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Pathogenesis of and immunity to melioidosis

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Abstract

While *Burkholderia pseudomallei*, the causative agent of melioidosis, is becoming increasingly recognized as a significant cause of morbidity and mortality in regions to which it is endemic, no licensed vaccine preparation currently exists for immunization against the disease. Therefore, one of the primary goals of our research has been to identify and characterize antigens expressed by *B. pseudomallei* isolates for the intended purpose of developing a vaccine construct that can be used to actively immunize specific high risk populations against the disease. By utilizing a combination of biochemical, immunological and molecular approaches, our studies now indicate that some of the most promising candidates for this task include flagellin proteins and the endotoxin derived *O*-polysaccharide (PS) antigens expressed by the organism. In this review, we have attempted to summarize the current status of *B. pseudomallei* research while endeavoring to provide a rationale for our approach towards the development of a melioidosis vaccine.

Keywords: *Burkholderia pseudomallei*; Melioidosis; Pathogenesis; Virulence factors; Protective antigens; Conjugate vaccine

1. Introduction

Burkholderia pseudomallei, the etiological agent of melioidosis, is a Gram-negative, facultative anaerobic, motile bacillus that is responsible for a broad spectrum of illnesses observed in both humans and animals (Howe et al., 1971; Leelarasamee and Bovornkitti, 1989; Sanford, 1990). While epidemiological surveys have demonstrated that *B. pseudomallei* is endemic to regions which typically border 20° north and south of the equator, the incidence of disease is particularly high in South-East Asia and northern Australia (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989; Dance, 1991). In north-eastern Thailand alone, an estimated 20% of community acquired septicemia and approximately 40% of deaths due to the complications associated with bacterial sepsis can be attributed to *B. pseudomallei* (Chaowagul et al., 1989). Although the organism is not strictly confined to the equatorial regions, the probability of acquiring melioidosis outside of these geographic domains is exceedingly low (Howe et al., 1971). Since the microbe is nutritionally diverse and is capable of resisting a variety of environmental extremes, it is puzzling as to why a more uniform global distribution of *B. pseudomallei* is not more apparent (Smith et al.,

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1987). The organism can be readily isolated from environmental niches such as rice paddies, still or stagnant waters and moist soils which predominate in the tropics, and it is believed that these habitats are the primary reservoirs from which susceptible hosts acquire infections (Ellison et al., 1969; Leelarasamee and Bovornkitti, 1989).

The manifestations of melioidosis are commonly represented by acute, sub-acute and chronic illnesses, with the clinical indications of some forms of the disease often being mistaken for malaria, plague, pneumonia and miliary tuberculosis (Howe et al., 1971; Smith et al., 1987; Leelarasamee and Bovornkitti, 1989). Resistance to a variety of antimicrobial agents including penicillins, first- and second-generation cephalosporins and many of the aminoglycosides is characteristic of *B. pseudomallei* clinical isolates (Dance et al., 1988; Leelarasamee and Bovornkitti, 1989; Godfrey et al., 1991; Weinberg and Heller, 1997). With this in mind, accurate identification of the organism, evaluation of the severity of the infection and antibiotic susceptibility testing are of paramount importance in devising an effective chemotherapeutic strategy. While the newer therapies that utilize combinations of ceftazidime–cotrimoxazole or amoxicillin–clavulanate for treatment of disease are proving beneficial, the mortality rates associated with the acute septicemic and pulmonary forms of melioidosis are still unacceptably high (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989; Kanai and Kondo, 1994; Weinberg and Heller, 1997; Ho et al., 1997). Typically, prolonged oral therapy is also recommended to assure the full clinical resolution of infections while reducing the potential for recrudescence of disease.

