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Development of a DNA Vaccine Against Melioidosis

By

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ABSTRACT

Previous studies have demonstrated that the flagellin of *Burkholderia pseudomallei* is a highly immunogenic. Immunostaining of tissues harvested from Syrian golden hamsters infected with *B. pseudomallei* 1026b established that flagellin is expressed *in vivo*, confirming the potential of flagellin as a vaccine candidate. We constructed a mammalian expression vector containing the *fliC* gene named vector pCZF428. Tissue culture transfections with pCZF428 demonstrated that flagellin protein is expressed in mammalian tissues. Immunoprotection studies demonstrated that pCZF428 is capable of protecting infant diabetic rats from challenge with *B. pseudomallei* 1026b after only two immunizations. In contrast, four immunizations with pCZF428 were not sufficient to protect Syrian golden hamsters from subsequent challenge. The production of IgG antibody specific for flagellin was detected in sera collected from both hamsters and rats immunized with pCZF428, with an increase in titers following booster injections. RT-PCR was utilized to detect IFN-γ mRNA in spleen tissue of hamsters immunized with pCZF428 using RT-PCR. These observations suggest that a DNA vaccine containing the *fliC* gene of *B. pseudomallei* may be a suitable candidate for immunoprophylaxis against melioidosis.

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LIST OF ABBREVIATIONS

Ap	ampicillin
bp	base pair
β-gal	β-galactosidase
BGH	bovine growth hormone
CAT	chloramphenicol acetyltransferase
cDNA	complimentary deoxyribonucleic acid
CFU	colony forming units
CMV	cytomegalovirus
ConA	concanavalin A
CpG	cytosine-guanosine motifs
C-terminus	carboxyl-terminus
CTL	cytotoxic T lymphocyte
Da	Dalton
dH_20	distilled water
D-MEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum

FITC	fluoroscein isothiocyanate
g	gram
GAPDH	glyceraldehyde-phosphate dehydrogenase
HRP	horseradish peroxidase
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
IS	immunostimulatory sequences
kb	kilobase
kDa	kilodalton
kg	kilogram
Km	kanamycin
L	liter
LB	Luria-Bertani
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
M	molar
mA	milliamps
Mab	monoclonal antibody

MCS	multiple cloning site
mg	milligram
MHC	major histocompatibility complex
ml	milliliter
mM	millimolar
M_r	relative mobility
mRNA	messenger ribonucleic acid
mU	milliunits
Neo	neomycin
N-terminus	amino-terminus
nm	nanometer
OD	optical density
ori	origin of replication
pA	polyadenylation
PCR	polymerase chain reaction
PS	polysaccharide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PHA	phytohemagluttinin
rDNA	ribosomal deoxyribonucleic acid
RNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute

rRNA	ribosomal ribonucleic acid
RSV	rous sarcoma virus
RT-PCR	reverse-transcriptase PCR
SDS	sodium dodecyl sulphate
SI	stimulation index
STZ	streptozotocin
SV40	simian virus 40
Th1	T helper type 1
Th2	T helper type 2
TLC	thin liquid chromatography
TNF	tumor necrosis factor
U	units
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
UV	ultraviolet light
V	volts

I. INTRODUCTION

1. *Burkholderia pseudomallei*

Burkholderia pseudomallei is a Gram-negative bacillus that is the causative agent of melioidosis, a disease prevalent in tropical regions, especially Southeast Asia and Northern Australia [19], [34]. This non-spore-forming organism is a facultatively aerobic and grows readily on standard nutrient agar and mineral-base medium. *B. pseudomallei* is motile due to its multitrichous polar flagellae. The DNA of *B. pseudomallei* has a high molar percentage of guanine and cytosine (G+C) residues of 68% (34).

B. pseudomallei is an environmental saprophyte that is unevenly distributed in soil and water, both in the presence of the organism and the numbers of organisms in the soil. The sites that are most likely to yield positive culture for *B. pseudomallei* in Southeast Asia are cleared, cultivated and irrigated agricultural sites, as well as paddocks grazed by animals (19). The optimal temperature for growth of *B. pseudomallei* is 37-42°C, and the majority of wet rice fields are reported to attain a temperature of 40-43°C. Therefore, it is reasonable that these sites would be ideal for growth of *B. pseudomallei*. The incidence of melioidosis "hot spots" may also be due to the differences in virulence of indigenous strains of *B. pseudomallei*. It has become apparent in the past years that there is an organism, *Burkholderia thailandensis*, that is

morphologically, biochemically and serologically similar to *B. pseudomallei* but is avirulent (7).

Several putative virulence determinants of *B. pseudomallei* have been identified but not fully characterized. The variety of extracellular virulence determinants that have been investigated include a thermolabile toxin, protease, lipase, and lecithinase (30), (62), (2). Cell-associated virulence factors such as lipopolysaccharide (LPS), capsule, pili and flagella, have demonstrated potential to affect the pathogenesis of this organism (9), (49), (57). The ability of this organism to remain latent in the body and resist oxidative killing mechanisms highly suggests that *B. pseudomallei* behaves as an intracellular parasite, and is therefore able to evade the host immune system (35). *B. pseudomallei* is resistant to the cationic peptide protamine sulfate and purified human defensin HNP-1, found in lysosomes (36). This may facilitate intracellular survival. The type II O-polysaccharide (PS) moiety of LPS has been recognized as the molecular determinant required for serum resistance (23).

It is difficult to treat patients with melioidosis due to the intrinsic resistance of *B. pseudomallei* to a variety of antibiotics, including beta-lactams, aminoglycosides, macrolides and polymyxins (15), (34). Previous research has identified a multidrug efflux system (AmrAB-OprA) that confers aminoglycoside and macrolide resistance in *B. pseudomallei* (52). Currently, the treatment of choice for severe melioidosis is high–dose intravenous ceftazidime, with or without trimethoprim-sulphamethoxadole (14). Additionally, intensive care can be required to prevent fatality, from resuscitation to surgical drainage of

abscesses. Antibiotic treatment is recommended from 2 to 6 weeks parenterally, and then orally for up to 6 months following recovery. This is of particular importance in the presence of persistent visceral abscesses or osteomyelitis.

2. Melioidosis

Melioidosis was first recognized in 1912 by Whitmore and Krishnaswami in Rangoon (74) as a glanders-like disease. The name melioidosis originates from the Greek *melis* and its Latin derivation *malleus*, which means "severe disease" (34). This disease was once considered to be relatively rare, as it is quite limited in its geographical distribution. Geographical surveys, however, have demonstrated that *B. pseudomallei* is widely distributed in tropical areas between 20°N and 20°S (64). The majority of cases have been described in Southeast Asia, but additional cases have been reported in military personnel who have performed service in endemic areas, specifically Vietnam (34). Presently, it is estimated that 2000-3000 cases of clinical melioidosis occur each year in Thailand (43). Infection is thought to occur by ingestion, inhalation, or inoculation presumably through minor cuts and abrasions (15). The incubation period of the organism is unclear, but ranges from 2 days to 26 years (44).

Burkholderia pseudomallei can also cause melioidosis in animals. This organism has been documented to cause an epizootic infection in sheep, goats, and pigs living on Caribbean islands (68). In 1975, 24 dolphins died of melioidosis at a Hong Kong oceanarum (44). Similarly, multiple outbreaks of

melioidosis have been recorded in Northern Australian lamb flocks. Water buffalo appear to be immune to infection, presumably due to the continual interaction with the organism in the soil when used to plow rice paddy fields. Melioidosis is a polymorphic disease. The clinical manifestations of this disease include an inapparent infection, transient bacteremia, asymptomatic pulmonary infiltration, acute localized suppurative infection, acute pulmonary infection, disseminated septicemic infection, nondisseminated septicemic infection, or chronic suppurative infection. A definitive diagnosis is difficult to achieve as this requires both clinical suspicion and confirmation in a bacteriologic laboratory. There are relatively few modern laboratory facilities present in endemic areas, and this limitation can affect proper diagnosis. Following clinical suspicion, rapid diagnosis is essential so that effective chemotherapy can be instituted as soon as possible, particularly in the more severe forms of the disease. Ultimately, confirmation of infection is achieved by positive culture of *B. pseudomallei* from the patient.

There are several tests available for the serodiagnosis of *B. pseudomallei*. These include the indirect hemagglutination (IHA) test and enzyme-linked immunosorbent assay (ELISA) to demonstrate the presence of the organism. The IHA test employs crude antigens but lacks sensitivity for diagnosis of active disease due to background cross-reactivity (60). The ELISA was developed for the detection of specific IgG and IgM antibody to *B. pseudomallei,* however, differentiation between IgM and IgG responses do not distinguish between active and inactive forms of the disease. Negative serological results do not exclude

the presence of the disease, as it has been found that culture-positive patients sometimes have negative serologies at the time of diagnosis. Presently, there are numerous developments in techniques for diagnosis, including the use of monoclonal antibodies specific for exopolysaccharide in a latex agglutination test, and the use of polymerase chain reaction (PCR) to detect 16S rRNA and 23S rDNA in clinical specimens (65), (25), (45).

Clinical diagnosis often results from the observation of the acute disease, which manifests frequently in the form pulmonary infection. This infection ranges in severity from a mild bronchitis to an overwhelming necrotizing pneumonia (60). Mild pulmonary infections are either self-limiting or are successfully treated with prompt antibiotic therapy if the infection is confined to the lungs (34). The more severe form of pulmonary infection is quite similar to tuberculosis in clinical suspicion. In these cases, the presence of residual pulmonary lesions after treatment poses the potential risk of relapse.

Melioidosis can also manifest as a progressively fatal septicemia despite antibiotic treatment. Whitmore described the more severe forms of melioidosis among narcotic addicts in 1912, in which malnutrition and debilitation were prominent conditions. The acute septicemic form occurs primarily in patients with underlying conditions, including renal failure, diabetes mellitus, steroid-treated lupus erythematosus, and antineoplastic therapy (60). Symptoms of this disease include disorientation, the development of cutaneous pustular lesions, diarrhea, high fever, and muscle tenderness. The mortality rate of the septicemic form is approximately 90%. Surviving patients generally have evidence of visceral

abscesses in the liver and/or spleen, in addition to the presence of soft tissue abscesses, osteomyelitis, and septic arthritis (20).

Sub-acute melioidosis is characterized as a prolonged febrile illness, in which the organism can be isolated from abscesses found in multiple organs (64). Chronic infections, which are most common in those residing in areas where the bacteria is prevalent in the environment, can be activated by the deterioration of host defense mechanisms (34). There have been reports of relapses of infection observed after an apparently successful antibiotic treatment for melioidosis (44). To this date, the mechanisms and sites to which *B. pseudomallei* is able to remain latent in the host are yet to be fully determined. The possibility of severe reactivation of melioidosis is a danger to any persons who have been in endemic areas and may have contracted the infection in any form.

3. Acute animal models of melioidosis

There are currently two animal models of acute melioidosis that are utilized in studying infection by *B. pseudomallei.* Syrian golden hamsters (*Mesocricetus auratus*) are exceedingly sensitive to infection by this organism. The 50% lethal dose (LD_{50}) in hamsters to *B. pseudomallei* is less than 10 bacteria (8), (21). *B. pseudomallei* infection in hamsters is typically characterized by the acute septicemic form of melioidosis, with death resulting within 2 to 3 days.

The second animal model of acute melioidosis is the infant diabetic rat model. Normal healthy rats (*Rattus norvegicus*) are quite resistant to *B. pseudomallei* infection, as the LD_{50} for nondiabetic rats to *B. pseudomallei* is > 1 x 10^8 bacteria. However, underlying conditions such as diabetes mellitus, can result in susceptibility to infection by *B. pseudomallei*. The LD_{50} of infant rats can be lowered by the onset of diabetes to approximately 1 x 10^4 bacteria (24). Diabetes can be induced in infant rats by the injection of the diabetogenic compound streptozotocin (STZ). The mechanism of STZ-induced diabetes is thought to be the damage of pancreatic β-cell DNA by the formation of alkylating radicals. The resulting necrosis of insulin-producing β-cells leads to the onset of the disease (77), (56). *B. pseudomallei* infection of infant diabetic rats leads to the development of acute septicemic melioidosis, and death usually results within 7 days.

4. Flagella of *Burkholderia pseudomallei*

Flagella are filamentous extracellular appendages that are employed by bacteria to move toward environments that promote their survival (75). Flagella are composed of 3 main elements: the flagellar cork-screwlike propeller (filament), the universal joint (hook), and the transmission shaft, motor and bushings (basal body), which is embedded in the cell envelope. Flagellar filaments consist of one or two repeating subunits of identical polypeptide monomers called flagellin (28). The flagella can be dissociated into flagellin by a

variety of agents, including acid, alkali, heat, urea, guanidine chloride, acetamide, sodium dodecyl sulfate, cetylpyridium chloride, and sonic oscillation (37). A structurally distinct sheath encloses the flagella of some Gram-negative bacteria, such as the polar flagella of *Vibrio* spp. (76). Little is known about the composition and function of the sheath, however, its role must be important in view of the high cost of its cellular production.

The filament is arranged in a left-handed macrohelical structure, whose handedness, pitch and diameter are determined by environmental factors such as pH, ionic strength and amino acid sequence of the constituent flagellins (75). The counter-clockwise rotation of the filament creates a thrust that, in combination with the effects of several flagella in a bundle, results in the propulsion of the bacterium (37). Upon clockwise rotation of the flagellum, a right-handed helix results and prevents further directional movement. Therefore, the bacterium alternates between "swimming" and "tumbling" via the basal body changing the direction of the rotation. This activity is driven by energy derived from electrochemical gradients.

Flagellin proteins have a characteristic three domain structure, consisting of a variable central domain and highly conserved N- and C-terminal regions. The N- and C-terminal amino acid sequence domains are composed of 140-145 and 85 residues, respectively, and are conserved among diverse species (75). Homma and colleagues proposed that the conserved regions of flagellin determine the inter- and intra-molecular interactions that define basic filament structure via the "hairpin" folding of flagellin (33). The central domain displays

the greatest variability between species in both length and amino acid composition, thus defining the antigenically and structurally diverse filament surface.

Flagellar filaments may possess functions other than motility. An association between flagella and virulence has been demonstrated for several pathogenic bacteria, including *Vibrio cholerae*, *Campylobacter jejeuni*, and *Salmonella typhi* (47, 53, 59). The role in virulence in these organisms is attributed to the prospect of the filaments carrying adhesions for attachment to the intestinal mucosa. Furthermore, Wilson et al. suggested that flagella may also assist bacteria in nutrient and waste exchange. This phenomena is proposed to occur due to the promotion of solute exchange by flagella, which disturb and mix the nutrient-poor and waste-rich local environment with the external milieu (75).

B. pseudomallei is motile by means of its polar lophotrichous flagella (34). The motility of *B. pseudomallei* has been studied extensively, with emphasis on the isolation and characterization of the flagellin proteins. The flagellar filaments of various strains of *B. pseudomallei* have been isolated by techniques involving mechanical shearing, differential centrifugation and purification by ammonium sulfate precipitation (9). The apparent molecular weight (M_r) of the flagellin monomer proteins isolated from *B. pseudomallei* was determined to be 43,400 (37). This is comparable to the M_r of other characterized flagellin molecules, which range in size from 15,000 to 62,000 (37). The presence of only one species of flagellin molecule is also consistent with the finding that unsheathed

flagellar filaments dissegregate to only one species (37). Amino acid analysis of flagellin monomers isolated from *B. pseudomallei* 319a demonstrated the presence of all biologically relevant amino acid residues, with the exception of tryptophan and cysteine (9). These findings are consistent with the amino acid composition of other characterized flagellins, and likewise, almost all external bacterial surface proteins lack cysteine and contain tryptophan and proline in low frequency (75).

Flagella are frequently associated with virulence due to their role in motility, they are also immunodominant antigens seen during infection by the host immune system. Previous research has been performed to elucidate the immunogenic properties of the flagellin protein of *B. pseudomallei.* Rabbit polyclonal antisera raised against purified *B. pseudomallei* 319a flagellin was utilized in immunoblotting studies to demonstrate that 64 out of 65 strains of *B. pseudomallei* had cross-reactive epitopes to the antiserum (9). Additionally, this polyclonal antiserum was shown to inhibit cell motility of strain 319a. The immobilization of bacteria by antibodies may prove to be an advantage in view of the fact that motility may be a contributing virulence determinant. Infant diabetic rats were protected from challenge by *B. pseudomallei* when passively immunized with immunoglobulins raised against purified 319a flagellin (9). The immunogenic properties of *B. pseudomallei* flagellin demonstrate its potential as a vaccine candidate against melioidosis.

Genetic studies have emphasized the characterization of the structural gene of flagellin, *fliC*. The full sequence of the *fliC* gene has been identified, as

well as the transcriptional start site at an A residue 83 bp upstream of the *fliC* ATG start codon and a σ^F-like promoter immediately upstream of the transcriptional start site (22). It has also been demonstrated that the NH_2 -terminal region of the *fliC* gene of strain 319a is identical to several other strains of *B. pseudomallei*, including 1026b (7). A transposon mutant containing a Tn5-OT182 integration in the *fliC* gene, called MM36, did not demonstrate any significant difference from 1026b (wild-type) in virulence in the diabetic rat or Syrian hamster animal models, indicating that flagella is probably not a virulence determinant in these cases (22).

5. Vaccine development

Prior studies have demonstrated that *B. pseudomallei* flagellin proteins and the O-polysaccharide (PS) are highly conserved molecules, both in structure and immunogenicity (9, 11, 12). The development of active immunization strategies against melioidosis has included the development of a conjugate molecule including both flagellin protein and LPS antigens (11). The rationale in this vaccine design was to combine two protective antigens that could enhance the immunological repertoire of the immunized individual. Additionally, the vaccine would evoke the desirable immunoglobulin (Ig) class-switching events and prevent the toxic side effects of LPS by eliminating the lipid A component. Three PS-flagellin protein glycoconjugates were constructed. One construct, PS-ADH-FLA, containing adipic acid dihydrazide (ADH) as a spacer molecule,

elicited a high titers to both LPS and flagellin protein molecules in New Zealand White rabbits after primary, immunization and two subsequent booster doses (11). The functional activity of the anti-PS-ADH-FLA antiserum was determined by motility inhibition assays, as the sera inhibited the motility of 38 out of 38 strains of *B. pseudomallei.* The purified IgG fraction from antiserum raised against the PS-ADH-FLA conjugate was utilized in passive immunoprotection studies and was shown to protect diabetic rats from challenge with a heterologous strain of *B. pseudomallei.*

The conjugate vaccine incorporating both flagellin and the polysaccharide portion of LPS is a reasonable vaccine candidate for immunization against melioidosis, as it afforded protection from challenge by *B. pseudomallei.* Cellular immunity is critical for protection against infection by an intracellular organism such as *B. pseudomallei*, and although the conjugate vaccine demonstrated a suitable humoral response, protein-based vaccines are unable to incite a Th1-type cytokine response. It has been well documented that T cell-independent type 2 antigens such as bacterial polysaccharides act poorly as immunogens in the elderly, in children under 2 years of age, and in immunocompromised populations (10). Additionally, the limitations of the acute animal models of melioidosis prevent further characterization and immunological investigation of the vaccine efficacy. Until an animal model is developed to study active immunization against melioidosis, studies with the conjugate vaccine have been discontinued.

