RESEARCH NOTE

HETEROLOGOUSLY TYPE IV PILUS EXPRESSED PROTEIN OF *BURKHOLDERIA PSEUDOMALLEI* IS IMMUNOGENIC BUT FAILS TO INDUCE PROTECTIVE IMMUNITY IN MICE

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Abstract. *Burkholderia pseudomallei* is the causative agent of melioidosis, a severe disease of humans and animals. At present, no effective vaccine against melioidosis exists. Bacterial type IV pilin proteins have been used successfully as subunit vaccines. In this study, we evaluated a heterologously expressed and purified type IV pilus protein (PilV) of *B. pseudomallei* strain K96243 as a candidate subunit vaccine. PilV protein was assessed for its ability to protect BALB/c mice against *B. pseudomallei* strain G207 infection. Mice subcutaneously immunized with purified PilV protein produced high titers of IgG antibodies, which were strongly biased towards IgG1, with lower levels of IgG2a. Even though the PilV protein was highly immunogenic, it could not induce protection against a lethal *B. pseudomallei* challenge. Possible mechanisms of this non-protection phenomenon are discussed.

Keywords: *Burkholderia pseudomallei*, type IV pili, PilV protein, subunit vaccine

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, a bacterial infection with high mortality rate in areas of endemicity of Southeast Asia and northern Australia (Leelarasamee and Bovornkitti, 1989). It is a potential agent

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for biological warfare and biological terrorism (Warawa and Woods, 2002). Besides humans, many animal species are susceptible to melioidosis (Sprague and Neubauer, 2004). Humans and animals are believed to acquire the infection by inhalation of dust, ingestion of contaminated water, and contact with contaminated soil especially through skin abrasions (Cheng and Currie, 2005). Treatment of *B. pseudomallei* infection requires specific antibiotic treatment because *B. pseudomallei* is resistant to many commonly used antibiotics. Moreover, antibiotics must

be administered over extended periods of time and relapse of the disease is frequent (White, 2003). There is currently no vaccine available for the prevention of disease. Therefore, vaccine candidates need to be investigated urgently. Various vaccine strategies have been explored. Significant protection in animal models has been demonstrated following immunization with vaccines of attenuated pathogens (Breitbach *et al*, 2008), recombinant proteins (Su *et al*, 2010), as well as DNA (Chen *et al*, 2006).

Type IV pili (TFP) are important for virulence in many pathogenic gram-negative bacteria. When used as subunit vaccines, type IV pilin proteins have been demonstrated to provide protection against several diseases in animals. For example, purified type IV pili from *Bacteroides nodosus* 256 protect sheep against footrot (Elleman and Stewart, 1988). Similarly, purified type IV pili of *Moraxella bovis* were used successfully to protect cattle against infectious bovine keratoconjunctivitis (Lepper *et al*, 1992). Gonococcal pilus vaccine was shown to induce antibodies capable of blocking the attachment of Gonococci to epithelial cells (Tramont *et al*, 1981). Taken together, these experiments suggest that type IV pilin proteins are effective candidates to be used as subunit vaccine against TFP-producing bacterial pathogens.

Eight TFP-associated loci have been identified in *B. pseudomallei* K96243 genome (Essex-Lopresti *et al*, 2005). Locus TFP7 contains a gene BPSS1593, encoding Type IVB minor pilus subunit PilV protein. In *Neisseria gonorrhoeae*, PilV has been shown to be essential for pilus-mediated adhesion to human epithelial cells (Winther-Larsen *et al*, 2001). Therefore, the aim of the present study was to assess whether a recombinant *B. pseudomallei* type IV PilV subunit vaccine can provide protection against *B. pseudomallei* infection in a mouse model.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and reagents

Escherichia coli JM109 (Stratagene, La Jolla, CA) was used for cloning and expression experiments. *B. pseudomallei* strain K96243 genomic DNA was used as template for PCR amplification of *pilV*. *B. pseudomallei* strain G207 was used in a mouse challenge study. PolyHis expression vector pCI30 (Moore *et al*, 2001) was used for cloning and expression of *pilV*. Bacteria were cultured in Luria-Bertani (LB) medium at 37ºC. All reagents were obtained from commercial sources, and the manufacturers' instructions were followed.

Animal

All animal experimental procedures were performed according to guidelines of the Animal Ethics Research Committee of Khon Kaen University, Thailand. BALB/c mice, 6 to 8 weeks old (National Laboratory Animal Center, Nakhon Pathom, Thailand) were housed in polypropylene cages and had free access to food and water during the study.

Cloning, expression, and purification of the *B. pseudomallei* PilV protein

Genomic DNA of *B. pseudomallei* K96243 was isolated as described previously (Srilunchang *et al*, 2009). The non-signal peptide region of the open reading frame encoding PilV was PCR amplified using primers, 5' CCAGGATCCGC-GTTCGACGCGTGGCG 3' designed to contain *Bam*HI site and 5' GGGGTACCT-

TAGGTGTACCCGCACGAGAAATCC 3' with *Kpn*I site in order to facilitate insertion into pCI30 expression vector. The authenticity of the cloned DNA was confirmed by nucleotide sequencing (ABI 373 DNA sequencer, Applied Biosystems, Carlsbad, CA).

