The Type IV Pilin of \textit{Burkholderia mallei} Is Highly Immunogenic but Fails To Protect against Lethal Aerosol Challenge in a Murine Model\textsuperscript{†}

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Received 29 January 2007/Returned for modification 23 February 2007/Accepted 21 March 2007

\textit{Burkholderia mallei} is the cause of glanders and a proven biological weapon. We identified and purified the type IV pilin protein of this organism to study its potential as a subunit vaccine. We found that purified pilin was highly immunogenic. Furthermore, mice infected via sublethal aerosol challenge developed significant increases in titers of antibody against the pilin, suggesting that it is expressed in vivo. Nevertheless, we found no evidence that high-titer antipilin antisera provided passive protection against a sublethal or lethal aerosol challenge and no evidence of protection afforded by active immunization with purified pilin. These results contrast with the utility of type IV pilin subunit vaccines against other infectious diseases and highlight the need for further efforts to identify protective responses against this pathogen.

Glanders, a contagious and fatal disease of odd-toed ungulates that has zoonotic potential, has been known since antiquity. It is caused by infection with the bacterium \textit{Burkholderia mallei}. In solipeds (horses, mules, and donkeys), \textit{B. mallei} can cause disease in two forms, respiratory (glanders) and subcutaneous (farcy) (26). Glanders remains a problem in parts of Asia, South America, and Africa (2). Humans may become infected with \textit{B. mallei} through contact with infected animals, through laboratory accidents, and through inhalation (24). Spread from animals to humans in the natural setting is apparently inefficient, as animal handlers are uncommonly infected. In contrast, \textit{B. mallei} poses a considerable risk to laboratory workers. Both O. Kalning and K. Helmann, who independently developed diagnostic tests for glanders in 1891, died of the disease within a year (26). A 1947 study described six cases of glanders in researchers who had worked with the organism at Camp Detrick, MD (12). More than 50 years later, glanders was diagnosed in a researcher who worked with the same organism at the same institute (now the U.S. Army Medical Research Institute of Infectious Diseases at Fort Detrick) (20). Most of these cases occurred in the absence of a definable laboratory accident or breach of procedure. Thus, aerosols containing the organism are believed to be highly infectious (24). Untreated human glanders has an extremely high mortality rate (24). However, early and aggressive treatment with combinations of systemic antibiotics can be curative (20).

\textit{B. mallei} is one of the few pathogens that have been used as biological weapons. During World War I, undercover agents of the Central Powers infected animals destined for the Allies, as well as large numbers of Russian horses and mules on the eastern front, disrupting supply lines. The intent was to kill both the livestock and the humans with whom they came into contact (25). During World War II, the Japanese deliberately infected horses, civilians, and prisoners of war in China with \textit{B. mallei} (10). The potential reemergence of \textit{B. mallei} as a biological weapon is of great concern. \textit{B. mallei} is highly infectious via the respiratory route as an aerosol. The incubation period is long, making it difficult to trace the source of an outbreak or attack. The symptoms are nonspecific, and there is virtually no modern clinical experience to facilitate disease recognition. The illness has a high mortality rate if not recognized and treated. The organism is resistant to multiple antibiotics, making it likely that empirical therapy will fail (11). Therapy must be continued for prolonged durations, potentially taxing antimicrobial supplies in the case of large numbers of exposures. There is no vaccine.

Type IV pili, or fimbriae, are produced by many species of pathogenic gram-negative bacteria (4). Type IV pilin proteins have been used successfully as subunit vaccines for the prevention of several diseases in animals. Purified \textit{Moraxella bovis} type IV pili protected cattle against keratoconjunctivitis induced by experimental \textit{M. bovis} infection (13). Comparable results were obtained when using recombinant pili expressed in \textit{Pseudomonas aeruginosa}, demonstrating that the pilin protein was the immunogen (14). Similarly, purified type IV pili from \textit{Dichelobacter nodosus} protected sheep against foot rot (21) and protection was also seen in animals vaccinated with recombinant pilin (7). A monoclonal antibody against the toxin-coregulated pilus provided passive protection against cholera in infant mice, indicating that antibodies alone can be protective (22). Additionally, a consensus sequence peptide conjugate vaccine derived from the receptor-binding domain of the \textit{P. aeruginosa} type IV pilin provided modest protection against death in a mouse model of infection (3). These experiments suggest that type IV pilin proteins are good candidates to serve as subunit vaccines against infections with bacteria that produce type IV fimbriae.

