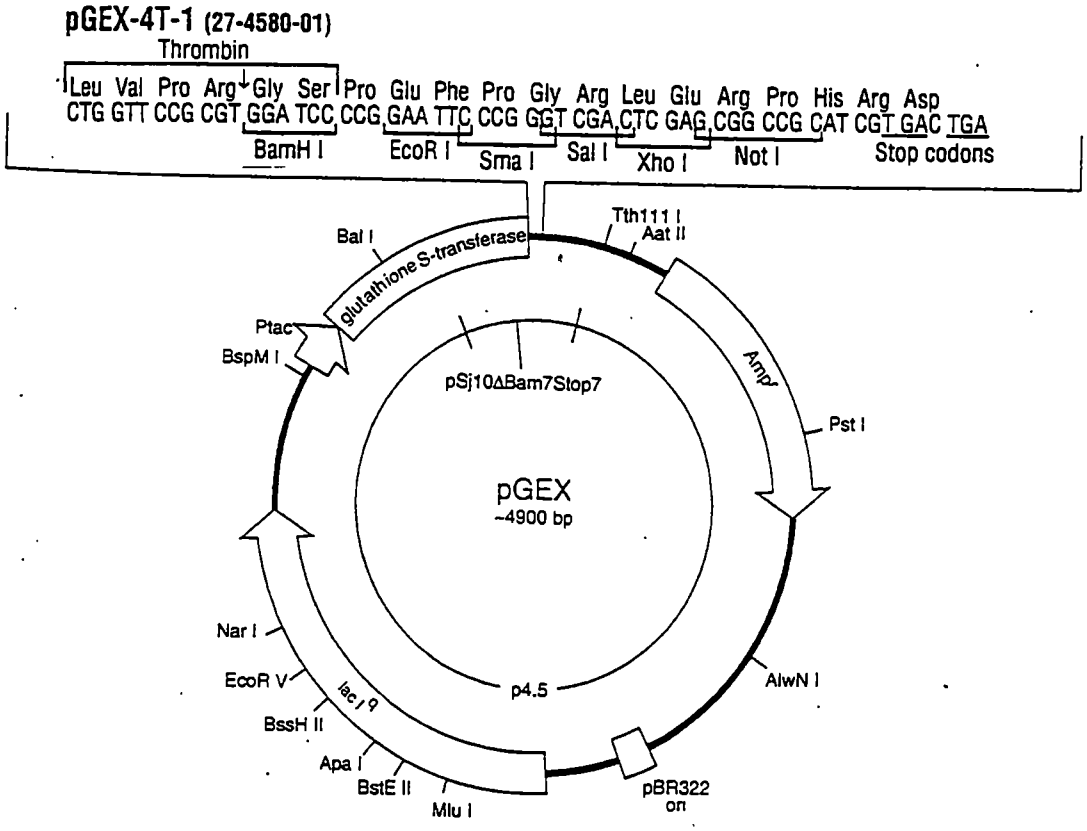

Figure 2. Diagram of pGEX-4T-1 vector used to generate GST fusion proteins.

reaction was run on a 1% agarose gel to confirm cleavage. Enzymes and salts were removed from the remaining mixtures by ethanol precipitation. Briefly, 0.1 volume of 3M sodium acetate (Fisher) and 2 volumes ice cold ethanol were added to the mixtures and incubated on dry ice for 30 min. Following centrifugation at 14,000 x g for 30 min at 4°C, the DNA was washed with 70% ice cold ethanol and recentrifuged. The DNA pellet was vacuum dried (Savant Instruments, Inc., Farmingdale, NY) and resuspended in sterile deionized water. The digested pcDNA3.1 and the digested pcr product were ligated in a mixture containing T4 DNA ligase and 5X ligase buffer (GIBCO) at 16°C overnight. The ligated product was used to transform competent *E. coli* DH5α cells.

Competent cells were prepared by the $CaCl_2$ method (215). Cultures were grown to an OD_{595} of 0.6, chilled for 10 min, and centrifuged at 5,000 x g at 4°C for 10 min. The pellet was resuspended in ice-cold 10mM magnesium sulfate (Mallinkrodt) and incubated on ice for 10 min. Cells were centrifuged as before and resuspended in 50mM calcium chloride (Fisher) and 10mM Tris, pH 8.0. After a 15 min incubation on ice, the cells were centrifuged, and the final pellet was gently resuspended in 50mM calcium chloride and 10mM Tris, pH 8.0.

The heat shock method was used to transform competent cells (215). The ligation mixture was added to the competent cells, placed on ice for 30 min, and then heat shocked at 42°C for 2 min. 2X yeast extract tryptone media (2X-YT, 16 g tryptone, 10 g yeast extract, 5 g sodium chloride per liter, pH 7.0) was added to the mixture and incubated at 37°C for 1 h shaking. Cells were then plated on 2X-YT agar with 100μg/ml

ampicillin (Sigma) and incubated overnight at 37°C. Individual colonies were subcultured and screened for the presence of pcDNA3.1 containing the inserted *lkt A* fragment.

Screening of the colonies was done by performing DNA mini-preps (Wizard™ Minipreps DNA Purification System, Promega). The isolated DNA was digested with *HindIII* and *XhoI* and electrophoresed on a 1% agarose gel. An excised DNA fragment of the appropriate size was assumed to be positive and was submitted for DNA sequencing.

The constructs containing the adjuvant sequences were generated in the pcDNA3.1 vector containing the *lkt A* fragment (pclkt). The unique *EcoRV* restriction site, which is cleaved between nucleotides 2163 and 2164 of the *lkt A* fragment, was used for an in-frame insertion of the adjuvant encoding oligonucleotides. The pclkt plasmid was digested with *EcoRV* for 2 h and then ethanol precipitated as previously described. Synthetic oligonucleotides were annealed in a mixture containing 2 µl 5' oligo (500 mM), 2 µl 3' oligo (500 mM), 5 µl dH20, and 1 µl sodium chloride (5 M). The mixture was incubated at 90°C for 2 min, 37°C for 5 min, and room temperature for 10 min. The annealed oligonucleotides were ligated into the linearized plasmid by T4 DNA ligase at 14°C overnight. The ligated product was used to transform competent DH5α cells as described previously.

Positive colonies were verified by pcr screening. Individual colonies were picked with sterile toothpicks and placed in a 25 µl reaction mixture containing 2 µl dNTP mix (10 mM each), 2.5 µl 5'primer (5 ng/µl), 2.5 µl 3' primer (5 ng/µl), 2.5 µl 10X pcr buffer,

0.75 µl MgCl (50 mM), 0.25 µl DNA *Taq* polymerase, and 14.5 µl dH_2O. The 5' oligonucleotides encoding the adjuvant sequence were used as the 5' primer in this reaction and the internal primer *XhoI* 936-943 was used as the 3' primer. The pcr cycles were the same as before and the resulting products were run on a 1% agarose gel and visualized with ethidium bromide. Samples with a band of the appropriate size were assumed to be positive and were submitted for DNA sequencing.

Constructs containing the *lkt A* fragment with and without the adjuvant sequences were also generated in the pGEX-4T-1 vector for the purpose of recombinant protein generation. Inserts could not be cleaved from the corresponding pcDNA 3.1 constructs utilizing the designed restriction sites *HindIII* and *XhoI* and inserted into the pGEX vector because a *HindIII* site is not contained in the multiple cloning site of pGEX. Therefore, a new 5' *EcoRI* site was designed by pcr primers in the same region as the *HindIII* site. The *lkt A* fragments were amplified from the respective pcDNA3.1 construct with the new 5' primer and a new 3' *XhoI* primer that extended from the leukotoxin fragment through the beginning of the multiple cloning site under pcr conditions as previously described.

Following amplification, the insert and pGEX plasmid were digested with the restriction enzymes *EcoRI* and *XhoI* and ethanol precipitated as described before. The digested insert and plasmid were ligated at 16°C overnight. The ligated product was transformed into *E.coli* JM109 and colonies were screened for the presence of the correct construct by Wizard™ Minipreps (Promega) followed by restriction enzyme digestion.

Samples with a fragment of the appropriate size when visualized on an ethidium bromide stained agarose gel were submitted for DNA sequencing.

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.).

Plasmid Purification

Large scale DNA preparation was performed by using Wizard™ Plus Maxipreps DNA Purification System followed by phenol-chloroform (Sigma) extraction and ethanol precipitation. The DNA concentration was determined using a GeneQuant spectrophotometer (Pharmacia). The purity of the samples was determined by a OD_{260}/OD_{280} ratio of 1.8 and by visualization on a 1% agarose gel.

Protein Purification

Recombinant proteins were expressed in *E.coli* JM109 cells containing the pGEX-4T-1 plasmid constructs. An overnight culture was grown in Luria-Bertani (LB) broth supplemented with 50 μg/ml ampicillin (Sigma). 500 ml of prewarmed media was inoculated with 5 ml of the overnight culture and incubated at 37°C in a shaking water

bath. At mid-log phase (OD_{595} approximately 0.6) the culture was induced for protein expression with isopropyl-β-D-thiogalactoside (IPTG, Gold Biotechnology, Inc., St. Louis, MO) adjusted to 0.1 mM. The culture was incubated for an additional 75 min under the same conditions. The culture was centrifuged at 5,000 x g for 10 min at 4°C, and the supernatant medium was discarded. The cell pellet was resuspended in ice-cold PBS, and cell lysis was obtained by sonication (Fisher Model 60 Sonic Dismembrator, Fisher Scientific). Following sonication, the suspension was centrifuged at 14,000 x g for 30 min at 4°C, and the supernatant fluid was filtered through an Acrodisc PF 0.8/0.2 μm Supor filter (Gelman Sciences, Ann Arbor, MI).

Glutathione Sepharose 4B (Pharmacia Biotech) was added to the filtered suspension and incubated overnight at 4°C. Suspensions were then added to a Poly-Prep chromatography column (BioRad Laboratories, Hercules, CA). After extensive washings with PBS, fusion proteins were eluted from the matrix with 10 mM reduced glutathione (Sigma). The eluate was dialyzed overnight in 4 L PBS at 4°C. The protein concentration was determined by BCA and the purity was determined by a Coomassie Blue stain (0.5% Coomassie Brilliant Blue R-250, BioRad, 5% methanol, and 7.5% acetic acid, Mallindrodt) of a SDS/PAGE gel. Fusion proteins were stored at 4°C for up to two weeks.

Western Immunoblot

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) using a Mini Protean II gel apparatus (BioRad) was performed according to the method described by Laemmli (141). Stacking gels of 4% and separating gels of 10% (acrylamine/ bisacrylamide 30:0.8, National Diagnostics, Atlanta, GA) were used. Protein samples were diluted in PBS and 2X sample buffer (2.5 ml 4X Tris-HCl pH 6.8, 2.0 ml glycerol,0.4 g SDS, 1 mg bromophenol blue, 5.5 ml dH_20) and electrophoresed at a constant amperage of 14 mA for approximately 2.5 h.

Proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) using a semi-dry protein transfer apparatus (Gelman) set at 175 mA for 90 min. Unbound sites on the membrane were blocked with 3% Nonfat Dry Milk (Carnation Co., Los Angeles, CA) in PBS with gentle agitation at 37°C for 30 min. The primary antibody (leukotoxin mAbs) used to detect the protein of interest was adjusted to 20 μg/ml in PBS-0.05% Tween-20 (Sigma) and incubated overnight at room temperature. After three washings with PBS-Tween, HRP recombinant Protein G (Zymed) at a dilution of 1:2000 was added to the membrane and incubated at 37°C for 90 min. The membrane was then washed three times with PBS-Tween and developed with HRP Color Development Reagent (BioRad).

Transfections

L929 cells (ATCC) were transiently transfected with pcDNA3.1 or pclkt using LIPOFECTAMINE™ Reagent (GIBCO) according to the manufacturer's protocol to ensure the expression of the leukotoxin fragment. To optimize transfections conditions, L929 cells were first transfected with the plasmid pCMVβ (Clontech, Palo Alto, CA) and stained for β-galactosidase activity. pCMVβ is a mammalian reporter vector designed to express β-galactosidase from the cytomegalovirus gene promoter. A six-well plate (Fisher) was seeded with the adherent fibroblast cell line and grown to 50-80% confluency. Plasmid DNA (1-2 μg) was diluted in 100 μl of serum-free DMEM and mixed with 2-25 μl of LIPOFECTAMINE™ Reagent also diluted in 100 μl of serum free media. The solution was incubated at room temperature for 30 min to allow the DNA-liposome complexes to form. Additional media (0.8 ml) was added to the mixture and then overlaid onto the cells. The cells with the complexes were incubated for 2-24 h at 37°C in 5% CO_2 and then assayed for gene activity at 24 h, 48 h, and 72 h post-transfection. Briefly, cells were washed twice with PBS, and fixed with 0.25% glutaraldehyde (Sigma). Following a 15 min incubation, the cells were washed three times with PBS. X-Gal solution (0.2% X-Gal, 2mM $MgCl_2$, 5mM $K_4Fe(CN)_6 \cdot 3H_20$, 5mM $K_3Fe(CN)_6$, Fisher) was then added to the washed cells and incubated at 37°C between 1-16 h until the cells were visibly stained. Once the transfection conditions were optimized, L929 cells were transfected with pcDNA3.1 or pclkt. Briefly, 1-2 μg of plasmid DNA and 12 μl of LIPOFECTAMINE™ were used as previously described. The

cells with the complexes were incubated for 5 h at 37°C in 5% CO_2, and then assayed for leukotoxin expression at 24 h, 48 h, and 72 h.

To verify gene activity, the cells were assayed for the expressed intracytoplasmic leukotoxin fragment. The tissue cultures were washed twice with PBS and overlaid with 100 µl of NTE buffer (20 mM Tris, pH 7.4, 1 mM EDTA, pH 7.5, 0.05% Aprotonin (Sigma), 0.2 mM phenylmethylsulfonylfluoride (PMSF, Sigma), 150 mM NaCl, and 0.1% SDS). Following a 5 min incubation on ice, 100 µl of 2% Triton X-100 (in NTE buffer) was added to the cells and incubated as before. The plate surface was scraped using a cell scraper (Fisher) to collect the cell lysates which were transferred to a microcentrifuge tube. The extracts were then centrifuged at 12,000 rpm for 5 min at 4°C and the supernatant was stored at -80°C.

An immunoblot was performed to detected the expressed leukotoxin fragment. The cell lysates were blotted onto a PVDF (Millipore) membrane and nonspecific protein binding sites were blocked with 3% Nonfat Dry Milk (Carnation). The membrane was then probed with mAb ltx-2, -4, and -35 followed by the addition of HRP recombinant Protein G. Color development was performed using HRP Color Development Reagent (BioRad).

Immunizations

For protein-based immunizations, female BALB/c mice were immunized i.p. with 100 µg of the indicated fusion proteins in 200 µl of PBS. LktA and JM-2 were

emulsified in CFA and served as positive controls. Mice were immunized on day 0 and boosted on day 21 and day 42. Serum samples were collected on days 12, 28, and 49, and assayed for leukotoxin-neutralizing antibodies and for anti-leukotoxin titers.

For DNA-based immunizations, pcDNA3.1 contructs were injected by the intramuscular (i.m.) or intranasal (i.n.) route. For i.m. immunizations, mice were anesthetized with metafane (Pitman-Moore, Inc.) and 100 μg of plasmid DNA in 50 μl sterile saline was injected into the quadriceps and tibialis muscles with a 28 gauge insulin syringe (Becton Dickinson & Company, Franklin Lakes, NJ) at day 0 and boosted with the same dose at day 28 when indicated. For i.n. immunizations, mice were anesthetized as before and 100 μg of plasmid DNA, with and without 2 μg of cholera toxin subunit B, was dropped into the nares at 20 μl per nare.

Blood Collection

Blood was collected from mice at the indicated time points using several techniques. Non-terminal bleeds were performed on anesthetized mice by introducing a microhematocrit tube (Fisher) into the medial canthus of the orbital sinus until blood entered the tube. Mice injected i.m. with plasmid DNA and with recombinant proteins were terminally bled by decapitation under metafane anesthesia. Intranasally immunized mice were terminally bled by eye removal under anesthesia and then sacrificed by cervical dislocation. Tracheal lavages were also performed on mice immunized in with plasmid DNA. Briefly, a midline incision was made and, with the skin retracted, the

trachea was exposed. Using a 1cc syringe with a 25 gauge needle (Fisher), 500 µl of sterile saline was washed through the trachea and the lavage fluid collected. Blood and tracheal lavage samples were incubated at 4°C overnight and centrifuged for 15 min at 10,000 g. Serum and tracheal fluids were collected and stored at -20°C.

Ammonium Sulfate Precipitation of Rabbit Antisera

The use of rabbit anti-leukotoxin polyclonal sera in enzyme linked immunosorbent assays (ELISA) required partial purification of IgG antibodies. The generation of the rabbit polyclonal sera was performed by Jack McPherson (179) of this laboratory. Large protein aggregates were preprecipitated by mixing 0.5 volume of saturated ammonium sulfate (Sigma) with the antisera. The mixture was incubated overnight at 4°C while stirring and then was centrifuged at 3,000 x g for 30 min. The remaining supernatant was again mixed with 0.5 volume of saturated ammonium sulfate and incubated as before. The suspension was centrifuged at 3,000 x g for 30 min and the pellet was resuspended in 0.3-0.5 the original antisera volume PBS. The antibody solution was dialyzed against three changes of PBS, aliquoted, and stored at -20°C.

Antibody Detection

Enzyme linked immunosorbant assays (ELISAs) were performed to determine the presence of leukotoxin-specific IgG antibodies in mouse sera. Direct ELISAs were used to detect protein-specific antibodies in sera obtained from mice immunized with plasmid

DNA. The leukotoxin A fragment-GST fusion protein was adjusted to 20 μg/ml, and 50 μl was added to each well of 96-well polyvinyl chloride plates (PVC plate, Dynatech Laboratories, Chantilly, VA) and incubated overnight at room temperature in a humid chamber. The plates were then washed three times with PBS-0.05% Tween-20 and the remaining protein sites were blocked with 100 μl 3% Nonfat Dry Milk (Carnation) for 2 h at 37°C. The plates were washed as before, and the desired serum dilutions were then added to the appropriate wells and serially diluted. After an incubation of 2 h at 37°C, a 1:3000 dilution of HRP rabbit anti-mouse IgG (gamma) (Zymed) was added. The plates were incubated at 37°C for 2 h and then developed by the addition of 2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS, Zymed). Following an incubation of 30 min in the dark, the absorbance at 405 nm was determined by a microtiter plate reader. All dilutions were performed in PBS and all washings were performed with PBS-0.05% Tween-20.