6. DNA-based Vaccination

DNA vaccination is a novel approach for immunoprophylaxis. Previous developments in vaccination strategies have failed to successfully protect against intracellular organisms, such as *B. pseudomallei,* by inducing a cellular response. Although vaccines composed of live attenuated organisms do induce cellular immunity, many practical issues of manufacturing and safety have prohibited their use. Plasmid DNA vaccines have been shown to induce both humoral and cellular immunity in a variety of murine and primate disease models. Protective immunity results from the endogenous expression of foreign proteins by host cells, and the antigen is therefore presented to the immune system in a manner that is similar to that which could occur during natural infection (29).

DNA immunization offers a number of attractive attributes. DNA vaccines are constructed with relative ease, producing pure, physiochemically stable plasmid DNA. The simplicity of DNA vaccines would facilitate rapid and large-scale production, potentially allowing for the formulation of combinations of immunogens that could be delivered in a single dose. DNA vaccines are likely to be less expensive to produce and deliver than current alternatives, as the necessity for cold-chain storage is eliminated. DNA-mediated vaccination appears to facilitate expression of vaccine antigens in their native form, thereby optimizing their presentation and processing within the immune system. The capacity of DNA vaccines to elicit both long-lived humoral and cellular cytolytic T lymphocyte (CTL) responses has been well documented (73). This feature may

possibly be due to the low level and persistent production of antigen *in vivo* by DNA vaccines, which would continuously stimulate the immune system. The response to the DNA-encoded antigen could potentially be enhanced or modulated by the co-delivery of plasmid-DNA-encoded cytokines or co-stimulatory molecules.

Another advantage in DNA immunization is natural adjuvanticity of the plasmid DNA backbone. Extensive research has been performed on the immunostimulatory (IS) effect of bacterial DNA containing sequences consisting of a cytosine-guanine (CpG) motif of the formula:5'-purine-purine-CG-pyrimidine-pyrimidine-3'. These motifs occur in bacteria DNA at a relative frequency of 1 in 16, and only 1 in 50 in eukaryotic DNA (70). Bacterial DNA and oligonucleotides containing CpG motifs have been shown to induce a humoral response via B-cell proliferation and immunoglobulin production, as well as secreting interleukin (IL)-6, gamma interferon (IFN-γ), IL-12, and IL-18. In contrast, eukaryotic DNA did not produce any such effects (70).

The type of immune response that is induced by DNA immunization is influenced by multiple factors. A gene of interest is cloned into a bacterial plasmid and engineered for optimal expression in eukaryotic cells. Firstly, a promoter sequence usually derived from a strong promoter, such as cytomegalovirus (CMV) or Rous sarcoma virus (RSV) to drive expression in mammalian cells is required. An mRNA stability polyadenlyation region, derived from sources such as the bovine growth hormone (BGH) or simian virus 40, is necessary at the 3' end of the insert to ensure proper post-transcriptional

modification and translation. An origin of replication for growth in bacteria is required for amplification of the plasmid vector, and the ColE1 origin of replication for *E. coli* from pUC plasmids is frequently used as it provides high copy numbers and high purification yields. Lastly, a bacterial antibiotic resistance gene is essential for selection of the vector during culture and purification. Kanamycin is the most common resistance gene for vectors that could potentially be for human use.

DNA vaccines are able to induce a full range of immune responses, including antibodies, major histocompatibility complex (MHC) class 1-restricted $CD8^+$ CTL and class II-restricted $CD4^+$ helper T cells (58). Protection against intracellular organisms, such as *B. pseudomallei*, depends predominantly upon CD4+ CTL generating cytokines such as, gamma interferon (IFN-γ), and other macrophage-activating factors to activate macrophages (69). T-helper cells are differentiated into two distinct populations, Th1 and Th2, depending on the types of cytokines produced. Th1 cells produce interleukin-2 (IL-2), IFN-γ, and tumor necrosis factor (TNF)-β, while Th2 cells predominantly produce IL-4, IL-5, IL-6 and IL-3 (42). These cell populations also differ in the type of immune response that they stimulate, as Th1 cells mediate delayed-type hypersensitivity (DTH) reactions, increase immunoglobulin (Ig) G2a and IgG3 isotype synthesis in murine models via IFN-γ secretion and are associated with a potent CTL response (1). In contrast, Th2 cells are associated with stimulating B cells to produce IgG1 and IgE subclasses due to IL-4 secretion, and activating eosinophils via IL-5. Adjuvants that are currently available for human use are

biased towards the induction of a Th2 response, and are therefore, insufficient to protect against intracellular organisms (67).

There are a variety of possibilities as to how DNA immunization results in the priming of MHC class I-restricted $CD8^+$ CTL: 1) a professional antigen presenting cell, specifically bone-marrow derived cells, are directly transfected by the plasmid DNA and presents the antigen via the intracellular pathway; 2) a somatic cell is transfected by the plasmid, produces the protein which is then ingested by a professional APC, and processes and presents the antigen exogenously; 3) A somatic cell, which is directly transfected, processes and presents the antigen intracellularly in conjunction with an adjacent cell produces the suitable costimulatory molecules; or 4) a nonprofessional APC processes and presents the antigen exogenously as an adjacent cell produces the appropriate costimulatory molecules (42). There is an abundance of evidence that suggests that bone marrow derived APCs, and not somatic cells, directly induce immune responses following DNA vaccination (18). Nevertheless, somatic cells such as myocytes or keratinocytes, are the cell populations that are predominantly transfected after DNA immunization via the intradermal (i.d.) or intramuscular (i.m.) route. These cell types may serve as a reservoir for the antigen.

Despite the potential advantages of DNA-based immunization, there are a variety of issues that need to be dealt with prior to introduction into clinical use. A major concern is the possibility of integration of the DNA vaccine into the host genome, and depending on the site of integration, the risk of altered cell growth or expression. Previous research has demonstrated that plasmids can persist at

the site of injection, or in alternate sites, for many months (73). Long term persistence may be common for self-antigen-encoding plasmids, as they do not produce an immune response against the transfected cell. Presently, there is no clear evidence that integration of plasmid DNA after immunization has taken place.

Another concern regard DNA vaccines is the possibility that an immune response could be induced against the transfected cell, and more specifically the immunostimulatory activity of CpG motifs in the plasmid backbone, thereby leading to the development of autoimmune disease. The effects of DNA vaccines have been evaluated in numerous autoimmune models. Present findings suggest that the level of autoantibody production elicited by plasmid DNA is insufficient to stimulate the onset of autoimmune disease (27). Additionally, human volunteers that have been exposed to plasmid DNA vaccines have not demonstrated any adverse or toxic effects. Immunization target age populations such as infants and children are potentially at risk for developing tolerance instead of immunity, as these populations are quite under-developed at the time of immunization. This concern arises from the idea that the DNA-encoded protein is produced endogenously and is expressed to the MHC in the perspective of "self". The induction of tolerance is dependent on the age of vaccine immunization, suggesting that DNA vaccines may be less immunogenic in the elderly as well as in young children (63). The co administration of plasmids encoding cytokines or costimulatory molecules could potentially improve the overall immunogenicity of DNA vaccines.

7. Thesis objectives

DNA vaccination is a promising approach for the development of immunoprophylaxis against intracellular organisms, such as *B. pseudomallei*. Plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models. DNA vaccines mimic the antigen presentation that results from natural infection or from the immunization of live attenuated organisms. This is extremely advantageous as DNA immunization eliminates the deleterious effects that occur with immunization with attenuated live bacterial vaccines or whole killed bacteria. It has been demonstrated that the flagellin proteins of *B. pseudomallei* are highly conserved, structurally and immunogenically. The construction of a DNA-based vaccine, encoding for flagellin proteins, is a reasonable initiative for the development of immunization strategies for melioidosis. Therefore, the hypothesis for this thesis is that a plasmid DNA vaccine containing the structural gene for flagellin, *fliC*, will be suitable for use in vaccination against melioidosis.

The objectives of this thesis are as follows: 1) Investigate the expression of the flagellin structural gene, *fliC*, of *B. pseudomallei* in vivo to assess the potential of this antigen to be a candidate gene for a DNA-based vaccine; 2) Assess the immunological response to purified flagellin in the Syrian golden hamster model of acute melioidosis, with emphasis in developing and employing immunological methods; 3) Construct a DNA-based vaccine vector containing the

flagellin structural gene, *fliC*, and evaluate its suitability for use in immunization against melioidosis.

II. MATERIALS AND METHODS

1. Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. The stock cultures of *Burkholderia pseudomallei* and *Escherichia coli* were maintained in 20% glycerol suspensions and stored at -70°C. These strains were grown at 37°C on regular or low-salt Luria-Bertani (LB) broth base or on LB agar plates (Becton Dickinson). When appropriate, antibiotics were added in the following concentrations: 100 μg/ml ampicillin (Ap; Sigma Chemical Co.) and 25 μg/ml kanamycin (Km; Sigma Chemical Co.) and 1.5 mg/ml trimethoprim (Tp; Sigma Chemical Co.) for *E. coli*. TSBDC culture medium was utilized for growth of bacteria for the purposes of animal studies. TSBDC was prepared by adding 60 g of tryptic soy broth (TSB; Difco Laboratories, Inc) to 180 ml of distilled water and stirring with 10 g of Chelex 100 resin (Bio-Rad Laboratories Inc.) for 4-6 hours at room temperature. The medium was then dialyzed against 2.0 litres of distilled water overnight at 4°C and autoclaved. Filter sterilized solutions of 1% glycerol and 50 mM monosodium glutamate (MSG; Sigma Chemical Co.) were then added.

2. Syrian golden hamster model of acute melioidosis

The Syrian golden hamster model of acute melioidosis has been described previously (8, 77). Bacteria were grown to late log phase in TSBDC culture media at 37°C. Female Syrian golden hamsters (*Mesocricetus auratus*) between 6-8 weeks old and weighing 80-90 grams (Charles River Laboratories, Canada) were utilized. Hamsters were injected intraperitoneally (i.p.) with 100 μl of the appropriate dilution of bacteria adjusted with sterile phosphate buffered saline (PBS). After infection, the hamsters were monitored for the appearance of symptoms of acute septicemia (e.g. sluggish behavior, purulent ocular exudates).

Immunization studies were performed using the Syrian golden hamster to examine the immune response and protective capacity of the DNA vaccine constructed. Groups of 5 Syrian golden hamsters, 6-8 weeks old were utilized. The hamsters were anesthesized by inhalation of methoxyfurane (Metofane; Janssen). The animals were injected intramuscularly (i.m.) four times at week 0, 3, 5, and 7 in both quadriceps muscles with pCZF428, the empty vector pCZ11, or purified *B. pseudomallei* 319a flagellin, using a 25-gauge needle. The hamsters received 50 μg of DNA or 10 μg of protein at each injection and hamster sera or spleen tissue were analyzed 2 weeks after the last DNA vaccination for the presence of an immune response.

The protective capacity of the plasmid vector pCZF428 was assessed in Syrian golden hamsters that were immunized through various regimens. Overnight cultures of *B. pseudomallei* 1026b were prepared in TSBDC broth and

incubated at 37°C at 250 rpm. Sub-cultures of 1026b were started on the day of infection and grown at 37°C at 250 rpm until the cell cultures were at mid-log phase (OD_{600}=0.5). The culture was diluted to the appropriate CFU/ml for infection prior to injection. Syrian golden hamsters were challenged two weeks after the fourth immunization with less than 10 CFU/ml of *B. pseudomallei* 1026b intraperitoneally. The days to death of the challenged groups of hamsters were recorded.

3. Infant diabetic rat model of acute melioidosis.

The second animal model of acute melioidosis is the infant diabetic rat model. Female Sprague-Dawley rats (*Rattus norvegicus*) that weigh 30-40 grams and that are recently weaned from the mother were utilized (20-22 days old). Streptozotocin (STZ; Sigma Chemical Co.) was utilized to make the infant rats diabetic. The STZ was dissolved in 100 mM sodium citrate (pH 4.2) and administered at a dose of 80 mg/kg of body weight via i.p. injection for 2 consecutive days. The onset and progression of diabetes was be checked daily by monitoring the urine glucose levels using Chemstrip glucose strips (Boehringer Mannheim). A concentration of 2g/dl (111 mM) glucose indicated the onset of diabetes.

Infant diabetic rats were also immunized with the DNA vaccine to determine the protective efficacy of the vector, as well as to detect an immune response. Groups of 4 infant Sprague-Dawley rats were weaned from the

mother at 20-22 days old (30-40 g in weight). The rats were immunized immediately after weaning with either pCZF428 or the control vector pCZ11 and a second time 10 days later. The rats received 50 μg of DNA at each injection in both quadriceps (i.m.). In between the two immunizations, the rats were made diabetic by i.p. injection of 80 mg/kg of body weight of stretozoticin (STZ; Sigma Chemical Co.) in 100 mM sodium citrate (pH 4.2) on two consecutive days. The onset of diabetes was confirmed by testing the urine with Chemstrip glucose strips (Boehringer Mannheim). The sera infant diabetic rats were analyzed 7 days after the second injection.

To determine the protective efficacy of the DNA vaccine in this animal model, infant diabetic rats were challenged 7 days after the second immunization with DNA with approximately 10^4 CFU/ml of *B. pseudomallei* 1026b. The challenged diabetic rats were observed for onset of disease and the number of days to death was recorded and compared for each group.

4. Immunostaining & fluorescent microscopy

The expression of the flagellin protein *in vivo* was assessed by the immunostaining of tissues from Syrian golden hamsters infected with *B. pseudomallei* 1026b. The lung, liver and spleen of each animal was harvested and homogenized in 5-10 ml of PBS. The homogenates were stored at -70°C if not used immediately.

The immunostaining slides were prepared as follows: 20-50 ul of tissue homogenate (lung, liver or spleen) was dropped on a glass microscope slide. A coverslip was used to spread the homogenate on the slide, creating a thin layer across the surface, and then dried in a laminar air hood. Fixation of the homogenate to the glass slide was performed via a series of alcohol dehydration-rehydration steps. Each slide was dipped in a 250 ml beaker containing various concentrations of alcohol and washes for a time period of 3 minutes. The series of solutions were: 100%, 95%, 90% and 70% absolute ethanol, followed by 2 washes of deionized distilled water. The slides were then treated with 0.1% trypsin solution in water with 0.1% calcium chloride at 4°C for 16 hours. The slides were washed 3 times with PBS. The slides were stored at 4°C prior to immunostaining.

The immunostaining was performed using mouse monoclonal antibody specific for purified *B. pseudomallei* 319a flagellin. The monoclonal antibodies were diluted 1:500 or 1:1000 in 3% skim milk-PBS and applied to the surface of the fixed tissues on the glass slides. Antibody penetration was permitted at 4°C overnight and then washed 3 times with PBS prior to exposure to secondary antibody. The secondary antibody utilized was goat anti-mouse IgG conjugated to fluoroscein isothiocyantate (FITC), which was diluted in 3% skim milk-PBS and Evan's blue counterstain. The Evan's blue was utilized to stain background tissue, which would then fluoresce red at 490 nm to contrast the green fluorescence of the FITC-conjugated secondary antibody. The secondary antibody was applied to the slide surface for 4 hours at room temperature or at

4°C overnight. The slides were then washed three times in PBS prior to visualization with a fluorescent microscope mounted with a Kodak camera apparatus. The immunostaining was recorded on Kodak 400 ASA color slide film or Kodak 200 ASA color film using the following settings: 35 mm format, reciprocity of 4, and exposure times ranging from 16 to 45 seconds.

5. Purification of *B. pseudomallei* 319a flagellin

The strategy for the purification of flagellin from *B. pseudomallei* strain 319a was described previously by Brett and colleagues (9). Briefly, LB plates containing 0.2% glucose were inoculated with *B. pseudomallei* 319a and incubated for 48 hours at 37°C. Bacterial colonies from these plates were then inoculated into 500 ml of LB plus 0.2% glucose and agitated overnight at 100 rpm at 37°C. The cells were centrifuged for 20 min at 7,000 x *g* at 4°C and subsequently resuspended in 300 ml of 50 mM sodium phosphate buffer (pH 7.0). The resuspended cells were then blended in a Waring commercial blender for 1.5 min at a low setting to shear the flagella from the cells, and then centrifuged at 12,000 x *g* at 4°C for 20 minutes to remove the cell debris. The supernatant was then subjected to 5% increments of ammonium sulfate saturation to a final concentration of 20%. After each addition, the supernatants were allowed to stir at room temperature for 4-5 hours and then centrifuged at 12,000 x *g* at 4°C to remove the insoluble material. The insoluble material was dissolved in a minimum volume of 50 mM sodium phosphate buffer (pH 7.0) and

then dialyzed against the same buffer overnight at 4°C. The fractions were subjected to a modified ultracentrifugation, acid-disassociation, differential centrifugation, acid-reassociation procedure to eliminate contaminants. The fractions were reduced in pH to 3.0 by addition of 2.0 M citric acid and stirring at 4°C for 10 min and then ultracentrifuged at 100,000 x *g* at 4°C for 30 min. The supernatant was removed and brought to pH 7.0 with 5 M NaOH and stirred at 4°C for 20 min prior to dialysis overnight against 50 mM sodium phosphate buffer (pH 7.0).

6. Delayed-type hypersensitivity assay

Delayed-type hypersensitivity (DTH) measurements to purified 319a flagellin and 5% formalin-killed *B. pseudomallei* 1026b were assessed in the Syrian golden hamster model. Hamsters were injected in the left rear footpad with 0.5 or 1.0 mg/ml purified 319a flagellin, or 10^3 or 10^6 dose of 5% killed 1026b. The right rear footpad was injected with PBS as a negative control. The footpad thickness was measured 24 and 72 hours after primary immunization using calipers capable of measuring 0.05-mm increments. The footpads were then exposed to the antigens a second time and the footpad measurements were taken 24 and 48 hours after. The difference between the left and right rear footpad thickness after secondary immunization was used as a measure of the DTH response.