Study of the closely related pathogen \textit{B. pseudomallei}, which is responsible for the disease melioidosis, revealed a type IV pilus required for virulence (8). Analysis of the \textit{B. mallei} ge-
nome reveals a putative type IV pilin gene. Given the sequence similarity between the predicted pilin proteins of \textit{B. mallei} and \textit{B. pseudomallei}, their relatedness to other type IV pilin proteins, the fact that there has been some success in type IV pilus-based vaccines in the past, and the urgent need for more research into potential biological weapons, we tested the hypothesis that a recombinant \textit{B. mallei} type IV pilin protein subunit vaccine can provide protection against \textit{B. mallei} disease in a relevant animal model.

MATERIALS AND METHODS

Expression and purification of the \textit{B. mallei} putative type IV pilin subunit.

We transformed plasmid pPF304 into competent cells of \textit{B. mallei} strain China 7 (ATCC 23344) to test for plasmid uptake. The transformation was performed in \textit{B. mallei} strain China 7 (ATCC 23344) competent cells, grown while shaking (225 rpm) at 37°C to an OD600 of 0.4. The strain was then cultured overnight in 60 ml of LB (1% yeast extract, 0.5% tryptone, 0.5% NaCl), and the pelleted cells were resuspended in 10 ml of LB and grown to an OD600 of 0.4. The culture was centrifuged, and the supernatant was removed. The pellet was resuspended in 50 ml of lysis buffer (20 mM NaH2PO4, pH 7.4) and incubated at 4°C overnight. After washing, the cells were resuspended in 50 ml of lysis buffer with 1 M MgCl2. The lysate was cleared by centrifugation, and the supernatant was applied to a 100 ml column of Ni-NTA beads. The beads were washed with 200 ml of wash buffer (20 mM NaH2PO4, 500 mM NaCl, pH 7.4), and the pilin protein was eluted with 10 ml of elution buffer (200 mM NaH2PO4, 500 mM NaCl, pH 7.4). The eluate was dialyzed against 100 ml of buffer (20 mM NaH2PO4, 500 mM NaCl, pH 7.4). The protein was then concentrated by centrifugation, and the purity was confirmed by SDS-PAGE and Western blotting.

RESULTS

\textit{B. mallei} pilin is highly immunogenic.

We have shown that the genome of \textit{B. mallei}, which at the time was available in an unfinished state on the website of the Institute for Genomic Research, for genes with homology to that encoding the type IV pilin of \textit{P. aeruginosa} strain PA01. We found a single open reading frame with characteristics of a type IV pilin gene (Fig. 1A). The putative mature type IV pilin protein of \textit{B. mallei} is identical to that of \textit{B. pseudomallei} and closely related to those of other members of the genus. It is also closely related to that of \textit{Legionella pneumophila}. The predicted protein has the characteristic prepilin peptidase cleavage motif (GFXXE, where cleavage occurs between G and F), followed by a typical hydrophobic N-terminal sequence, as well as two conserved carboxyl-terminal cysteine residues predicted to form a disulfide bond.

We overcame several technical difficulties to produce highly purified \textit{B. mallei} type IV pilin. To avoid problems with solubility due to the presence of the hydrophobic amino terminus, which is predicted to form a long alpha-helix buried within the pilus (4), we omitted the codons for the first 24 amino acids of the pilin gene. This approach allowed the pilin to be expressed in a soluble form, which was then purified by affinity chromatography. The purified pilin was then used in immunization experiments to test its immunogenicity.