2. Aspects of pathogenesis

Melioidosis is primarily acquired via the inoculation of compromised surface tissues by soils and waters contaminated with *B. pseudomallei;* the highest incidence of disease occurring during the monsoon and rainy seasons (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). It is believed that during these periods, rising water tables percolate the organism up through the underlying soils to the surface thus enhancing their potential for exposure to humans and animals. This route of transmission tends to explain the prevalence of disease amongst rice farmers and their families who labor in the rice paddies without the benefit of protective clothing (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). Another important route of infection appears to be the inhalation and aspiration of contaminated fomites. During the Vietnam war, a disproportionate number of helicopter crewmen succumbed to *B. pseudomallei* infections as compared to other soldiers stationed in the same regions. To explain this phenomenon, it has been proposed that the helicopter rotors acted to disturb infectious dust particles around landing zones and thus facilitated the pulmonary inoculation of the crewmen with *B. pseudomallei* (Howe et al., 1971; Sanford, 1990). Alternatively, ingestion of the organism and human to human transmission, although to much lesser extents, have been implicated as routes of inoculation (McCormick et al., 1975). To date, there have been no reports of transmission of disease between animals and humans (Leelarasamee and Bovornkitti, 1989; Dance, 1990).

A number of physiological abnormalities have been correlated with the predisposition of certain populations to *B. pseudomallei* infections. In particular, during a 1 year study of patients admitted to a hospital in north-eastern Thailand for treatment of septicemic melioidosis, 32% demonstrated pre-existing diabetes mellitus (Chaowagul et al., 1989). Similarly, it has been shown in a diabetic infant rat model of infection that such animals are far more sensitive to challenge with *B. pseudomallei* isolates than are the healthy, non-diabetic rats (Woods et al., 1993). The reasons for this increased susceptibility, however, are still being investigated. Other health related factors which appear to increase the probability of acquiring melioidosis include impaired cellular immunity, leukemia/lymphomas, HIV infections, renal disorders, and debilitating afflictions such as alcoholism and parenteral drug abuse (Whitmore and Krishnaswami, 1912; Whitmore, 1913; Leelarasamee and Bovornkitti, 1989; Tanphaichi-

tra, 1989). Although *B. pseudomallei* related illnesses are documented in apparently healthy individuals, the organism is probably still best described as an opportunistic pathogen.

The manifestations of the various forms of melioidosis can be loosely defined as either acute, sub-acute and chronic (Howe et al., 1971). While the incubation periods of *B. pseudomallei* infections are not well defined, a review of the literature does suggest that they can range from as little as a few days to upwards of 26 years (Mays and Ricketts, 1975; Leelarasamee and Bovornkitti, 1989). The acute form of the disease can be subdivided into two groups; the acute pulmonary and the acute septicemic forms. Acute pulmonary symptoms appear rapidly and are characterized by high fever and pulmonary distress. This is followed by the appearance of visceral abscesses and death within a few days if left untreated. The septicemic type illness is also rapidly fatal and displays a high mortality rate when similarly left untreated. Clinical indications of this form include malaise, meningitis, cellulitis, as well as cutaneous and subcutaneous lesions. In many instances the acute manifestations of melioidosis are associated with an appalling mortality rate, even when vigorous chemotherapeutic intervention is implemented (Howe et al., 1971; Leelarasamee and Bovornkitti, 1989; Dance, 1990; Sanford, 1990).

Sub-acute melioidosis is probably best characterized as a prolonged febrile illness. While multiple abscess formation can be observed on the viscera, brain abscesses are seldom found. During the latter stages of the disease, the organism can be readily cultured from blood, pus, urine and other bodily tissues and secretions (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989). In many instances, death occurs within a few weeks to months in the absence of clinical intervention. The sub-clinical or chronic form of the disease is considered to be the most common presentation of melioidosis. It generally remains undiagnosed, however, until activated by a traumatic event or upon post-mortem examination of the tissues (Weinberg and Heller, 1997).

B. pseudomallei causes melioidosis in animals as well as humans. In endemic areas, disease has been shown to manifest in dogs, cats, rats, rabbits and numerous other species. Animals reported to be immune to this organism are fowl, cattle and water buffalo (Smith et al., 1987). The latter example is rather interesting since these animals are frequently used to pull prows in rice paddies and are, therefore, expected to encounter the microbe on a regular basis. Epizootic outbreaks have been documented in various regions of the world. Notable examples include the death of 24 dolphins in a Hong Kong aquarium in 1975, and an outbreak which appeared in the Caribbean in 1957 resulting in the death of numerous sheep, pigs and goats. Accounts from northern Australia also relate multiple outbreaks of melioidosis in lamb flocks (Sutmoller et al., 1957; Ketterer and Bamford, 1967; Huang, 1976).