7. Plasmids, primers, enzymes

The plasmids and primers constructed and used in this study are described in Table 2 and 3, respectively. Plasmids were purified from overnight cultures using the Qiagen EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, California) or by large-scale cesium chloride preparations, followed by extractions by 1-butanol and phenol-chloroform and ethanol precipitation (3). Purified plasmid DNA was dissolved in saline at a concentration of 1 mg/ml. The DNA concentration was determined by spectrophotometry at 260 nm and the size of the plasmid was compared with DNA standards in an ethidium bromide-stained 1% agarose gel. Restriction enzymes, T4 DNA ligase and large fragment polymerase (Klenow) (Gibco BRL) were used according to standard cloning procedures (3). Ligation mixtures were chemically transformed into *E.coli* DH5α using standard protocols (3).

8. Polymerase chain reaction (PCR) amplification of *fliC*

The strain SURE (pPB400A) contains the plasmid pPB400A, which has the structural gene for flagellin, *fliC*. Plasmid DNA was isolated as previously described and used as a template to PCR-amplify the *fliC* gene. The oligodeoxyribonucleotide primers used in the PCR, *fliC*-forward and *fliC*-backward (Table 3), were based upon a previously published *B. pseudomallei* *fliC* sequence (22). Both primers inserted a specific restriction site and the

forward primer replaced the bacterial start codon with a Kozak consensus sequence (GCCACCATG; positions –6 through +3) to enhance mammalian expression (40). The PCR reaction mix consisted of 10 ng plasmid DNA, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.5 μM of each primer, and 2.5 U of Taq DNA polymerase (Gibco BRL). The PCR was performed using a GeneAmp PCR System 9600 (Perkin Elmer Cetus) thermal cycler and subjected to the following conditions: 94°C for 3 minutes; 30 cycles of 94°C for 45 sec, 55°C for 30 sec, 72°C for 90 sec; 72°C for 10 minutes. The size and purity of the PCR products were assessed by 1% agarose gel electrophoresis, and visualized by ethidium bromide-staining and UV exposure. The PCR products were isolated from the reaction mixtures with the Wizard PCR Preps DNA Purification System (Promega).

9. Construction of a vaccine vector and reporter vectors

Fragments of the commercial vectors pCDNA™3.1(+) and pZErO™-2.1 were utilized to construct a mammalian expression vector. Primers were designed to amplify desired regions from both these vectors based on sequence described by the suppliers, and are illustrated in Table 3. The primers 3.1-forward and 3.1-reverse were utilized to amplify regions from pCDNA™ 3.1(+), and primers 2.0-forward and 2.0-reverse were used to amplify regions from pZErO™-2.1. The conditions used for PCR were the same as those used to amplify *fliC*. The resultant PCR products were double digested using *Bst*BI and

*Sma*I restriction endonucleases and purified using the GeneClean II Kit (Bio 101, Inc.). The purified products were ligated in a 20 μl reaction volume with T4 DNA ligase at 16°C overnight. Chemically competent *E. coli* DH5α were harvested and transformed with 2 μl of the ligation mixture and screened for antibiotic resistance (3). Resultant clones were screened for the presence of the 2.1 kB plasmid, and this construct was named pCZ11. This vector was utilized as a control vector for all expression and immunization studies.

The 1.2 kb PCR product containing *fliC* was cut using the restriction enzymes *Hind*III and *Eco*RI and cloned into pCZ11, which had been similarly digested. The resulting construct, pCZF428, was chemically transformed into *E. coli* DH5α and selected for (Km^r). This vector was utilized for all expression and immunization studies as the test vector. The reporter vector, pBL305, was constructed by inserting the 1.2 kb PCR *fliC* product into the TOPO-cloning vector pBLUE-TOPO™ (Invitrogen) as per manufacturers' instructions.

The structure of the purified plasmids was confirmed by separating restriction endonuclease digestion products of the plasmid in 1% agarose gels. Partial automated DNA sequencing was performed by the University Core DNA Services (University of Calgary) with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System and AmpliTaq DNA polymerase (Perkin Elmer Cetus). The sequencing confirmed that the CMV promoter was intact and that the *fliC* gene was inserted in frame in both pCZF428 and pBL305.

10. Tissue culture lines, media, and growth conditions

Cell lines utilized in this study originated from American Type Culture Collection (ATCC; Rockville, MD). The three tissue culture cell lines used were: human epitheloid cervical carcinoma (HeLa). African green monkey kidney (COS-1) and Chinese hamster ovary (CHO). The HeLa and COS-1 cell lines were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Gibco Canada Inc.) and CHO.K1 cells were maintained in D-MEM combined with F-12 media. In all cases, the cell culture media contained a standard antibiotic mixture (100 U penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin B; Sigma Chemical Co.) and 10% fetal bovine serum (FBS; Gibco Canada Inc.). All cell lines were maintained at 37°C in 5% CO_2.

11. Tissue culture transfections

The expression of protein product from the vector containing *fliC,* pCZF428, was assessed by transfection of the cell lines, HeLa and CHO.K1. The transfections were performed using the cationic lipid reagent, Lipofectamine PLUS (Gibco Canada Inc), as per manufacturer's instructions. The cells were seeded in 6-well plates the day before transfection at a quantity that would result in 70-90% confluency the day of transfection. Plasmid DNA dilutions were prepared for each individual wells in 100 μl of media without serum (FCS), from 0.5 to 3 μg of DNA per well and incubated at room temperature for 15 minutes.

In a second microfuge tube, Lipofectamine reagent was diluted in similar medium without serum. The DNA and Lipofectamine dilutions were then combined, mixed, and incubated for 15 minutes at room temperature. The medium was replaced on the tissue culture cells and the DNA-Lipofectamine complexes were added to the cells in fresh medium. The transfection mixture was incubated at 37°C with 5% CO_2 for 3-5 hours. After the incubation, the transfection medium was removed and completely replaced with fresh medium containing serum. The transfected cells were allowed transient expression for 24-72 hours.

After 24-72 hours of expression, cells were harvested. Firstly, each well was rinsed in Dulbecco's Phosphate Buffered Saline (D-PBS) with calcium and magnesium (Gibco Canada Inc.). Lysis buffer, consisting of 0.1% Triton X-100 and 0.1m Tris-HCl (pH 8.0), was added in enough volume to cover the well and allow for easy transfer of the lysed cells. The cells were lysed by freezing the plate at -70°C for at least 1 hour and thawing at 37°C, before transfer to a centrifuge tube. The lysate was centrifuged for 5 minutes at 9,000 x *g* in a microcentrifuge to pellet the insoluble material. The supernatant was collected and stored at -20°C if not used immediately.

12. β-galactosidase assay

β-galactosidase assays were performed on extracts of cells that were transfected with the vector pBL305. This was accomplished using the β-gal

Assay Kit (Invitrogen), as per manufacturer's protocols.

13. Chloramphenicol acetyl transferase (CAT) assay

CAT assays were performed on extracts of cells that were transfected with the vector pCDNA™ 3.1/CAT (Invitrogen). Successful transfection conditions were monitored using CAT assay protocols that were slightly modified from standard protocols (3). To assay 50 µl of cell extract, an 80 µl reaction mixture was prepared, consisting of: 50 µl of 1 M Tris-Cl (pH 7.8), 10 µl of ^{14}C-labeled chloramphenicol (diluted in water to 0.1 mCi/ml) and 20 µl of a freshly prepared 3.5 mg/ml acetyl coenzyme A solution. Purified CAT enzyme was used as a positive control at 40 or 50 mU in quantity. Extracts from cells transfected with the vector pCDNA™ 3.1(+) (Invitrogen), was used as a negative control. Each sample was mixed with CAT reaction mixture and incubated at 37°C for 30 minutes to 2 hours. After incubation, 1 ml of ethyl acetate was added to each sample and mixed thoroughly before centrifugation at 12,000 x *g* for 5 minutes at room temperature. At this step, the acetylated forms of chloramphenicol separate into an upper (organic) phase and the unacetylated chloramphenicol remains in the lower (aqueous) phase. The upper phase (approximately 900 µl) was transferred to a fresh tube and a rotating evaporator (Savant SpeedVac) was used to evaporate the ethyl acetate under vacuum. The reaction products were redissolved in 25 µl of ethyl acetate and 10-15 µl of this reaction mix was

applied to the origin of a 25-mm silica gel, thin-layer chromatography (TLC) plate (Kodak Eastman). The TLC plate was then placed in a TLC chamber prepared with 200 ml of chloroform:methanol (95:5) and sealed. The solvent front was allowed to move to approximately 75% of the distance to the top of the plate before it was removed and allowed to dry. The plate was then exposed to X-ray film.

14. Preparation of *B. pseudomallei* cell lysates

Cell lysates of *B. pseudomallei* 1026b were prepared to be probed by individual sera collected from immunized Syrian hamsters. The lysates were prepared by methods modified from Hitchcock and Brown (31). An overnight culture of *B. pseudomallei* 1026b in LB broth was started and incubated at 37°C at 250 rpm. A 0.5 ml portion of this suspension was centrifuged in a Beckman microcentrifuge for 2 minutes to precipitate the cells. The cell pellet was then solubilized in 50 μl of lysing buffer (2% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris pH 6.8, and a little bromphenol blue). The lysates were heated to 100°C for 10 min and approximately 50 μl was used for each sample of sera for western blotting.

15. Electrophoresis and western blotting

Transfection cell extracts and sera collected from immunized or infected animals were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% stacking and 12.5% separating (wt/vol) acrylamide slab gel system (3). Denaturing buffer was added to the samples prior to heating in a boiling water bath for 5-10 minutes. The samples were loaded into the gel wells and electrophoresed for 3 hours under 45 mA of current. Samples were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) via electrophoretic transfer using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories) overnight at 4°C at 40 V, as described by Towbin *et al* (71).

Western blotting was performed as described by Catty et al. (13). Mouse monoclonal antibody directed towards purified *B. pseudomallei* 319a flagellin was used to probe cells extracts from transfections of HeLa and CHO.K1 cells with pCZF428 or the empty vector pCZ11. The secondary antibody utilized in these studies was a goat anti-mouse whole IgG conjugated to horseradish peroxidase (HRP) (Sigma Chemicals).

Individual hamster sera collected from animals immunized with pCZF428 or pCZ11 were used to probe *B. pseudomallei* cell lysates to detect the production of antibodies specific for flagellin protein. The secondary antibody utilized in the detection of anti-flagellin antibodies was a goat anti-hamster whole IgG conjugated to HRP (Sigma Chemicals). All western blots were developed

using the HRP Color Development Reagent (Bio-Rad Laboratories), which contains 4-chloro-1-napthol as the substrate for horseradish peroxidase.

16. Enzyme-linked immunosorbent assay (ELISA)

Vaccinated hamsters were sacrificed 2 weeks after the last DNA injection, while vaccinated infant diabetic rats were sacrificed 7 days after the second DNA injection. The production of specific antibodies directed against purified *B. pseudomallei* 319a flagellin was assessed by indirect enzyme-linked immunosorbent assay (ELISA) (3). Individual sera from groups of five hamsters immunized with either pCZF428, control vector pCZ11, or purified 319a flagellin, were analyzed. Individual sera from groups of three infant diabetic rats immunized with either pCZF428 or pCZ11 were also analyzed.

Briefly, microtiter plates were coated with 100 µl (10 µg/ml) of purified 319a flagellin in 0.1 M carbonate buffer (pH 9.6) and incubated for 2 hours at 37°C or overnight at 4 °C. The plates were then washed with 0.05% Tween 20 in PBS and saturated with skimm milk (3 % in PBS-Tween 20) for 2 hours at 37°C. After washing, 100 µl of serial twofold dilutions of serum (1:50 to 1:3200) in 1% skim milk-PBS-Tween 20 were added for 1 h at 37°C. The plates were washed and peroxidase-labeled goat anti-hamster whole IgG (ICN Biomedicals Inc., Aurora, OH) or peroxidase-labeled sheep anti-rat whole IgG (ICN Immunobiologicals, UK) was added for 1 h at 37°C. Finally, the plates were washed and developed by the addition of an ABTS substrate solution

(Kirkegaard and Perry Laboratories, Inc.) for 20 minutes and read at an absorbance of 405 nm. The highest dilution of immune serum that reacted against a specific antigen was expressed as the reciprocal value of that dilution.

17. Splenocyte proliferation assays

Hamsters were sacrificed 2 weeks after the fourth immunization of pCZF428, control vector pCZ11 or purified 319a flagellin. The spleens were harvested and passed through a 60-mesh stainless steel sieve. Erythrocytes were removed by Ficoll-Hypaque separation as described by Boyum (5). The cells were then washed three times in Hanks balanced salt solution (Gibco Canada Inc.) by centrifugation (800 x *g*) for 10 min at 4°C and suspended in culture medium containing RPMI 1640, 5% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U penicillin per ml, and 100 μg/ml of streptomycin (all from Gibco BRL, Burlington, Ontario, Canada) and 5 x 10^{-3} 2-mercaptoethanol (Sigma). Cell viability and quantity were assessed by trypan blue exclusion and approximately 2 x 10^{5} cells were plated in presence or absence of stimuli in 96-well flat-bottomed tissue culture plates (Nunclon; Nunc, Roskilde, Denmark). The stimuli used were purified 319a flagellin (0.01 to 10 μg/ml), concanavalin A (0.5-3.0 μg/ml) (Con A; Sigma) and phytohemagluttinin (0.5-3.0 μg/ml) (PHA; Sigma). The plates were incubated at 37°C in 5% CO_2 for 3, 5, or 7 days. Eighteen to twenty hours before the end of incubation, 1 μCi of [^{3}H]TdR (ICN, Montreal, Quebec, Canada) was added to

each well. Cells were harvested onto glass fiber filters with a PHD cell harvester (Cambridge Technology Inc., Watertown, MA) and a liquid scintillation counter was used to determine the number of counts per minute. This was done in quadruplicate for each group on each day. The stimulation index (SI) was calculated by dividing the stimulated counts per minute by the unstimulated counts per minute.

18. Reverse-transcriptase polymerase chain reaction (RT-PCR)

The mRNAs for gamma interferon (IFN-γ), *fliC*, and GAPDH were detected in immunized hamster spleen tissue by using reverse-transcriptase PCR (RT-PCR). Hamster spleen samples were frozen using dry ice and ethanol at the time of euthanasia. Spleen RNA was purified according to manufacturer's protocol by using TRIzol reagent (Gibco Life Technologies). First strand (cDNA) synthesis was performed in a 20-μl volume containing 2 μg total RNA, 250 ng random hexamers (Roche Diagnostics), 0.5 mM of each dNTP, 20 U RNaseOUT Rnase inhibitor, and 100 U Superscript II reverse transcriptase (all Life Technologies). The mixture was incubated at room temperature for 10 min and then at 42°C for 50 min. The reaction was inactivated by heating to 70°C for 15 minutes. The removal of excess RNA complementary to the cDNA was accomplished by incubating the reaction with 2 U of *E. coli* Rnase A for 20 min at 37°C.

The primers used to amplify the various cytokines, *fliC* transcript, and GAPDH are listed in Table 3. PCR was conducted in a 100 μl volume consisting of 2 μl cDNA, 10 μl 10x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 U Taq DNA polymerase (all Gibco BRL, Burlington, Ontario, Canada). An initial denaturation for 12 min at 95°C was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Each PCR product was subjected to electrophoresis in 2% agarose, stained with ethidium bromide and visualized by UV transillumination.

Table 1. Bacterial strains used in this study.

Strain	Description	Source/Reference
E. coli		
DH5α	F$^-$φ80d*lacZ*ΔM15Δ(*lacZYA-argF*) U169 *enda*1 *recA*1 *hsdR17 deoR* *thi*-1 *supE*44 *gyrA*96 *relA*1; Ap^s Tc^s	Bethesda Research Laboratories
SURE	e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171* *endA1 supE44 thi-1 gyrA96 relA1 lac* *recB recJ sbcC umuC*:: Tn5 *uvrC* [F' *proABlacI^{q}ZΔM15* Tn*10*]; Km^{r}Tc^r	Stratagene
B. pseudomallei	Clinical isolate: Gm^{r}Km^{r}Pb^{r}Sm^{r}Tc^s	D.A.B. Dance[a]
1026b		
319a	Clinical isolate	D.A.B. Dance[a]

[a] Wellcome-Mahidol-Oxford Tropical Medicine Research Programme, Bangkok, Thailand.

Table 2. Plasmids used in this study.