In order to express PilV protein, overnight culture of the bacterial clone was inoculated into LB medium supplemented with ampicillin (100 µg/ml) and grown with shaking (250 rpm) at 37ºC until an optical density at 600 nm of 0.5 was obtained. Expression was induced with 2 mM isopropyl-β-D-thiogalactoside (IPTG) (Promega, Madison, WI) at a final concentration for 4 hours. Protein expression was analyzed by SDS-PAGE with Coomassie brilliant blue dye staining. Protein purification was performed using immobilized metal ion affinity chromatography (Protino Ni-IDA) system. In brief, 1 gram of cell pellet was suspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Lysozyme (1 mg/ml) was added and the solution was stirred on ice for 30 minutes before sonication. The crude lysate was centrifuged to collect inclusion bodies, which were suspended in 2 ml of denaturing solubilization buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0) and centrifuged. The supernatant was applied onto the affinity column, washed with denaturing solubilization buffer and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 250 mM imidazole, pH 8.0). Imidazole was subsequently removed by dialysis against phosphate-buffered saline (PBS). Protein concentration was determined by Bradford reagent (Biolab, Hercules, CA).

Mouse immunization and antibody analysis

Three groups of five female 6-8 week old BALB/c mice were immunized subcutaneously with 100 µg of purified PilV protein in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Booster immunizations of 50 µg in incomplete Freund's adjuvant were given on days 14 and 28. Control mice received injections of adjuvant alone. Four weeks after the final immunization, mice were challenged intraperitoneally with 5-30 x LD_{50} dose of *B. pseudomallei* strain G207. Survival of mice was observed for 30 days.

For anti-PilV antibody analysis, mouse serum samples were collected one week prior to the first injection and once one week prior to challenge injection. Antibody titer of IgG1 and IgG2a subclasses were analyzed by an enzyme-linked immunosorbent assay (ELISA). In short, 96-well microplates were coated with 2 µg per well of purified PilV recombinant protein in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated overnight at 4 ºC. The plates were incubated with 3% BSA in PBS pH 7.2, washed with 0.05% Tween 20 in PBS pH 7.2, then incubated with serial dilutions of serum samples at 37ºC for 1 hour. Following washing, goat anti-mouse IgG1 or IgG2a conjugated with HRP (1:2,000) was added and the plates were further incubated at 37ºC for 1 hour. Plates were washed and TMB was added. Reaction product was determined by measuring optical density at 450 nm using a microplate reader (Labsystems, USA).

RESULTS

Analysis of *B. pseudomallei* PilV

pilV (BPSS1593) of *B. pseudomallei* strain K96243 is located on chromosome 2 and encodes a 557 amino acid protein containing a 32 amino acid signal peptide with a predicted molecular mass of 58.58 kDa.

Blast analysis showed that this PilV protein is homologous to type IV prepilin or type IV pilus protein of *B. pseudomallei* strain 1710b, 1106a and 668 (99%), *B. mallei* strain NCTC 10247, NCTC 10229 and ATCC 23344 (99%) and *B. thailandensis* strain E264 (83%). It also has a limited amino acid homology to *Escherichia coli* minor fimbrial subunit PilV and to *Salmonella typhi* PilV protein. *B. pseudomallei pilV* is part of a pilus operon containing at least 8 genes that are involved in type IV pilus protein biosynthesis.

Immunogenicity of PilV protein

In order to evaluate the immunogenicity of purified recombinant PilV protein, groups of 5 female BALB/c mice were immunized subcutaneously three times (2 week intervals) with purified recombinant PilV protein in adjuvant. Pre-immune sera collected prior to immunization and immune sera collected one week prior to challenge injection in the immunized group and the control group were pooled and analyzed by ELISA. Mice immunized with purified PilV protein developed high-titer antibodies. To identify the type of immune response induced, the specific IgG1 and IgG2a subclasses were determined, which indicated a much higher titer of IgG1 (1:7,812,500) over IgG2a (1:312,500) when compared to that of pre-immune sera from the same mice and of immune sera from the control mice (Fig 1). The ratio of IgG2a/IgG1 was 0.04.
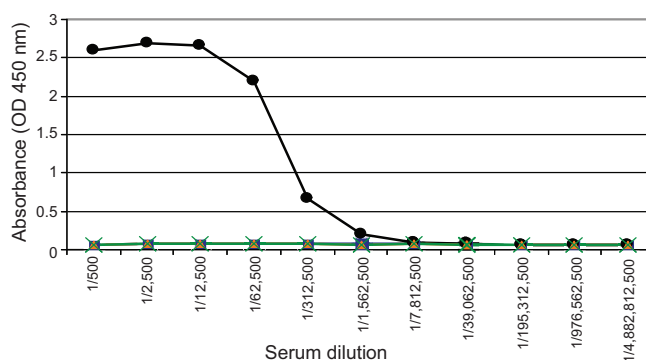
A. IgG1 response
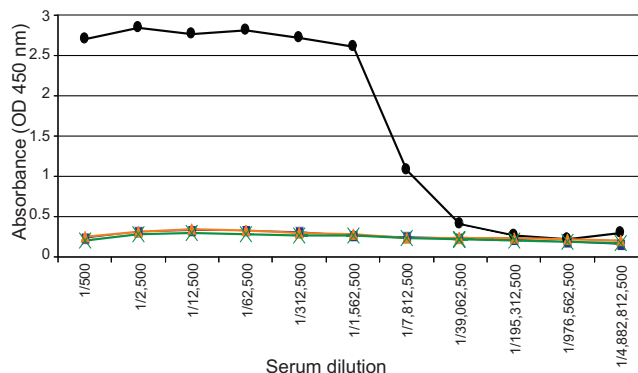
B. IgG2a response

