Immunization with Polyamine Transport Protein PotD Protects Mice against Systemic Infection with Streptococcus pneumoniae

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The human pathogen Streptococcus pneumoniae contains genes for a putative polyamine ABC transporter which are organized in an operon and designated potABCD. Polyamine transport protein D (PotD) is an extracellular protein which binds polyamines and possibly other structurally related molecules. PotD has been shown to contribute to virulence in both a murine sepsis model and a pneumonia model with capsular type 3 pneumococci. The protective efficacy of recombinant PotD was evaluated by active immunization and intravenous challenge with capsular type 3 pneumococci in CBA/N mice. Immunized mice had 91.7% survival following lethal pneumococcal challenge, compared with 100% mortality in the control group. Immunized animals had high-titer anti-PotD antibodies following three immunizations with alum. Protection in a sepsis model was also seen after passive administration of rabbit antiserum raised against PotD (P < 0.004). These results suggest that antibodies to PotD confer protection against invasive pneumococcal disease and that this protein should be studied further as a potential vaccine candidate for protection against invasive pneumococcal infections.

Streptococcus pneumoniae (pneumococcus) is an important human pathogen and is a leading cause of bacterial pneumonia, otitis media, and bacterial meningitis. This pathogen causes significant morbidity and mortality worldwide in both children and adults (12, 21, 35). Management of pneumococcal disease is also complicated by the increasing rate at which this organism is developing resistance to multiple classes of antimicrobial agents on a global scale (3, 25).

Current immunization strategies for pneumococcal disease are directed against capsular polysaccharide. It is known that antibodies to capsular polysaccharide confer a high level of protection against invasive disease (15, 18). Two vaccine formulations are presently licensed in the United States: a 23-valent polysaccharide vaccine and a heptavalent polysaccharide-protein conjugate. The efficacy of the polysaccharide vaccine is only about 60% for preventing invasive diseases in groups for which it is indicated (11, 13). A heptavalent protein-polysaccharide conjugate vaccine that is protective in children less than 2 years old and reduces nasopharyngeal colonization has been developed (4, 10). However, because of the limited immunogenicity and serotype distribution, as well as the expense, of current vaccines there is a need for improved pneumococcal vaccine antigens.

Pneumococcal proteins are being studied as potential vaccine candidates because they have several advantages over polysaccharide antigens. Proteins are, generally, antigenically conserved across all serotypes and stimulate a T-cell-dependent immune response resulting in more robust immunological memory (28). Several pneumococcal surface proteins have been shown to be effective immunogens and can prevent sepsis, pneumonia, and carriage in animal models (1, 2, 5, 6, 23, 26).

Polyamines such as spermidine and putrescine are small, ubiquitous, polycationic molecules that are involved in many aspects of cellular physiology in both eukaryotes and prokaryotes. Polyamines interact extensively with nucleic acids and consequently have a significant role in DNA replication, transcription, and translation (9, 16, 29). Intracellular polyamines in bacteria are derived both from de novo synthesis from amino acids and from the environment. In Escherichia coli a four-gene operon designated potABCD encodes an ABC transporter for putrescine and spermidine. This transport system is required for normal cell growth in vitro (17, 20). An operon with significant homology to the E. coli Pot operon at the amino acid level has been described in pneumococci, and mRNA has been detected during in vitro growth, suggesting a role for this transporter in cellular physiology (33).

PotD from pneumococci shows significant homology to E. coli PotD, and the pneumococcal protein possesses a characteristic gram-positive signal peptide, suggesting an extracellular location for this molecule. Interestingly, it does not possess an amino acid motif characteristic of sortase-processed proteins, lipoproteins, or choline-binding proteins. However, flow cytometry and cell fractionation experiments have shown that PotD in pneumococci is surface exposed (27a). Previous studies have shown that a potD mutation in a mouse-virulent capsular type 3 strain significantly attenuates the natural history of infection in systemic and pulmonary murine models, indicating a role for PotD in pneumococcal pathogenesis (32).

In the present study immunization with recombinant PotD was used to immunize mice, and protection against lethal bacteremia was assessed. Passive protection with hyperimmune serum was also performed to focus on the role of immunoglobulin in protective immune responses elicited by PotD.
MATERIALS AND METHODS

Bacterial strains, media, and DNA isolation. Pneumococcal strain WU2 that expresses a serotype 3 capsule was used as the inoculum for all infections. This strain is virulent in a mouse model of sepsis (7). The genome of TIGR4, a capsular type 4 strain, has been sequenced (31) and served as the template for amplification of potD. Bacteria were grown in THY medium (Todd-Hewitt broth supplemented with 0.5% yeast extract; Difco Laboratories, Detroit, MI) or on solid media containing blood agar base and 5% sheep erythrocytes at 37°C in 5% CO2. Plasmid DNA was isolated from E. coli with QIAGEN columns according to the manufacturer's instructions (QIAGEN, Valencia, CA), and pneumococcal chromosomal DNA was isolated with a MasterPure gram-positive DNA purification kit (Epicenter, Madison, WI). Cloning and transformation of E. coli were performed using previously described protocols (27). Plasmids were maintained in E. coli host strain TOP10F+ grown at 37°C on Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin.