Thus, melioidosis should be considered in any febrile patient with a history of residence in a major endemic region. If Gram-negative bipolar staining bacilli are observed in sputum, the organism can be readily cultured and identified (Sanford, 1990). In acute cases, blood and urine cultures are frequently positive, whereas, if chronic or sub-acute forms of the disease are suspected, biopsy may be required. (Sanford, 1990). Serological studies can be helpful for diagnosing active and recrudescent disease, and an immunoglobulin M (IgM) immunofluorescence test is often positive in recent infections (Dance, 1991). Also, indirect hemagglutination and complement fixation and tests are available, but require the testing of paired sera over several weeks to confirm the presence of an active infection (Smith et al., 1987; Chaowagul et al., 1989).

3. Virulence factors and protective antigens

Although *B. pseudomallei* isolates are capable of expressing an impressive array of both secreted and cell-associated antigens, the role(s) of these products in the pathogenesis of disease have to date been relatively ill defined. One of the primary reasons for this has been due to the lack of suitable techniques for genetically manipulating the organism. Due to the recent application of a Tn5 transposon based mutagenesis system for use

in *B. pseudomallei*, we have begun to identify and characterize genetic loci which encode a number of these putative virulence determinants and protective antigens (DeShazer et al., 1997, 1998). Therefore, in the following sections we have attempted to summarize the results of our most current studies, as well as those from other groups, in order to provide an overview of the antigens expressed by this *B. pseudomallei*.

3.1. Secreted antigens

The ability to acquire iron from host sources is a prerequisite for the successful establishment and maintenance of most bacterial infections. Yang et al. have demonstrated that 84/84 *B. pseudomallei* strains examined during their studies tested positive for siderophore production using the chrome azurol S (CAS) assay. A structural and chemical analysis of the siderophore synthesized by *B. pseudomallei* U7 confirmed that the molecule was approximately 1000 Da in size, water soluble with a yellow–green fluorescence and that it belonged to the hydroxamate class (Yang et al., 1991). Furthermore, studies have also demonstrated the siderophore was capable of scavenging iron from both lactoferrin and transferrin in vitro (Yang et al., 1993). The name malleobactin has been proposed for this compound (Yang et al., 1991).

It has been previously shown that *B. pseudomallei* isolates are capable of secreting antigens that demonstrate biological activities consistent with proteases, lecithinases, lipases and hemolysins (Esselman and Liu, 1961; Ashdown and Koehler, 1990; Sexton et al., 1994). However, while these factors have been implicated as important factors in the pathogenesis of the disease, only the protease has been characterized to date. Studies conducted by Sexton et al. have confirmed the presence of a 36 000 kDa antigen with associated proteolytic activities in *B. pseudomallei* culture supernatants. In particular, a protease expressed by *B. pseudomallei* 319a was found to be a metalloenzyme requiring iron for maximal protease activity and demonstrated optimally activity at pH 8.0 and 60°C (Sexton et al., 1994). Furthermore, monoclonal antibodies (MAb) raised against a *Pseudomonas aeruginosa* alkaline protease were cross-reactive with this antigen (Sexton et al., 1994).

Most recently, via transposon mutagenesis, we have identified an 11.8 kb chromosomal locus in *B. pseudomallei*, that demonstrates a high degree of homology to operons which encode for the products of the main terminal branch of general secretory pathway (GSP) (Pugsley, 1993). Further characterization of the open reading frames in this locus have confirmed that their orientation and physical arrangement are virtually identical to the *pul* gene cluster of *Klebsiella oxytoca* (Pugsley, 1993). Not surprisingly, the phenotypic analysis of the individual transposon mutants has also confirmed their inability to secrete antigens associated with protease, lipase and lecithinase into the extracellular milieu. Interestingly, while we screened more than 30 000 mutants for the loss of one or more of the enzymatic activities, no protease, lipase or lecithinase structural genes were identified. In order to asses the significance of GSP secreted products in the pathogenesis of melioidosis, we compared the virulence of the secretion mutants to the wild type strain in the Syrian hamster model of infection. The results of these studies indicated that while the protease, lipase and lecithinase may play a small role in the pathogenesis of acute melioidosis, mutants deficient in their ability to secrete these particular exoenzymes were not severely attenuated in their ability to cause a fulminating illness (Woods, unpublished data).