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trated eluate from gel filtration chromatography (lane 4) are shown.
2), eluate from nickel affinity chromatography (lane 3), and concen-
lysate (lane 1), flowthrough from nickel affinity chromatography (lane
is overlined. (B) Coomassie-stained gel of purified pilin. The total cell
mallei mature pilin omitted in the recombinant soluble purified protein
highlighted in black, and the hydrophobic N-terminal region of the
conserved prepilin peptidase cleavage sites and cysteine residues are
indicated by two dots and one dot, respectively. The
sequences are indicated by asterisks, and highly similar and similar
CLUSTAL W version 1.83. Residues identical among all of these
proteins from B. mallei, B. pseudomallei, L. pneumophila strain Phila-
phia 1, and
aeruginosa strain PAO1 was performed with
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lysate (lane 1), flowthrough from nickel affinity chromatography (lane
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trated eluate from gel filtration chromatography (lane 4) are shown.
The relative mobility of molecular mass markers (masses are shown in
thousands on the left) is indicated.

FIG. 1. Identification and purification of the B. mallei type IV pilin protein. (A) Multiple-sequence alignment of predicted type IV pilin proteins from B. mallei, B. pseudomallei, L. pneumophila strain Phila-
phia 1, and P. aeruginosa strain PAO1 was performed with
CLUSTAL W version 1.83. Residues identical among all of these
sequences are indicated by asterisks, and highly similar and similar
residues are indicated by two dots and one dot, respectively. The
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the protein. To ensure proper disulfide bond formation and folding in the periplasm (27), we fused the truncated protein to the signal peptide of DsbA. For purification, we added an
amino-terminal hexahistidine tag, reasoning that this location
would allow the tag to protrude away from the rest of the
protein (17). We overcame a limitation in expression levels
that was apparently due to the high G+C codon usage of B.
mallei by addition of a plasmid encoding specific tRNA mole-
cules. Finally, we eliminated contaminating proteins by two
rounds of gel filtration chromatography. These strategies al-
lowed us to obtain highly purified protein for further study
(Fig. 1B). The identity of the purified protein was verified by
immunoblotting with a monoclonal antibody that recognizes the histidine tag (data not shown).

We immunized three mice with purified pilin and injected three control mice with adjuvant alone. All three of the mice immunized with the purified pilin developed strong IgG responses, with titers exceeding 1:320,000. In contrast, the control mice had titers below 1:100.

Inhalational challenge with live B. mallei induces an immune response to pilin. To determine whether pilin are produced in vivo and elicit an immune response, five mice were challenged via small-particle aerosol with a sublethal dose of 230 CFU of live B. mallei. Sera were collected weekly and analyzed by ELISA. Four of the five mice developed greater-than-fourfold titer increases (data not shown), and there was a significant increase in the response with time ($P = 0.02$; Fig. 2). This result suggests that mice infected via the respiratory route with live B. mallei are exposed to pilin protein and develop antibody responses to the antigen.

Antibodies against pilin do not protect mice against respiratory B. mallei infection. Mice immunized with purified pilin protein produced high-titer antibodies. To determine whether these antibodies can protect against infection, we conducted two passive-immunotherapy experiments. In each experiment, groups of five or six mice received 50 μl of pooled hyperimmune serum or pooled control serum from the mice that received adjuvant alone via i.p. injection. An additional group of mice received an i.p. injection of 50 μl of HBSS. One hour later, we challenged all of the animals via small-particle aerosol with live B. mallei. In the first experiment, despite a challenge dose of 1,990 CFU (~2 LD$_{50}$), no mice had succumbed to the infection by day 20. Therefore, all of the mice were sacrificed and the viable B. mallei bacteria in their livers and spleens were enumerated by plate counting. There was no significant difference ($P = 0.37$) in the geometric mean numbers of CFU recovered from the livers of the mice that received hyperimmune serum (2.9 × 10$^7$), control serum (3.4 × 10$^7$), or HBSS (2.6 × 10$^7$). Similarly, there was no significant difference ($P = 0.32$) in the geometric mean numbers of CFU recovered from the spleens of these mice (2.2 × 10$^5$, 1.6 × 10$^5$, and 7.4 × 10$^4$, respectively). In a second experiment, similar groups of six
mice received 14,261 CFU (approximately 14 LD$_{50}$s) via small-particle aerosol. All succumbed to infection on either the fourth or the fifth day after the challenge (Fig. 3). Thus, we are unable to conclude that passive immunization with high-titer antipilin serum protects mice against either lethal or sublethal 