A trap ELISA was performed to assay for protein-specific IgG antibodies in mice immunized with recombinant proteins. These animals were injected with GST-fusion proteins, and, therefore, the direct ELISA protocol using the leukotoxin A fragment-GST fusion protein as the antigen could not be used. Rabbit anti-leukotoxin antibodies generated by Jack McPherson (179) of this laboratory and further purified by ammonium sulfate precipitation were used as the trapping antibody. Briefly, rabbit anti-leukotoxin antibodies at a dilution of 1:3000 in PBS were added to PVC plates and incubated overnight at room temperature. Plates were washed and blocked with 3% Nonfat Dry

Milk. Following a 2 h incubation at 37°C, leukotoxin at a dilution of 1/100 was added to the plates which were incubated for 2 h at 37°C. Serum dilutions were then added, and the remaining assay was carried out as described above. All dilutions and washing were performed in PBS-0.05% Tween-20.

A trap ELISA was also performed to determine the presence of leukotoxin specific-IgA in tracheal lavages. Briefly, PVC plates were coated with mAb 35 at a concentration of 20 μg/ml and incubated overnight at room temperature. Plates were washed and blocked with 3% Nonfat Dry Milk. Following a 2 h incubation at 37°C, leukotoxin at a dilution of 1/100 was added to the plates and incubated for 2 h at 37°C. Tracheal lavage dilutions were added and incubated for an additional 2 h as before. Finally, goat anti-mouse IgA-HRP (Fisher) at a dilution of 1/6000 was added and plates were incubated at 37°C for 2 h. Plates were developed as previously described. All dilutions were performed in PBS, and all washings were performed in PBS-0.05% Tween.

Ltx-2 Standard Titration Curve

To determine the concentration of leukotoxin-specific antibodies generated by DNA-based immunizations, a standard ltx-2 titration curve was used. This curve was generated by direct ELISA as previously described. Briefly, 50 μl of JM-2 at 20 μg/μl was added to each well of a 96-well PVC plate and incubated overnight at room temperature. After plates were washed, 3% Nonfat Dry Milk was added and incubated

for 2 h at 37°C. Plates were washed as before, and ltx-2 at 10 μg/ml was added to the appropriate wells and serially diluted. After a 2 h incubation, plates were washed and HRP rabbit anti-mouse IgG (gamma) at 1:3000 was added. Following a 2 h incubation, plates were developed with ABTS as before.

Cytotoxicity Assay

A modification of the MTT cytotoxicity assay (189) was used to determine the activity of the 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 x 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% CO_2 for 1 h. Following this incubation, 50 μl of complete RPMI-1640 (media + 10% FBS + 2% psg) and 10 μl 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 h. Cells were then lysed and the tetrazolium dye solubilized by the addition of 100μl acid alcohol (0.04 N HCl in isopropanol, Mallinckrodt). The absorbance was read at 565 nm on a microtiter plate reader.

For leukotoxin neutralization assays, mouse sera were adjusted to the desired starting dilution and serially diluted two-fold in RPMI-1640. One unit of leukotoxin was then added to each well and incubated on ice for 45 min. BL-3 cells were then added to the plate and the remainder of the assay was performed as previously described.

Statistical Analysis

One way ANOVA analysis was used where applicable. Results were considered significant if $P \leq 0.05$.

CHAPTER 3

RESULTS

Monoclonal Antibody Characterization

A panel of six monoclonal antibodies reactive with the 101.9 kDa leukotoxin of *Pasteurella haemolytica* was generated by Donald Gerbig and Mark Cameron of this laboratory (92,93). Three of these monoclonal antibodies, ltx-2, ltx-4, and ltx-35, have leukotoxin-neutralizing capabilities with ltx-2 as the most potent neutralizer (76). Flow cytometry results indicated that ltx-2 prevents the binding of leukotoxin to susceptible BL-3 cells (92). Initial epitope mapping with CNBr cleavage fragments, mutant deletion proteins, and alkaline phosphatase/leukotoxin fusion proteins indicated the region between LktA amino acids 768 and 939 to be important in ltx-2 binding to target cells (92). Additional studies further defined the ltx-2 epitope as the region between amino acids 876-939 in the carboxyl end of the leukotoxin which is not involved in LktC mediated activation of LktA (76,179). It is also known from previous work that the presence of "ltx-2-like" antibodies correlates with a reduction of disease severity in cattle (R. Moore, personal communication).

Generation of pcDNA3.1 Constructs

To examine the efficacy of DNA-based vaccination, constructs encoding a *lktA* gene fragment were generated in pcDNA3.1. Based on antibody absorption assays, Jack

McPherson of this laboratory defined a leukotoxin fragment between amino acids 713-939 as potentially capable of inducing "ltx-2-like" antibodies when immunized with fusion partner GST. This protein construct (JM-2) produced high rabbit anti-leukotoxin serum titers as well as high rabbit serum leukotoxin neutralizing titers compared to other fragments examined (179). Based on these findings, this portion of the leukotoxin A gene was incorporated into a plasmid suitable for DNA vaccine applications.

A fragment encoding amino acids 710-953 was amplified from genomic DNA of *P. haemolytica A1* and cloned into the *HindIII* and *XhoI* sites of pcDNA3.1. This plasmid is a nonfusion vector therefore a Kozak sequence was engineered upstream of the transcriptional start site based on changes made by pcr primers in the toxin insert. Studies by Marilyn Kozak show that these consensus sequences enhance mRNA and protein expression (132). A schematic representation of the resulting construct, pclkt, is shown in Figure 3.

To examine the potential adjuvant effectiveness of human IL-1β 163-171 (9mer) and human acidic isoferritin 172-185 (14mer), corresponding synthetic oligonucleotides were cloned into pclkt at a unique *EcoRV* site at amino acids 721 and 722. In addition to the adjuvant encoding oligonucleotides, oligonucleotides encoding two glycine residues were added at the 5' and 3' ends of the adjuvant insert. This was done in an attempt to separate the immunogenic and immunostimulatory functional regions. Glycine is a strong disrupter of α-helix secondary structure formation and indifferent toward β-sheet secondary structure formation, therefore, a two-glycine residue spacer would effectively prevent any secondary structural overlap between the adjuvant sequence and the LktA

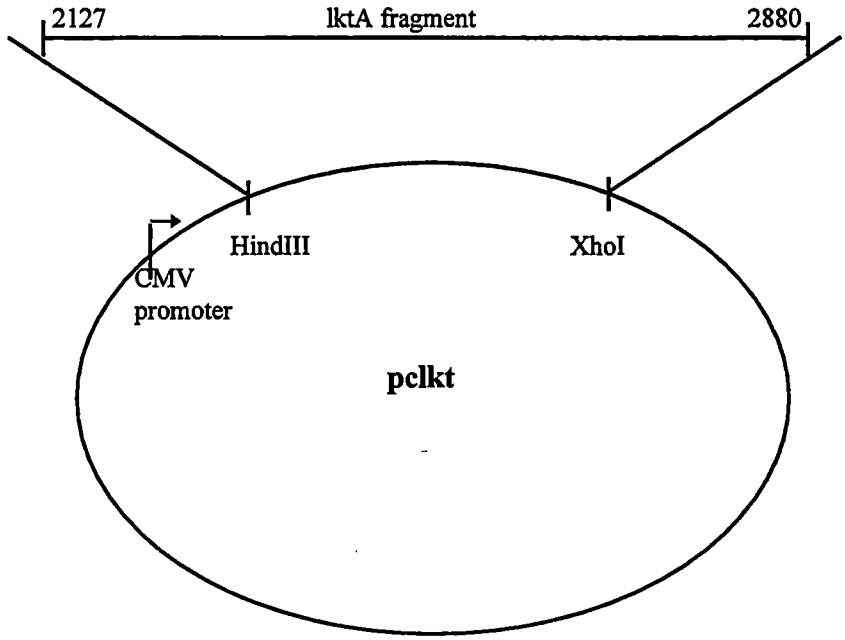

Figure 3. Diagram of pclkt construct. Numbers shown are nucleotides. Expression of the gene is under the control of the CMV promoter.

fragment. In addition, the Gly residues should have minimal influence on the hydrophilicity profile of the immunogen given its zero hyrophilicity value and should not add any novel antigenic feature since they lack a side chain (206). Oligos encoding isoleucine were also added at the 5' end of the adjuvant inserts to recreate the *EcoRV* site. The resulting pclkt construct encoding hIL-1β 163-171, pclkt.9, is depicted in Figure 4. The construct encoding acidic isoferritin 172-185, pclkt.14 is shown in Figure 5. An additional construct was made in pclkt as a control plasmid for pclkt.9 and pclkt.14. The amino acid sequence of the ferritin 14mer, 172-185,was scrambled, and the encoding oligonucleotides with flanking glycine spacers were cloned into pclkt. The resulting construct, pclkt.14scr is shown in Figure 6.

Generation of pGEX4T-1 Constructs

To examine the effectiveness of the adjuvant sequences in recombinant protein vaccines, constructs were generated in the bacterial expression vector pGEX-4T-1. Because the multiple cloning site did not contain a *HindIII* site, inserts from the corresponding pclkt constructs could not be cleaved and ligated directly into pGEX. Rather, the inserts were amplified from the appropriate pclkt construct using pcr primers that engineered a new *EcoRI* site at the 5' end of the insert. The amplified fragments were then cloned into the *EcoRI* and *XhoI* site of pGEX. The resulting constructs in the pGEX expression vector are pGlkt.9, pGlkt.14, and pGlkt.14scr. The pGEX construct

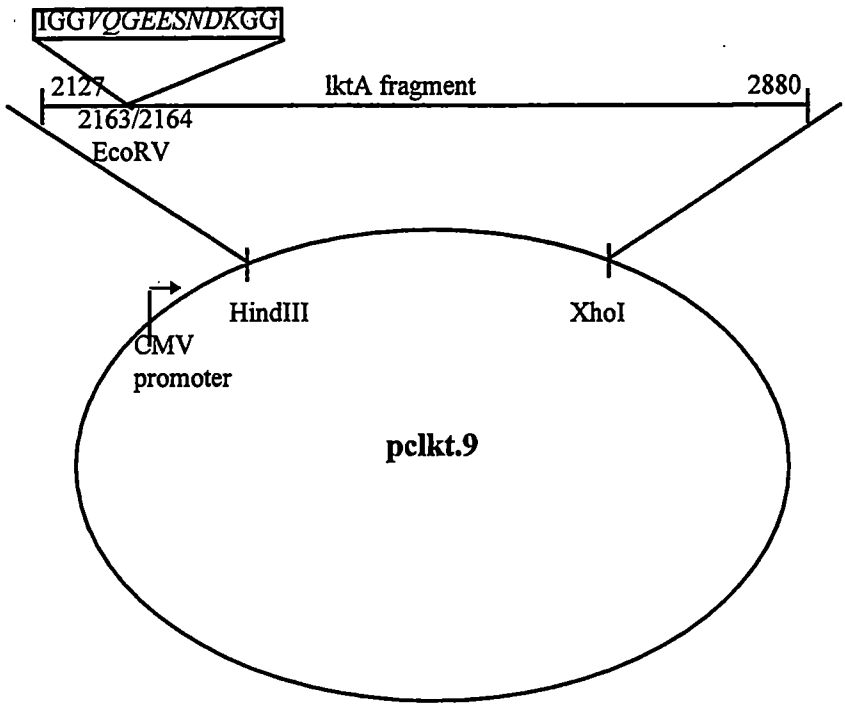

Figure 4. Diagram of pclkt.9 construct. The sequence in the box represents the amino acids encoded by the synthetic oligonucleotides which includes an isoleucine to recreate the restriction site, glycine linkers, and hIL-1β 163-171. Numbers shown are leukotoxin bases. Expression of the gene is under the control of the CMV promoter.

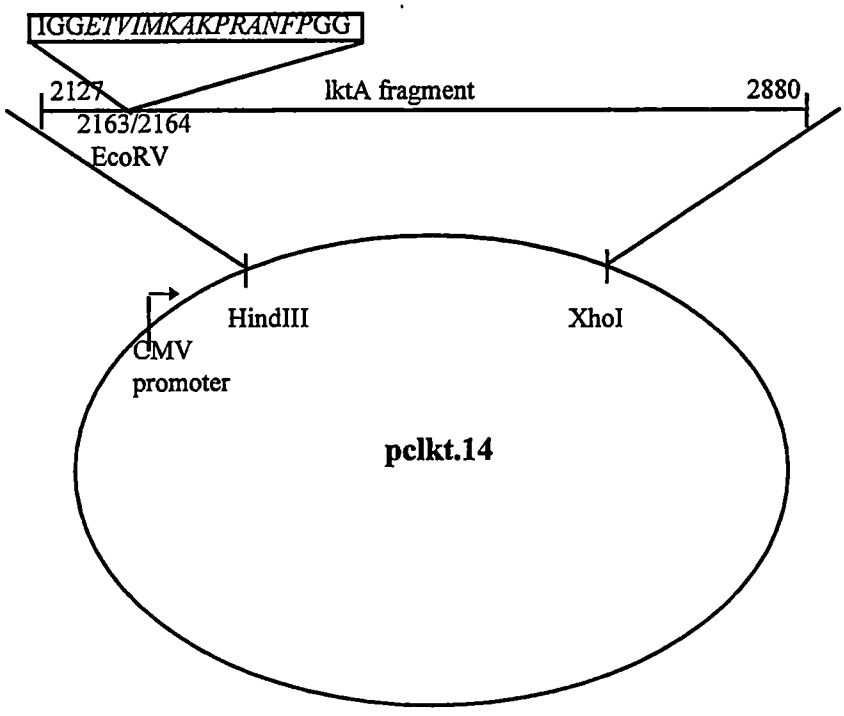

Figure 5. Diagram of pclkt.14 construct. The sequence in the box represents the amino acids encoded by the synthetic oligonucleotides which includes an isoleucine to recreate the restriction site, glycine linkers, and apoferritin 172-185. Numbers shown are leukotoxin bases. Expression of the gene is under the control of the CMV promoter.

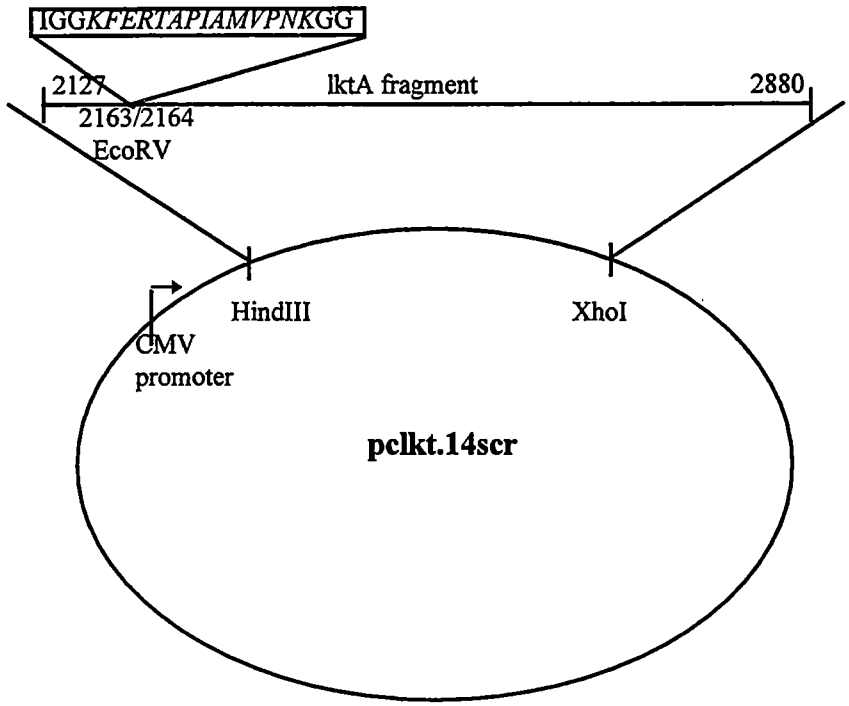

Figure 6. Diagram of pclkt.14scr construct. The sequence in the box represents the amino acids encoded by the synthetic oligonucleotides which includes an isoleucine to recreate the restriction site, glycine linkers, and scrambled apoferritin 172-185. Numbers shown are leukotoxin bases. Expression of the gene is under the control of the CMV promoter.

that contains the leukotoxin A fragment without any adjuvant sequence (JM-2) was generated by Jack McPherson and was used in this study.

DNA Sequencing

The DNA sequence of the leukotoxin fragment in the constructs was compared to the sequence published by Lo *et al.* (149). The DNA sequences of the adjuvant fragments were compared to their respective published sequences (7,47,48,169). All constructs were identical to these sequences.

Generation and Reactivity of Fusion Proteins

Constructs generated in pGEX-4T-1 were used to transform JM109 cells for protein expression. This *E. coli* strain was used because the $lacI^q$ repressor contained on the F' episome produces 10-fold more repressor than is found in most host strains. This ensures a more stringent control of fusion protein expression. The resulting proteins were expressed as C-terminal fusions to glutathione-S-transferase of *S. japonicium* which were purified by affinity chromatography. Schematic representation of the fusion proteins are shown in Figure 7.

The ability of mAbs ltx-2, ltx-4, and ltx-35 to recognize each fusion protein was tested by western immunoblots (Figures 8-10). In each case, all three mAbs recognized the 101.9 kDa intact leukotoxin and failed to recognize the 29 kDa GST protein. In addition, all fusion proteins were recognized by the panel of mAbs indicating that the insertion of the adjuvant peptides did not significantly effect the conformation of the

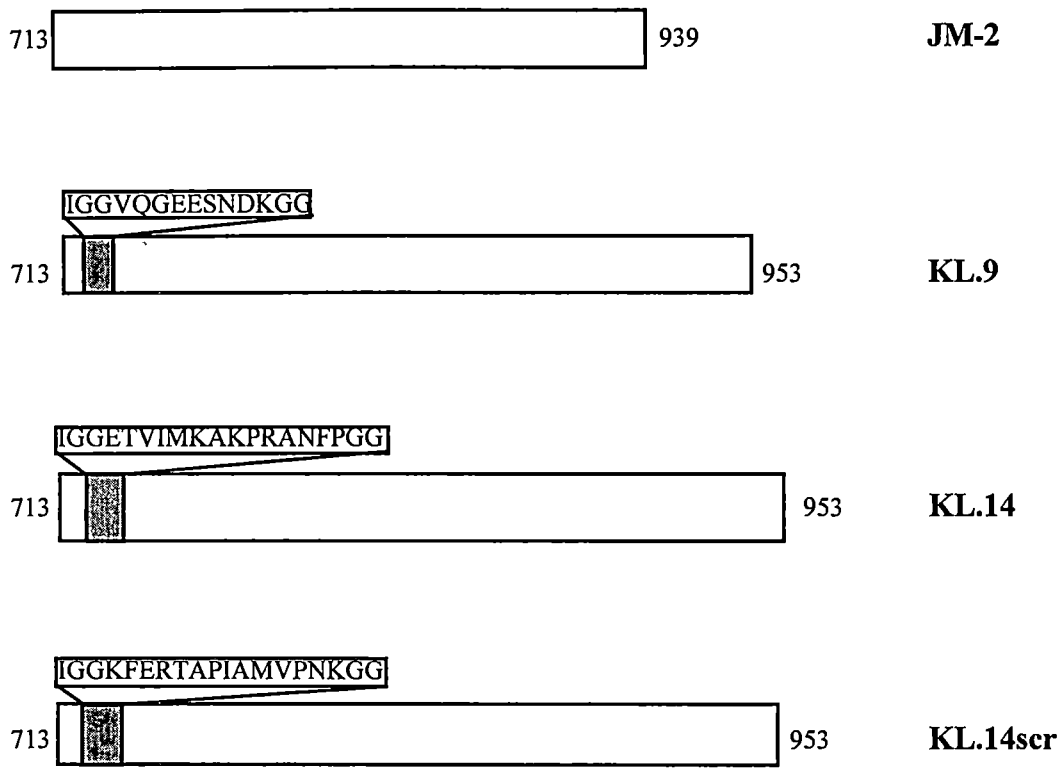

Figure 7. Schematic representation of LktA fragments with incorporated adjuvant peptides. Numbers shown are leukotoxin amino acids. Shaded areas indicate adjuvant sequences which are inserted between amino acids 721 and 722. KL.9 contains the 9mer peptide, KL.14 the contains 14mer peptide, and KL.14scr contains the scrambled 14mer. Proteins are fused at their amino end with GST.