In the mid 1950s, several studies demonstrated that filter sterilized *B. pseudomallei* culture supernatants were lethal for mice and hamsters when administered parenterally (Nigg et al., 1955; Heckly and Nigg, 1958; Heckly, 1964). These results were consistent with the fulminating illnesses observed in animals following inoculation with viable bacteria, and suggested that *B. pseudomallei* strains might be capable of secreting a lethal toxin. In studies conducted by Ismail et al. (1987) mouse lethal, thermolabile toxin was reportedly purified to homogeneity and characterized as a 31 000 MW protein. Haase et al. (1997) have also described the presence of cytotoxic activity in culture filtrates. Their results, however, suggest that the antigen is only ~ 3 kDa in size

and that the cytotoxic activity is this instance is most likely due to the presence of a small peptide. Recently it has been reported that a rhamnolipid purified from *B. pseudomallei* culture supernatants demonstrates a cytotoxic effect against HL60 and HeLa cell lines (Haubler et al., 1998). Since this activity can be neutralized by albumin, however, it is unlikely to be of consequence in the pathogenesis of *B. pseudomallei* infections. Curiously, while it has also been our experience that filter sterilized *B. pseudomallei* culture supernatants are lethal for a variety of tissue culture cell lines, we have been unable to reproduce these effects in animal models, even when using preparations concentrated by lyophilization (Brett et al., 1997, 1998).

3.2. Cell-associated antigens

A number of previous studies have confirmed that *B. pseudomallei* is capable of synthesizing an acid phosphatase. It now appears that the molecule is a glycoprotein with optimal substrate activity around pH 7.2 (Kanai and Kondo, 1991, 1994; Kondo et al., 1996). While it has been reported by Kanai and Kondo (1994) and Kondo et al. (1996) that the acid phosphatase can be readily isolated from both whole cell and supernatant fractions, we have found the enzyme to be predominantly cell-associated (unpublished data). Kanai et al. (1996) have also proposed that the cell-associated form of the enzyme is a high affinity receptor for insulin and that this receptor/ligand interaction may be responsible for modulating the enzymatic profiles of *B. pseudomallei* isolates. Recently, while screening transposon mutants for their inability to hydrolyze the chromogenic substrate 5-bromo-4-chloro-3-indoyl phosphate (X-P), we successfully identified a mutant devoid of acid phosphatase activity (unpublished data). Sequence analysis of the chromosomal DNA flanking the transposon insertion demonstrated the presence of an open reading frame whose translated product was highly homologous to an acid phosphatase expressed by *Francisella tularensis* (var. novicida).

Capsular polysaccharides are a common feature of many bacterial pathogens including *Haemophilus influenzae* and *Streptococcus pneumoniae*. These extracellular moieties enable bacteria to evade host defense mechanisms by inhibiting complement activation and phagocytic mediated killing (Joiner, 1988). A review of the literature also indicates that *B. pseudomallei* strains are capable of synthesizing capsular antigens (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989), and that they may play an important role in the pathogenesis of melioidosis. While in vitro studies have determined that encapsulated *B. pseudomallei* strains are as susceptible to phagocytic uptake by polymorphonuclear leukocytes (PMN) as non-encapsulated variants, evidence tends to suggest that the presence of exopolysaccharide confers upon them the ability to resist the bactericidal effects of the phagolysosomal environment (Smith et al., 1987; Pruksachartvuthi et al., 1990). This is a feature of *B. pseudomallei* strains that may help to explain why these organisms are capable of remaining latent in a host for upwards of 26 years.