Fig 1–Analysis of specific IgG subclass response of *B. pseudomallei* PilV-immunized mice. Serial dilutions of the sera collected prior to and after immunization with PilV were analyzed for the levels of specific IgG1 (A) and IgG2a (B) antibodies by ELISA. Specific IgG level in pre-immune serum (■), immune serum (●) of immunized mice or pre-immune serum (▲) and immune serum (X) of control mice.

Vaccine potential of the purified PilV protein in BALB/c mice

In order to evaluate the vaccine potential of purified recombinant PilV protein, three groups of five BALB/c mice were immunized subcutaneously with purified PilV protein as described above, and four weeks after the last
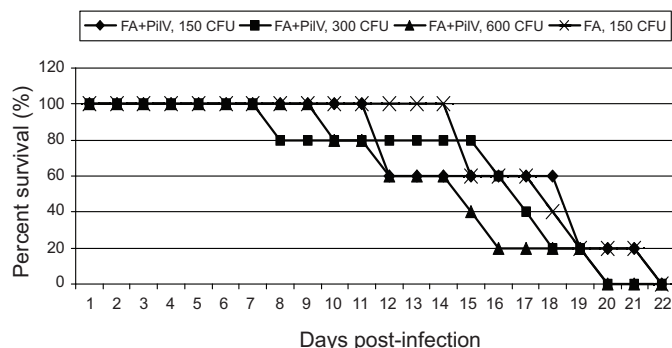
Fig 2–Survival of BABL/c mice immunized with recombinant PilV protein against challenge of *B. pseudomallei* strain G207. Control mice immunized with vehical alone were challenged with 150 cfu of *B. pseudomallei* strain G207. FA, Freund's adjuvant.

booster dose immunized mice were challenged intraperitoneally with 150 cfu (*ca* 5 LD_{50}) (group1), 300 cfu (*ca* $10LD_{50}$) (group 2), and 600 cfu (*ca* $20LD_{50}$) (group 3) of *B. pseudomallei* strain G207. Control mice were challenged with 150 cfu of *B. pseudomallei* strain G207. No protection was seen in immunized mice against the lowest challenge dose and all succumbed to the infection by day 22 (Fig 2). The experiment was repeated once more and the same results were obtained (data not shown).

DISCUSSION

As type IV pili have been used successfully as subunit vaccines to prevent various infections in animals (Elleman and Stewart, 1988; Lepper *et al*, 1992), we are interested in assessing the immunogenicity of purified recombinant PilV protein from *B. pseudomallei* strain K96243 and its ability to protect against *B. pseudomallei* infection in a BALB/b mouse model. Although the purified recombinant PilV protein was highly immunogenic in BALB/c mice, it failed to induce protection against lethal *B. pseudomallei* challenge. Similar results were observed with type IV pilin of *B. mallei*, the causative agent of glanders (Fernandes *et al*, 2007).

There could be several reasons for the non-protection phenomenon. Firstly, the production of this minor subunit protein during infection could be rapidly downregulated rendering the anti-PilV antibodies unable to prevent the pilus-mediated adhesion to the host cells. Secondly, subcutaneous immunization could result in antibody response directed predominantly against non protective epitopes of the proteins. More importantly, subcutaneous immunization with PilV protein resulted in a Th2-driven immune response as demonstrated by the elevated IgG1/IgG2a ratio. This type of response is important to fight against extracellular organism (Kidd, 2003). *B. pseudomallei* is a facultative intracellular pathogen and eradication of this bacterium and other intracellular organisms has been shown to be dependent on the generation of a Th1 type immune response (Abbas *et al*, 1996). Nonetheless, monoclonal antibodies against lipopolysaccharide have been shown to confer passive protection against *B. pseudomallei* (Jones *et al*, 2002). Therefore, both antibody-mediated and cell-mediated immunity are important to fight against melioidosis. Thirdly, the subcutaneous route of immunization did not result in the quality and quantity of antibodies sufficient for protection against intraperitoneal challenge. Intraperitoneal immunization with recombinant outer membrane protein has been shown to induce a Th1 type response and to confer

significant protection against peritoneal challenge (Hara *et al*, 2009).

Although the PilV protein failed to induce protection in a BALB/c, Th2-prone, mouse model of infection, it might be successful in a different mouse strain. For instance, it will be of interest to evaluate how PilV protein performs in Th1-prone C57BL/6 mice (Mills *et al*, 2000).

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