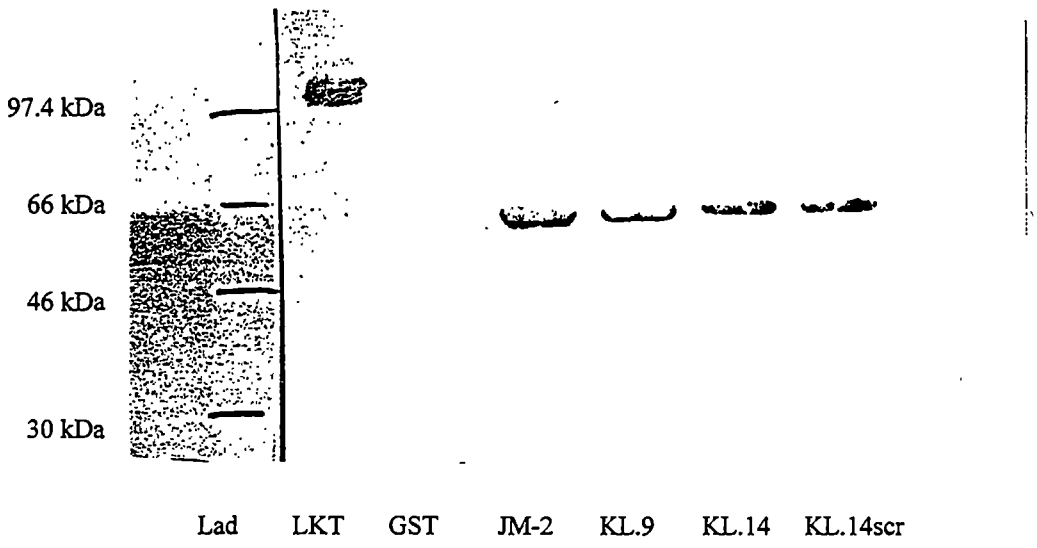

Figure 8. Western blot of fusion proteins reacted with Ltx-2.

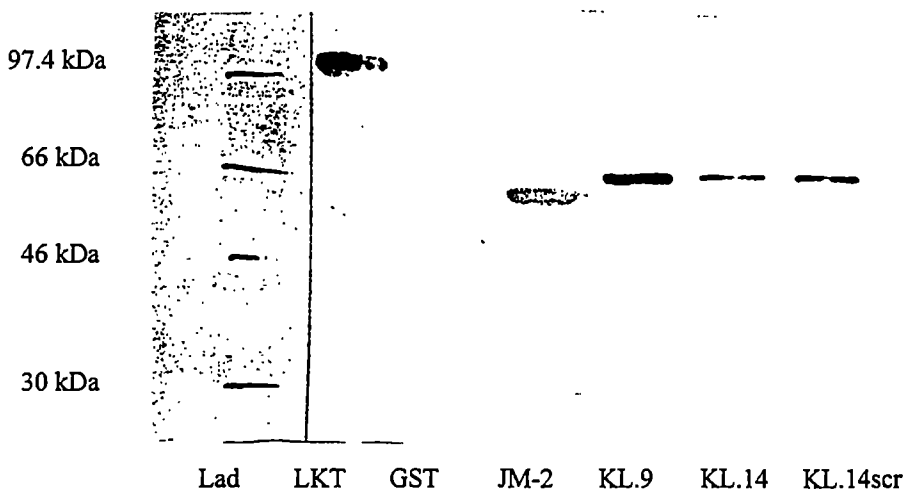

Figure 9. Western blot of fusion proteins reacted with Ltx-4.

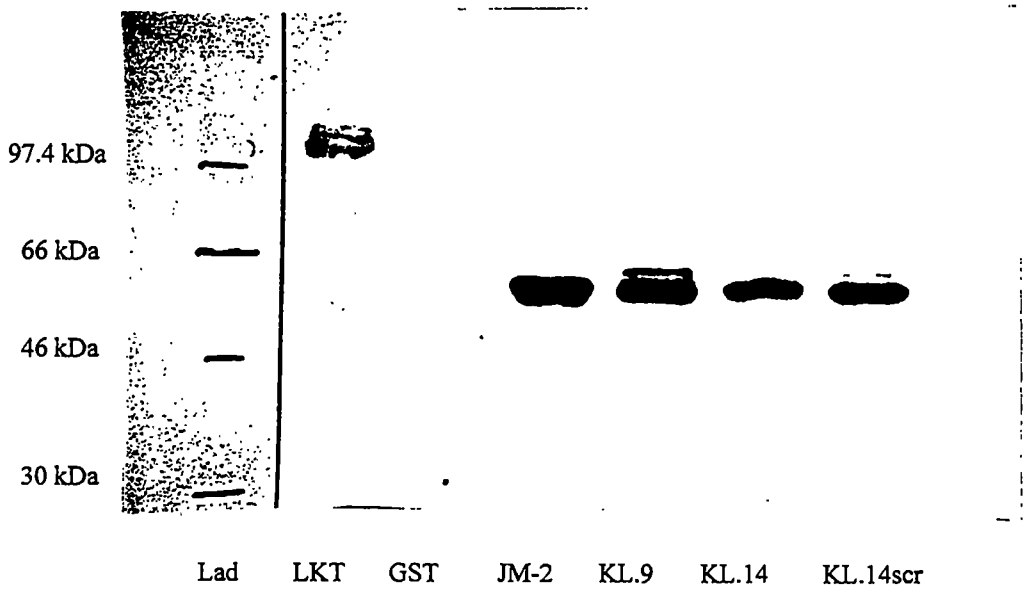

Figure 10. Western blot of fusion proteins reacted with Ltx-35.

leukotoxin fragment. JM-2 produced a band at 53.8 kDa, KL.9 produced a band at 56.3 kDa, and KL.14 and KL.14scr produced bands at 56.8 kDa.

***In vitro* expression of pclkt**

To verify expression of the leukotoxin fragment encoded by pclkt, L929 cells were transiently transfected, and intracytoplasmic extracts were assayed by immunoblot procedures. Optimal conditions were first defined by transfecting cells with pCMVβ and staining with an X-Gal solution to detect beta-galactosidase activity. L929 cells were transfected with pcDNA3.1 or pclkt. Cell lysates were then immobilized on PVDF membranes and then probed with mAbs ltx-2, ltx-4, and ltx-35. Leukotoxin fragment expression by pclkt was detected by ltx-35 (Figure 11). However, ltx-2 and ltx-4 did not detect protein expression which may reflect the low levels of leukotoxin expression by transfected cells or a conformational change in the leukotoxin fragment that may affect binding of the mAbs.

Humoral Responses Induced by Recombinant Protein Immunizations

BALB/c mice were used to determine the immunogenicity of the generated fusion proteins. Mice were immunized i.p. with 100 μg/100 μl of fusion proteins JM-2, KL.9, KL.14, KL.14scr. In addition, mice were immunized with PBS alone, GST, whole LktA in complete Freund's adjuvant, and JM-2 in complete Freund's adjuvant. Primary immunizations were given on day 0 with subsequent boosts on day 21 and day 42. All

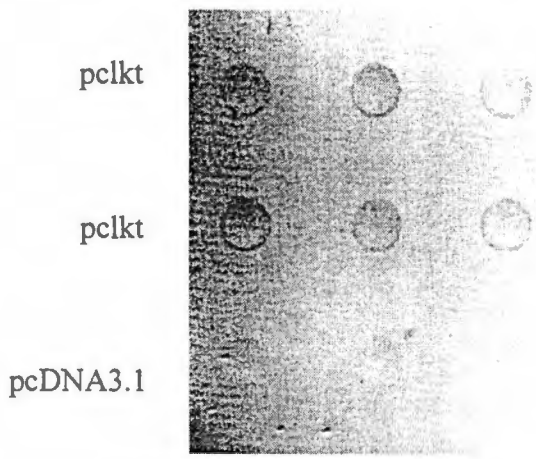

Figure 11. Immunoblot of cell lysates from pclkt or pcDNA3.1 transfected l929 cells reacted with ltx-35.

second and third immunizations were given in PBS. Terminal bleeds were performed on six mice per group throughout the duration of the experiment on days 12, 28 (7 days after the second immunization), and 49 (7 days after the third immunization).

Total antibody response was determined by trap ELISA. Ammonium sulfate precipitated rabbit anti-leukotoxin antibodies were used to trap intact LktA. Serum dilutions were then added and diluted two-fold. Results are shown in Tables 3 and 4 . Animals immunized with LktA and JM-2 in CFA exhibited increasing protein-specific antibody titers in the three bleeds with the highest titers on day 49. The PBS and GST immunized animals produced no detectable levels of such antibodies throughout the experiment. The fusion proteins in PBS, JM-2, KL.9, KL.14, and KL.14scr, produced detectable levels of antibodies in all serum samples with the exception of one KL.14 injected animal on day 12, one KL.9 injected animal on day 28, and one KL.14scr injected animal on day 28. However, all groups immunized with fusion proteins in PBS showed comparable antibody titers with no observed immunomodulatory effects by the adjuvant peptides. Lkt/CFA and JM-2/CFA immunized animals were significantly different than the fusion proteins in PBS ($p \leq 0.05$).

MTT leukotoxin neutralization assays were performed to detect the presence of neutralizing antibodies in the collected sera of recombinant protein-immunized mice. Values reported are $\log_2$ of the serum dilution that allow 75% of cells to survive challenge by 1U of LKT (Tables 5 and 6). Results from this set of experiments indicate that the fusion proteins with the incorporated adjuvant sequences were not able to

Table 3. Serum anti-leukotoxin titers of control mice as determined by trap ELISA.

Immunogen	Mouse #	Day 12	Day 28	Day 49
PBS	1-6	<4.3	<4.3	<4.3
GST	1-6	<4.3	<4.3	<4.3
LktA/CFA	1	<4.3	15.8	17.9
	2	11.3	16.3	18.5
	3	11.3	17.8	17.9
	4	10.4	17.8	16.3
	5	10.7	17.4	17.5
	6	11.3	13.5	17.0
	Mean	11.0±0.2	16.4±0.7	17.5±0.3
JM-2/CFA	1	7.2	12.8	15.4
	2	7.9	11.8	15.8
	3	6.8	10.8	16.3
	4	8.7	12.6	15.4
	5	7.5	13.1	15.5
	6	7.8	10.2	16.0
	Mean	7.7±0.3	11.9±0.5	15.7±0.2

Titers are expressed as reciprocal $\log_2$ of serum dilutions that gives OD_{405} of 0.3.
Means are expressed as average titers of responding mice ± SEM.

Table 4. Serum anti-leukotoxin titers of fusion protein immunized mice as determined by trap ELISA.

Immunogen	Mouse #	Day 12	Day 28	Day 49
JM-2	1	6.7	7.7	7.4
	2	6.8	8.0	7.4
	3	6.7	7.1	8.9
	4	5.4	7.2	8.9
	5	7.3	7.3	9.9
	6	7.1	7.6	9.9
	Mean	6.7±0.3	7.5±0.2	8.7±0.5
KL.9	1	6.9	7.6	8.2
	2	7.3	7.5	8.2
	3	7.8	7.2	9.2
	4	7.2	<4.3	9.2
	5	6.6	7.9	8.4
	6	5.7	6.8	8.2
	Mean	6.9±0.3	7.4±0.2	8.6±0.2
KL.14	1	5.9	6.2	8.0
	2	5.6	6.7	8.6
	3	6.6	6.5	8.9
	4	6.3	7.5	8.9
	5	<4.3	8.1	9.4
	6	6.7	8.1	7.9
	Mean	6.2±0.2	7.2±0.3	8.6±0.2
KL.14scr	1	5.7	8.6	7.3
	2	6.0	<4.3	9.1
	3	5.3	7.8	9.1
	4	7.1	7.1	9.7
	5	6.5	7.3	9.2
	6	5.0	7.4	9.2
	Mean	5.9±0.3	7.6±0.3	8.9±0.3

Titers are expressed as reciprocal $\log_2$ of serum dilutions that gives OD_{405} of 0.3.
Means are expressed as average titers of responding mice ± SEM.

Table 5. Mouse serum leukotoxin neutralizing titers induced by protein immunizations in control groups.

Second Immunization[a]			Third Immunization[b]		
Immunogen	Mouse Number	Neutralizing Titer[c]	Immunogen	Mouse Number	Neutralizing Titer
PBS	1-6	<4.3	PBS	1-6	<4.3
GST	1-6	<4.3	GST	1-6	<4.3
LktA/CFA	1	8.2	LktA/CFA	1	11.2
	2	9.0		2	10.7
	3	10.2		3	11.4
	4	9.6		4	9.2
	5	12.6		5	10.6
	6	6.3		6	10.3
	Mean[d]	9.3±0.9		Mean	10.3±0.3
JM-2/CFA	1	6.3	JM-2/CFA	1	12
	2	9.4		2	12.8
	3	10.0		3	11.9
	4	7.4		4	11.0
	5	7.7		5	10.2
	6	7.0		6	10.3
	Mean	7.9±0.6		Mean	11.3±0.4

[a]Sera taken 7 days post secondary immunization at Day 28.

[b]Sera taken 7 days post third immunization at Day 49.

[c]Titers are expressed as reciprocal $\log_2$ of serum dilution that protects 50% of target cells.

[d]Means are expressed as average titers of responding mice ± SEM.

Table 6. Mouse serum leukotoxin neutralizing titers induced by recombinant fusion protein immunizations.

Second Immunization[a]			Third Immunization[b]		
Immunogen	Mouse Number	Neutralizing Titer[c]	Immunogen	Mouse Number	Neutralizing Titer
JM-2	1	5.9	JM-2	1	<4.3
	2	5.2		2	5.0
	3	4.5		3	6.6
	4	<4.3		4	5.1
	5	4.5		5	8.4
	6	<4.3		6	9.1
	Mean	5.0±0.3		Mean	6.9±0.8
KL.9	1	<4.3	KL.9	1	<4.3
	2	<4.3		2	<4.3
	3	<4.3		3	4.5
	4	<4.3		4	5.1
	5	<4.3		5	7.7
	6	<4.3		6	6.9
	Mean	<4.3		Mean	6.1±0.8
KL.14	1	<4.3	KL.14	1	<4.3
	2	<4.3		2	<4.3
	3	<4.3		3	4.6
	4	<4.3		4	5.8
	5	<4.3		5	4.5
	6	<4.3		6	<4.3
	Mean	<4.3		Mean	4.9±0.4
KL.14scr	1	<4.3		1	6.4
	2	<4.3		2	<4.3
	3	<4.3		3	5.1
	4	<4.3		4	5.5
	5	<4.3		5	6.4
	6	<4.3		6	4.7
	Mean	<4.3		Mean	5.6±0.3

[a] Sera taken 7 days post secondary immunization at Day 28.

[b] Sera taken 7 days post third immunization at Day 49.

[c] Titers are expressed as reciprocal $\log_2$ of serum dilution that protects 50% of target cells.

[d] Means are expressed as average titers ± SEM.

augment the production of neutralizing antibodies upon immunization. Following the second immunization, JM-2 began to induce detectable levels of neutralizing antibodies which increased following the third immunization. However, KL.9, KL.14, and KL.14scr did not induce detectable levels of neutralizing antibodies until after the third immunization. All fusion proteins elicited comparable levels of neutralizing antibodies by day 49. As expected, PBS and GST were unable to induce detectable levels of neutralizing antibodies. Furthermore, JM-2 and LKT in CFA elicited significantly higher neutralizing titers compared to immunizations of the fusion proteins in PBS alone.

Humoral Responses Induced by Intramuscular DNA Immunizations

To examine the possibility of inducing a leukotoxin specific-immune response following genetic immunization, BALB/c mice were injected with various plasmid DNA constructs in the quadriceps and tibialis muscles and the serum was assayed for protein specific antibodies at the indicated time points. Initial experiments involved immunizing mice with 100 μg of plasmid DNA and assaying the serum by ELISA at weeks 2, 3, and 4. Results from these experiments showed no detectable antibodies. Therefore, the length of the experiment was extended because of the possibility of a delayed immune response.

Based on the results from the previous experiment, the length of the next set of experiments was extended to eight weeks (day 56). Mice were immunized i.m. on day 0 with 100 μg of plasmid DNA. At day 28 (week four), half the mice in each group were

boosted with 100 μg of the appropriate plasmid DNA construct. Terminal bleeds were performed at Day 56 (week eight) on all mice. Total leukotoxin specific IgG was analyzed by direct ELISA using the fusion protein JM-2 as antigen. Serum dilutions were added to JM-2 coated plates and bound IgG was detected by rabbit anti-mouse IgG (gamma). To verify the sensitivity of this assay, ELISA were performed using GST as the antigen to ensure that nonspecific binding to this moiety would not occur. Results from these assays indicate that background levels were negligible.

Data obtained from direct ELISA are shown in Table 7. Antigen-specific IgG are expressed as microgram/milliliter based on values obtained from a standard curve of ltx-2. The standard ltx-2 curve is shown in Figure 12. This method was employed because the sera titered at low dilutions. In addition, a comparison can be made between the levels of antibodies induced by DNA immunizations and the known concentration of ltx-2. The leukotoxin fragment inserted into the pcDNA3.1 vector, pclkt, was able to elicit a protein specific immune response when injected intramuscularly with a mean antigen specific antibody concentration of 19.6 μg/ml. In addition, the 9mer and the 14mer adjuvant sequences were able to significantly enhance this immune response generating 100.1 μg/ml and 129.8 μg/ml of leukotoxin specific antibodies, respectively, which is significantly different ($p \leq 0.05$) than the response generated by pclkt. However, mice that received booster immunizations, did not produce detectable levels of protein specific antibodies indicating that not only are additional immunizations not necessary, they may have a negative effect on the outcome of the immune response (data not shown).