Plasmids	Description	Reference/Source
pPB400A	pUCP28T containing 2.0-kb *Hind*III-*EcoR*I fragment: *rpsU*$^+$-*fliC*$^+$; Tp^r	(7)
pZErO™-2.0	Negative selection cloning vector; ColE1 *ori*; Km^r Neo^r	Invitrogen
pCDNA™3.1(+)	Mammalian expression vector; ColE1 *ori*; SV40 *ori*; Ap^r Neo^r	Invitrogen
pDVAC	pCDNA™3.1/His containing 1.2-kb *Kpn*I-*EcoR*I fragment from pPB400A: *fliC*; Ap^r Neo^r	(6)
pBLUE-TOPO™	Positive selection cloning vector; pMB1 *ori*; *lacZ*; SV40 *ori*; Ap^r Neo^r	Invitrogen
pBL305	pBLUE-TOPO™ containing 1.2 fragment from pPB400A: *fliC*; Ap^r Neo^r	This study
pCDNA™3.1/CAT	Positive selection reporter vector; ColE1 *ori*; SV40 *ori*; *cat*; Ap^r Neo^r	Invitrogen
pCZ11	1.2-kb fragment from pCDNA™3.1(+): pCMV; 1.8-kb fragment from pZErO™-2.0: ColE1*ori*, Km^r	This study
pCZF428	pCZ11 containing 1.2-kb fragment from pPB400A: *fliC*; Km^r	This study

Table 3. Primers used in this study

Primer	Description	Reference/ Source
3.1-forward	5'-ATCCCCCGGGAGGCGTTTTGCG CTGCTTCG-3'	This study
3.1-backward	5'-TAGGAAGCTTAAGCCATAGAG CCCACCGCA-3'	This study
2.0-forward	5'-ATCCTTCGAAGGATGTTGCCG ATTTCGGCC-3'	This study
2.0-backward	5'-TAGGGGGCCCGAATCAGGGGAT AACGCAGG-3'	This study
fliC-forward	5'-TTAAGCTTACCATGCTCGGAATC AACAGC-3'	This study
fliC-backward	5'-CGGAATTCTTATTGCAGGAGC TTCAGCAC-3'	This study
RT-PCR		
IFN-γ-forward	5'-GGATATCTGGAGGAACTGGC-3'	(50)
IFN-γ-backward	5'-CGACTCCTTTTCCGCTTCCT-3'	(50)
fliC-PE	5'-GTTGCTGTTGATTCCGAGCAT-3'	(22)
fliC-reverse	5'-GTTGCCTGCAGATTGTTG-3'	This study
GAPDH-forward	5'-CGGAGTCAACGGATTTGGTCGAT-3'	(66)
GAPDH-reverse	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	(66)

III. RESULTS

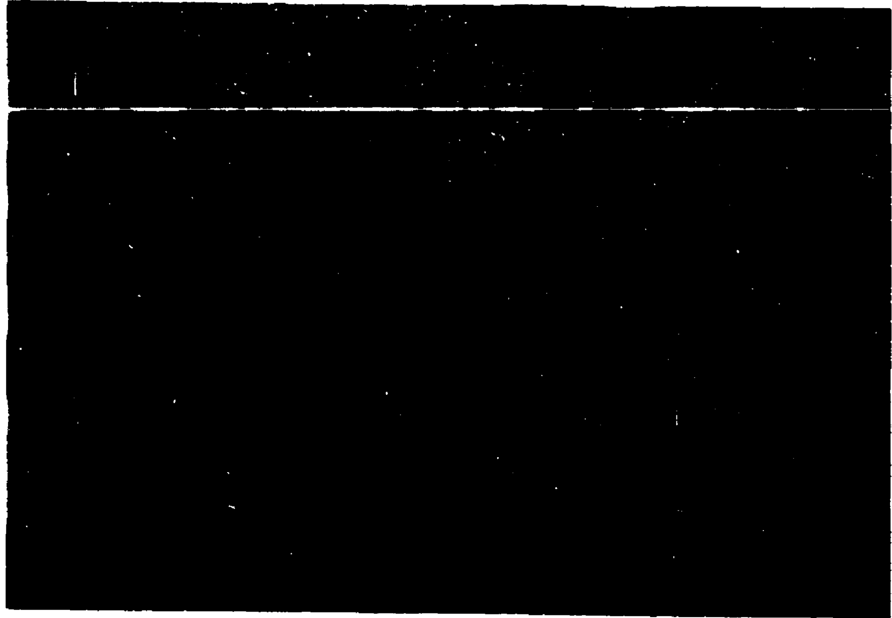
1. Fluorescent microscopy

The expression of the *Burkholderia pseudomallei* structural gene, *fliC*, was assessed in vivo using fluorescent microscopy of infected tissues. The lung, liver, and spleen were harvested from Syrian golden hamsters that were infected with *B. pseudomallei* strain 1026b, homogenized, and prepared on glass microscope slides. A mouse monoclonal antibody directed against purified *B. pseudomallei* strain 319a flagellin was used to probe the tissues and a fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG was used as a secondary antibody against background stained with Evan's blue dye. Positive samples fluoresced green at 490 nm against the red background tissue stained with Evan's blue.

Fluorescent microscopy performed on lung, liver, and spleen, tissue harvested from 1026b-infected hamsters demonstrated the expression of flagellin protein via reactivity to the monoclonal antibody and FITC-conjugated secondary antibody (Fig. 1a, 2a, 3a, respectively). The results of these studies demonstrated that flagellin is expressed in all three tissue types in infected hamsters, in contrast to lung, liver and spleen tissues harvested from healthy uninfected hamsters (Fig. 1b, 2b, 3b, respectively). The use of Evan's blue dye to cause background tissue to fluoresce red at 490 nm helped to distinguish the

Figure 1. Expression of flagellin in lung tissue of *B. pseudomallei* 1026b-infected Syrian golden hamsters. (a) Immunostaining of homogenized lung tissue from 1026b-infected hamsters reacted with a 1/500 dilution of mouse monoclonal antiserum specific for purified *B. pseudomallei* strain 319a flagellin and 1/1000 dilution of FITC-conjugated secondary antibody (anti-mouse IgG). (b) Immunostaining of homogenized lung tissue from healthy, uninfected hamsters reacted with 1/500 dilution monoclonal antibody to flagellin and 1/1000 FITC-anti-mouse-IgG.

A



B

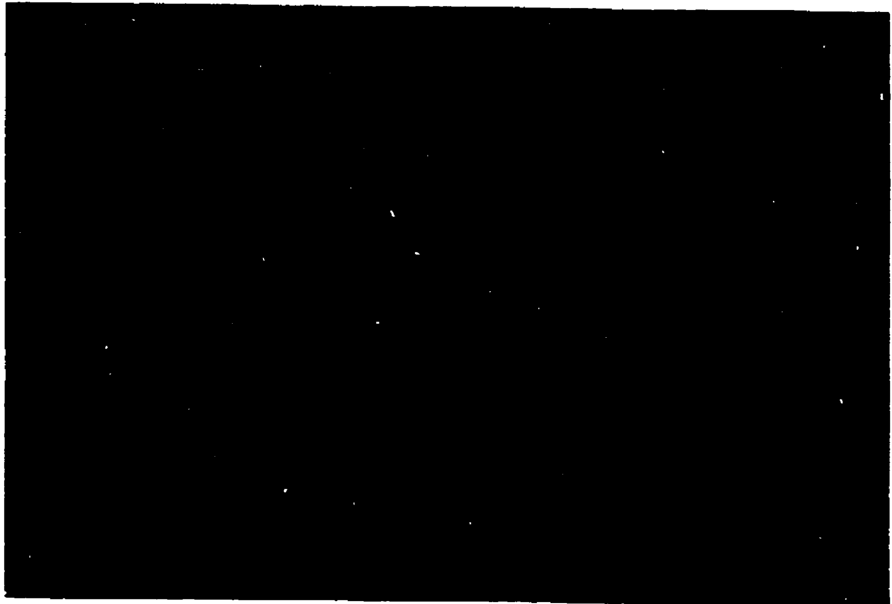
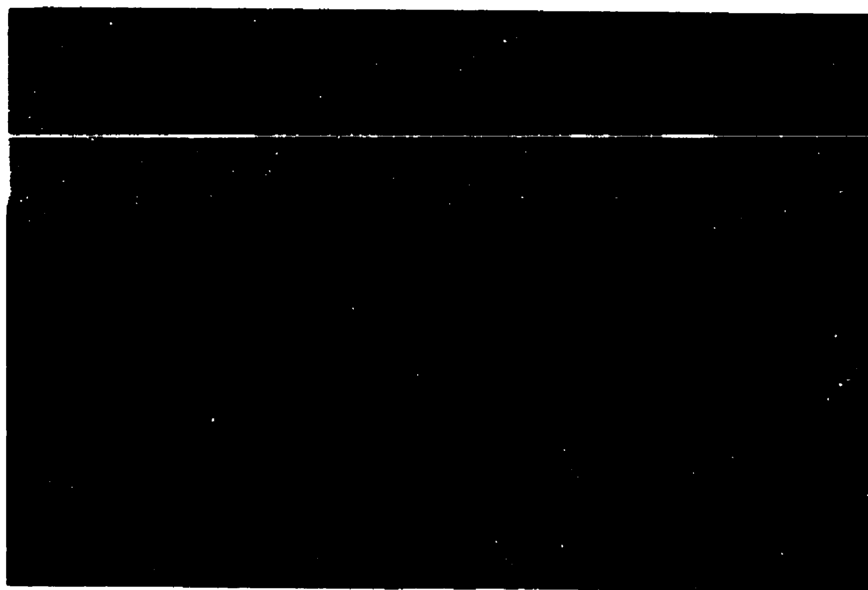


Figure 2. Expression of flagellin in liver tissue of *B. pseudomallei* 1026b-infected Syrian golden hamsters. (a) Immunostaining of homogenized liver tissue from 1026b-infected hamsters reacted with a 1/500 dilution of mouse monoclonal antiserum specific for purified *B. pseudomallei* strain 319a flagellin and 1/1000 dilution of FITC-conjugated secondary antibody (anti-mouse IgG). (b) Immunostaining of homogenized liver tissue from healthy, uninfected hamsters reacted with 1/500 dilution monoclonal antibody to flagellin and 1/1000 FITC-anti-mouse-IgG.

A



B

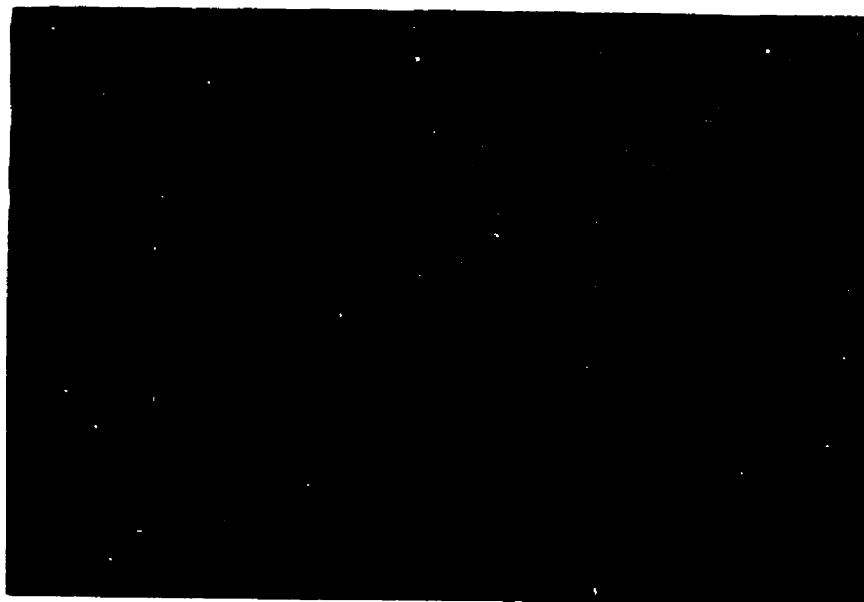
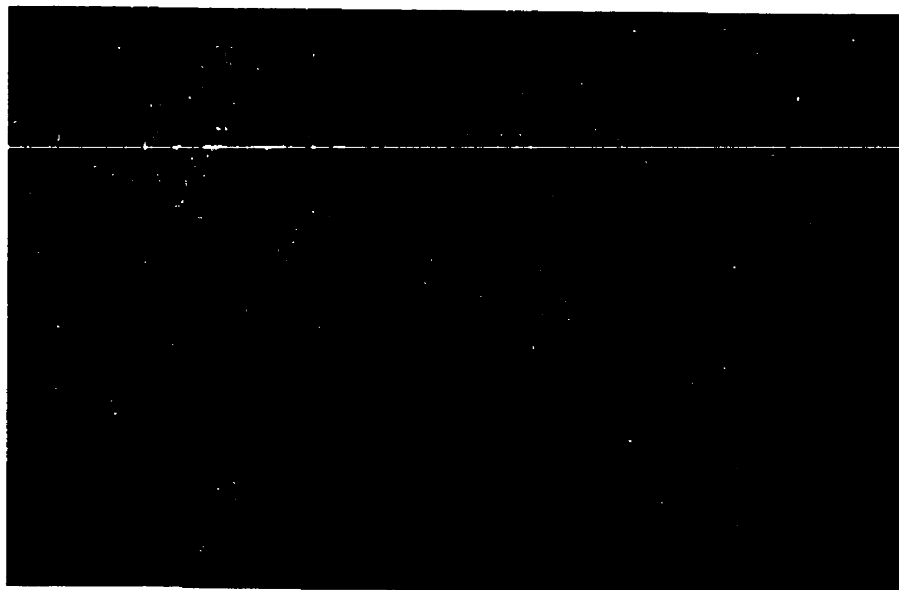


Figure 3. Expression of flagellin in spleen tissue of *B. pseudomallei* 1026b-infected Syrian golden hamsters. (a) Immunostaining of homogenized spleen tissue from 1026b-infected hamsters reacted with a 1/500 dilution of mouse monoclonal antiserum specific for purified *B. pseudomallei* strain 319a flagellin and 1/1000 dilution of FITC-conjugated secondary antibody (anti-mouse IgG). (b) Immunostaining of homogenized spleen tissue from healthy, uninfected hamsters reacted with 1/500 dilution monoclonal antibody to flagellin and 1/1000 FITC-anti-mouse-IgG.

A



B

green fluorescence of the FITC-stained flagellin protein. The color of background tissue ranged from red to yellow, depending on the time of exposure, thickness of tissue homogenate on the slide, and the concentration of antibodies applied to the slide. These factors served to influence the "bleaching" effect of the background tissue in the figures. To further confirm that the immunostaining was specific for the flagellin protein, the tissues harvested from infected animals did not demonstrate any fluorescence when stained with FITC-conjugated secondary antibodies alone (data not shown).

2. Delayed-type hypersensitivity assay

To determine if purified flagellin could produce a specific cellular immune response, we examined the ability of Syrian golden hamsters to mount a delayed-type hypersensitivity (DTH) response. A DTH response was examined by inoculation of the left rear footpad of hamsters with either purified 319a flagellin, 5% formalin-killed *B. pseudomallei* 1026b, or PBS. Measurements of left rear footpad thickness were taken and compared to the thickness of the right rear footpad at various time points after primary and secondary exposure to the antigens (Fig. 4). The difference between footpad thickness at 24 and 72 hours after primary (1°) immunization was attributed to non-specific inflammation, as compared to control-inoculated hamsters. The difference between footpad

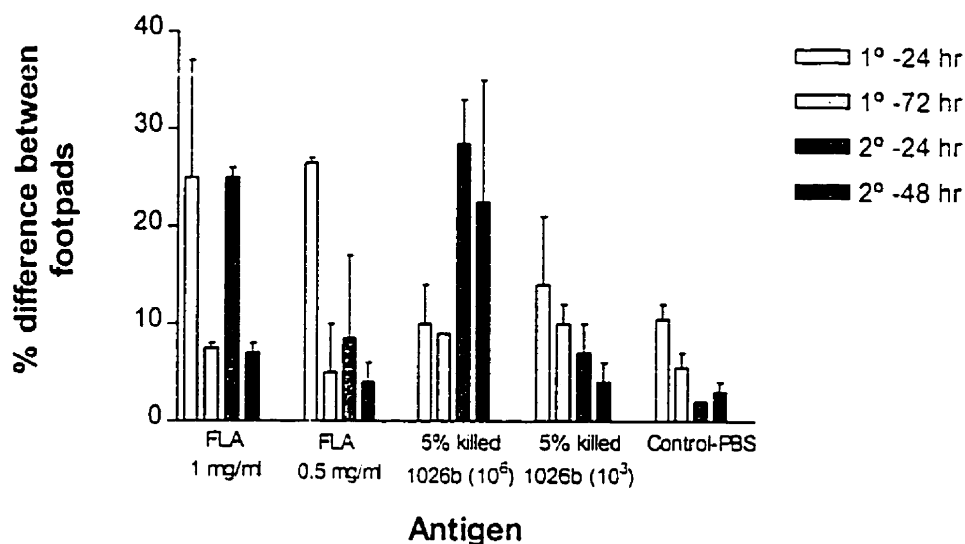


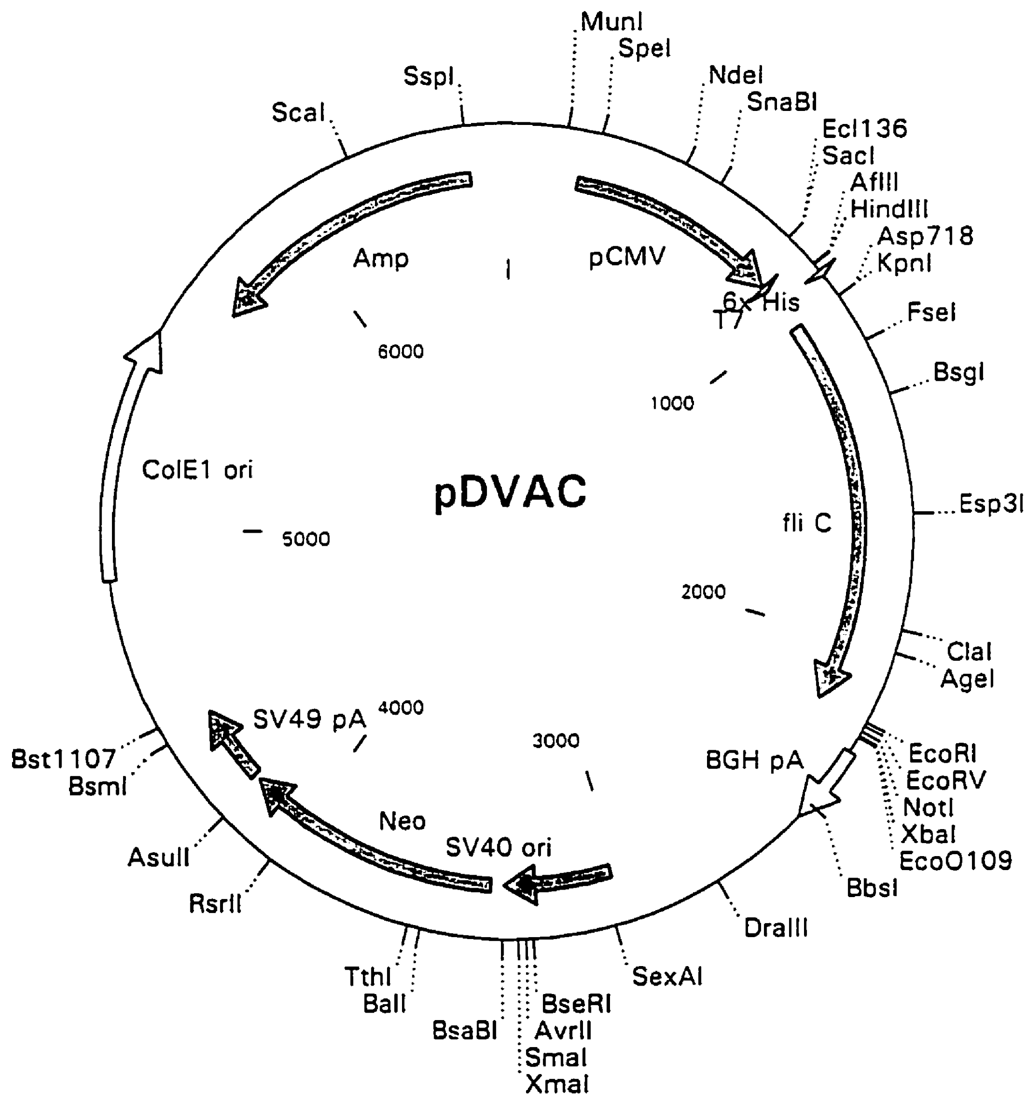
Figure 4. Delayed-type hypersensitivity (DTH) response of Syrian golden hamsters to various antigens. Two hamsters per immunization group were injected with antigen in the left rear footpad two times (1° and 2° immunization) with purified *B. pseudomallei* 319a flagellin (FLA), 5% formalin-killed *B. pseudomallei* 1026b (5% killed 1026b) or phosphate buffered saline (control-PBS). The % difference between footpads was calculated as: (L-R)/L x 100%, where L=width of the left rear footpad and R=width of the right rear footpad. Footpad width was measured using calipers calibrated to 0.5 mm.

thickness 24 and 48 hours after secondary (2°) immunization was recognized as a memory or DTH response to the antigen. The DTH responses to the antigens administered to the hamster footpads were calculated as the % difference between footpads (Fig. 4). Flagellin was inoculated in two doses, 0.5 and 1 mg/ml, and did not demonstrate a characteristic DTH response at either inoculum, as the % difference between footpad diameter was not higher after secondary exposure as compared to measurements taken after primary exposure. The 5% formalin-killed whole organism was inoculated at two doses, at 10^3 or 10^6 organisms. The 10^3 dose did not produce a DTH response, as the difference between footpad diameter decreased linearly at each measurement. In contrast, the 10^6 dose produced a significant DTH response, as there was a 3-fold increase in the difference between footpad thickness at 24 h after secondary immunization, and greater than 2-fold difference at 72 h. The animals inoculated with control PBS also did not demonstrate a DTH response.