**Immunization with pilin does not protect mice against lethal respiratory** 

**B. mallei** infection. The failure of passive immunization to protect mice against sublethal or lethal respiratory 

**B. mallei** infection does not preclude protection by active immunization, which could involve cell-mediated, as well as humoral, responses or could require higher antibody levels than are attainable by passive immunization. Therefore, we immunized groups of 10 mice with either purified 

**B. mallei** pilin or irradiated 

**B. mallei** organisms, administered adjuvant alone, or injected them with HBSS. The animals received a booster dose at week 4. Ten weeks after the initial injection, all of the mice were challenged with 21,558 CFU (approximately 22 LD$_{50}$s) of live 

**B. mallei** via small-particle aerosol. The number of mice surviving on each day postchallenge is shown.

**FIG. 3.** Effect of passive immunization on the survival of mice challenged via aerosol with live 

**B. mallei**. Groups of six mice each received i.p. injections of 50 µl of HBSS (solid triangles), pooled serum from three mice sham immunized with adjuvant alone (open squares), or pooled hyperimmune serum from three mice immunized with purified 

**B. mallei** pilin (solid circles). One hour later, mice were challenged with 14,261 CFU (approximately 14 LD$_{50}$s) of live 

**B. mallei** via small-particle aerosol. The number of mice surviving on each day postchallenge is shown.

**FIG. 4.** Effect of active immunization on the survival of mice challenged via aerosol with live 

**B. mallei**. (A) Ten mice each received HBSS (solid triangles), adjuvant alone (open squares), 50 µg of purified 

**B. mallei** pilin (solid circles), or 100 µg of irradiated 

**B. mallei** (open diamonds). Booster injections were given at week 4. Ten weeks after the initial injection, all of the mice were challenged via small-particle aerosol with 6,965 CFU (approximately 7 LD$_{50}$s) of live 

**B. mallei** via small-particle aerosol. The number of mice surviving on each day postchallenge is shown. (B) Mice were injected as described for panel A but received an additional booster injection and a different adjuvant. They were challenged with 21,558 CFU (approximately 22 LD$_{50}$s) of live 

**B. mallei** via small-particle aerosol. The number of mice surviving on each day postchallenge is shown.

**DISCUSSION**

**B. mallei** causes diseases with important economic impact in many parts of the world and is a proven biological weapon. It is highly infectious when aerosolized and causes a potentially lethal infection in humans that requires prolonged antibiotic therapy. An effective vaccine against 

**B. mallei** would be desirable. Type IV fimbriae are important virulence factors for many species of gram-negative bacteria (4). Type IV pilin proteins have proven to be efficacious as subunit vaccines to
prevent a variety of infections in animals due to bacteria that produce these fimbriae (3, 7, 13, 14, 21, 22). Therefore, we tested the hypothesis that immunization with the type IV pilin protein of \( B. mallei \) would be effective at curtailling morbidity and mortality in an animal model of infection. Although the pilin protein proved to be highly immunogenic, we did not find support for this hypothesis.