Table 7. IgG serum response in mice following intramuscular DNA immunization (Group A).

Immunogen	Mouse Number	Antigen Specific Antibodies (μg/ml)[a]
Preimmune Serum	----	ND[b]
pcDNA3.1	1-6	ND
pclkt	1	8.4
	2	12.5
	3	16.2
	4	15.3
	5	11.5
	6	53.7
	Mean[c]	19.6±6.9
pclkt.9	1	90.8
	2	76.8
	3	134.5
	4	124.2
	5	102.6
	6	71.8
	Mean	100.1±10.3
pclkt.14	1	152.5
	2	163.1
	3	188.1
	4	119.4
	5	49.3
	6	106.1
	Mean	129.8±20.2

[a] Leukotoxin specific IgG is represented as μg/ml based on values obtained from the Ltx-2 standard curve.
[b] IgG levels not detected.
[c] Means are expressed as average concentrations ± SEM.

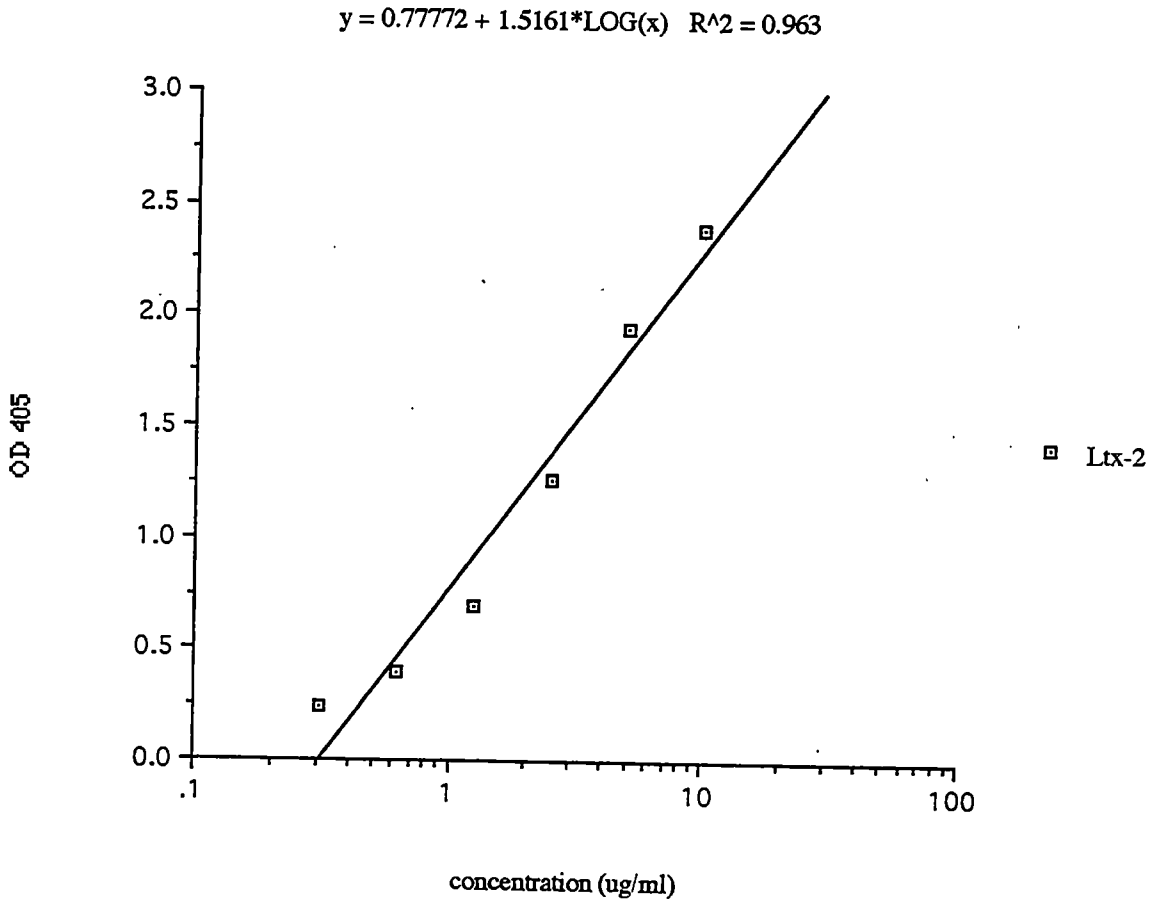

Figure 12. Ltx-2 standard titration curve.

To verify the results of the previous set of experiments, mice were once again immunized with the pcDNA3.1 constructs with the addition of pclkt.14scr as a control for the adjuvant encoding plasmids. Mice were injected with DNA on day 0 and terminal bleeds were performed on day 56 (week 8). Direct ELISA were performed as before. Results are displayed in Table 8. All plasmids encoding the leukotoxin A fragment were able to elicit a protein specific immune response. The construct pclkt elicited 18.6 μg/ml of protein specific antibodies whereas such antibodies were undectected in animals immunized with plasmid alone. Furthermore, the plasmids pclkt.9 and pclkt.14 with the incorporated adjuvant sequences were able to enhance the immune response over two- and three-fold higher, respectively, compared to pclkt, which is a significant difference ($p \leq 0.05$). The construct pclkt.14scr was able to generate an immune response in the immunized mice which was comparable to the response generated by pclkt. Furthermore, this response differed significantly than that generated by pclkt.9 and pclkt.14 ($p \leq 0.05$) indicating that enhanced response generated by the latter constructs were due to the activity of the adjuvant sequences.

A final set of DNA immunized mice was examined in which the time course following injection was extended. BALB/c mice were once again immunized i.m. with 100 μg of plasmid DNA. Retroorbital bleeds were performed on day 56 and terminal bleeds on these mice were performed on day 84. Serum was assayed for leukotoxin specific antibodies as previously described. Results are displayed in Table 9. At day 56, the construct pclkt was able to elicit an average immune response of 35.5 μg/ml of

Table 8. IgG serum response in mice following intramuscular DNA immunization (Group B).

Immunogen	Mouse Number	Antigen Specific Antibodies (μg/ml)[a]
Preimmune Serum	----	ND[b]
pcDNA3.1	1-6	ND
pclkt	1	11.5
	2	21.4
	3	26.3
	4	19.9
	5	24.6
	6	8.1
	Mean	18.6±3.0
pclkt.9	1	34.7
	2	69.5
	3	37.2
	4	45.9
	5	56.4
	6	43.4
	Mean	47.9±5.3
pclkt.14	1	85.6
	2	112.9
	3	39.9
	4	69.5
	5	49.1
	6	52.7
	Mean	68.3±11.1
pclkt.14scr	1	22.9
	2	15.1
	3	26.3
	4	32.4
	5	15.1
	6	30.3
	Mean	23.7±3.0

[a] Leukotoxin specific IgG is represented as μg/ml based on values obtained from the Ltx-2 standard curve.
[b] IgG levels not detected.
[c] Means are expressed as average concentrations ± SEM.

Table 9. IgG serum response in mice following intramuscular DNA immunization (Group C).

Immunogen	Mouse Number	Antigen Specific Antibodies (μg/ml)[a] Day 56	Day 84
Preimmune Serum	----	ND[b]	ND
pcDNA3.1	1-6	ND	ND
pclkt	1	52.7	15.1
	2	39.9	9.3
	3	45.9	11.4
	4	6.6	7.6
	5	28.2	ND
	6	39.9	18.6
	Mean	35.5±6.7	12.4±2.0
pclkt.9	1	105.3	28.2
	2	74.4	39.9
	3	79.8	24.6
	4	85.6	45.9
	5	69.5	28.2
	6	52.7	15.1
	Mean	77.9±7.2	30.3±4.5
pclkt.14	1	120.9	52.7
	2	74.5	39.9
	3	91.7	32.4
	4	79.8	26.3
	5	74.5	18.6
	6	69.5	21.4
	Mean	85.2±7.8	31.9±5.2
pclkt.14scr	1	34.7	ND
	2	38.5	16.2
	3	52.7	15.3
	4	42.8	21.4
	5	42.3	14.1
	6	74.5	17.4
	Mean	47.6±5.9	16.9±1.3

[a] Leukotoxin specific IgG is represented as μg/ml based on values obtained from the Ltx-2 standard curve.
[b] IgG levels not detected.

antigen specific antibodies whereas no detectable levels of leukotoxin specific antibodies were produced by animals immunized with plasmid alone. The constructs, pclkt.9 and pclkt.14, generated an increased immune response of 77.9 μg/ml and 85.2 μg/ml, respectively, which is not only significantly different ($p \leq 0.05$) than the pclkt response but also different ($p \leq 0.05$) from the response generated by pclkt.scr. This latter response, although producing a higher response than pclkt at 47.6 μg/ml, is still comparable to the leukotoxin fragment without an adjuvant sequence.

Antigen specific antibodies were still detected on day 84, however, to a lesser extent. The construct pclkt produced 12.4 μg/ml of leukotoxin specific antibodies which is almost three times less than what was detected on day 56. Constructs pclkt.9 and pclkt.14 also showed a decreased immune response from day 56 at 30.3 μg/ml and 31.9 μg/ml, respectively. However, this response was still significantly different than that of pclkt ($p \leq 0.05$). The control construct, pclkt.14scr, also elicited almost three times fewer antibodies than day 56. In addition, the pclkt.14scr response was comparable to the responses produced by other three constructs.

To determine the presence of leukotoxin neutralizing antibodies in DNA immunized mice, cytotoxic assays were performed. Serum dilutions were incubated with 1U of LktA to allow for antibody binding and then BL-3 target cells were added. Cytoxicity was measured as previously described. In all three experiments, no detectable levels of neutralizing antibodies were produced. This held true for all groups immunized with the various leukotoxin constructs, even those augmented with the adjuvant

sequences. Therefore, although an immune response is generated and detected by ELISA, this does not guarantee that these antibodies will be neutralizing.

Humoral Responses Induced by Intranasal DNA Immunizations

BALB/c mice were immunized i.n. with plasmid DNA to examine the effectiveness of the pcDNA3.1 construct vaccines via a different route of immunization. Mice were immunized with 100 μl of plasmid DNA in 40 μl by applying 20 μl per nare on day 0. Two sets of mice were used. One set received plasmid DNA alone whereas the second set received plasmid DNA with 2 μg of cholera toxin subunit B. Terminal bleeds and tracheal lavages were performed on day 21, day 35, and day 56.

Tracheal lavage samples were assayed for leukotoxin specific IgA and IgG to examine the potential mucosal immune response. Not much is known about the mucosal response following DNA immunizations as such research has met with varying degrees of success. Trap ELISAs were performed to detect IgA in tracheal washings. mAb 35 was used to trap intact leukotoxin and tracheal lavage dilutions were then added and serially diluted two-fold. Goat anti-mouse IgA-HRP was used to detect leukotoxin bound IgA. In all cases, no leukotoxin specific IgA was detected (data not shown).

Direct ELISAs were performed to assay for protein specific IgG in tracheal lavages. JM-2, the leukotoxin A fragment-GST fusion protein, was used as antigen. Tracheal lavage samples were added, diluted two-fold, and bound IgG was detected with rabbit anti-mouse IgG (gamma). In all cases, no leukotoxin specific IgG was detected

(data not shown). Once again, ELISAs were performed with GST as antigen to control for any nonspecific binding of proteins to this fusion partner. In all cases, background was negligible.

Mucosal immune responses were not detected following intranasal immunizations, however, systemic responses were observed. Direct ELISA were performed with the antigen JM-2 as before. Results from these experiments are shown in Table 10. Values are expressed as microgram/milliliter of leukotoxin specific IgG based on values obtained from a standard Ltx-2 curve. As expected, mice injected with pcDNA3.1 had no detectable levels of antibodies at any time point. Mice injected with pclkt, pclkt.9, pclkt.14, and pclkt.14scr demonstrated a delayed systemic immune response with consistent level of IgG expression achieved by day 56. Mice immunized with pclkt generated a mean response of 23.2 μg/ml which was comparable to the responses produced by pclkt.14, 23.6 μg/ml, and pclkt.14scr, 29.9 μg/ml. However, mice injected with pclkt.9 displayed elevated levels of inducible antibodies at 62.3 μg/ml, which is significantly different from the other groups ($p \leq 0.1$), indicating the potential adjuvant effect of IL-1β 163-171. pclkt.14 did not augment the immune response in this study.

A systemic response was also detected in mice immunized with plasmid DNA and CTB. Comparable levels of leukotoxin-specific antibodies were detected in responding mice in all groups with the exception of pcDNA3.1 mice in which no antibodies were detected. However, antibody levels were very low and not all the mice were responding

Table 10. IgG serum response in mice following intranasal DNA immunization[a].

Immunogen	Mouse #	Day 21	Day 35	Day 56
pcDNA3.1	1-6	ND[b]	ND	ND
pclkt	1	30.9	ND	33.4
	2	ND	ND	18.4
	3	ND	ND	17.5
	4	ND	ND	17.8
	5	ND	ND	29.9
	6	----[c]	8.7	22.2
	Mean[d]			23.2±2.8
pclkt.9	1	11.5	ND	31.8
	2	ND	ND	57.7
	3	ND	ND	138.8
	4	ND	ND	43.7
	5	ND	ND	48.9
	6	----	ND	52.7
	Mean			62.3±15.7
pclkt.14	1	ND	ND	8.1
	2	ND	ND	ND
	3	ND	ND	45.9
	4	ND	ND	15.0
	5	ND	ND	27.8
	6	----	ND	20.9
	Mean			23.5±6.5
pclkt.14scr	1	ND	ND	8.7
	2	ND	ND	12.2
	3	ND	ND	15.0
	4	ND	23.4	83.0
	5	ND	ND	40.6
	6	ND	ND	20.6
	Mean			29.9±11.5

[a] Leukotoxin specific IgG is represented as μg/ml based on values obtained from the Ltx-2 standard curve.
[b] No detectable levels of IgG.
[C] On Day 21, 5 mice per group were sacrificed.
[d] Means are expressed as average concentrations ± SEM.

by day 56 (data not shown). These results may be a reflection of the cholera toxin subunit B that was used in the immunizations. The wrong CTB vial was used which may have expired, affecting the outcome of this set of experiments. Therefore, this data will not be used.

MTT neutralization assays were also performed with intranasal immunized mice to screen for the presence of leukotoxin-neutralizing antibodies in the collected serum. These cytotoxicity assays were performed as previously described. In all cases, no detectable levels of neutralizing antibodies were observed.

Isotyping of Humoral Responses Generated by DNA Immunizations

Direct ELISA were performed on selected sera to examine the IgG isotyope profile which is indicative of the type immune response (Th1vs Th2). ELISA were conducted as previously described. Leukotoxin specific antibodies were detected using rabbit anti-mouse IgG1 and IgG2a. Serum samples from mice immunized intramuscularly (Group B) and intranasally were examined. Results are shown in Tables 11 and 12. Assays were performed in PBS-0.05% Tween 20 to minimize background levels. However, this buffer may have been too stringent, decreasing the level of serum antibody binding to the JM-2 coated plates. These experiments were not repeated using PBS alone due to the availability of the selected serum samples. An accurate concentration of the bound antibody could not be obtained because the serum did not titer, therefore, the data is presented as the OD_{405} at a reciprocal $\log_2$ serum dilution of 4.9.

Table 11. IgG isotype examination of antibody response in mice following intramuscular DNA immunization (Group B)[a].

Immunogen	Mouse #	IgG1	IgG2a
pcDNA3.1	1-6	ND[b]	ND
pclkt	1	0.061	0.211
	2	0.034	0.413
	3	0.045	0.131
	4	0.052	0.233
	5	0.031	0.182
	6	0.039	0.194
pclkt.9	1	0.072	0.213
	2	0.117	0.342
	3	0.017	0.453
	4	0.025	0.264
	5	0.037	0.302
	6	0.035	0.260
pclkt.14	1	0.038	0.245
	2	0.044	0.274
	3	0.047	0.316
	4	0.101	0.202
	5	0.085	0.303
	6	0.072	0.296
pclkt.14scr	1	0.050	0.232
	2	0.026	0.286
	3	0.103	0.199
	4	0.082	0.192
	5	0.077	0.123
	6	0.071	0.189

[a]Values are expressed as OD_{405} at a reciprocal $\log_2$ serum dilution of 4.9.
[b]Not done.

Table 12. IgG isotype examination of antibody response in mice following intranasal DNA immunization[a].

Immunogen	Mouse #	IgG1	IgG2a
pcDNA3.1	1-6	ND[b]	ND
pclkt	1	0.044	0.150
	2	0.043	0.253
	3	0.039	0.183
	4	0.041	0.201
	5	0.059	0.210
	6	0.035	0.171
pclkt.9	1	0.046	0.111
	2	0.020	0.271
	3	0.041	0.201
	4	0.021	0.264
	5	0.031	0.272
	6	0.035	0.311
pclkt.14	1	0.010	0.025
	2	0.033	0.071
	3	0.027	0.059
	4	0.033	0.081
	5	0.025	0.067
	6	0.025	0.057
pclkt.14scr	1	0.053	0.098
	2	0.032	0.081
	3	0.033	0.078
	4	0.035	0.176
	5	0.041	0.223
	6	0.040	0.125

[a]Values are expressed as OD_{405} at a reciprocal $\log_2$ serum dilution of 4.9.
[b]Not done.

Following intramuscular DNA immunizations, ELISA revealed a predominance of IgG2a antibodies indicating a Th1 type response. These results were consistent in all samples including those mice immunized with the adjuvant encoding constructs. The same results were observed in mice following i.n. DNA immunization. When the two isotypes IgG1 and IgG2a were measured, only the latter isotype could be detected predominately in all tested groups.

CHAPTER 4

DISCUSSION

In spite of new and advanced vaccines, bovine respiratory disease is still the most important disease affecting feedlot cattle in North America, and its economic impact is underestimated. This disease syndrome is influenced by a variety of factors including stress and viral and bacterial infections which are often difficult to reproduce under laboratory conditions. One bacterium, *Pasteurella haemolytica*, has emerged as a key agent in "shipping fever" as it is the most frequent isolate from the lungs of cattle that die from acute respiratory disease. Many believe that if this single factor was eliminated, losses from bovine respiratory disease would be reduced drastically.

With the recognition of the primary involvement of *P. haemolytica* A1 in the disease process, attempts have been made to prevent the disease by vaccinating against *P. haemolytica*. Many laboratories have studied the efficacy of a variety of vaccine preparations including live bacteria (37,42), bacterins (41), capsular polysaccharides (44), outer-membrane proteins (38,51), culture supernatant of *P. haemolytica* (45,223), and recombinant leukotoxin (45). All of these attempts have met with varying degrees of success.