3. DNA-based vectors and reporter vectors

Several plasmid vectors were utilized to study the expression of the flagellin structural gene, *fliC*, in tissue culture transfections. A previously constructed vector, named pDVAC, consisted of the *fliC* gene cloned into the commercial vector pCDNA™3.1/HisB (Invitrogen) (Fig. 5). This vector contained all features necessary for expression in mammalian hosts, including the promoter

Figure 5. Physical and genetic map of the mammalian expression vector pDVAC. The 1.2-kb *fliC* flagellin structural gene was inserted into the multiple cloning site of commercial vector pCDNA3.1/HisB™ (Invitrogen) using the restriction enzymes *Kpn*I and *Eco*RI. pCMV, promoter from cytomegalovirus (CMV); BGH pA, polyadenlyation site from bovine growth hormone (BGH); SV40 ori, origin of replication from simian virus 40 (SV40); Neo, neomycin resistance marker; SV40 pA, polyadenylation signal from SV40; ColE1 ori, origin of replication for amplification in *E. coli*; Amp, ampicillin resistance marker.


MunI
SpeI
NdeI
SnaBI
Ecl136
SacI
AflII
HindIII
Asp718
KpnI
FseI
BsgI
Esp3I
ClaI
AgeI
EcoRI
EcoRV
NotI
XbaI
EcoO109
BbsI
DraIII
SexAI
BseRI
AvrII
SmaI
XmaI
BsaBI
TthI
BalI
RsrII
AsuII
Bst1107
BsmI
ScaI
SspI
Amp
pCMV
6x His
T7
6000
1000
ColE1 ori
pDVAC
fli C
5000
2000
SV49 pA
4000
3000
BGH pA
Neo
SV40 ori

from cytomegalovirus (CMV), a polyadenylation (poly A) signal from bovine growth hormone (BGH), simian virus 40 (SV40) origin of replication and poly A site, ColE1 origin of replication (ori) for amplification of the vector in *E. coli*, and resistance markers (ampicillin and kanamycin). The vector, pDVAC was utilized for transfections of the cell lines, HeLa and CHO.K1.

A reporter vector, pBL305, consisted of the *fliC* gene cloned into the TOPO™-cloning vector pBLUE-TOPO™ (Invitrogen) (Fig. 6). This vector contained the origin of replication and poly A site from SV40, a poly A site from BGH, a pMB1 ori for amplification in *E. coli*, as well as Neo^R and Amp^R resistance markers. The vector, pBL305, was utilized in transfections of the COS-1 epithelial cell line. Successful transfection conditions and expression of the *fliC* gene was monitored via β-galactosidase assays of transfected cell extracts.

Another reporter vector, pCDNA™3.1/CAT (Invitrogen) was utilized to monitor successful transfection conditions (Fig. 7). This commercial vector includes a gene for the enzyme chloramphenicol acetyl transferase, and therefore can be used as a positive control for transfection conditions as monitored by CAT assays. Successful transfection conditions were elucidated using pCDNA™3.1/CAT, which could subsequently be employed for transfections of the plasmid DNA vector constructed for immunization studies.

A plasmid DNA vector, pCZ11, was constructed for expression in mammalian hosts (Fig. 8). This vector was constructed by the PCR

Figure 6. Physical and genetic map of mammalian expression vector pBL305. The 1.2-kb *fliC* locus was inserted into the commercial vector pBLUE-TOPO™ (Invitrogen). T7, promoter from virus T7; 6X His, histidine tag for purification of expressed protein; *lacZ*, promoterless reporter gene for detection of protein expression via β-galactosidase assays; BGH poly A, polyadenlyation signal from bovine growth hormone (BGH); SV40 ori, origin of replication from simian virus 40 (SV40) for expression of the vector in SV40-transformed tissue culture cell lines; Neomycin, neomycin resistance marker; SV40 pA, polyadenylation signal from Sv40; pMB1 ori, origin of replication for amplification of the vector in *E. coli*; Ampicillin, ampicillin resistance marker.

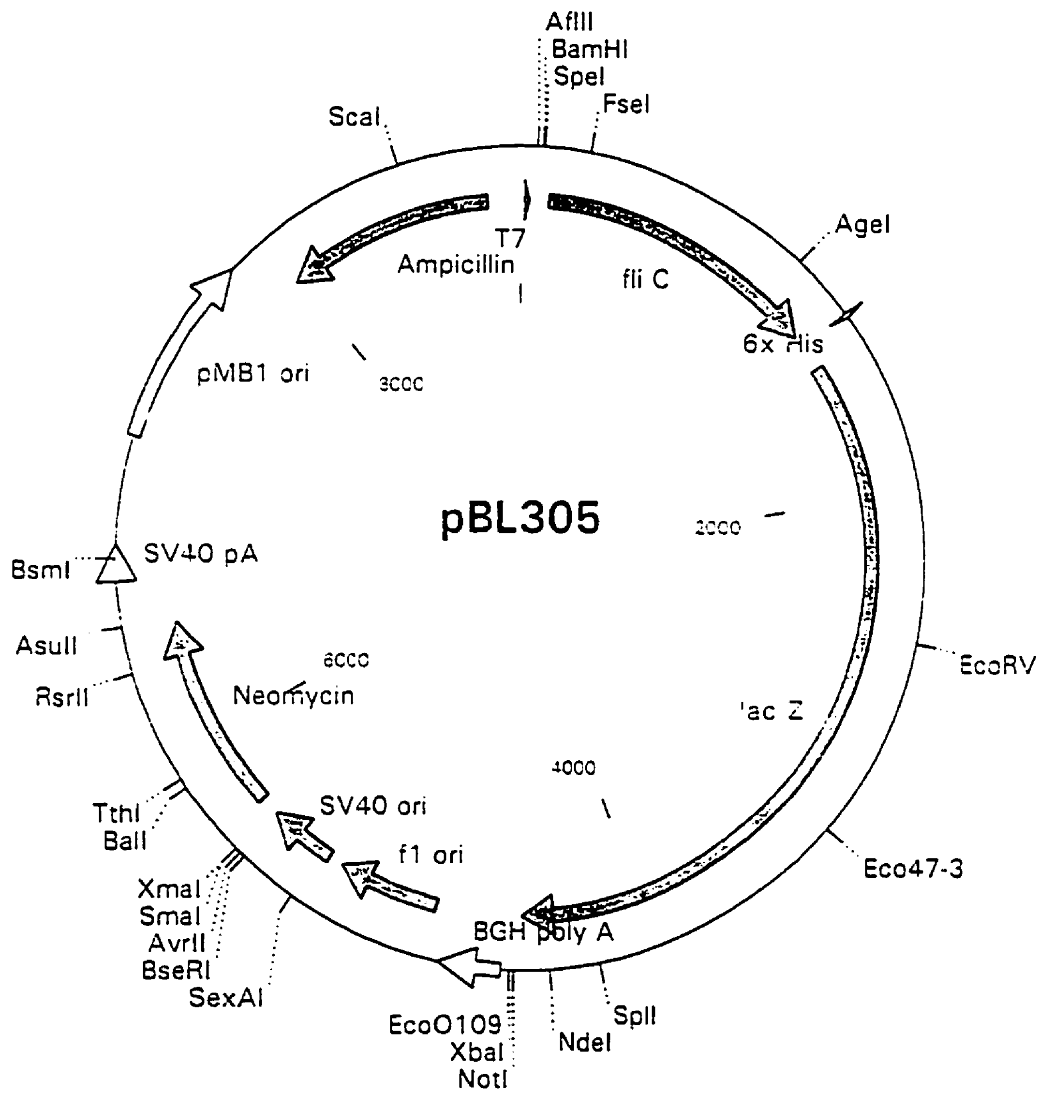

AflII
BamHI
SpeI
FseI
ScaI
AgeI
T7
Ampicillin
fli C
6x His
pMB1 ori
pBL305
SV40 pA
BsmI
AsuII
RsrII
Neomycin
lac Z
EcoRV
TthI
BalI
SV40 ori
f1 ori
Eco47-3
XmaI
SmaI
AvrII
BseRI
SexAI
BGH poly A
EcoO109
XbaI
NotI
NdeI
SplI

Figure 7. Physical and genetic map of mammalian expression vector pCDNA3.1/CAT™ (Invitrogen). pCMV, promoter from cytomegalovirus (CMV); T7, promoter from T7 virus; CAT, chloramphenicol acetyltransferase gene; BGH pA, polyadenylation signal from bovine growth hormone (BGH); SV40 ori, origin of replication from simian virus 40 (SV40) for replication of the vector in SV40-transformed tissue culture cell lines; Neo, neomycin resistance marker; SV40 pA, polyadenylation signal from SV40; ColE1, origin of replication for amplification of the vector in *E. coli*; Amp, ampicillin resistance marker.

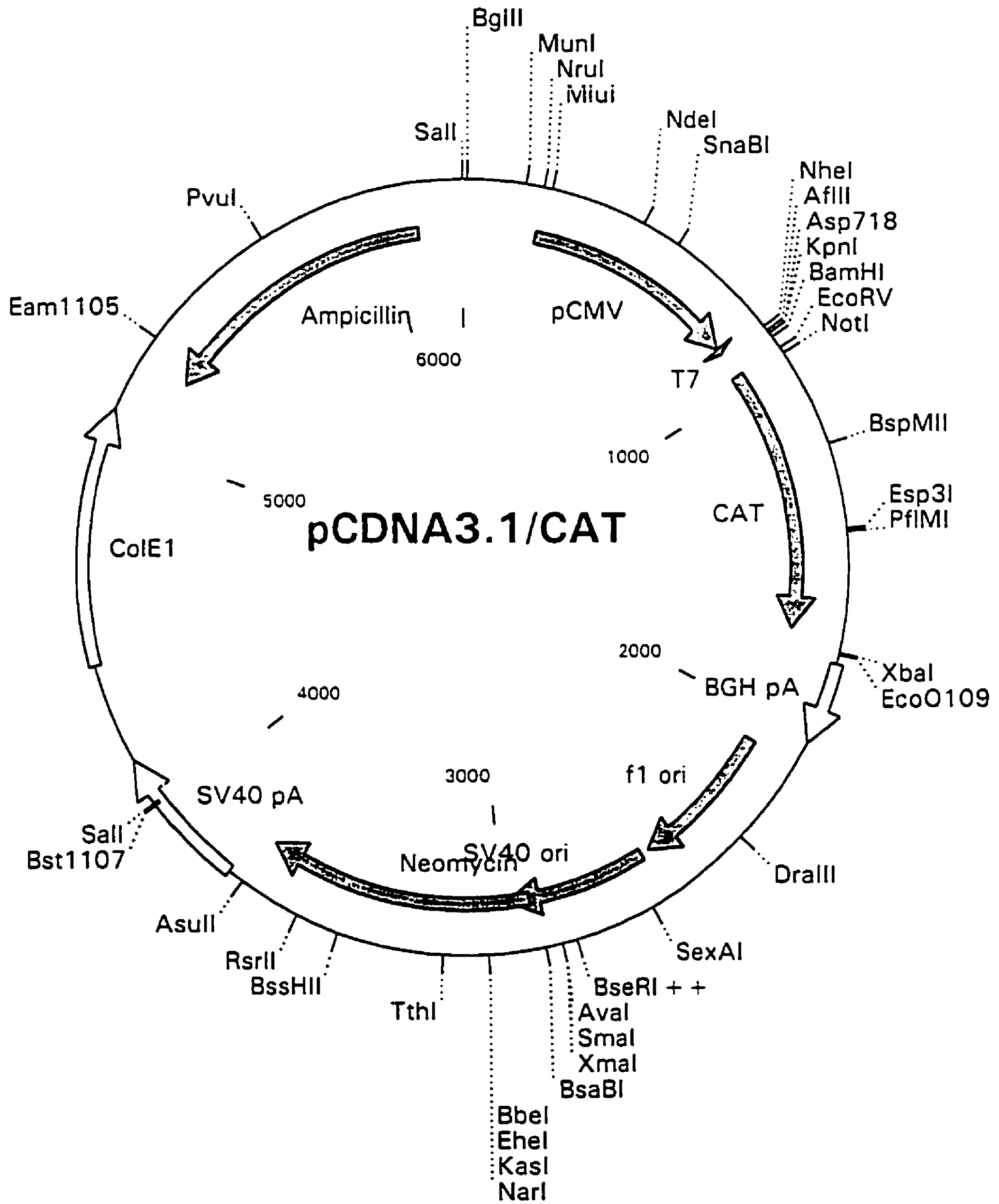
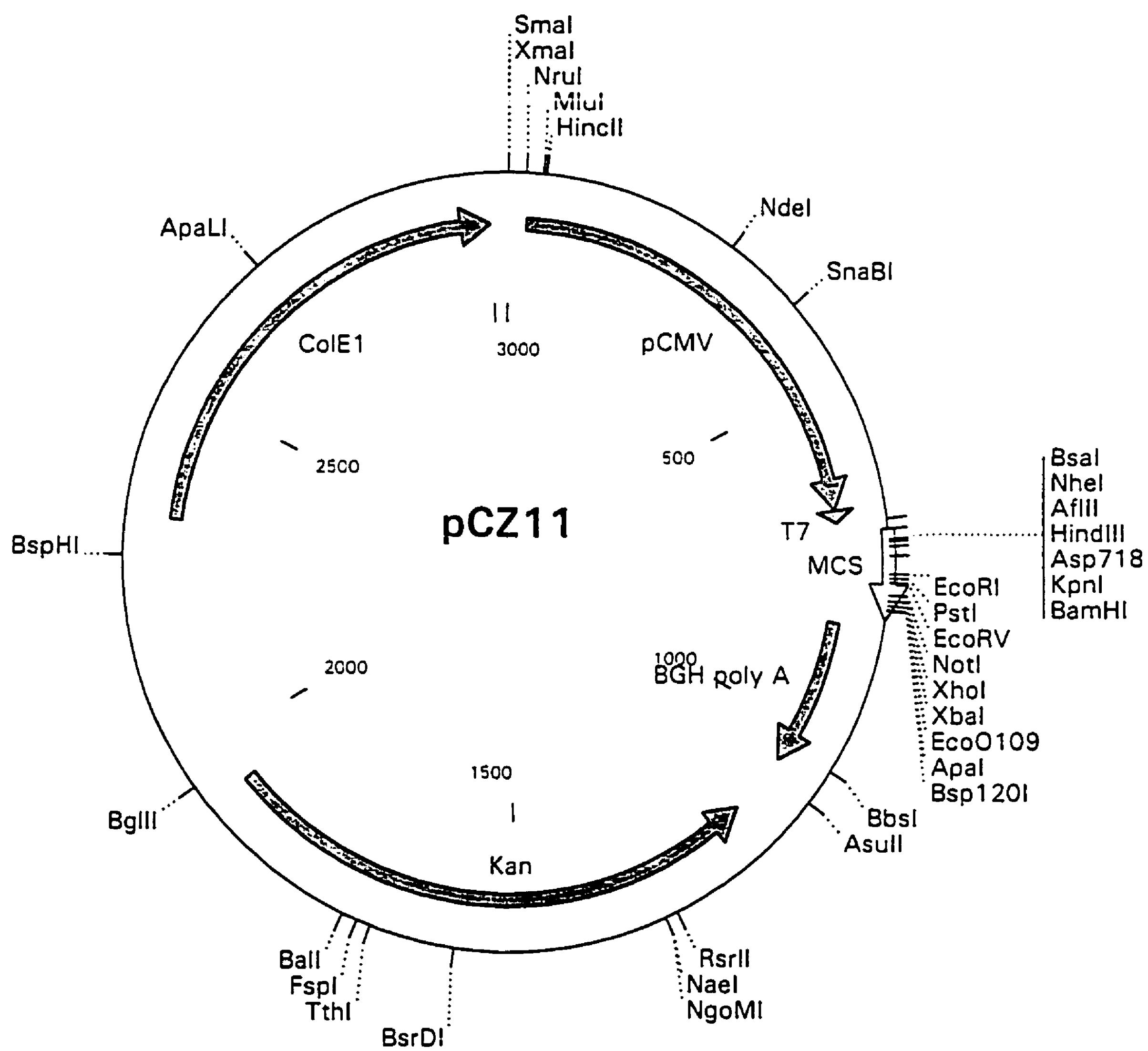

pCDNA3.1/CAT
BglII
MunI
NruI
MluI
NdeI
SnaBI
SalI
PvuI
NheI
AflII
Asp718
KpnI
BamHI
EcoRV
NotI
Eam1105
Ampicillin
pCMV
6000
T7
BspMII
1000
5000
Esp3I
PflMI
CAT
ColE1
2000
XbaI
EcoO109
BGH pA
4000
3000
f1 ori
SV40 pA
SV40 ori
Neomycin
SalI
Bst1107
DraIII
AsuII
SexAI
RsrII
BssHII
TthI
BseRI + +
AvaI
SmaI
XmaI
BsaBI
BbeI
EheI
KasI
NarI

Figure 8. Physical and genetic map of mammalian expression vector pCZ11. Primers were designed to amplify regions from two commercial vectors, pCDNA3.1™(+) and pZErO™-2.0 (both Invitrogen) and ligated to create this vector. Regions from pCDNA3.1(+) included: pCMV, promoter from the cytomegalovirus (CMV) for expression in mammalian tissues; T7, promoter from virus T7; MCS, multiple cloning site; BGH pA, polyadenylation signal from bovine growth hormone (BGH). Regions from pZErO™-2.0 included: Kan, kanamycin resistance marker; ColE1, origin of replication for amplification of the vector in *E. coli*.