Recently, Steinmetz et al. isolated and purified a high molecular weight capsular antigen (>150 kDa) from *B. pseudomallei* NCTC 7431 and succeeded in raising a MAb against it (Steinmetz et al., 1995). Via ELISA based techniques, they were able to demonstrate the reactivity of both mucoid and non-mucoid strains with the MAb, thus suggesting that the capsular antigen is constitutively expressed by *B. pseudomallei* strains. Interestingly, temperature appeared to have little effect on the synthesis of the exopolysaccharide since *B. pseudomallei* strains grown at both 15 and 37°C reacted with the MAb. Furthermore, an assay utilizing a variety of *Pseudomonas* and *Burkholderia* spp. as controls was able to confirm the specificity of the MAb for *B. pseudomallei* and *B. mallet* strains only (Steinmetz et al., 1995). More recently, Masoud et al. (1997) have been successful at elucidating the chemical and structural characteristics of a capsular polysaccharide isolated from the virulent clinical isolate *B. pseudomallei* 304b. Their results demonstrated that the exopolysaccharide was a linear unbranched polymer of repeating tetrasaccharide units having the structure (-3)-2-*O*-Ac-β-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(2-). Similarly,

Nimtz et al. (1997) have demonstrated that a structurally identical capsular antigen is expressed by *B. pseudomallei* NCTC 7431. Studies by both groups have also shown that patient sera reacted strongly with the purified carbohydrate antigens indicating that this carbohydrate polymer is most likely expressed in vivo (Steinmetz et al., 1995; Masoud et al., 1997).

Previous studies have confirmed that the lipopolysaccharide (LPS) antigens expressed by *B. pseudomallei* strains are highly conserved throughout this species (Pitt et al., 1992). In fact, serological evidence suggests that there may be only one serotype of *B. pseudomallei* (Bryan et al., 1994). In order to investigate this phenomenon, Perry et al. has characterized the LPS antigens isolated from a number of *B. pseudomalle*i strains (Perry et al., 1995). Their results demonstrated that *B. pseudomallei* strains coordinately express two distinct somatic *O*-antigens (PS) on their cell surface. The Type I antigen consists of a high-molecular weight unbranched 1,3-linked homopolymer of 2-*O*-acetylated 6-deoxy-β-D-manno-heptopyranosyl residues, while the Type II antigen is an unbranched heteropolymer consisting of (-3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranosyl-(1-disaccharide repeats (L-6dTal*p*: ~33% *O*-4 acetylated and *O*-2 methylated; ~66% *O*-2 acetylated) (Knirel et al., 1992; Perry et al., 1995). While the simultaneous expression of two or more LPS moieties is not an uncommon feature associated with Gram-negative bacteria, the degree to which the two PS antigens are conserved amongst *B. pseudomallei* strains is quite remarkable (Perry et al., 1995).

It has been previously reported that *B. pseudomallei* strains are resistant to the bactericidal effects of normal human serum (Ismail et al., 1988). Recently, we have established that the presence of Type II PS is essential for conferring this resistance phenotype. Via the application of the transposon mutagenesis system, we have identified a number of mutants that demonstrate a marked sensitivity, in comparison to the parental strain, to the bactericidal effects of normal serum. Sequence analysis of the chromosomal DNA flanking these transposon insertions has enabled us to identify a 17.5 kb region of the chromosome that is required for the synthesis of the Type II antigen and conferring the serum resistance phenotype (DeShazer et al., 1998). Using the infant diabetic rat model, we have also confirmed that Type II PS is probably a significant determinant in the pathogenesis of melioidosis since the LD50 value associated with a Type II PS mutant is approximately 140 fold higher than that of the wild type strain (Woods et al., 1993; DeShazer et al., 1998).

Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens since the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease (Penn and Luke, 1992; Moens and Vanderleyden, 1996). Therefore, we have focused a great deal of attention towards determining the relative importance of motility in the pathogenesis of melioidosis. In previous studies we have demonstrated that a significant degree of size and antigenic homogeneity exists amongst flagellins expressed by *B. pseudomallei* isolates. Furthermore, we have also shown that flagellin specific antiserum is capable of passively protecting diabetic infant rats against a *B. pseudomallei* challenge (Brett et al., 1994). Curiously, however, in recent studies we have found that there was no significant difference between the virulence capacities associated with a wild-type strain of *B. pseudomallei* and non-motile mutants in either the diabetic infant rat or Syrian hamster models of infection (DeShazer et al., 1997). Thus, taken together, these results indicate that while flagella and/or motility may not be major virulence determinants in the pathogenesis of melioidosis, purified flagellin may still serve as a protective immunogen against *B. pseudomallei* infections.