Understanding the mechanisms of the pathogenesis of *P. haemolytica* A1 continues to increase. As understanding continues, more effective vaccines will develop. Mounting evidence indicates that not only is the leukotoxin considered the most

important virulence factor, it is also the most important immunogen (39,43). High levels of LktA antibodies are consistently correlated with resistance to disease (91). Because of this, the incorporation of LktA into an effective vaccine seems inevitable.

Due to the importance of the leukotoxin in the pathogenesis of pneumonic pasteurellosis, this laboratory has focused on defining important immunogenic epitopes of the leukotoxin. A panel of monoclonal antibodies, ltx -2, -4, -35, was generated which recognize potential protective epitopes within the leukotoxin molecule (92,93). All three monoclonal antibodies have leukotoxin-neutralizing capabilities with ltx-2 as the most potent neutralizer. Initial epitope mapping with CNBr cleavage fragments, mutant deletion proteins, and alkaline phosphatase/leukotoxin fusion proteins indicate that the region between amino acids 768 and 939 is important in Ltx-2 binding to target cells (92). It was further determined that the epitope recognized by Ltx-2 is in the region between amino acids 876 and 939 (179). Since the presence of "Ltx-2-like" polyclonal antibodies correlates with a reduction of disease severity in cattle (R. Moore, personal communication), inclusion of the Ltx-2 epitope appears to be essential for an effective vaccine.

Vaccination against *P. haemolytica* A1 has met with varying degrees of success. A novel method of vaccination was examined in this study for its potential ability to generate protective antibodies against the leukotoxin. Within the past decade, DNA-based vaccines have emerged with demonstrated ability to provide long lasting cellular and humoral immune responses to numerous antigens, including bacterial antigens. In this study, plasmid DNA encoding an immunogenic fragment of *P. haemolytica*

leukotoxin was used to immunize mice and the resulting immune response was examined. In addition, the immunomodulatory activity of two adjuvant peptides, IL-1β 163-171 and acidic isoferritin 172-185, was examined for the ability to augment this developing immune response.

A recombinant protein vaccination approach was applied to examine the ability of the adjuvant sequences to augment an immune response in immunized mice. Constructs were generated in the pGEX 4T-1 plasmid and protein expression was achieved by induction using IPTG. The resulting proteins were all recognized by mAbs ltx -2, -4, -35 in western immunoblot procedures. The 101.9 kDa leukotoxin was recognized by all three mAbs whereas the 29 kDa GST molecule was not recognized. The fusion proteins JM-2 at 53.8 kDa, KL.9 at 56.3 kDa, and KL.14 and KL.14scr at 56.8 kDa were all detected by the panel of mAbs. These results indicated that the incorporation of the adjuvant peptides just inside the 5' end of the leukotoxin fragment did not significantly effect the monoclonal antibody binding epitopes in the leukotoxin molecule. Therefore, these proteins were used to immunized BALB/c mice.

Sera from recombinant protein immunized mice were analyzed for leukotoxin reactive antibodies by ELISA and toxin-neutralization assays. Native leukotoxin and JM-2 with CFA served as positive controls; PBS and GST served as negative controls. Anti-leukotoxin antibody titers on day 12, day 28 and day 49 were determined by trap ELISA. The negative controls, PBS and GST, did not elicit detectable anti-leukotoxin titers throughout the duration of the experiment. The positive controls, Lkt A and JM-2 with

CFA, elicited increasing antibody titers in the three bleeds with the highest titers on day 49. Mice receiving fusion proteins in PBS were compared to examine the potential ability of the adjuvant sequences to augment the immune response and to possibly replace CFA. Antibody titers were detected on day 12, day 28, and day 49. However, the fusion proteins with the incorporated adjuvant sequences produced titers that were comparable with JM-2, the GST-leukotoxin fusion protein alone. This may indicate the possibility that the adjuvant sequences were masked between the 29 kDa GST molecule and the 25.9 kDa leukotoxin fragment. These hidden sequences would then not be exposed as the immune response develops and would, therefore, not be able to elicit immunopotentiating effects.

MTT neutralization assays displayed similar results. After the second and third immunizations, the positive control groups LKT and JM-2 with CFA were producing neutralizing titers with the highest titers on day 49. However, the only fusion protein which elicited a neutralizing antibody response after the second immunization was JM-2. This response was weak and not all mice were responding at this point. By day 49, all fusion proteins were able to generate neutralizing antibodies, however, not all mice in these groups were responding. In addition, the responses in these groups were comparable as determined by statistical analysis. Again, these results would suggest that the short adjuvant sequences are unavailable due to the conformation of the GST-leukotoxin fragment molecule which may mask these areas. The negative controls, PBS and GST, did not elicit detectable anti-leukotoxin titers.

Data from the DNA-based vaccine studies produced some important findings. However, these results are somewhat limited by the nature of the immune response that was generated. Three groups of intramuscular immunized mice were examined which produced confirming results. Group A contained mice that were immunized with plasmid alone, pclkt, pclkt.9, and pclkt.14. The leukotoxin specific antibody response was determined at week 8. The construct with the leukotoxin fragment elicited low levels of protein specific antibodies with a mean value of 19.6 μg/μl. The incorporation of the adjuvant sequences significantly enhanced this immune response, producing an antibody level over five times higher than the leukotoxin fragment alone. Human IL-1β 163-171 (9mer) and human acidic isoferritin 172-185 (14mer) produced comparable levels of antigen specific antibodies. The difference between the response generated by the DNA-based vaccines and the protein-based vaccines may be due to the location of the adjuvant sequences in the expressed molecules. In the recombinant protein, the immunomodulatory sequences are located between the GST moiety and the leukotoxin fragment. However, in the DNA construct which does not contain a fusion partner, the sequences are located just inside the 5' end of the leukotoxin molecule. This latter configuration increases the probability that the adjuvant sequences will be exposed once the protein is expressed, and not hidden within the molecule.

Groups B and C were immunized with the same test immunogens in Group A, with the addition of the control construct, pclkt.14scr. In Group B, pclkt was able to generate an average antigen specific antibody concentration of 18.6 μg/μl which was

comparable to the response generated by the control group, pclkt.14 scr at 23.7 μg/μl. The immunogens pclkt.9 and pclkt.14 were able to further augment this immune response greater than two- and three-fold, respectively, from the pclkt and pclkt.14scr mice. Results are shown in Table 8. Similar results were also observed in mice from Group C at week 8. By week 12, an antibody response was still detectable in Group C mice, however, this response had significantly decreased from the response observed at week 8. Results are shown in Table 9. This may indicate that the immune response elicited in immunized animals peaks earlier, around week 8, and decreases while maintaining a low level of long lasting antibody expression characteristic of DNA-based vaccination.

One set of i.m. immunized mice received a booster injection at Week 4. These mice produced no detectable levels of leukotoxin specific antibodies in any of the indicated groups. Several possible explanations may account for this phenomenon. The amount of DNA that these animals received may have been at levels high enough to produce a state of tolerance to the immunogen. It is also possible that following the booster immunization, the expressed proteins in the host were bound by low levels of antibodies generated by the first injection. These exogenous proteins would then not be in a form that APCs could take up, process, and present on MHC class II molecules. Therefore, a subsequent immune response would not develop. Due to these results, booster immunization were not performed in latter experiments.

The systemic antibody response in i.n. DNA-immunized mice was also examined. Sera from each group was examined on day 21, day 35, and day 56. A consistent

response was not observed until day 56 (week 8). The mean-leukotoxin specific IgG level in pclkt immunized mice was 23.2 μg/μl which was comparable with the responses of mice immunized with pclkt.14scr at 29.9 μg/μl and pclkt.14 at 23.5 μg/μl. The pclkt.9 construct produced antibody levels 2-2.5 times higher than the response produced by the former three constructs. This difference is most likely due to the activity of the 9mer adjuvant sequence. Surprisingly, the 14mer sequence in pclkt.14 was not able to augment the immune response in i.n. immunized mice which is in contrast to the results this construct produced in i.m. immunized mice. This may be due to the mechanism of adjuvanicity by this 14mer peptide which has yet to be defined. It is possible that the musocal environment is not optimal for the 14mer adjuvant activity, however, this issue needs further study. Another explanation is that there was a problem with the pclkt.14 plasmid itself. This issue could be addressed by repeating the experiment.

Tracheal lavage samples from i.n. immunized mice were also examined for the presence of IgG and IgA antibodies. No such antibodies were detected in mice immunized without the CTB adjuvant. A set of mice was also immunized with CTB, however, the wrong vial was used, producing inconsistent results. CTB is a well known mucosal adjuvant that is able to alter the regulatory environment within the mucosal immune system to favor responsiveness (137,272). Therefore, it is likely that if these experiments were repeated with a new vial of CTB, levels of IgG and IgA may be detected.

A systemic response was observed in mice immunized i.m. and i.n. with the various DNA constructs, with the exception of mice immunized with pcDNA3.1 alone. Unfortunately, no neutralizing antibodies were detected in any of the immunized mice including those with elevated antibody levels. It is possible that the bacterial leukotoxin antigen, which is expressed by the injected animal host and not *P. haemolytica*, may undergo different types of posttranslational modification resulting in non-native conformation. Therefore, epitopes necessary to produce neutralizing antibodies may not be maintained. It is also possible that due to the low levels of inducible antibodies, potential neutralizing antibodies may be at a level that cannot be detected by MTT neutralization assays. Finally, the ltx-2 epitope within the encoded leukotoxin fragment has recently been shown to be unstable (179). After DNA vaccination, it is possible that the minimal sequences required to stabilized this epitope are not present and, therefore, the ltx-2 epitope is not maintained.

Finally, the IgG isotype of the immune responses in DNA immunized mice was examined. It was hoped that these results would help define the type of immune response that was elicited by the two routes of immunization, and in addition, indicate the type of response generated by the adjuvant sequences. Direct ELISA were performed in PBS-0.05% Tween to increase the stringency of antibody binding of the leukotoxin moiety of JM-2. However, these conditions may have been too stringent as OD_{405} readings were exceptionally low. Due to the low amount of remaining serum, these experiments were not repeated and conclusions were drawn from the resulting data. In both sets of mice, i.m. and i.n., the data indicate a predominance of IgG2a antibodies as opposed to IgG1

antibodies. These results indicate a predominant Th1-type response. This is characteristic of DNA vaccines applied by the i.m. route (73,199,218,258). Responses to i.n. immunizations have not been adequately characterized, but studies using CTB elicit a higher IgG1 response indicating that a Th2-type response may predominate (137,234,276). It was surprising, however, that the constructs with the incorporated adjuvant sequences elicited a similar type response in both routes of immunization. Initial thoughts were that the two adjuvants might elicit different types of immune response, Th1 vs. Th2, which might account for the inability of the 14mer in pclkt.14 to augment the immune response when applied intranasally. This did not hold true indicating that further study is needed to characterize that mechanism of action of the two adjuvant sequences.

Although information pertaining to the 14mer adjuvant is limiting, knowledge of the activity of the 9mer is accumulating. Recent studies suggest that the IL-1β fragment enters cells through a receptor-independent mechanism, possibly by passive diffusion through the cell membrane, similar to that seen for steroid hormones and for cyclosporin A (20). After internalization, this peptide is localized in the cytosol where it can bind to cytoplasmic proteins and initiate an intracellular pathway of cell activation that is IL-1R independent (20). The identification of this immunostimulatory cytokine domain has many potential applications; however, further study is needed to define its mechanism of action.

In this study, every effort has been made to maintain the proper controls. However, two issues need to be addressed. First is the presence of the GST moiety in the fusion protein constructs. This molecule may have an effect on the conformation and presentation of the leukotoxin fragment and, as shown by the data, an effect on the exposure of the adjuvant sequences to the surface of the molecule. This problem could be addressed by cleaving the fusion protein and removing the GST portion. This has been attempted previously in this laboratory with limited success. The resulting protein epitopes cleaved of GST were unstable suggesting that the GST may aid in stabilizing the native fragment conformation. The second issue that needs to be addressed is the development of a consistent, standardized ELISA to detect the presence of protein specific antibodies in DNA immunized mice. A trap ELISA, as was used with sera samples from protein immunized mice, was attempted but proved unsuccessful. This assay has an additional step which may decrease its sensitivity. Some groups of DNA immunized mice elicited low levels of antibodies that were undetected by this approach. Because intact leukotoxin does not adhere well to ELISA plates, the fusion protein JM-2 was used. This approach was more successful, however, it was necessary to repeat some ELISA several times until background levels were acceptable. This is not ideal because serum samples are often limited. Results from all ELISA were not utilized until background levels, which may result from nonspecific binding to GST, were acceptable.

Current strategies to prevent pneumonic pasteurellosis have met with varying degrees of success. Many approaches produce promising results, however, often times these results are not repeatable. This may be due to the many factors that play a role in

the development of this disease. Therefore, it is necessary to continue to study and characterize the organisms and events that contribute to the severity of the disease. Many researchers believe that the best vaccine strategy would include the incorporation of antigenic regions of the leukotoxin which is believed to be one of the primary virulence factors of *P. haemolytica*. This study has investigated several approaches to the development of potentially efficacious toxin vaccine component. Although these approaches in themselves were not effective in eliciting protective neutralizing antibodies, the information learned from these studies has potential applications. First, based on data obtained from DNA-based vaccination, the adjuvant sequences from IL-1β 163-171 and apoferritin 172-185 are able to augment the immune response in immunized mice. Second, DNA vaccination with an immunogenic fragment of the leukotoxin of *P. haemolytica* is able to generate protein specific antibodies, however, these antibodies are not neutralizing. Therefore, an approach that might prove successful would be a protein based vaccine in which the adjuvant sequences are located at the 5' end or 3' end of the molecule so that they would not be masked by large flanking moieties. In addition, the i.n. route of immunization could be further characterized following protein immunization with the more accessible immunomodulatory sequences. It may prove important to elicit both a systemic as well as a mucosal response to achieve high levels of protection. This type of approach may also have potential applications as a multifactorial subunit vaccine with the incorporation of immunogenic epitopes from other virulence factors associated with the disease.

LIST OF REFERENCES

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1. Acsadi, G., G. Dickson, D. R. Love, A. Jani, F. S. Walsh, A. Gurusinghe, J. Wolff, and K. E. Davies. 1991. Human dystriphin expression in mdx mice after intramuscular injection of DNA constructs. *Nature*. **352**:815-818.

2. Adlam, C. 1989. P. 75-92. The structure, function, and properties of cellular and extracellular components of *Pasteurella haemolytica*. In C. Adlam and J. R. Rutter, eds., Pastuerella and pasteurellosis. Academic Press. London.

3. Adlam, C., J. M. Knights, A. Mugridge, J. C. Lindon, P. R. W. Baker, J. E. Beesley, B. Spacey, G. R. Craig, and L. K. Nagy. 1984. Purification, characterization and immunological properties of the serotype-specific capsular polysaccharide of *Pasteurella haemolytica* (serotype A1) organisms. *J. Gen. Microbiol.* **130**: 2415-2426.

4. Antoni, G., R. Presentini, . Perin, A. Tagliabue, P. Ghiara, S. Censini, G. Volpini, L. Villa, and D. Boraschi. 1986. A short synthetic peptide fragment of human interleukin1 with immunostimulatory but not inflammatory activity. *J. Immunol.* **137**:3201-3204.

5. Arnold, D., S. Faath, H. G. Rammensee, and H. Schild. 1995. Cross priming of minor histocompatibility antigen specific cytotoxic T cells upon immunization with the heat shock protein gp 96. *J. Exp. Med.* **182**:885-890.

6. Atansasiu, P. 1962. Production de tumeurs chez le hamster par inoculaton d'acid deoxynucleique extrait de cultures de tissues infectees parc le curs de polyome. *Acad. Sci.* **254**:4228-4233.

7. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, S. M. Wolff, and C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* **81**:7907-7911.

8. Babiuk, L. A., M. J. P. Lawman, and P. Griebel. 1989. pp. 141-171. Immunosuppression by bovine herpesvirus-1 and other selected herpesviruses. In: S. Specter, M. Bendinelli, and H. Friedman, eds., Virus-induced Immunosuppression. Plenum Publishing Corp., New York.

9. Baker, J. C., J. A. Ellis, and E. G. Clark. 1997. Bovine respiratory syncytial virus. *Vet. Clin. North Am. Food Anim. Pract.* **13**: 425-454.

10. Balauyut, C. S., R. R. Simonson, W. J. Bemrick, W. J. Maheswaran, and S. K. Maheswaran. 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: Identification and partial characterization of a cytotoxin. *Am. J. Vet. Res.* **41**: 1920-1926.

11. Banyard, S. H., D. K. Stammers, and P. M. Harrison. 1978. Electron density map of apoferritin at 28-A resolution. *Nature*. **271**:282-284.

12. Beckers, W., L. Villa, S. Gonfloni, L. Castagnoli, S. M. C. Newton, G. Cesareni, and P. Ghiara. 1993. Increasing the immunogenicity of protein antigens through the genetic insertion of VQGEESNDK sequence of human IL-1β into their sequence. *J. Immunol.* **151**:1757-1764.

13. Berggen, K. A., C. S. Baluyut, R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran. 1981. Cytotoxin effects of *Pasteurella haemolytica* on bovine neutrophils. *Am. J. Vet. Res.* **42**: 1383-1388.

14. Bhakdi, S., N. Mackman, J. M. Nacaud, and I. B. Holland. 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infect. Immun.* **52**: 63-69.

15. Biberstein, E. L. 1990. Our understanding of the Pasteurellaceae. *Can. J. Vet. Res.* **54**: S78-S82.

16. Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature*. **321**:209-213.

17. Boehm, D. F., R. A. Welch, and I. S. Snyder. 1990. Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. *Infect. Immun.* **58**: 1951-1958.

18. Boehm, D. F., R. A. Welch, and I. S. Snyder. 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. *Infect. Immun.* **58**: 1959-1964.

19. Bohn, E., J. Heeseman, S. Ehlers, and I. B. Autenrieth.1994. Early gamma interferon mRNA expression associated with resistance of mice against *Yersinia entercolitica*. *Infect. Immun.* **62**:3027-3032.

20. Boraschi, D., P. Ghiara, G. Scapigliati, L. Villa, A. Sette, A. Taglibue. 1998. Binding and internalization of the 163-171 fragment of human IL-1β. *Cytokine*. **4**:201-204.

21. Bourne, N., L. R. Stanberry, D. I. Bernstein, and D. Lew. 1996. DNA immunization against experimental genital herpes-simplex virus-infection. *J. Inf. Dis.* **173**:800-807.

22. Broaddus, V. C., C. A. Hebert, R. V. Vitangcol, J. M. Hoeffel, M. S. Bernstein, and A. M Boylan. 1992. Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema. *Am. Rev. Respir. Dis.* **146**: 825-830.