There could be several reasons why we did not detect a protective effect of passive or active immunization with the purified pilin protein. For example, there is no doubt that the model we used has limitations. In the natural setting, glanders is spread among solipeds through respiratory secretions, which contaminate feeds and are ingested by other animals, or by inoculation through abraded skin (15). We infected animals via small-particle aerosol because it mimics the route likely to be encountered if \( B. mallei \) is used as a biological weapon. However, this route may bypass colonization of the upper respiratory tract, where type IV pili may be expressed and important for initial adherence. Thus, a pilin vaccine might still have utility against infection in the natural setting. Other aspects of the model may also preclude a role for antipilin immunity in protection. For example, type IV fimbriae may not be important in mice but could play an important role in natural hosts or in accidental or intentional human infection.

Alternatively, it could be that active or passive immunization with purified pilin failed to provide protection because type IV pili are not produced during infection and therefore the bacteria are not recognized by antipilin antibodies. However, this does not appear to be the case, as we were able to detect a significant rise in antipilin antibody titer in mice infected via the aerosol route with a sublethal dose of \( B. mallei \). This result suggests that the pili are indeed produced in vivo, although it is conceivable that the mice were exposed to another antigen related to pilin during infection that elicited cross-reactive antibodies. It is also possible that the infecting organisms had preformed pili but that production of pili is rapidly down-regulated during infection. In this case, antibodies against pilin could be generated but would not be able to protect against infection once the bacteria no longer produce the protein. However, the fact that the pilin from the closely related pathogen \( B. pseudomallei \) is required for virulence suggests that the \( B. mallei \) pili might also be produced during infection (8). Furthermore, though not successful against \( B. mallei \), an antipilin vaccine strategy might be successful in preventing disease due to \( B. pseudomallei \), which expresses an identical pilin protein. Since animals infected with \( B. mallei \) produce antibodies against the pilin protein, the purified pilin might also have utility as a serological test for infection with \( B. mallei \) or \( B. pseudomallei \). As current methods are inadequate, a sensitive and specific diagnostic test would be welcome (24).

Another possible explanation for the failure of antipilin antibodies to protect against experimental infection concerns the route of immunization. We found that the purified soluble pilin protein was highly immunogenic when administered subcutaneously. However, we did not measure antibody levels in the respiratory tract. It is conceivable that local antibody concentrations were insufficient for protection. Vaccination by another route, such as topical administration to the nasal mucosa, might elicit a protective local response. Alternatively, the antibody response to the pilin may be directed predominantly against epitopes that are not protective.

Immunization with purified pilin elicited robust levels of specific IgG antibodies, but we did not determine whether the antigen also stimulated a potent cell-mediated immune response. \( B. mallei \) appears to be a facultative intracellular pathogen that is capable of surviving and multiplying in macrophage-like cell lines (18). It is therefore likely that a cytolytic, cell-mediated immune response is necessary to control the infection and antibodies may play a limited role in protection. Nevertheless, monoclonal antibodies that recognize \( B. mallei \) lipopolysaccharide were able to confer passive protection against a \( B. mallei \) challenge in a model similar to the one that we used (23). Such antibodies might prevent initial infection before the organism adopts an intracellular lifestyle.

It is noteworthy that mice immunized with irradiated \( B. mallei \) organisms plus Freund’s adjuvant survived significantly longer than did mice immunized with purified pilin plus Freund’s adjuvant or than control mice that received only saline injections. This result requires confirmation, as there was no significant difference between the survival time of mice that received the irradiated organisms and that of the group that received adjuvant alone. There was also no effect of the same antigen with a different adjuvant and administration schedule. Nonetheless, this observation appears to be novel, as previous attempts at \( B. mallei \) immunization have not resulted in significant protection (1). If confirmed by further study, efforts to determine which components of the organisms are responsible for the modest degree of protection observed should be pursued in an effort to develop a safe and effective vaccine against this potentially dangerous pathogen.

ACKNOWLEDGMENTS

We thank Virginia Lockatell for technical assistance. This work was supported by Public Health Service awards R03 AI-54690 and R01 AI-37606 from the National Institutes of Health.

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