4. Conjugate vaccines

Previous studies have demonstrated that both polyclonal antiserum and MAb's raised against *B. pseudomallei* flagellin proteins, LPS and a tetanus toxin-PS glycoconjugate are capable of passively immunizing diabetic infant rats against challenge with *B. pseudomallei* (Brett et al., 1994; Bryan et al., 1994). While these initial results were encour-

aging, it had become evident to us, however, that an active vaccine preparation would be the most practical for immunizing high risk populations against melioidosis. Therefore, with this in mind, we synthesized a glycoconjugate molecule that incorporated both flagellin protein and PS antigens. Based upon the preliminary success of these studies, we believe that this preparation is a reasonable vaccine candidate for actively immunizing against the disease (Brett and Woods, 1996).

It has been well documented that T cell-independent type 2 (TI-2) antigens such as bacterial polysaccharides are capable of eliciting protective antibody responses in adults (Dintzis, 1992; Jennings, 1992), but act poorly as immunogens in the elderly, in immunocompromised populations and in children less than 18 months of age (Gold et al., 1977; Robbins, 1978; Jennings, 1983). In adult populations, TI-2 antigens typically stimulate the synthesis of antigen specific immunoglobulin M (IgM), but often fail to evoke augmented immune responses and isotype conversions following boosting with carbohydrate preparations. This phenomenon is primarily due to the inability of activated B lymphocytes to recruit $CD4^+$ T cell (Th) involvement via major histocompatibility complex type II (MHC II) restricted events (Hodgkin and Basten, 1995; Mond et al., 1995). In order to remedy this situation, a number of bacterial carbohydrates (such as capsular polysaccharides or somatic *O*-antigens) have been covalently coupled to protein or peptide carrier molecules in order to facilitate T cell-dependent (TD) immune responses against the particular carbohydrate moieties (Cryz et al., 1986; Insel and Anderson, 1986; Winter et al., 1988; Watson et al., 1992; Fattom et al., 1993; Konadu et al., 1994; Lett et al., 1994). The presence of T cell epitopes inherent to peptide or proteinacious carriers is believed to facilitate these events (Braley-Mullen, 1980).

Therefore, by conjugating the PS moieties to the flagellin carrier, we have been able to elicit augmented immune responses against the PS components of the vaccine while concomitantly evoking desirable immunoglobulin (Ig) class switching events (Brett and Woods, 1996). More importantly, the use of an active vaccine containing only the PS portion of LPS and not the toxic component of LPS, namely lipid A, conjugated to flagellin protein obviates the toxic side effects of LPS but takes advantage of the protective response to the PS moiety. Furthermore, the coordinate presentation of multiple protective antigens has served to enhance the immunological repertoire of the vaccine recipient while providing immunity against a number of *B. pseudomallei* strains.

Since the size of the saccharide components displayed by a conjugate molecule appears to influence the immunogenicity of the preparation, a heterogeneous combination of PS antigens were covalently linked to the flagellin protein carrier (Dintzis et al., 1983, 1985; Jennings, 1992). The incorporation of larger PS fragments act to increase the size of the conjugate molecules such that the cross-linking of B cell mIg's is maximized. At the same time, the larger PS molecules also tend to retain conformationally stabilized epitopes which appear to be critical in the design of a successful vaccine (Jennings, 1992). The smaller oligosaccharide fragments appear to be critical for stimulating immunoglobulin responses against terminal epitopes of the carbohydrate molecules.

We have demonstrated that immune serum raised against the glycoconjugate conjugate reacts with both purified flagellin protein and both Type I and II PS; a situation that is obviously critical if the immune serum is to be effective against a *B. pseudomallei* challenge. This confirmed that native epitopes displayed by the precursor molecules were maintained during the synthesis of the conjugate molecule. Furthermore, passive immunization studies have demonstrated that the IgG fraction purified from the immune serum was capable of protecting diabetic infant rats from a challenge with *B. pseudomallei* (Brett and Woods, 1996). Based upon the success of these preliminary studies, we are currently characterizing the efficacy of this vaccine preparation in active immunization studies.

Since *B. pseudomallei* isolates have been shown to express both the Type I and II PS antigens (an exception being 824a which displays only the Type I antigen) and the flagellin proteins appear

to be antigenically conserved, we expect the conjugate molecule to afford protection against the majority of *B. pseudomallei* strains that we will encounter in our future studies.

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