23. Brogden, K. and C. Clarke. 1997. Increase of glycocalx and altered lectin agglutination profiles of *Pasteurella haemolytica* A1 after incubation in bovine subcutaneous tissue chambers in vivo or in ruminant serum in vitro. *Infect. Immun.* **65**: 957-963.

24. Cardella, M. A., M. A. Adviento, and R. M. Nervig. 1987. Vaccination studies against experimental bovine Pasteurella pneumonia. *Can. J. Vet. Res.* **51**: 204-211.

25. Carriere, P. D., M. G. Maxie, B. N. Wilkie, M. Savan, V. E. O. Vallie, and J. A. Johnson. 1983. Exposure of calves to aerosols of parainfluenza-3 virus and *Pasteurella haemolytica*. *Can. J. Comp. Med.* **47**:422-432.

26. Caswell, J. L., D. M. Middleton, S. D. Sorden, and J. R. Gordon. 1998. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. *Vet. Pathol.* **35**: 124-131.

27. Chae, C. H., M. J. Gentry, A. W. Confer, and G. A. Anderson. 1990. Resistance to host immune defense mechanisms afforded by capsular material of *Pasteurella haemolytica* serotype 1. *Vet. Microbiol.* **25**: 241-251.

28. Chang, Y. F. C., R. Young, and D. K. Struck. 1989. Cloning and characterization of a hemolysin gene from *Actinabacillus (Haemophilus) pleuropneumoniae*. *DNA*. **8**: 635-647.

29. Chang, Y.-F., H. W. Renshaw, and A. B. Richards. 1986. *Pasteurella haemolytica* leukotoxin: Physiochemical characteristics and susceptibility of leukotoxin to enzymatic treatment. *Am. J. Vet. Res.* **47**: 716-723.

30. Chang, Y.-F., H. W. Renshaw, and R. Young. 1987. Pneumonic pastuerellosis: Examination of typable and untypable *Pasteurella haemolytica* strains for leukotoxin production, plasmid content, and antimicrobial susceptibility. *Am. J. Vet. Res.* **48**: 378-384.

31. Chapman, B. S., R. Thayer, K. Vincent, and N. Haigwood. 1991. Effect of intron A from human cytomegalovirus immediate-early gene on heterologous expression in mammalian cells. *Nuc. Acids Res.* **19**:3979-3986.

32. Chow, Y. -H., W. -L. Huang, W. -K. Chi, Y. -D. Chu, and M. -H. Tao. 1997. Improvement of hepatitis B virus DNA vaccine by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. *Virol.* **71**:169-178.

33. Clarke, C. R., A. W. Confer, and D. A. Mosier. 1998. In vivo effect of *Pasteurella haemolytica* infection on bovine neutrophil morphology. *Am. J. Vet. Res.* **59**:588-592.

34. Clinkenbeard, K. D., D. A. Mosier, and A. W. Confer. 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. *Infect. Immun.* **57**: 420-425.

35. Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. J. Falo. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* **2**:1122-1128.

36. Confer, A. W. and K. R. Simons. 1986. Effects of *Pasteurella haemolytica* lipopolysaccharide on selected functions of bovine leukocytes. *Am. J. Vet. Res.* **47**: 154-157.

37. Confer, A. W., R. J. Panciera, R. W. Fulton, M. J. Gentry, and J. A. Rummage.1985. Effect of vaccination with live or killed *Pasteurella haemolytica* on resistance to experimental bovine pneumonic pasteurellosis. *Am. J. Vet. Res.* **46**: 342-347.

38. Confer, A. W., B. A. Lessley, R. J. Panciera, R. W. Fulton, and J. A. Kreps. 1985. Serum antibodies to antigens derived from saline extract of *Pasteurella haemolytica*: correlation with resistance to experimental bovine pneumonic pasteurellosis. *Vet. Immunol. Immunopathol.* **10**: 265-278.

39. Confer, A. W., R. J. Panciera, and D. A. Mosier. 1988. Bovine pneumonic pasteurellosis: Immunity to *Pasteurella haemolytica. JAVMA.* **193**: 1308-1316.

40. Confer, A. W., R. J. Panciera, K. D. Clinkenbeard, and D. A. Mosier. 1990. Molecular aspects of virulence of *Pasteurella haemolytica. Can. J. Vet. Res.* **54**: S48-S52.

41. Confer, A. W., R. J. Panciera, M. J. Gentry, and R. W. Fulton. 1987. Immunologic response to *Pasteurella haemolytica* and resistance against experimental bovine pneumonic pasteurellosis, induced by bacterins in oil adjuvants. *Am. J. Vet. Res.* **48**: 163-168.

42. Confer, A. W., R. J. Panciera, R. E. Corstvet, J. A. Rummage, and R. W. Fulton. 1984. Bovine pneumonic pasteurellosis: effect of culture age of *Pasteurella haemolytica* used as a live vaccine. *Am. J. Vet. Res.* **45**: 2543-2545.

43. Confer, A. W. and R. W. Fulton. 1995. Evaluation of *Pasteurella* and *Haemophilus* Vaccines. 27th Annual Convention Proceeding American Association of Bovine Practitioners. **27**:136-141.

44. Conlon, J. A. R. and P. E. Shewen. 1993. Clinical and serological evaluation of a Pasteurella haemolytica A1 capsular vaccine. *Vaccine*. **11**:767-772.

45. Conlon, J. A. R., P. E. Shewen, and R. Y. C. Lo. 1991. Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. *Infect. Immun.* **59**:589-591.

46. Corstvet, R. E., M. J. Gentry, R. P. Newman, J. A. Rummage, and A. W. Confer. 1982. Demonstration of age-dependent capsular material on *Pasteurella haemolytica* serotype 1. *J. Clin. Micro.* **16**: 1123-1126.

47. Costanzo, F., C. Santoro, V. Colantuoni, G. Bensi, G. Raugei, V. Romano, and R. Cortese. 1984. Cloning and sequencing of a full length cDNA coding for a human apoferritin H chain: evidence for a multigene family. *EMBO.* **3**: 23-27.

48. Costanzo, F., M. Colombo, S. Staempfi, C. Santoro, M. Marone, F. Frank, H. Delius, and R. Cortese. 1986. Cloning of genes and pseudogenes of human apoferritin H. *Nucleic Acids Res.* **14**:721-736.

49. Costerton, J. W. and R. T. Irvin. 1981. The bacterial glycocalyx in nature and disease. *Ann. Rev. Microbiol.* **35**: 299-324.

50. Cox, G. J. M., T. J. Zamb, and L. A. Babiuk. 1993. Bovine herpesvirus 1: immune response in mice and cattle injected with plasmid DNA. *J. Virol.* **67**:5664-5667.

51. Craven, R. C., A. W. Confer, and M. J. Gentry. 1992. Cloning and expression of a 30 kDa surface antigen of *Pasteurella haemolytica. Vet. Microbiol.* **27**: 63-78.

52. Cruz, W. T., R.Young, Y. F. Chang, and D. K Struck. 1990. Deletion analysis resolves cell-binding and lytic domains of the Pasteurella leukotoxin. *Mol. Microbiol.* **4**: 1933-1939.

53. Czuprynski, C. J. and J. F. Brown. 1987. Recombinant murine interleukin -1α enhancement of nonspecific antibacterial resistance. *Infect. Immun.* **55**:2061-2065.

54. Danko, I. And J. A. Wolff. 1994. Direct gene transfer into muscle. *Vaccine.* **12**:1499-1502.

55. Davies, H. L., M. L. Michel, M. Mancini, M. Schleef, and R. G. Whalen. 1994. Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B surface antigen. *Vaccine.* **12**:1503-1509.

56. Davies, R. L. and W. Donachie. 1996. Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide, and outer membrane proteins. *Microbiology.* **142**:1895-1907.

57. Davis, H. L., M. Schleef, P. Moritz, M. Mancini, J. Schorr, and R. G. Whalen. 1996. Comparison of plasmid DNA preparation methods for direct gene transfer and genetic immunization. *Biotechiques.* **21**:92-94.

58. Davis, H. L., R. G. Whalen, and B. A. Demeneix. 1993. Direct gene transfer into skeletal muscle in vivo: factors affecting efficience of transfer and stability of expression. *Hum. Gene Ther.* **4**:151-159.

59. Deck, R. R., C. M. Dewitt, J. J. Donnelly, M. A. Liu, and J. B. Ulmer. 1997. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine.* **15**:71-78.

60. Dinarello, C. A. 1984. Interleukin-1 and the pathogenesis of the acute-phase response. *N. Engl. J. Med.* **311**:1413-1418.

61. Dinarello, C. A. 1984. Interleukin-1. *Rev. Infect. Dis.***6**:51-95.

62. Donnelly, J. J., A. Friedman, D. Martinez, D. L. Montgomery, J. W. Shriver, S. L. Motzel, J. B. Ulmer, and M. A. Lui. 1995. Preclinical efficacy of a protrtype DNA vaccine-enhanced protection against antigenic drift in influenza-virus. *Nature Med.* **1**:583-587.

63. Donnelly, J. J., J. B Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol.* **15**:617-648.

64. Donnelly, J. J., J. B. Ulmer, and M. A. Liu. 1994. Immunization with DNA. *J. Immunol. Meth.* **176**:145-152.

65. Dowty, M. E., P. Williams, G. Zhang, J. E. Hagstrom, and J. A. Wolff. 1995. Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. *Proc. Natl. Acad. Sci. USA.* **92**:4572-4576.

66. Durham, J. A., S. M. Antone, M. W. Cunningham, and A. W. Confer. 1988. Monoclonal antibodies to *Pasteurella haemolytica* serotype 1 lipopolysaccharide: Demonstration of antigenic similarities among several serotypes. *J. Clin. Micro.* **26**: 885-889.

67. Eberspacher, B., F. Hugo, M. Pohl, and S. Bhakdi. 1990. Functional similarity between the haemolysins of *Escherichia coli* and *Morganella morganii. J. Med. Microbiol.* **33**: 165-170.

68. Edelman, R. 1997. Adjuvant for the future. In M. M. Levine, G. C. Woodrow, J. B. Kaper, and G. S. Cobon, eds., New Generation Vaccines. Marcel Dekker, Inc., New York.

69. Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes. 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol.* **12**:791-797.

70. Ertl, H. C. J. and Z. Q. Xiang. 1996. Genetic Immunization. *Viral Immunol.* **9**:1-9.

71. Felmlee, T. and R. A. Welch. 1988. Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytolytic activity and secretion. *Proc. Natl. Acad. Sci. USA.* **85**: 5269-5273.

72. Felmlee, T., S. Pellet, and R. A. Welch. 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol.* **163**: 94-105.

73. Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* **158**:2278-2284.

74. Fenner, F., R. Witlek, and K. R. Dumbell. 1989. The Orthopoxyvirus. Academic Press, San Diego, CA.

75. Filion, L. G., R. L. McGuire, and L. A. Babiuk. 1983. Nonspecific suppressive effect of bovine herpesvirus type 1 on bovine leukocyte functions. *Infect. Immun.* **42**:106-112.

76. Fish, Elizabeth. 1993. Protective epitopes in the leukotoxin of *Pasteurella haemolytica*. Dissertation. The University of Tennessee. Knoxville, TN.

77. Forestier, C. and R. A. Welch. 1990. Nonreciprocal complementation of the *hlyC* and *lktC* genes of the *Escherichia coli* hemolysin and *Pasteurella haemolytica* leukotoxin determinants. *Infect. Immun.* **58**: 828-832.

78. Francis, M. J. 1990. Peptide vaccines for viral disease. *Sci. Prog.* **74**:115-130.

79. Frank, G. H. 1986. The role of *Pasteurella haemolytica* in the bovine respiratory disease complex. *Vet. Med.* **September**: 838-846.

80. Frank, G. H. 1989. pp. 197-222. Pasteurellosis in cattle. In C. Adlam and J. M. Rutter, eds., Pasteurella and Pasteurellosis. Academic Press, London.

81. Frank, G. H. and L. B. Tabatabai. 1981. Neuraminidase activity of *Pasteurella haemolytica* isolates. *Infect. Immun.* **32**:1119-1122.

82. Frank, G. H. and P. C. Smith. 1983. Prevalence of *Pasteurella haemolytica* in transported calves. *Am. J. Vet. Res.* **44**: 981-985.

83. Friend, S. C. E., B. N. Wilkie, R. G. Thompson, and D. A. Barnum. 1977. Bovine pneumonic pasteurellosis: Experimental induction in vaccinated and nonvaccinated calves. *Can. J. Comp. Med.* **41**: 77-83.

84. Fu, T. M., J. B. Ulmer, M. J. Caulfield, R. R. Deck, A. Friedman, S. Wang, X. Liu, J. J. Donnelly, and M. A. Liu. 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Med.* **3**:362-371.

85. Fuller, D. H. and J. R. Haynes. 1994. A qualitative progression in HIV type I glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA based glycoprotein 120 vaccine. *AIDS Res. Hum. Retrovir.* **10**:1433-1441.

86. Fuller, D. H., L. Simpson, K. S. Cole, J. E. Clements, D. L. Panicalli, R. C. Montelaro, M. Murphey-Corb, and J. R. Haynes. 1997. Gene gun-based nucleic acid immunization alone or in combination with recombinant vaccinia suppresses virus burden in rhesus macaques challenged with a heterlogous SIV. *Immun. Cell Biol.* **75**:389-396.

87. Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA *Vaccines*: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. U.S.A.* **90**:11478-11482.

88. Geissler, M., A. Gesien, K. Takushige, and J. R.. Wands. 1997. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol.* **158**:1231-1237.

89. Gentry, M. J. and S. Srikumaran. 1991. Neutralizing monoclonal antibodies to *Pasteurella haemolytica* leukotoxin affinity-purify the toxin from crude culture supernatants. *Microbial. Path.* **10**: 411-417.

90. Gentry, M. J., A. W. Confer, and J. A. Kreps. 1985. Simple visual assay for determination of *Pasteurella haemolytica* cytotoxin neutralizing antibody titers in cattle sera. *J. Clin. Micro.* **22**: 968-972.

91. Gentry, M. J., A. W. Confer, and R. J. Panciera. 1985. Serum neutralization of cytotoxin from *Pasteurella haemolytica*, serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. *Vet. Immunol. Immunopathol.* **9**: 239-250.

92. Gerbig, D. G., 1991. Generation of monoclonal antibodies to *Pasteurella haemolytica* leukotoxin: Characterization of a neutralizing monoclonal antibody. Dissertation. The University of Tennessee. Knoxville, TN.

93. Gerbig, D. G., Jr., M. R. Cameron, D. K. Struck, and R. N. Moore. 1992. Characterization of a neutralizing monoclonal antibody to *Pasteurella haemolytica* leukotoxin. *Infect. Immun.* **60**: 1734-1739.

94. Gilkerson, G. S., P. Ruiz, and A. M.M. Pippen. 1996. Modulation of renal disease in autoimmune NZB/NZW mice immunization with bacterial DNA. *J. Exp. Med.* **183**:1289-1387.

95. Gilkeson, G. S., A. J. Pritchard, and D. S. Pisetsky. 1991. Specificity of anti-DNA antibodies induced in normal mice by immunization with bacterial DNA. *Clin. Immunol. Immunopathol.* **59**:288-300.

96. Glaser, P., D. Ladent, O. Sezer, F. Pichot, A. Ullman, and A. Danchin. 1988. Sercretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO.* **7**: 3997-4004.

97. Glaser, P., D. Ladent, O. Sezer, F. Pichot, A. Ullman, and A. Danchin. 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. *Mol. Micobiol.* **2**: 1-30.

98. Glorioso, J. C., G. W. Jones, H. G. Rush, L. J. Pentler, C. A. Darif, and J. E. Coward. 1982. Adhesion of type A *Pasteurella multocida* to rabbit pharyngeal cells and its possible role in rabbit respiratory tract infections. *Infect. Immun.* **35**: 1103-1109.

99. Granick, S. 1951. Structure and physiological function of ferritin. *Physiol. Rev.* **31**:489-511.

100. Gray, L., N. Mackman, J. M. Nicad, and I. B. Holland. 1986. The carboxyl-terminal region of hemolysin 2001 is required for secretion of the toxin from *Escherichia coli*. *Mol. Gen. Genet.* **205**: 127-133.

101. Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. *Vet. Clin. North Am. Food Anim. Pract.* **13**: 367-377.

102. Hagstrom, J. E., I. N. Rybakova, T. Staeva, J. A. Wolff, and J. M. Ervasti. 1996. Nonnuclear DNA binding proteins in striated muscle. *Biochem. Mol. Med.* **58**:113-121.

103. Halpern, M. D., R. J. Kurlander, and D. S. Pisetsky. 1996. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell Immunol.* **167**:72-78.

104. Hartikka, J., M. Sawdey, F. Cornefert-Jensen, M. Margabeth, K. Barnhart, M. Nolasco, H. Vahlsing, J. Meek, M Marquet, P. Hobart, J. Norman, and M. Manthorpe. 1996. An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum. Gene Ther.* **7**:1205-1217.

105. Hartlein, M., S. Schiefl, W. Wagner, U. Rdest, J. Kreft, and W. Goebel. 1983. Transport of hemolysin by *Escherichia coli*. *J. Cell. Biochem.* **22**: 87-97.

106. Henry, C. W. 1984. Shipping fever pneumonia: A new look at an old enemy. *Vet. Med.* **September**: 1200-1206.

107. Highlander, S. K., M. Chaidambaram, M. J. Engler, and G. M. Weinstock. 1989. DNA sequence of the *Pasteurella haemolytica* leukotoxin gene cluster. *DNA*. **8**: 15-28.

108. Highlander, S. K., M. J. Engler, and G. M. Weinstock. 1990. Secretion and expression of the *Pasteurella haemolytica* leukotoxin. *J. Bacteriol.* **172**: 2343-2350.

109. Himmel, M. E., M. D. Yates, L. H. Lauerman, and P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from *Pasteurella haemolytica*. *Am. J. Vet. Res.* **43**: 764-767.

110. Hohfeld, R. and A. G. Engel. 1994. The immunobiology of muscle. *Immunol. Today*. **15**:269-274.

111. Hughes, C. J. P. Issartel, K. Hardie, P. Stanley, E. Koronakis, and V. Koronakis. 1992. Activation of *Escherichia coli* prohemolysin to the membrane-targeted toxin by Hly-C directed ACP-dependent fatty acylation. *FEMS Microbiol. Immunol.* **105**: 37-44.

112. Hui, K. M., T. K. Sabapathy, A. Oei, and T. F. Chia. 1994. Generation of allo-reactive cytotoxic T lymphocytes by particle bombardment-mediated gene transfer. *J. Immunol. Meth.* **171**:147-155.