SmaI
XmaI
NruI
MluI
HincII
NdeI
SnaBI
ApaLI
ColE1
3000
pCMV
2500
500
pCZ11
T7
MCS
BspHI
BsaI
NheI
AflII
HindIII
Asp718
KpnI
BamHI
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
EcoO109
ApaI
Bsp120I
2000
1000
BGH poly A
1500
BglI
BbsI
AsuII
Kan
BalI
FspI
TthI
BsrDI
RsrII
NaeI
NgoMI

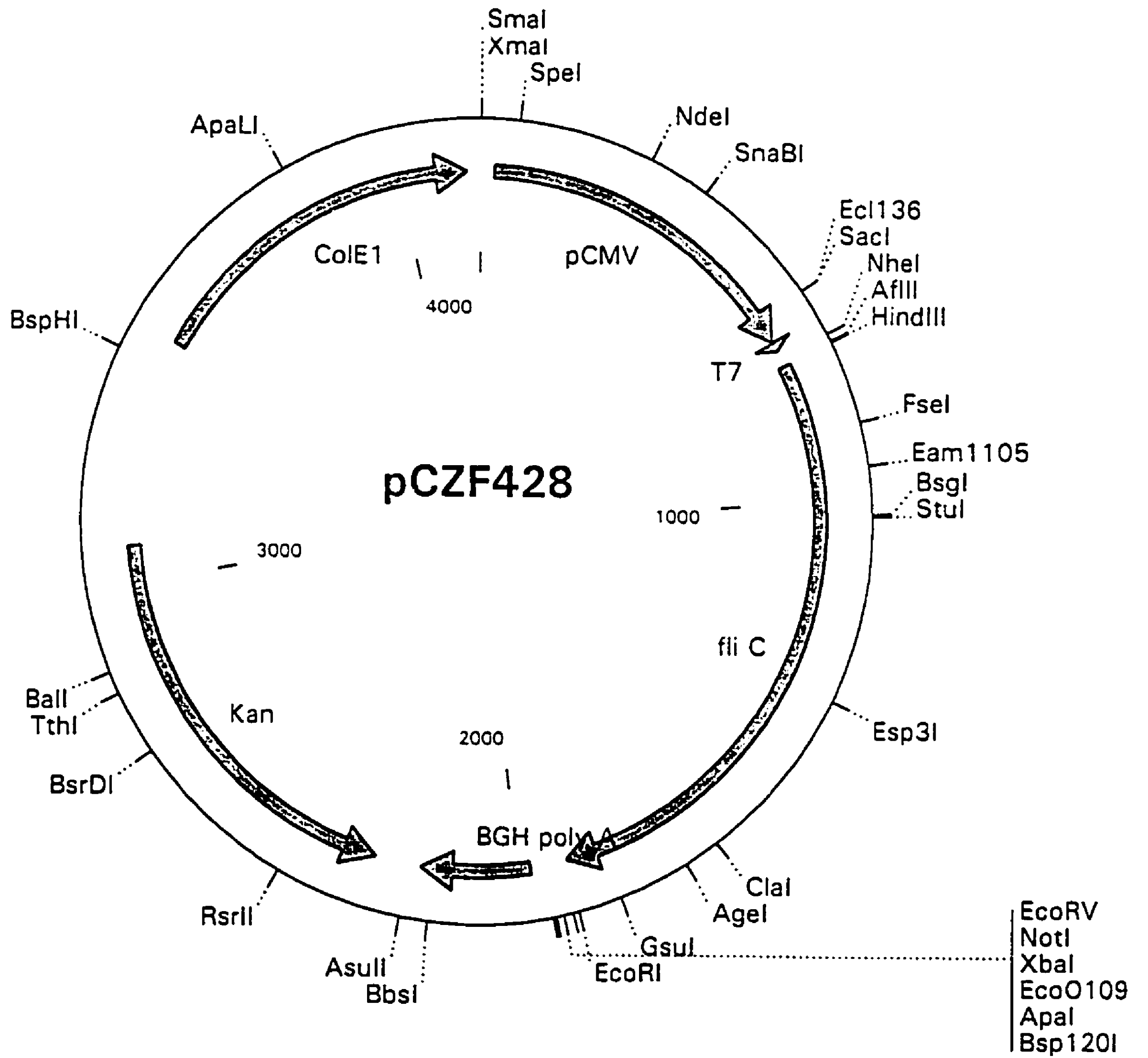
amplification of regions from two commercial vectors, pCDNA™3.1(+) and pZErO™-2.0 (Invitrogen), using primers designed from sequence published by the manufacturers (Table 3). The resultant PCR products were digested with restriction enzymes and ligated into one plasmid vector, pCZ11. This vector contains the promoter from CMV, multiple cloning site (MCS), BGH poly A site, ColE1 origin of replication, and kanamycin resistance (Kan^R) marker. The vector, pCZF428, was constructed by cloning the *fliC* into the multiple cloning site of pCZ11 (Fig. 9). Both pCZ11 and pCZF428 were used as control and test vectors, respectively, for tissue culture transfections, as well as subsequent animal studies and immunological assays.

4. Tissue culture transfections

The expression of flagellin protein from the structural gene, *fliC*, encoded on various plasmid vectors was investigated via lipid-mediated tissue culture cell line transfections. The cell lines used in this study included the human epitheloid cervical carcinoma (HeLa), Chinese hamster ovary (CHO.K1) and the SV40-transformed African green monkey kidney cell line (COS-1).

Prior experimentation using the mammalian expression vector pDVAC demonstrated that transfection of HeLa, CHO.K1, and COS-7 cell lines with this vector resulted in expression of flagellin as analyzed by fluorescent microscopy (6). Therefore, cell extracts from tissue culture transfections using pDVAC

Figure 9. Physical and genetic map of DNA vaccine vector pCZF428. The 1.2-kb *fliC* locus was inserted into the multiple cloning site (MCS) of mammalian expression vector pCZ11.


pCZF428
SmaI
XmaI
SpeI
NdeI
SnaBI
Ecl136
SacI
NheI
AflII
HindIII
FseI
Eam1105
BsgI
StuI
Esp3I
ClaI
AgeI
GsuI
EcoRI
EcoRV
NotI
XbaI
EcoO109
ApaI
Bsp120I
BbsI
AsuII
RsrII
BsrDI
TthI
BalI
BspHI
ApaLI
ColE1
pCMV
T7
fli C
BGH poly A
Kan
4000
1000
2000
3000

in this study was examined for the production of flagellin protein. Cell extracts from HeLa, CHO.K1 and COS-1 cell lines transfected with pDVAC were analyzed via SDS-PAGE and western blotting using a mouse monoclonal antibody against purified *B. pseudomallei* 319a flagellin as a probe. A variety of tissue culture transfection conditions were examined and protein expression was not detected by western blotting (data not shown).

Two different approaches were taken to attempt to study flagellin expression from a plasmid vector, and to elucidate the optimal conditions for lipid-mediated tissue culture transfections of the three cell lines used in this study. The first approach was to clone the *fliC* locus into a promoterless reporter vector capable of expression in mammalian tissues, and the resultant vector was pBL305. This vector contained a *lacZ* gene, therefore expression could be monitored by β-galactosidase assay. Cell extracts from tissue culture transfections of the COS-1 cell line with pBL305 did not result in higher levels of activity than untransfected cell lines, as measured by β-galactosidase assays (data not shown).

The second approach was to use the control vector pCDNA™3.1/CAT to transfect the cell lines and elucidate the most favorable conditions for successful transfection by a mammalian expression vector. Cell extracts from transfections of HeLa and CHO.K1 with this control vector were assayed for chloramphenicol acetyltransferase (CAT) activity via CAT assays (Fig.10). CAT assays were

Figure 10. Expression of chloramphenicol acetyltransferase (CAT) in cell lysates of transfected CHO.K1 and HeLa cells. Autoradiograph of chloramphenicol acetyl transferase assay. Lanes 1, 2, 4-cell lysates of CHO.K1 cells transfected with pCDNA™3.1/CAT; lanes 3, 5-cell lysates of HeLa cells transfected with pCDNA™3.1/CAT; lane 6-cell lysate of CHO.K1 cells transfected with a negative control vector (does not have CAT gene); lane 7- cell lysate of HeLa cells transfected with negative vector; lanes 8, 9-positive control of CAT enzyme (40 and 50 mU, respectively). M, mono-acetylated ^{14}C-chloramphenicol; D, di-acetylated ^{14}C-chloramphenicol; E, excess ^{14}C-chloramphenicol.

1 2 3 4 5 6 7 8 9

D

M

E

performed on cell extracts of pCDNA™3.1/CAT-transfected cells, cells transfected with a vector without the CAT gene, as well as with purified CAT enzyme as a positive control. The lower bands in lanes 3, 5, 7, 8, and 9 represent excess ^{14}C-labelled chloramphenicol. The intermediate band represents mono-acetylated ^{14}C-labelled chloramphenicol, while the upper band represents di-acetylated ^{14}C-labelled chloramphenicol. Cell extracts from transfections performed on CHO.K1 cells with the plasmid vector demonstrated CAT activity in the form on mono- and di-acetylated ^{14}C-labelled chloramphenicol in lanes 1, 2, and 4 of the autoradiograph. HeLa cells transfected with pCDNA™3.1/CAT also demonstrated CAT activity by the presence of mono-acetylated ^{14}C-labelled chloramphenicol in lanes 3 and 5 of the autoradiograph. Both CHO.K1 and HeLa cells transfected with the plasmid vector lacking the CAT gene did not demonstrate any CAT enzyme activity, as seen in lanes 6 and 7. Purified CAT enzyme was loaded on the silica gel as a positive control at 40 and 50 mU in lanes 8 and 9, respectively, and exhibited mono-acetylated ^{14}C-labelled chloramphenicol in the presence of mid-distance bands.

The results of the CAT assay demonstrated that certain conditions were optimal for transfections using plasmid vectors under the control of the promoter from CMV. To be more precise, lipid-mediated transfections were successful when 0.5 to 3.0 μg of plasmid DNA and 1.0 to 2.0 μl of Lipofectamine PLUS reagent (Gibco Canada, Inc.) was utilized in 6-well plates. Optimal incubations times were between 3 to 5 hours, with protein expression permitted for 24 to 72 hours without the presence of a selective antibiotic.

There are a variety of conditions that did not result in successful transfection of the tissues. The use of 24-well plates is not suggested, as the number of cells in these plates that can be manipulated is below optimal levels. Additionally, it is recommended that initial seeding does not exceed 75-80% confluency, as these cell lines multiply rapidly and space for transfected cells to grow is essential. During the transfection period, it was determined that the presence of serum was not required, and should only be added after the transfection has occurred. Additionally, the presence of a selective antibiotic is not suggested, as the growth of transfected cells was retarded in the presence of antibiotic.

5. Flagellin protein expression

The expression of flagellin protein from the plasmid vector, pCZF428, was investigated by SDS-PAGE analysis and subsequent western immunoblotting of cell extracts from HeLa and CHO.K1 cells transfected with this vector, the control vector pCZ11, and untreated cells. The samples were loaded onto a 12.5% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with a mouse monoclonal antibody specific for purified *B. pseudomallei* 319a flagellin at a dilution of 1:1000, and then a 1:5000 dilution of a goat anti-mouse IgG antibody conjugated to horseradish peroxidase.

Western blotting of cell extracts from HeLa cells transfected with pCZF428 (plasmid vector containing *fliC*) demonstrated the presence of a single protein

that reacted with the mouse monoclonal antibody specific for 319a flagellin and had an apparent relative mobility consistent with purified flagellin (M_r = 43,400) (Fig. 11). The purified 319a flagellin that was present as a positive control had been overloaded, and the presence of two bands was observed. The lower band was approximately 43 kDa in size and consistent with the size of flagellin monomers, while the upper band was over 80 kDa in size, most likely the

Figure 11. Expression of flagellin protein in transfected HeLa cells. Cell extracts from HeLa cells transfected with pCZF428 (containing *fliC*), pCZ11 (control vector), and purified *B. pseudomallei* 319a flagellin were analyzed by 12.5% SDS-PAGE. Western blotting was performed using a mouse monoclonal antibody specific for purified 319a flagellin (1:1000) and a peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000). Lanes 1–4, extracts from cells transfected with pCZ11; lanes 5-7, extracts from cells transfected with pCZF428; lanes 8-10, extracts from untransfected cells; lanes 11 and 12, purified 319a flagellin (monomers ≈ 43 kDa; dimers ≈ 80 kDa).

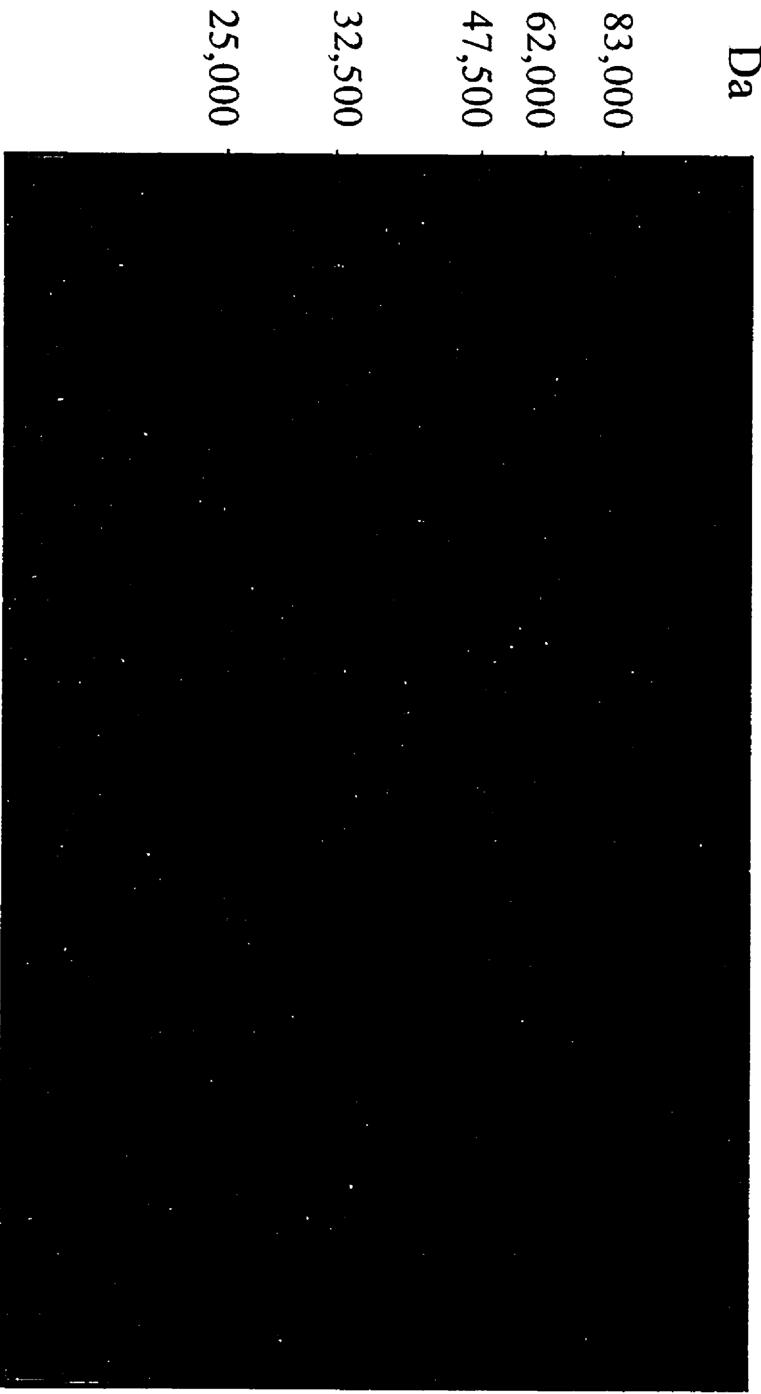

Da
83,000
62,000
47,500
32,500
25,000
MW 1 2 3 4 5 6 7 8 9 10 11 12

Figure 12. Expression of flagellin protein in transfected CHO.K1 cells. Cell extracts from CHO.K1 cells transfected with pCZF428 (containing *fliC*), pCZ11 (control vector), and purified *B. pseudomallei* 319a flagellin were analyzed by 12.5% SDS-PAGE. Western blotting was performed using a mouse monoclonal antibody specific for purified 319a flagellin (1:1000) and a peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000). Lanes 1–3, extracts from untransfected cells; lanes 4-6, extracts from cells transfected with pCZF428; lanes 7-11, extracts from cells transfected with pCZ11; lanes 12 and 13, purified 319a flagellin (monomers ≈ 43 kDa).

Da
83,000
47,500
32,500
25,000
MW 1 2 3 4 5 6 7 8 9 10 11 12 13

presence of flagellin dimers. HeLa cells transfected with the control vector pCZ11 or untransfected did not exhibit any protein reactive to the monoclonal antibody. A single protein was also produced in CHO.K1 cells transfected with pCZF428 that was reactive to the monoclonal antibody and was approximately 43 kDa in size, consistent with bands resulting when purified 319a flagellin is also probed (Fig. 12). There was no protein reactive to the monoclonal antibody specific for flagellin in cell extracts from untransfected cells or CHO.K1 cells transfected with pCZ11.