113. Iovane, G., M. Galdiero, M. Vitiello, and L. De Martino. 1998. Effect of *Pasteurella haemolytica* outer membrane proteins on bovine neutrophils. *FEMS Immunol. Med. Microbiol.* **20**: 29-36.

114. Issartel, J. P., V. Koroakis, and C. Hughes. 1991. Activation of *Escherichia coli* prohemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature*. **351**: 759-761.

115. Jain, S. K., K. J. Barrett, D. Boyd, M. F. Faveau, J. Crampton, and J. W. Drysdale. 1985. Ferritin H and L chains are derived from different multigene families. *J. Biol. Chem.* **260**:11762-11768.

116. Jericho, K. W. F., and E. V. Langford. 1982. Aerosol vaccination of calves with *Pasteurella haemolytica* against experimental respiratory disease. *Can. J. Comp. Med.* **46**: 287-292.

117. Jericho, K. W. F., C. I Q. Darcel, and E. V. Langford. 1982. Respiratory disease in calves produced with aerosols of parainfluenza-3 virus and *Pasteurella haemolytica*. *Can. J. Comp. Med.* **46**: 293-301.

118. Jiao, S., P. Williams, R. K. Berg, B. A. Hodgeman, L. Liu, G. Repetto, and J. A. Wolffe. 1992. Direct gene transfer into nonhuman primate myofibers in vivo. *Hum. Gene Ther.* **3**:21-33.

119. Justewicz, D. M., M. J. Morin, H. L. Robinson, and R. G. Webster. 1995. Antibody-forming cell response to virus challenge in mice immunized with DNA encoding the influenza virus hemagglutinin. *J. Virol.* **69**:7712-7717.

120. Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat, and D. W. Johnson. 1980. Evidence of cytocidal effects of *Pasteurella haemolytica* on bovine peripheral blood mononuclear leukocytes. *Am. J. Vet. Res.* **41**: 1690-1693.

121. Kaehler, K., R. J. F. Markham, C. C. Muscoplat, and D. W. Johnson. 1980. Evidence of species specificity in the cytocidal effects of *Pasteurella haemolytica*. *Infect. Immun.* **30**: 615-616.

122. Kapil, S. and Randall J. Basaraba. 1997. Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus. *Vet. Clin. North Am. Food Anim. Pract.* **13**: 455-469.

123. Katsumi, A. N. Emi, A. Abe, Y. Hasegawa, M. Ito, and H. Saito. 1994. Humoral and cellular immunity to an encoded protein induced by direct DNA injection. *Hum. Gene Ther.* **5**:1335-1339.

124. Keiss, R. E., D. H. Will, and J. R. Collier. 1964. Skin toxicity and hemodynamic properties of endotoxin derived from *Pasteurella haemolytica. Am. J. Vet. Res.* **25**: 935-941.

125. Kim, J. J., V. Ayyavoo, M. L. Bagarazzi, M. A. Chattergoon, K. Dang, B. Wang, J. D. Boyer, and D. B. Weiner. 1997. In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *J. Immunol.* **158**:816-826.

126. Klemm, P. 1985. Fimbrial adhesins of *Escherichia coli*. Rev. *Infect. Dis.* **7**: 321-340.

127. Klinman, D. M., A. K. Yi, S. L. Beaucage, J. Conover, A. M. Krieg. 1996. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. U.S.A.* **93**:2879-2883.

128. Klinman, D. M., G. Yamshchikov, and Y. Ishigatsubo. 1997. Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* **158**:3635-3639.

129. Koronakis, V., E. Koronakis, and C. Hughes. 1989. Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO.* **8**: 595-605.

130. Koronkis, V., M. Cross, B. Senior, E. Koronakis, and C. Hughes. 1987. The secreted hemolysins of *Proteus mirabilus*, *Proteus vulgaris*, and *Morganella morganii* alpha-hemolysin of *Escherichia coli. J. Bacteriol.* **169**: 1509-1515.

131. Kovacsvics-Bankowsky, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigen presented on MHC-class I molecules. *Science.* **267**:243-246.

132. Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**:8125-8148.

133. Kraig, E., T. Dailey, and D. Kolodrubetz. 1990. Neucleotide sequence of the leukotoxin gene from Actinobacillus actinomycetemcomitans: homology to the alpha-hemolysin/leukotoxin gene family. *Infect. Immun.* **58**: 920-929.

134. Kreisberg, R., M. S. Detrick, A. P. Osmand, and R. N. Moore. 1993. Cytokines mediation of the suppressive effect of ferritin on CSF-1 dependent monocytopoiesis. *J. Immunol.* **150**: 5094-5103.

135. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. **374**:546-549.

136. Kucera, C. J., J. C. S. Wong, and T. J. Feldner. 1983. Challenge exposure of cattle vaccinated with a chemically altered strain of *Pasteurella haemolytica*. *Am. J. Vet. Res.* **44**: 1848-1852.

137. Kuklin, N, M. Daheshia, K. Karem, E. Manickan, and B. T. Rouse. 1997. Induction of musocal immunity against herpes simples virus by plasmid DNA immunization. *J. Virol.* **71**:3138-3145.

138. Kumar, V. and E. Sercarz. 1996. Genetic vaccination: the advantage of going naked. *Nat. Med.* **2**:857-859.

139. Kuper, C. F., P. J. Koornstra, and D. M. H. Haneleers. 1992. The role of nasoopharyngeal lymphoid tissue. *Immunol. Today*. **13**:219-224.

140. Kurar, E.and G. A. Splitter. 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. *Vaccine*. **17**:1851-1857.

141. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

142. Lafleur, R. L., M. S. Abrahasen, and S. K. Maheswaran. 1998. The biphasis mRNA expression pattern of bovine interleukin-8 in *Pasteurella haemolytica* lipopolysaccharide stimulated alveolar macrophages is primarily due to tumor necrosis factor alpha. *Infect. Immun.* **66**:4087-4092.

143. Lally, E. T., E. E. Golub, I. R. Kieba, N. S. Taichman, J. Rosenbloom, J. C. Rosenbloom, E. W. Gibson, and D. R. Demuth. 1989. Analysis of the *Actinbacillus actinomycetemcomitans* leukotoxin gene. *J. Biol. Chem.* **264**: 15451-15456.

144. Lally, E. T., I. R. Keiba, D. R. Demuth, J. Rosenbloom, E. E. Golub, N. S. Taichman, and C. W. Gibson. 1989. Identification and expression of the *Actinobacillus actinomycetemconitans* leukotoxin gene. *Biophys. Res. Comm.* **159**: 256-262.

145. Leake, E. S., M. J. Wright, and A. S. Dregar. 1978. In vitro effect of purified protease of *Pseudomonas aeurginosa* on rabbit lung macrophages. *Exp. Mol. Path.* **29**.241-252.

146. Levine, M. M., D. M. Hone, B. A. Stocker, and M. Cadoz. 1990. pp. 269-287. Vaccines to prevent typoid fever. In G Woodrow and M. M. Levine, eds., New generation Vaccines. Marcel Dekker, New York.

147. Lillie, L. E. 1974. The bovine respiratory disease complex. *Can. Vet. J.* **15**: 233-242.

148. Lo, R. Y. C. 1990. Molecular characterization of cytotoxins produced by Haemophilus, Actinobacillus, Pasteurella. *Can. J. Vet. Res.* **54**: S33-S35.

149. Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen. 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect. Immun.* **55**: 1987-1996.

150. Lo, R. Y. C., P. E. Shewen, C. A. Strathdee, and C. N. Greer. 1985. Cloning and expression of the leukotoxin gene of *Pasteurella haemolytica* A1 in *Escherichia coli* K-12. *Infect. Immun.* **50**: 667-671.

151. Lofthouse, S. A., A. E. Andrews, M. J. Elhay, V. M. Bowles, E. N. T. Meeusen, and D. Nash. 1996. Cytokines as adjuvants for ruminant vaccines. *Internatl. J. Parasitol.* **26**: 835-842.

152. Lomedico, P. T., U. Gubler, C. P. Hellmann, M. Dukovich, J. G. Giri, Y. C. Pan, K. Collier, R. Semionow, A. O. Chua, and S. B. Mizel. 1984. Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*. *Nature*. **312**:458-462.

153. Lu, S. J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M. Wyland, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. *J. Virol.* **70**:3978-3991.

154. Lu, S. J. C. Santoro, D. H. Fuller, J. R. Haynes, and H. L. Robinson. 1995. Use of DNAs expressing HIV-1 env and noninfectious HIV-1 particles to raise antibody responses in mice. *Virology*. **209**:147-154.

155. Ludwig, A., M Vogel, and W. Goebel. 1987. Mutations affecting activity and transport of haemolysin in *Escherichia coli*. *Mol. Gen. Genet.* **206**: 238-245.

156. Ludwig, A., T. Jarchae, R. Benz, and W. Goebel. 1988. The repeat domain of *Escherichia coli* haemolysin (Hly A) is responsible for its Ca2+-dependent binding to erythrocytes. *Mol. Gen. Genet.* **214**: 553-561.

157. Luke, C. J., K. Carner, X. Liang, and A. G. Barbour. 1997. An OspA-based DNA vaccine protects mice against infection with *Borrelia burgdorferi*. *J. Infect. Dis.* **175**:91-97.

158. Mackman, N, J. M. Nicaud, L. Gray, and I. B. Holland. 1986. Secretion of haemolysin by *Escherichia coli*. *Curr. Top. Microbiol. Immunol.* **125**: 15-181.

159. Mackman, N. and I. B. Holland. 1984. Functional characterization of a cloned haemolysin determinant from *E. coli* of human origin, encoding information for the secretion of a 107 K polypeptide. *Mol. Gen. Genet.* **196**: 129-134.

160. Mackman, N. and I. B. Holland. 1984. Secretion of a 107 kDa polypeptide into the medium from a haemolytic E. coli K12 strain. *Mol. Gen. Genet.* **193**: 312-315.

161. MacLeod, C. M. and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. *J. Exp. Med.* **92**: 1-9.

162. Maeland, J. A. and E. Wedge. 1989. Serum antibodies to cross-reactive Neisseria outer membrane antigens in healthy persons and patients with meningococcal disease. *APMIS* Sect. C 97, 774-780.

163. Majury, A. L. and P. E. Shewn. 1991. The effect of *Pasteurella haemolytica* A1 leukotoxin culture supernatant on the in vitro proliferative response of bovine lymphocytes. *Vet. Immunol. Immunopath.* **29**:41-56.

164. Malone, J. G., P. J. Bergland, P. Liljestrom, G. H. Rhodes, and R. W. Malone. 1997. Mucosal immune response associated with polynucleotide vaccination. *Behring. Inst. Mitt.* **98**:63-72.

165. Mandrell, R. E. and W. D. Zollinger. 1989. Human immune response to meningococcal outer membrane epitopes after natural infection and vaccination. *Infect. Immun.* **57**: 1590-1598.

166. Manickan, E. R. Rouse, Z. Y. Zu, W. S. Wire, B. T. Rouse. 1995. Genetic immunizaation against herpes-simplex-virus protection is mediated by CD4+ T-lymphocytes. *J. Immunol.* **155**:259-265.

167. Manickan, E. Z. Yu, R. D. J. Rouse, W. S. Wire, and B. T. Rouse. 1995. Induction of protective immunity against the herpes simplex virus with DNA encoding the immediate early protein ICP 27. *Viral Immunol.* **8**:53-61.

168. Manickan, E., K. L. Karem, and B. T. Rouse. 1997. DNA vaccines-A modern gimmick or a boon to vaccinology. *Crit. Rev. Immunol.* **17**:139-154.

169. March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature*. **315**:641-647.

170. Markham, R. J. F. and B. N. Wilkie. 1980. Interaction between *Pasteurella haemolytica* and bovine alveolar macrophages: Cytotoxic effect on macrophages and impaired phagocytosis. *Am. J. Vet. Res.* **41**: 18-22.

171. Martin, W. 1983. Vaccination: Is it effective in preventing respiratory disease of influencing weight gains in feedlot calves? *Can. Vet. J.* **24**: 10-19.

172. Martin, W., P. Wilson, R. Curtis, B. Allen, and S. Acres. 1983. A field trial, of preshipment vaccination, with intranasal infectious bovine rhinotracheitis parainfluenza-3 vaccines. *Can J. Comp. Med.* **47**: 245-249.

173. Martin, W., S. Acres, E. Jansen, P. Williams, and B. Allen. 1984. A field trial of preshipment vaccination of calves. *Can. Vet. J.* **25**: 145-147.

174. Mason, E. O., Jr., S. L. Kaplan, B. L. Wiedermann, E. P. Norrod, and W. A. Stenback. 1985. Frequency and properties of naturally occurring adherent piliated strains of Haemophilus influenzae type b. *Infect. Immun.* **49**: 98-103.

175. Matsumoto, M., J. A. Schmitz, B. Syuto, B. J. Watrous, and D. E. Mattson. 1984. Immunogenicity of a soluble antigen against *Pasteurella haemolytica* in calves. *Vet. Res. Com.* **8**: 117-130.

176. Maul, G. G., A. M. Ishov, and R. D. Everett. 1995. Nuclear domain 10 (ND10) are the preferred sites where the nuclear replication cycle of herpes simplex virus type 1 begins. *Virology*. **59**:498-513.

177. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. Eldridge, M. Hirasawa, and H. Klyono. 1992. The mucosal immune system from fundamental concepts to vaccine development. *Vaccine*. **10**:75-88.

178. McKee, K. T.,W. H. Bancroft, K. H. Eckels, R. R. Redfield, P. L. Summers, and R. K. Russell. 1987. Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. *Am. J. Trop. Med. Hyg.* **36**:435-442.

179. McPherson, Jackson. 1998. Localization of neutralizing epitopes in the leukotoxin of *Pasteurella haemolytica*. Dissertation. The University of Tennessee. Knoxville, TN.

180. McVey, D. S., R. W. Loan, C. W. Purdy, and W. J. Shuman. 1990. Specificity of bovine serum antibody to capsular carbohydrate antigens from *Pasteurella haemolytica. J. Clin. Microbiol.* **28**: 1151-1158.

181. Menestrina, G., C. Moser, S. Pellet, and R. Welch. 1994. Pore-forming by *Escherichia coli* hemolysin (HlyA) and other members of the RTX toxins family. *Toxicology*. **87**: 249-267.

182. Michel, M. L., H. L. Davis, M. Schleef, M. Mancini, P. Tiollaris, and R. G. Whalen. 1995. DNA-mediated immunization to the hepatitis-B surface-antigen in mice—aspects of the humoral response mimic hepatitis-B viral-infection in humans. *Proc. Natl. Acad. Sci. USA.* **92**:5307-5311.

183. Montgomery, R. I., J. W. Shiver, K. R. Leander, H. C. Perry, A. Friedman, D. Martinez, J. B. Ulmer, J. J. Donnelly, and M. A. Liu. 1993. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol.* **12**:777-783.

184. Moore, R. N., R. D. Walker, G. A. Shaw, F. M. Hopkins, and E. P. Shull. 1985. Antileukotoxin antibody producted in the bovine lung after aerosol exposure to viable *Pasteurella haemolytica. Am. J. Vet. Res.* **46**: 1949-1952.

185. Morck, D. W., T. J. G. Raybould, S. D. Acres, L. A. Babiuk, J. Nelligan, and J. W. Costerton. 1987. Electron microscopic description of glycocalx and fimbriae on the surface of *Pasteurella haemolytica. Can. J. Vet. Res.* **51**:83-88.

186. Morton, R. J., R. J. Panciera, R. J. Fulton, G. H. Franks, S. A. Ewing, J. T. Homer, and A. W. Confer. 1995. Vaccination of cattle with outer membrane protein-enriched fractions of *Pasteurella haemolytica* and resistance against experimental challenge exposure. *Am. J. Vet. Res.* **56**: 875-879.

187. Mosier, D. A., A. W. Confer, and R. J. Panciera. 1989. The evolution of vaccines for bovine pneumonic pasteurellosis. *Res. Vet. Sci.* **47**: 1-10.

188. Mosier, D. A., B. A. Lessley, A. W. Confer, S. M. Antone, and M. J. Gentry. 1986. Chromatographic separation and characterization of *Pasteurella haemolytica* cytotoxin. *Am. J. Vet. Res.* **47**: 2233-2241.

189. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65**: 55-63.

190. Muraguchi, A., T. Hirano, B. Tang, T. Matsuda, Y. Horii, K. Nakajima, and T. Kishimoto. 1988. The essential role of B cell stimulatory factor 2 (B SF-2-IL-6) for the terminal differentiation of B cells. *J. Exp. Med.* **167**:332-340.

191. Najjar, V. A. 1985. Tuftsin: a natural activator of phagocytic cells with antibacterial and antineoplastic activity. In P. F. Torrence, ed., Biological Response Modifiers: New Approaches to Disease Intervention. Academic Press.

192. Nencioni, L., L. Villa, A. Tagliabue, G. Antoni, R. Presentini, F. Perin, S. Silvestri, and P, Boraschi. 1987. In vivo immunostimulating activity of the 163-171 peptide of human IL-1β. *J. Immunol.* **137**:800-804.

193. Nicaud, J. M., N. Mackman, L. Gray, and I. B. Holland. 1986. The C-terminal 23kDa peptide of *E. coli* haemolysin 2001 contains all the information necessary for its secretion by the hemolysin export machinery. *FEBS Lett.* **204**: 331-335.

194. Nichols, W. W., B. J. Ledwith, S. V. Manam, and P. J. Troilo. 1995. Potential DNA vaccine integration into host cell genome. *Ann. NY Acad. Sci.* **772**:30-39.

195. Nyarko, K.A., B. L. Coomber, A. mellors, and P. A. Gentry. 1998. Bovine platelet adhesion is enhanced by leukotoxin and sialoglycoprotease isolated from *Pasteurella haemolytica* A1 cultures. *Vet. Microbiol.* **61**: 81-91.

196. Okada, E., S. Sasaki, N. Ishii, I. Aoki, T. Yasuda, K. Nishioka, J. Fukushima, J. Miyazaki, B. Wahren, and K. Okuda. 1997. Intranasl immunization of DNA vaccine with IL-12 and granulocyte-macrophage colony-stimulating factor expressing plasmids in liposomes induces strong mucosal and cell mediated immune responses against HIV-1 antigens. *J. Immunol.* **159**:3638-3647.

197. Otulakowski, G. L., P. E. Shewen, A. E. Udoh, A. Mellors, and B. N. Wilkie. 1983. Proteolysis of sialoglycoprotein by *Pasteurella haemolytica* cytotoxic culture supernatant. *Infect. Immun.* **42**: 64-70.

198. Ozaki, Y., T. Ohashi, A. Minami, and S. I. Nakamura. 1987. Enhanced resistance of mice to bacterial infection induced by recombinant human interleukin-1α. *Infect. Immun.* **55**:1436-1440.