6. Animal protection studies

Immunoprotection studies were performed on Syrian golden hamsters and infant diabetic rats that were immunized through various regimens. The Syrian golden hamsters were immunized at 0, 3, 5, and 7 weeks with either pCZF428 (with *fliC*), the empty vector pCZ11, or with purified *B. pseudomallei* 319a flagellin via i.m. injection in each quadriceps muscles, and were challenged two weeks after the last DNA vaccination with less than 10 organisms of *B. pseudomallei* 1026b via intraperitoneal (i.p.) injection. All immunized groups succumbed to disease and death due to infection within 48 hours, therefore, the plasmid vector pCZF428 did not confer any protection to the Syrian golden hamsters from challenge by *B. pseudomallei* 1026b.

Table 4. Immunoprotection by pCZF428 in infant diabetic rats challenged with *B. pseudomallei* 1026b

Group	Animal	Days to Death Post-Challenge	CFU/ml of harvested spleens
pCZ11	1	1	ND
	2	2	ND
	3	5 [a]	> 10^7
	4	5 [a]	> 10^7
pCZF428	1	6	> 10^7
	2	8	10^3
	3	8 [a*]	10^3
	4	8 [a*]	0

[a] - spleen harvested from carcasses and plated out on LB;

* - of the group of 3 test rats on Day 8, one rat was dead, one was very sick and the third rat looked healthy; two rats still alive were cardiac bled and sacrificed

ND – done

Sprague-Dawley infant rats were immunized immediately after weaning from their mother at roughly 20 days of age, and then immunized for a second time ten days later. The two immunization groups consisted of infant rats immunized with either pCZF428 (with *fliC*) or with pCZ11 (empty vector). In the interval between immunizations, the rats were rendered diabetic by two consecutive injections of the diabetogenic compound streptozotocin (STZ). The infant diabetic rats were then challenged intraperitoneally (i.p.) with approximately 10^4 *B. pseudomallei* 1026b 7 days after the second immunization. The challenged rats were monitored for onset of sickness and the number of days before death was recorded for each group (Table 4). The control rats (immunized with pCZ11) succumbed to death within 5 days after challenge with 1026b, which was significantly quicker than the test rats (immunized with pCZF428), which survived 6 to 8 days after challenge ($P < 0.0.5$, Table 4). Upon death, the spleen were harvested from the animals and plated on Luria-Bertani (LB) agar to determine the colony forming units per ml (CFU/ml). Of the rats in the test group, 50% were cardiac bled and sacrificed in day 8, revealing a significantly lower amount of CFU/ml in the spleens as compared to the control rats.

7. Splenocyte proliferation in immunized animals

To investigate the generation of a cytotoxic T lymphyocyte (CTL) response, the proliferative responses to purified 319a flagellin in splenocytes of

Syrian golden hamsters were measured following immunization with either DNA or protein. Splenocytes were harvested from hamsters immunized four times with either pCZF428 (with *fliC*), pCZ11 (empty vector), or purified 319a flagellin protein on day 3, 5 and 7 after harvest. The CTL response was analyzed by measuring the incorporation of [^{3}H]-thymidine in response to purified flagellin, the mitogenic stimuli phytohemagluttinin (PHA), or no stimuli. The proliferation due to stimuli was determined as the stimulation index (SI). The SI was calculated by dividing the counts per minute (cpm) as determined by a scintillation counter of the stimulated cells by the cpm of naïve splenocytes. Stimulation of splenocytes from immunized hamsters with purified 319a flagellin or PHA resulted in the highest SI when the splenocytes were harvested on day 5 (Fig. 13). On day 5, there was no significant difference between the proliferation of splenocytes from hamsters immunized with either DNA or protein. More specifically, splenocytes from hamsters immunized with the test vector pCZF428 (with *fliC*) did not show significantly different SI values than splenocytes from control-immunized (pCZ11) hamsters.

8. Immune responses to DNA vaccination

The immune serum titers of infant diabetic rats and Syrian golden hamsters immunized with either pCZF428 (*fliC*) or pCZ11 (empty vector) were monitored throughout the immunization regimen to study the production of antibodies specific for flagellin. The production of antibodies specific for flagellin was also investigated in sera collected from infant diabetic rats that were

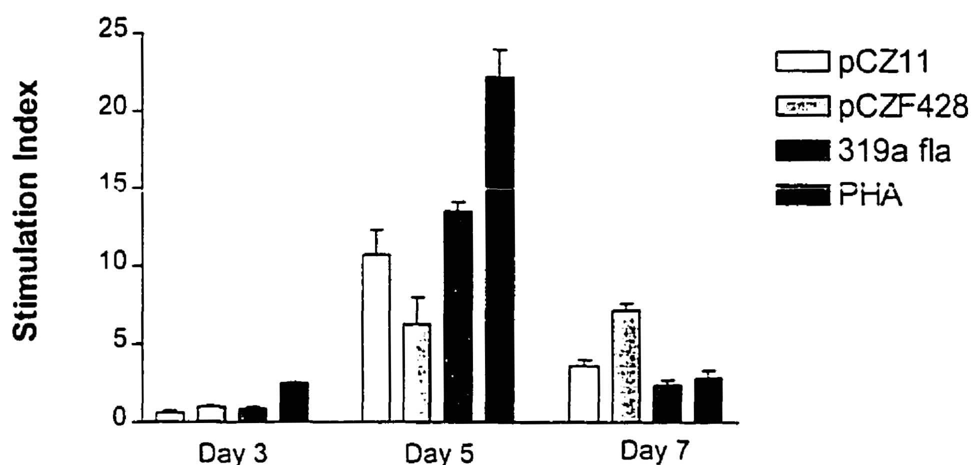


Figure 13. Proliferation of splenocytes from Syrian golden hamsters immunized with DNA or protein. Splenocytes harvested from hamsters immunized with pCZ11 (plasmid vector without *fliC* gene), pCZF428 (plasmid vector with *fliC* gene), or purified *B. pseudomallei* 319a flagellin were stimulated with 0.1 or 0.01 ug of purified 319a flagellin. Splenocytes stimulated with 2.0 ug phytohemagluttinin (PHA) were used as a positive control. Stimulated cells were pulsed with [^{3}H]-thymidine 18-22 hours before harvest of cells. Splenocytes were harvested on day 3, 5, and 7, and radioactivity was counted using a scintillation counter. Stimulation index is calculated as the counts per minute (cpm) of stimulated cells divided by the cpm of naïve cells. The data is representative of cpm performed in quadruplicate.

immunized with either pCZF428 or pCZ11. The rats were bled after the first and second immunizations, as well as after infection by *B. pseudomallei* 1026b and the reciprocal value of the highest dilution of sera that reacted in an ELISA against purified 319a flagellin was determined to be the titer (Table 5). The sera from hamsters immunized with the control vector pCZ11 did not demonstrate an increase in IgG titers specific for flagellin, whereas the sera from pCZF428-immunized hamsters demonstrated an 8-fold increase in IgG titer from the 1st to the 2nd immunization. Sera collected from rats after infection by 1026b demonstrated high levels of IgG antibody to flagellin.

Immunized hamsters with DNA or protein were bled at week 5 (after 3rd immunization) and week 9 (two weeks after 4th immunization). The highest dilution of immune serum that reacted in an ELISA against purified 319a flagellin was expressed as the value of that dilution (Table 6). In contrast to pre-immune hamster serum, the serum from hamsters immunized with pCZF428 clearly demonstrated an increase in IgG titers specific for 319a flagellin after immunization and booster doses. The sera from hamsters immunized with the control vector pCZ11 showed the least amount of reactivity to purified flagellin, with levels of IgG titers similar to serum from non-immunized animals. The sera from hamsters immunized with purified 319a flagellin exhibited the highest immune response, as the IgG titers were consistently the highest measured.

Table 5. Evaluation of immune responses to DNA vectors pCZF428 and pCZ11 in immunized infant diabetic rats.

Immunization Group	Pre-infection Titer		Post-infection Titer
	1st immunization	2nd Immunization	
pCZF428 (fliC)	1:100	1:800	>1:3200
pCZ11 (empty vector)	<1:50	<1:50	>1:3200

- sera was diluted 1:50 to 1:3200 in doubling dilutions
- ELISA plates were coated with 10 μg/well purified *B. pseudomallei* 319a flagellin
- secondary antibody used was goat anti-rat IgG conjugated to horseradish peroxidase (HRP)

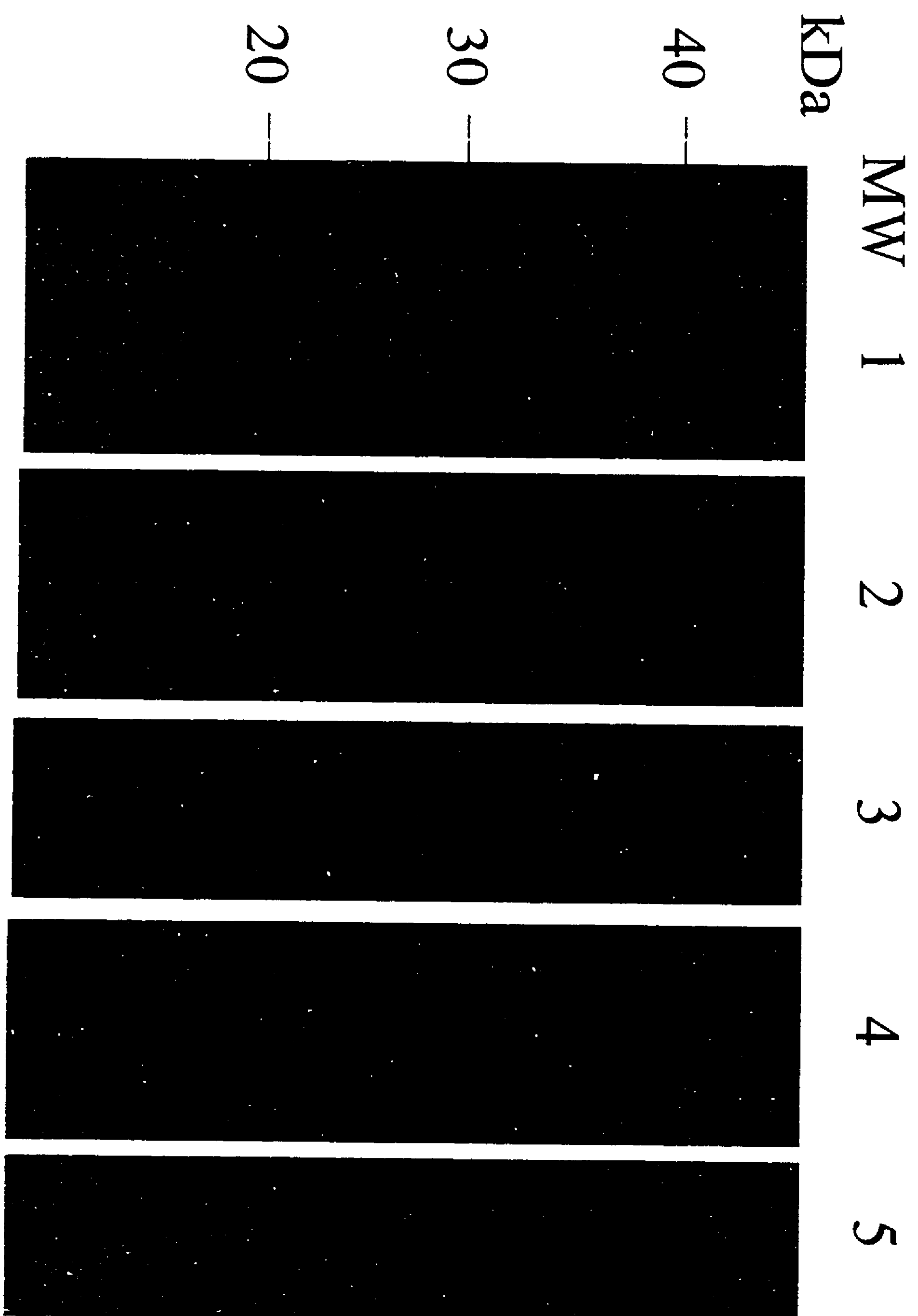
Table 6. Evaluation of immune responses to DNA and protein immunization in Syrian golden hamsters.

Immunization Group	Week 5 Titer	Week 9 Titer
Control (pCZ11)	<1:50	<1:50
Test (pCZF428)	1:200	1:800
B. pseudomallei 319a flagellin	>1:3200	>1:3200
(-) non-immunized hamster sera	<1:50	<1:50

- sera from immunized/control animals was diluted 1:50 to 1:3200 in doubling dilutions.
- ELISA plates were coated with 10 ug/well 319a flagellin.
- Secondary antibody used was goat anti-hamster IgG conjugated to horseradish peroxidase (HRP)

To further demonstrate the specificity of the antibodies present in immunized hamster sera for the flagellin of 1026b, 1026b cell lysates were run on an SDS-PAGE and probed on nitrocellulose membrane sera collected from hamsters immunized with pCZF428 (*fliC*), pCZ11 (control vector), and purified 319a flagellin (Fig. 14). Hamsters immunized with pCZF428 and purified 319a flagellin demonstrated production of antibodies reactive to a protein within 1026b cell lysate that has a relative mobility similar to flagellin protein ($M_r \approx 43$ kDa) in lanes 1 and 2, respectively. The mobility of flagellin protein was confirmed by the use of mouse monoclonal antibody specific for 319a flagellin to probe the cell lysate in lane 4, as well as to probe purified 319a flagellin in lane 5. Sera from hamsters immunized with pCZ11 did not react with any protein in the cell lysate.

Figure 14. The production of antibodies specific for *B. pseudomallei* 319a flagellin in immunized Syrian golden hamsters. Western blot of *B. pseudomallei* 1026b cell lysate probed by individual sera collected from hamsters immunized with DNA or protein. Lane 1, sera from hamsters immunized with pCZF428 (*fliC*); lane 2, sera from hamsters immunized with purified 319a flagellin; lane 3, sera from hamsters immunized with pCZ11 (control vector); lane 4, sera containing mouse monoclonal antibody specific for 319a flagellin (positive control); lane 5, purified 319a flagellin probed with mouse monoclonal antibody specific for purified 319a flagellin.


kDa
40
30
20
MW
1
2
3
4
5

9. Detection of cytokine mRNAs in immunized hamsters

The expression of cytokine mRNAs in spleen tissue of Syrian golden hamsters immunized with either DNA or protein was investigated in this study using reverse-transcription PCR (RT-PCR). We utilized cross-species DNA amplification RT-PCR techniques using published primers (Table 3), which targeted regions of similarity among mouse, rat and human sequences (50). In this study, primers for interferon gamma (IFN-γ) amplification were utilized following cDNA synthesis performed on total RNA isolated from hamsters immunized with pCZF428, pCZ11 and purified 319a flagellin. Additionally, primers were used to amplify *fliC* and glyceraldehydes-phosphate dehydrogenase (GAPDH) from the immunized animals (Table 3). The resultant PCR products were run on 2% agarose gel electrophoresis and visualized by UV transillumination (Figure 15). Gamma interferon, approximately 300 bp in size, was detected in spleen tissue of hamsters immunized with pCZF428, but was not detected in the spleen tissue of pCZ11- or flagellin-immunized hamsters. The primers were designed to amplify a product 309 nucleotides in length, which represents a partial-length cDNA that represented regions highly homologous to cytokines from other species.

The PCR products resulting from use of the *fliC* primers had a relative mobility of 1.2 kb on agarose gel, which represents the entire structural gene of flagellin. A band corresponding to the size of flagellin was demonstrated in both the tissue harvested from pCZF428-immunized and protein-immunized animals.

The presence of a band in the protein-immunized animals is a false-positive, and therefore, this must be repeated. Additionally, RT-PCR was performed using primers specific for GAPDH, which is constitutively expressed and acted as a positive control. A 306-bp product resulted in PCR reactions using cDNA synthesized from all three tissues (data not shown).

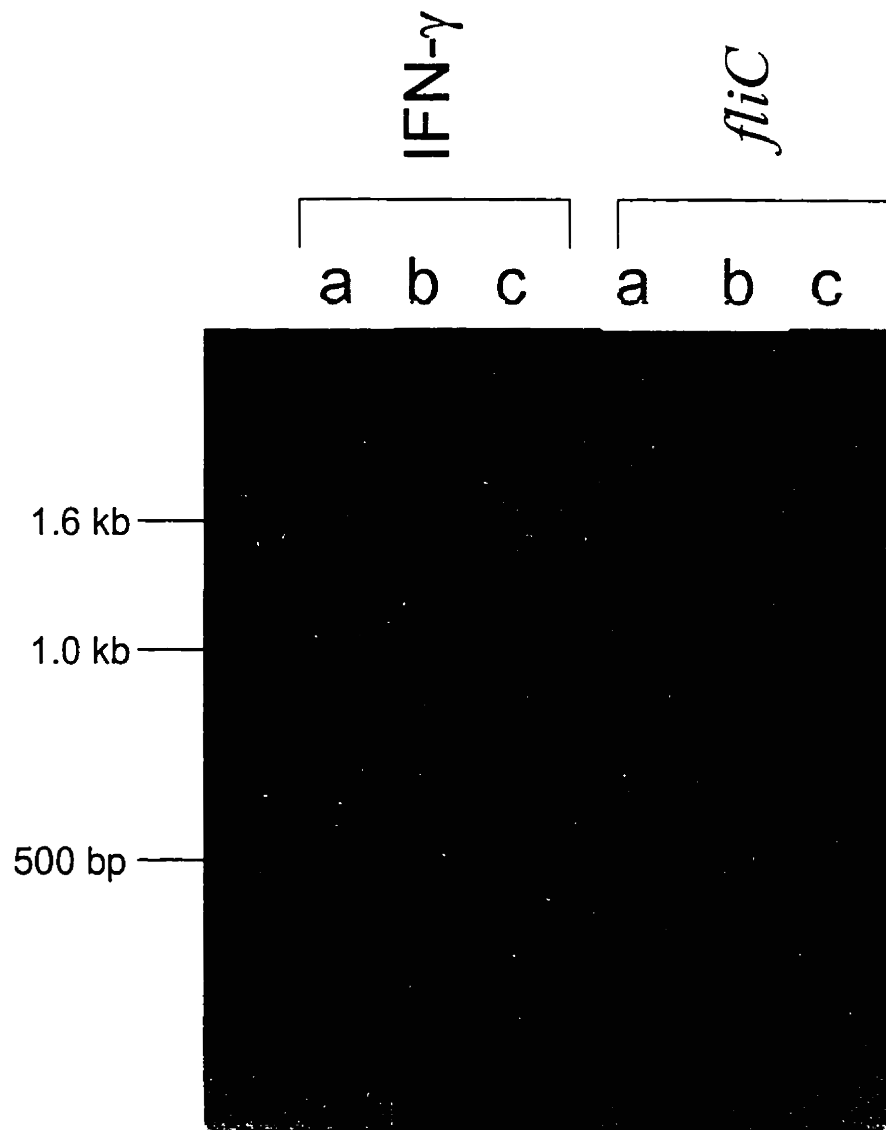


Figure 15. Detection of PCR products in spleen tissue of Syrian golden hamsters immunized with DNA or protein after RT-PCR. After RT-PCR with IFN-γ and *fliC* primers, PCR products were loaded on 2% agarose gel electrophoresis and stained with ethidium bromide for ultraviolet visualization. a) *B. pseudomallei* 319a flagellin-immunized tissue, b) pCZF428- immunized tissue, c) pCZ11-immunized tissue.

IV. DISCUSSION

Melioidosis is a disease that is increasingly being recognized as an important cause of morbidity and mortality (15). The modernization of facilities in endemic areas has resulted in the development of more accurate diagnosis and an appreciation for the significance of melioidosis and its causative agent, *Burkholderia pseudomallei.* Due to the widespread exposure of endemic populations to the organism, the susceptibility of certain populations, and the increase in travel to endemic areas, it is becoming more evident that an immunization strategy is needed for protection against melioidosis.

Previous work has identified that *B. pseudomallei* flagellin is quite immunogenic and may be an excellent candidate for development of a vaccine. Flagella have been shown to be an essential element for full virulence of a variety of organisms, including *Pseudomonas aeruginosa* (32). More importantly, immunization of burned mice using flagella preparations protects animals from subsequent challenge by *P. aeruginosa strains* and prevents the spread of organisms from the site of infection (51).

Research in developing immunization strategies has demonstrated the need for a vaccine that stimulates cell-mediated immunity but is also safe for use in humans. In this study, a DNA-based vaccine encoding the structural gene for *B. pseudomallei* flagellin, *fliC*, was evaluated for its potential for immunization against melioidosis. The *fliC* gene was inserted into the mammalian expression vector, pCZ11 and was named pCZF428. The vector pCZF428 was evaluated

for its ability to produce a cellular immune response and protect Syrian golden hamsters and infant diabetic rats from challenge with *B. pseudomallei* 1026b.

Results from immunostaining of lung, liver and spleen tissues from Syrian golden hamsters infected with *B. pseudomallei* 1026b demonstrated that flagellin is expressed *in vivo*. The expression of flagellin *in vivo* further confirms that this antigen may be an excellent candidate for the development of a DNA vaccine. The construction of a DNA vaccine plasmid vector encoding the structural gene for flagellin, *fliC*, would also require elements essential for expression in mammalian tissues. There are a number of commercially available mammalian expression vectors that are designed for use in DNA vaccination. A vector containing the *fliC* gene was previously constructed in our laboratory by the insertion of the gene in the multiple cloning site of pCDNA™3.1/HisB (Invitrogen), and this vector was named pDVAC. This vector was previously shown to express flagellin protein in tissue culture transfections of the cell lines COS-1, HeLa, and CHO.K1 via fluorescent immunostaining. Our efforts to demonstrate flagellin protein expression from this vector via tissue culture transfections and subsequent western blotting of cell extracts were unsuccessful.

An alternative approach was taken to studying the expression of flagellin from a mammalian expression vector, as *fliC* was cloned in a commercial vector pBLUE-TOPO™ (Invitrogen), which contains a promoterless *lacZ* reporter gene and named pBL305. This vector was utilized to measure flagellin expression in tissue culture transfections via β-galactosidase assays. The vector pBL305 contained the mammalian promoter from the SV40 virus, and for that reason,

only cell lines that are transformed with the SV40 virus, such as COS-1 could be transfected. Unfortunately, enzyme activity in the cell extracts of COS-1 cells transfected with pBL305 was not detected. This may be due to the unsuccessful transfection of the cells with pBL305 via lipid-mediated entry, or to a low level of enzyme activity produced which could not be detected by the assay.

The failure of previous transfections led us to concentrate on establishing defined conditions that would permit expression of protein from transfected cells. The mammalian expression vector pCDNA3.1/CAT was utilized as it contains the gene for chloramphenicol acetyltransferase (CAT). This vector was used to transfect HeLa, CHO.K1 and COS-1 cells under a variety of different conditions and evaluated via CAT assays. The results of these assays demonstrated that HeLa and CHO.K1 cells were successfully transfected when using specific concentrations of DNA and lipid reagent, particular incubation and expression time periods, as well as the elimination of selective antibiotic in transient transfections. Further studies were performed using these two cell lines only.

An alternative mammalian expression vector was constructed which simplified the vector by combining essential elements from two different commercial vectors, and this vector was named pCZ11. The *fliC* gene was then cloned into the multiple cloning site of pCZ11, and this vector was named pCZF428. The transfection conditions established from the CAT assays for successful transfection of HeLa and CHO.K1 cell lines were applied to transfections executed with pCZF428 (with *fliC*) and pCZ11 (empty vector). The transfections of HeLa and CHO.K1 cells with pCZF428 expressed protein which

was reactive to mouse monoclonal antibody specific for purified 319a flagellin and also had a similar relative mobility as purified flagellin ($M_r \approx 43$ kDa). Transfections with the empty vector pCZ11 did not result in expression of protein. Therefore, pCZF428 was a suitable mammalian expression vector that produced protein encoded by the *fliC* gene.

The analysis of serum antibody production demonstrated that IgG antibodies specific for flagellin were produced in Syrian golden hamsters and infant diabetic rats after immunization with pCZF428 (with *fliC*), indicating that the construct was immunogenic. In contrast, there was no detectable production of immunoglobulin reactive against flagellin in animals immunized with the control plasmid vector, pCZ11. Due to the limitation of commercially available immunological reagents, we were unable to further analyze the serum for other immunoglobulin isotypes. The antibody responses were detected in sera of Syrian golden hamsters after the second immunization with pCZF428, and the titers increased four-fold after the fourth immunization. The sera collected from the immunized hamsters were also used to probe cell lysates of *B. pseudomallei* 1026b. Sera from hamsters immunized with pCZF428 and 319a flagellin were specific for flagellin protein, whereas sera from pCZ11-immunized animals did not show any reactivity to the cell lysates. A humoral response was detected in the infant diabetic rats after the first immunization with pCZF428, and this increased in titer 8-fold after the second immunization.

Immunoprotection studies with pCZF428 demonstrated that four immunizations were not sufficient to protect Syrian golden hamsters, likely due to

the fact that hamsters are exceptionally susceptible to infection by *B. pseudomallei*. In contrast, two immunizations of the vector pCZF428 were adequate to protect infant diabetic rats from challenge with *B. pseudomallei* 1026b, while immunization with the empty vector (pCZ11) could not protect the animals. Additionally, immunization with pCZF428 appeared to decrease the bacterial burden in the spleen, as the data demonstrated lower values of CFU in pCZF428-immunized rats in contrast to rats immunized with pCZ11. These studies demonstrate the limitations of the two acute animal models of melioidosis in reference to active immunization. As stated earlier, the LD_{50} of Syrian golden hamsters to infection by *B. pseudomallei* is tremendously low at less than 10 organisms, and this acute sensitivity prevents accurate measurement of protection. Diabetic rats are only susceptible to infection by *B. pseudomallei* when infants (9); therefore, this stringent requirement precludes the use of this animal model in long-term active immunization studies.

Another major disadvantage of the Syrian golden hamster model of melioidosis is the lack of commercially available reagents, which limits immunology studies in this animal model. The innate immune response in hamsters is insufficient for protecting the animal for a long enough period to establish a specific cellular immune response which is essential for combating an intracellular organism like *B. pseudomallei*. To circumvent this limitation, whole-killed organisms were used to challenge the hamsters as these do not cause lethal infection and this allows for the study of the delayed-type hypersensitivity response (DTH).

In this study, Syrian golden hamsters were immunized in the left rear footpad with 5% formalin-killed *B. pseudomallei* 1026b, purified *B. pseudomallei* 319a flagellin or PBS as a negative control. The results of this experiment demonstrated that 5%-formalin-killed bacteria produced a DTH response, as the difference between the footpads was significantly higher after secondary exposure to the killed organism. The DTH response is apparently dependent on the dose of organism inoculated, as the 10^6 dose of killed bacteria produced a characteristic DTH response, while the 10^3 dose did not produce a significantly higher secondary response. Immunization with either purified 319a flagellin or PBS did not produce a DTH response. Presumably, immunization with flagellin protein was insufficient to produce a DTH response.

Generally, peptides from exogenous molecules are processed via the endosomal compartments of antigen presenting cells (APC) and displayed via MHC class II to be recognized by $CD4^+$ cells. The stimulation of a $CD8^+$ cytolytic T lymphocyte response typically requires the utilization of the endogenous pathway of antigen processing and subsequent display on MHC class I APC. Further informative studies may include the measurement of DTH response in hamsters that have been immunized with pCZF428 to determine if a specific memory response would be stimulated by the injection of flagellin protein.

To further study the cellular immune response in hamsters, immunological assays developed for measuring immune response in murine models of infectious disease were adapted for study using Syrian golden hamsters. The splenocyte proliferation assay is valuable for demonstrating the memory

response of lymphocytes after immunization with DNA. Splenocytes harvested from Syrian golden hamsters were utilized for analyzing the proliferative response due to stimulation by purified flagellin. Hamster splenocytes exhibited peak incorporation of [^{3}H]-thymidine on day 5, identifying this time point as the optimal time for cell harvest and measurement of proliferation. Mitogenic lectins such as concanavalin A and phytohemagluttinin typically peak on day 2 to 4, while recall antigens frequently peak after 7 days of culture (4). The splenocytes from hamsters immunized with pCZF428 demonstrated lower levels of proliferation than the hamsters immunized with the empty vector pCZ11 or with purified 319a flagellin.

The apparent nonspecific response to purified *B. pseudomallei* 319a flagellin as a stimulant may be due to several reasons. It is possible that the response could be a result of contaminating lipopolysaccharide (LPS) in the flagellin preparation. Studies could be undertaken to determine whether lymphocyte proliferation was due to contaminating LPS, including the use of *R. sphaeroides* lipid A, which antagonizes the response to LPS, to block the response to purified flagellin (4). Additionally, research on the antigenic nature of flagellin has identified this protein to be highly immunogenic, which may explain the ability of this antigen to cause splenocytes from control-immunized animals to proliferate. It has been demonstrated that flagella from gram-negative bacteria are potent inducers of proinflammatory cytokines by human blood monocytes (16). In a murine model of typhoid fever, it was demonstrated that flagellin protein encoded by *S. typhimurium fliC* gene is the dominant recall antigen in

proliferation assays in contrast to other antigens present in fractionated whole bacteria (17).

Protective immunity against intracellular organisms, such as *B. pseudomallei*, is dependent on the interaction of antigen-specific $CD4^+$ and $CD8^+$ T-lymphocytes with activated macrophage effector cells harboring the bacteria (38). The release of cytokines is essential to the interaction between these cells and can have a direct impact on disease pathogenesis and progression. The regulation of proinflammatory cytokine production is critical in maintaining a balance between antimicrobial activity and the extent of host immunopathology. The cytokines produced by the two subsets of $CD4^+$ T helper cells, Th1 and Th2, can inhibit the actions of the opposite cell type and therefore, determine the differentiation pathway of T-helper cells. The resistance to intracellular pathogens has been closely associated with Th1-type cytokines which stimulate cell-mediated immunity (76). The Th2-type profile is typically involved with anti-inflammatory and humoral immunity which are more important for resistance to extracellular pathogens (76). Within this classification, the Th1-type cytokine profile generally includes IFN-γ, IL-2 and TNF-β, (54). A Th2-type is represented by the release of IL-5, IL-6, IL-10, and IL-13. The cytokines present early in infection influences the differentiation of naïve $CD4^+$ T helper cells, as IL-12 is associated with a Th1-type response, while early IL-4 is correlated to Th2-type T cell development (39).

Studies on many intracellular pathogens have identified gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and several interleukins

(IL) are important in establishing innate response to infection (72). The production of IFN-γ is particularly important in controlling the growth of bacteria as demonstrated in mice depleted of IFN-γ by gene deletion or neutralizing antibody (55). More specifically, it has been demonstrated that a rapid IFN-γ response within the first day of infection by *B. pseudomallei* in a murine model determines whether the infection proceeds to an acute lethal outcome or becomes chronic (61). Melioidosis is similar to diseases caused by the pathogens *M. tuberculosis* and *Toxoplasma gondii*, as IFN-γ-mediated resistance does not lead to complete and protective sterilizing immunity. Instead, a successful IFN-γ response is still unable to eliminate the organism, permitting the possibility of latency and reactivation at a later time.

To determine if immunization with DNA results in production of IFN-γ in the Syrian golden hamster model, reverse-transcription PCR was performed on spleen tissue harvested from hamsters immunized with DNA or protein. The RT-PCR of spleen tissue from hamsters immunized with pCZF428 demonstrated the presence of IFN-γ mRNA, whereas none was detected in spleen tissue from hamsters immunized with either pCZ11 or purified 319a flagellin. Additionally, flagellin mRNA was detected using primers specific for the *fliC* gene in tissue of animals immunized with pCZF428 and purified 319a flagellin, but not in animals immunized with pCZ11. The sequence analysis of hamster cytokines by Melby et al. demonstrated that hamster IFN-γ had an additional 17 amino acids at the C terminus upon comparison with mouse and rat homologs (50). This excess in

length may decrease the biological activity of this molecule and therefore, contribute to the extreme susceptibility to intracellular pathogens, such as *B. pseudomallei.*

The limitation of immunological reagents in the two animal models described here results in the development of alternate protocols to study immune responses. The development of the RT-PCR protocol to monitor expression of cytokines and other immunostimulatory molecules is important in the analysis of DNA vaccine efficacy. This method will be significant in determining the route of cellular immunity in the Syrian golden hamster and infant diabetic rat models. Additionally, the further development of assays, such as the splenocyte proliferation assay and delayed-type hypersensitivity assay, will aid in study with these animal models.

V. FUTURE STUDIES

Further studies are required to elucidate the mechanism by which immunization mediates protection and to clarify and improve the efficacy and safety of this vaccine. Although organism clearance was accelerated by DNA vaccination in the infant diabetic rats, a minority of the rats were still infected by challenge infection, indicating the requirement for improved efficacy.

To demonstrate the preference of DNA vaccines for stimulating a Th1-type cytokine response, future studies should include the use of the RT-PCR system to investigate expression of Th1-type cytokines in DNA-immunized animals. It would be interesting to investigate the presence or absence of IFN-γ throughout infection by *B. pseudomallei.* The examination of production of IFN-γ in the infant diabetic rats immunized with pCZF428 may also help to explain the protective capacity of the vector in this animal model.

The inability of the immunization with plasmid encoding the flagellin structural protein to protect Syrian golden hamsters may also be the limitation of the DNA vaccine encoding only one antigen. The delivery of one plasmid encoding several antigens or the co-delivery of multiple DNA plasmids encoding individual antigens is a possibility that could be explored. Inconsistent delivery of the plasmid DNA to the muscle cells may have resulted in the failure to provide complete protection to the DNA-immunized animals. Several methods for improving plasmid DNA delivery and uptake by muscle cells could be investigated to enhance the immune response to flagellin. It is still unclear

exactly how naked DNA is taken up, mRNA is expressed, and protein is presented to the immune system. The relatively large amount of immunizing DNA (approximately 50-100 μg DNA per injection) required suggests that the mechanisms for antigen display after intradermal or intramuscular injection are relatively inefficient. Gene gun delivery of plasmid DNA has been shown to elicit an immune response with considerably less DNA than is required for i.m. injection and could be used to deliver pCZF428 in future experiments (26).

Other approaches to increasing the efficacy of DNA vaccines include the co-immunization with vectors expressing cytokines, such as gamma-interferon or interleukin-12 (58), or the use of co-stimulatory molecules. The co-administration of plasmids expressing desired cytokines could shift the immune response induced by these vaccines from a mixed T-helper population toward the desired Th1 phenotype with a potential increase in protective effectiveness. It may also be possible to manipulate the DNA plasmid backbone to increase its adjuvanticity, perhaps by the addition of multiple immunostimulatory sequences (41). Recent studies in which the effectiveness of DNA vaccines fused to tissue plasminogen activator (TPA) signal sequences were evaluated demonstrated that there were higher levels of TPA-fused proteins in the host cells, which lead to increased secretion of the proteins and a more generalized activation of the immune system (46). A combination of these approaches may be necessary to obtain clinically significant long-lived protective efficacy for the use of DNA immunization in humans.

Research in DNA immunization is still in its infancy and there are many questions and concerns that need to be addressed before DNA vaccines can be employed for use in humans. Immunization with DNA-based plasmid vectors encoding bacterial antigens clearly has the potential to induce biologically relevant immune responses against a variety of bacterial pathogens. Although safety considerations may limit the use of DNA vaccines in humans, DNA immunization may be useful against bacterial diseases in animals. DNA immunization also has a variety of other uses, including the use of this system to produce antisera against potential bacterial antigens, thereby eliminating the necessity to obtain purified preparations of antigens of interest. DNA immunization may also provide a useful tool for determining the nature of the immune response against pathogenic bacteria via manipulation of T-helper responses. Another application is the combination of immunotherapy with DNA vaccines with antimicrobial therapy to combat the persistence of and eliminate the presence of bacteria, which can occur after resolution of the initial infection (48).

VI. CONCLUDING REMARKS

There is currently no licensed strategy of immunoprophylaxis for melioidosis. Our laboratory has been successful in identifying numerous antigens that are responsible for the virulence of *B. pseudomallei*, as well as developing a conjugate vaccine that incorporates both flagellin and polysaccharide that poses a reasonable vaccine candidate. The need for induction of cell-mediated immune response to combat infection by an intracellular pathogen led us to investigate the prospect of DNA immunization. We have demonstrated that a DNA vaccine incorporating the flagellin structural gene, *fliC*, provides a humoral immune response and has the capacity to protect infant diabetic rats from challenge. More studies are required to elucidate the capacity of this immunogenic construct to fully initiate a cell-mediated immune response. Additionally, a more suitable animal model must be developed for studying the sub-acute and chronic forms of melioidosis. More importantly, an animal model is essential for active immunization studies. Based upon initial observations and the potential of this strategy, a DNA vaccine may be a rational candidate for active immunization against melioidosis.

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