199. Pal, S., K. A. Barnhart, Q. Wei, A. M. Abai, E. M. Peterson, and L. M. Maza. 1999. Vaccination of mice with DNA plasmids coding for the *Chlamydia trachomatis* major outer membrane protein elicits an immune response but fails to protect against a genital challenge. *Vaccine*. **17**:459-465.

200. Panciera, R. J., R. E. Corstvet, A. W. Confer, and C. N. Gresham. 1984. Bovine pneumonic pasteurellosis: Effect of vaccination with live Pasteurella species. *Am. J. Vet. Res.* **45**: 2538-2542.

201. Pardoll, D. M. and A. M. Beckerleg. 1995. Exposing the immunology of naked DNA vaccines. *Immunity*. **3**:165-169.

202. Pike, B. L., G. J. V. Nossal. 1985. Interleukin-1 can act as a B-cell growth and differentiation factor. *Proc. Natl. Acad. Sci.* USA. **82**:8153-8157.

203. Potgieter, L. 1997. Bovine respiratory tract disease caused by bovine viral diarrhea virus. **13**: 471-481.

204. Potgieter, L. N., M. D. McCracken, F. M. Hopkins, R. D. Walker, and J. S. Guy. 1984. Experimental production of bovine respiratory tract disease with bovine viral diarrhea virus. *Am. J. Vet. Res.* **45**:1582-1585.

205. Ramon, G. 1926. Procedes pour accroitre la production des antitoxines. *Ann. Inst. Pasteur*. **40**:1-10.

206. Rao, K. V. S. and A. R. Nayak. 1990. Enhanced immunogenicity of a sequence derived from hepatitis B virus surface antigen in a composite peptide that includes the immunostimulatory region from human interleukin 1. *Proc. Natl. Acad. Sci. USA*. **87**:5519-5522.

207. Reed, S. G., D. L. Pihl, and K. H. Grabstein. 1989. Immune deficiency in chronic Trypansoma cruzi infection: Recombinant IL-1 restores Th function for antibody production. *J. Immunol.* **142**:2067-2071.

208. Rehmtulla, A. J. and R. J. Thomson. 1981. A review of the lesions in shipping fever of cattle. *Can. Vet. J.* **22**: 1-8.

209. Rimsay, R. L., J. E. Coyle-Dennis, L. H. Lauerman, and P. G. Squire. 1984. Purification and biological characterization of endotoxin fractions from *Pasteurella haemolytica*. *Am. J. Vet. Res.* **42**: 2134-2138.

210. Robertson, C. R. and D. S. Pisetsky. 1992. Immunochemical properties of anti-DNA antibodies in the sera of patients with *Escherichia coli* bacteremia. *Int. Arch. Allergy Immunol.* **98**:311-316.

211. Robinson, H. L., L. A. Hunt, and R. G. Webster. 1993. Protection against a lethal influenza virus challenge by immunization with a haaemagglutinin-expressing plasmid DNA. *Vaccine*. **11**:957-90.

212. Romagnani, S. 1995. Biology of human TH1 and TH2 cells. *J. Clin. Immunol.* **15**:121-129.

213. Roman, M., E. M. Orozco, J. S. Goodman, M. Mguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. *Nat. Med.* **3**:849-854.

214. Rowe, G. E., S. Pellet, and R. A. Welch. 1994. Analysis of toxinogenic functions associated with the RTX repeat region and monoclonal antibody D12 epitope of *Escherichia coli* hemolysin. *Infect. Immun.* **62**: 579-588.

215. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

216. Sasaki, S., K. Sumino, K. Hamajima, J. Fukushima, N. Ishii, S. Kawamoto, H. Mohri, C. R. Kensil, and K. Okuda. 1998. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 Saponin adjuvant via intramuscular and intranasal routes. *J. Virol.* **72**:4931-4939.

217. Sato, Y., m. Roman, H. Tighe, D. Lee, M. Corr, M. -D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science*. **273**:352-354.

218. Schrijver, R. S., J. P. M. Langedijk, G. A. Keil, W. G. J. Middel, M. Maris-Veldhuis, J. T. Oirshot, and F. A. M. Rijsewijk. 1998. Comparison of DNA application methods to reduce BRSV shedding in cattle. *Vaccine*. **16**:130-134.

219. Schlor, S., A. Schmidt, E. Maier, R. Benz, W. Goebel, and I. Gentschev. 1997. In vivo and in vitro studies on interactions between the components of the hemolysin (HlyA) secretion secretion machinery of *Escherichia coli*. *Mol. Gen. Genet.* **256**: 306-319.

220. Sedegah, M. R. Hestrom, P. Hobart, and S. L. Hoffman. 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. USA.* **91**:9866-9870.

221. Shewen, P. E. and B. N. Wilkie. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect. Immun.* **35**: 91-94.

222. Shewen, P. E. and B. N. Wilkie. 1985. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. *Am. J. Vet. Res.* **46**: 1212-1214.

223. Shewen, P., A. Sharp, and B. N. Wilkie. 1988. Efficacy testing of a *Pasteurella haemolytica* extract vaccine. *Vet. Med.* **October**: 1078-1084.

224. Shewn, P. E. and B. N. Wilkie. 1983. *Pasteurella haemolytica* cytotoxin neutralizing activity in sera from Ontario beef cattle. *Can. J. Comp. Med.* **47**: 497-498.

225. Siddiqui, M. Z., A. K. Sharma, S. Kumar, A. Kumar, and P. N. Bhat. 1994. Conformation of a T-cell stimulating peptide of interleukin-1β protein: circular dichroism studies. *Int. J. Biol. Macromol.* **16**:259-263.

226. Singh, S. P., Y. U Williams, W. H. Benjamin, P. E. Klebba, and D. Boyd. 1996. Immunoprotection by monoclonal antibodies to the porins and lipopolysaccharide of *Salmonella typhimurium*. *Microb. Pathogen.* **21**: 249-263.

227. Slocombe, R. F., J. Malark, R. Ingersoll, F. J. Derksen and N. E. Robison. 1985. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. *Am. J. Vet. Res.* **46**:2253-2258.

228. Smith, C. K., J. N. Davidson, and C. W. Henry. 1985. Evaluating a live vaccine for *Pasteurella haemolytica* in dairy calves. *Vet. Med.* **80**. 78-88.

229. Smith, E. R. 1961. The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions in sheep. *J. Path. Bact.* **81**: 431-440.

230. Smith, K. A., L. B. Lachman, J. J. Oppenheim, and M. F. Favata. 1980. The functional relationship of the interleukins. *J. Exp. Med.* **151**:1551-1556.

231. Sreevatson, S., T. R. Ames, E.Werdin, H. S. Yoo, and S. K. Maheswaran. 1996. Evaluation of three experimental subunit vaccines against pneumonic pasteurellosis in cattle. *Vaccine*. **14**:147-154.

232. Srinand, S., S. L. Hsuan, H. S. Yoo, S. K. Maheswaran, T. R. Ames, and R. E. Werdin. 1996. Comparative evaluation of antibodies induced by commercial *Pasteurella haemolytica* vaccines using phase immunoassays. *Vet. Microbiol.* **49**: 181-195.

233. Srinand, S., T. R. Ames, S. K. Maheswaran, and V. L. King. 1996. Efficacy of various vaccines against pneumonic pasteurellosis in cattle:a meta-analysis. *Prevent. Vet. Med.* **25**:7-17.

234. Staats, H. F., W. G. Nichols, and T. J. Palker. 1996. Systemic and vaginal antibody responses after intranasl immunization with the HIV-1 C4/V3 peptide T1Sp10 MN(A). *J. Immunol.* **157**:462-472.

235. Standiford, T. J., S. L. Kunkel, M. J. Greenberger, L.L. Laichalk, and R. M. Streiter. 1996. Expression and regulation of chemokines in bacterial pneumonia. *J. Leukocyte Biol.* **59**: 24-29.

236. Stanley, P. L., C. Packman, V. Koronakis, and C. Hughes. 1994. Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science*. **266**: 1992-1996.

237. Staruch, M. J. and D. D. Wood. 1983. The adjuvanicity of interleukin 1 in vivo. *J. Immunol.* **130**:2191-2194.

238. Stockdale, P. H. G., E. V. Langford, and C. I. Q. Darcel. 1979. Experimental bovine pneumonic pasteurellosis I. Prevention of the disease. *Can. J. Comp. Med.* **43**: 262-271.

239. Stockdale, P. H. G., K. W. F. Jericho, W. D. G. Yates, C. I. Q. Darcel, and E. V. Langford. 1979. Experimental bovien pneumonic pasteurellosis II. Genesis and prevention. *Can. J. Comp. Med.* **43**: 272-279.

240. Strathdee, C. A. and R. Y. C. Lo. 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-haemolysin of *Escherichia coli*. *Infect. Immun.* **55**: 3233-3236.

241. Strathdee, C. A. and R. Y. C. Lo. 1989. Regulation of expression of the *Pasteurella haemolytica* leukotoxin determinant. *J. Bacteriol.* **171**: 5955-5962.

242. Strathdee, C. A. and R. Y. C. Lo. 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* determinant. *J. Bacteriol.* **171**:916-928.

243. Straus, D. C. and C. W. Purdy. 1994. In vivo production of neuraminidase by *Pasteurella haemolytica* A1 (Ph A1) in goats following transthoracic bacterial challenge. Proc. 94th Ann. Meet. Amer. Soc. Microbiol., Las Vegas. B-128.

244. Street, N. E., and T. R. Mosmann. 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J.* **5**:171-177.

245. Sulzer, B. M, J. P. Craig, and R. Castagna. 1987. pp. 113-123. Immunomodulation by outer membrane proteins associated with the endotoxin of Gram-negative bacteria. In J. A. Majde. Immunopharmacology of non-specific resistance. Alan R. Liss, Inc., New York.

246. Suto, R. and P. K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science*. **269**:1585-1588.

247. Tagliabue, A. and D. Boraschi. 1993. Cytokines as vaccine adjuvants: Interleukin 1 and its syntheic peptide 163-171. *Vaccine*. **11**: 594-595.

248. Tang, D. C., M. DeVit, and S. A. Johnson. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature*. **356**:152-154.

249. Tartaglia, J., M. Perkus, J. Taylor, E. K Norton, J. C. Audonnet, W. I. Cox, S. W. Davis, V. D. Hoeven, B. Meignier, M. Rivere, B. Languet, and E. Paoletti. 1992. NYVAC: a highly attenuated strain of vaccinia virus. *Virology*. **188**:217-232 .

250. Torres, C. A. T., A. Iwasaki, B. J. Barber, and H. L. Robinson. 1997. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* **158**:4529-4532.

251. Tzschaschel, B. D., C. A. Guzman, K. N. Timmis, V. deLorenzo. 1996. An *Escherichia coli* hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like toxin IIeB subunit by *Salmonella typhimurium* aroA. *Nat. Biotechnol.* **14**: 765-769.

252. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felger, J. J. Dwarki, S. H. Gromkowski, R. Deck, C. M. Dewitt, A. Friedman, L. A. Hawe, K. R. Laender, D. Martinez, H. C. Perry, J. Shiver, D. L. Montgomery, and M. A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*. **254**:1745-1749.

253. Ulmer, J. B., R. R. Deck, C. M Dewitt, J. J. Donnelly, and M. A. Liu. 1996. Generation of MHC class-I restricted cytotoxic T lymophocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunol.* **89**:59-67.

254. Ulmer, J. B., R. R. Deck, C. M. Dewitt, A. Friedman, J. J. Donnelly, and M. A. Liu. 1994. Protective immunity by intramuscular injection of low-doses of influenza virus DNA vaccines. *Vaccine*. **12**:1541-1544.

255. Uyttenhove, C., P. G. Couli, and J. Van Snick. 1988. T cell growth and differentiation induced by interleukin -HP1/IL-6, the murine hybidoma/plasmocytoma growth factor. *J. Exp. Med.* 167:1417-1427.

256. Van Dijk, W. C., H. A. Verbrugh, R. Peters, M. E. Van Der Tol, P. K. Peterson, and J. Verhoef. 1977. *Escherichia coli* K antigen in relation to serum-induced lysis and phagocytosis. *J. Med Microbiol.* **12**: 123-130.

257. Walker, R. D., F. M. Hopkins, T. W. Schultz, M. D. McCraken, and R. N. Moore. 1985. Changes in leukocyte populations in pulmonary lavage fluids of calves after inhalation of *Pasteurella haemolytica. Am. J. Vet. Res.* **46**: 2429- 2433.

258. Wang, B., J. Boyer, V. Srikantan, L. Coney, R. Carrano, C. Phan, M. Merva, K. Dang, M. Agadjanyan, L. Gilbert, K. E. Ugen, W. V. Williams, and D. B. Weiner. 1993. DNA inoculation induces neutralizing immune-responses against human immunodeficiency-virus type-1 in mice and nonhuman primates. *DNA Cell Biol.* **12**:799-805.

259. Wang, B. M. Merva, K. S. Dang, K. E. Ugen, J. Boyer, W. V. William, and D. B. Weiner. 1994. DNA inoculation induces protective in-vivo immune-responses against cellular challenge with HIV-1 antigen-expressing cells. *Aids Res. Human Retroviruses*. **10**:S35-S41.

260. Wang, B., K. Dang, M.G. Agadjanyan, V. Srikantan, F. Li, K. E. Ugen, J. Boyer, M. Merva, W. V. Williams, and D. B. Weiner. 1997. Mucosal immunization with a DNA vaccine induces immune response against HIV-1 at mucosal site. *Vaccine*. **15**:821-825.

261. Wang, B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. U.S.A.* **90**:4156-4160.

262. Wang, Y., Z. Xiang, S. Pasquini, and H. C. J. Ertl. 1997. Immune response to neonatal genetic immunization. *Virology*. **288**:278-284.

263. Welch, R. A. 1987. Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect. Immun.* **55**: 2183-2190.

264. Welch, R. A. 1990. Pore-forming cytolysins of Gram-negative bacteria. *Mol. Microbiol.* **5**: 521-528.

265. Whiteley, L. O., S. K. Maheswaran, D. J. Weiss, and T. R. Ames. 1991. Morphological and morphometrical analysis of the acute response of the bovine alveolar wall to *Pasteurella haemolytica* A1-derived endotoxin and leucotoxin. *J. Comp. Path.* **104**: 23-32.

266. Whitley, L. O., S. K. Mahesaran, D. J. Weiss, T. R. Ames, and M. S. Kannan. 1992. *Pasteurella haemolytica* A1 and bovine respiratory disease: pathogenesis. *J. Vet. Intern. Med.* **6**:11-22.

267. Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* **1**:363-369.

268. Wolff, J. A., M. E. Dowty, S. Jiao, G. Repetto, R. K. Berg, J. J. Ludtke, P. Williams, and D. B. Slauttterback. 1992. Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle. *J. Cell Sci.* **103**:1249-1259.

269. Wolff, J. A., P. Williams, G. Acsadi, S. Jiao, A. Jani, and W.Chong. 1991. Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques.* **11**:474-485.

270. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. *Science.* **247**:1465-1468.

271. Wood, D. D. 1985. pp.183-201. Interleukin-1 in arthritis. In G. A. Higgs and T. J. Williams,eds., Inflammatory Mediators. MacMillan Press Ltd., Houndmills.

272. Wu, H. Y., and M. W. Russell. 1998. Induction of mucosal and systemic immune response by intranasal immunization using recombinant cholera toxin B subunit as as adjuvant. Vaccine. 16:286-292.

273. Xiang, Z. Q., S. L. Spitalnik, J. Cheng, J. Erikson, B. Wojczyk, and H. C. J. Ertl. 1995. Immune responses to nucleic acid vaccines to rabies virus. *Virology.* **209**:569-579.

274. Xiang, Z. Q., Spitalnik, M. Tran, W. H. Wunner, J. Cheng, and H. C. J. Ertl. 1994. Vaccination with plasmid vector carrying the rabies virus glyoprotein gene induces protective immunity against rabies virus. *Virology.* **199**:132-140.

275. Xin, K, -Q., K. Hamajima, S. Sasaki, A. Honsho, T. Tsuji, N. Ishii, X. -R. Cao, Y. Lu, J. Fukushima, P. Shapshak, S. Kawamoto, and K. Okuda. 1998. Intranasal administration of HIV-1 DNA vaccine with IL-2 expression plasmid enhances cell-mediated immunity against HIV-1. *Immunol.* **94**:438-444.

276. Yamamoto, M., J. L. Vancott, N. Okahashi, M. Marinaro, H. Kiyono, K. Fujihashi, R. J. Jackson, S. N. Chatfield, H. Bluethmann, and J. R. McGhee. 1996. The role of Th1 and Th2 cells for mucosal IgA responses. *Ann. NY Acad. Sci.* **778**:64-71.

277. Yang, N. -S. and W. S. Sun. 1995. Gene gun and other non-viral approaches for cancer gene therapy. *Nature Med.* **1**:481-483.

278. Yankauckas, M. A., J. E. Morrow, S. E. Parker, A. Abai, G. H. Rhodes, V. J. Dwarki, and S. H. Gromkowski. 1993. Long-term antinucleoprotein cellular and humoral immunity is induced by intranmuscular injection of plasmid DNA containing NP gene. *DNA Cell Biol.* **12**:771-776.

279. Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral bacterial synergism in respiratoy disease of cattle. *Can. J. Comp. Med.* **46**: 225-263.

280. Yates, W. D. G., P. H. G. Stockdale, L. A. Babiuk, and R. J. Smith. 1983. Prevention of experimental bovine pnuemonic pasteurellosis with an extract of *Pasteurella haemolytica*. *Can. J. Comp. Med.* **47**: 250-256.

281. Yokoyama, M., D. E. Hassett, J. Zhang, and J. L. Whitton. 1997. DNA immunization can stimulate florid local inflammation and the antiviral immunity induced varies depending on injection site. *Vaccine*. **15**:553-560.

282. Yokoyama, M., J. Zhang, and J. L. Whitton. 1995. DNA immunization confers protection against lethal lymphocyte choriomeningitis virus infection. *J. Virology*. **69**:2684-2688.

283. Younan, M. and I. Fodor. 1995. Characterization of a new *Pasteurella haemolytica* serotype (A17). *Res. Vet. Sci.* **58**: 98-103.

284. Yu, Zhiya. 1997. Immunity against cutaneous herpes simples virus infection: host defense mechanisms mediated by IFN-γ and active protection induced by DNA vaccination. Dissertation. The University of Tennessee. Knoxville, TN.

285. Zhan, Y. and C Cheers. 1995. Differential induction of macrophage derived cytokines by live and dead intracellualr bacteria in vitro. *Infect. Immun.* **63**:1387-1390.

286. Zhang, F., Y. Yin, C. H. Arrowsmith, and V. Ling. 1995. Secretion and circular dichromisn analysis of the C-terminal signal peptides of HlyA and LktA. *Biochem.* **34**: 4193-4201.

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