Cloning, expression, and purification of His6-PotD fusion protein. The coding sequence for PotD was amplified using high-fidelity polymerase with a 5′ primer starting at the first codon downstream from the 31-amino-acid signal peptide-coding sequence: potD forward (5′-CACCATGTTGATATGAAAATCAAT-3′) and potD reverse (5′-CTTCCGATACATTTAAAACGTAA-5′). The amplified DNA was ligated into the vector pET101/D-TOPO, which added a C-terminal hexahistidine sequence to the expressed protein. The resulting plasmid was used to chemically transform competent E. coli cells. The orientation and sequence of the cloned gene were verified by DNA sequencing of the recombinant plasmid. This plasmid was then used to transform E. coli BL21Star(DE3) for protein expression. The transformed cells were grown overnight at 37°C in 10 ml of LB medium containing 100 μg/ml of ampicillin. The overnight culture was used to inoculate 200 ml of LB broth containing ampicillin, which was incubated at 37°C with shaking until the optical density at 600 nm reached 0.6 to 0.8. Induction of PotD was achieved by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM, and the culture was incubated for an additional 6 h. The cells were harvested by centrifugation, and recombinant PotD was purified with a B-Per 6His fusion protein purification kit (Pierce, Rockford, IL) used according to the manufacturer's protocol. Briefly, the bacterial pellet was resuspended in 10 ml of lysis buffer and centrifuged at 14,000 rpm using a Beckman JA-17 rotor. The pellet was discarded, and the supernatant was loaded onto 1-ml nickel-chelated resin columns previously equilibrated with 10 column volumes of lysis buffer. The columns were washed with 6 column volumes of 10 mM imidazole buffer, followed by 6 volumes of buffer containing 25 mM imidazole. The protein was eluted with 6 volumes of buffer containing 200 mM imidazole. Fractions (1 ml) were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to identify the fractions containing PotD. The selected fractions were dialyzed extensively against phosphate-buffered saline (PBS) (pH 7.0) to remove the imidazole, and the protein was stored at −20°C. A similar procedure was used to treat E. coli host strain BL21 without the recombinant plasmid for use as a control in the immunization experiments.

Active immunization. PotD purity was confirmed by SDS-PAGE, and the concentration was determined by using Bradford reagent (Pierce, Rockford, IL). All immunization experiments were performed with 8- to 12-week-old CBA/CaHN-B6K-129(CBA/N) mice (Jackson Laboratory, Bar Harbor, ME). For each immunization experiment mice were immunized subcutaneously with 5 μg PotD suspended in PBS with 1 mg/ml of alum. Each animal received three immunizations at 7- to 14-day intervals, and sera were collected from the mice by retro-orbital bleeding before immunization (day 0) and 1 week after the third immunization (day 35). Control mice received an identical course of immunization with E. coli BL21 lysate plus alum or PBS with alum. All experimental and control groups contained 12 animals. Intravenous infection was performed 1 week after the third immunization using 106 CFU of WU2. The mice were closely monitored for 10 days, and the survival time of each mouse was recorded. All experiments were approved by the Institutional Animal Care and Use Committee. Data on survival of mice for the active immunization experiment were analyzed by using the Kaplan-Meier graph and log rank test.

ELISA and immunoblotting. Specific antibody titers were determined by enzyme-linked immunosorbent assays (ELISA) using 96-well plates (Nunc, Chantilly, VA) coated with 5 μg/ml purified recombinant PotD. Individual mouse or rabbit sera were incubated with coated plates for 1 h at room temperature, and bound antibodies were detected by using alkaline phosphates-conjugated goat-anti-mouse or anti-rabbit immunoglobulin G (Southern Biotech, Birmingham, AL) and disodium p-nitrophenol phosphate as the substrate. Absorbance at 405 nm was measured, and the endpoint titer was defined as the reciprocal of the dilution of serum showing absorbance above the background level (optical density, >0.07). For immunoblotting, purified protein or pneumococcal culture lysates were separated by SDS-PAGE with 3 to 12% gels, electroblotted onto nitrocellulose membranes, and reacted with specific antiserum using standard protocols (27).

Rabbit antiserum and passive immunization. Eight- to 12-week-old New Zealand White rabbits were immunized subcutaneously with recombinant PotD at biweekly intervals, and subsequently serum was collected by ear bleeding as previously described (14). Pooled rabbit antiserum was titrated by ELISA as described above. Pre- and postimmune sera were diluted 10−2 and 10−3 in PBS, and 200 μl was administered intraperitoneally to groups of six mice. Four hours after administration of serum, animals were challenged by intravenous infection with 104 CFU of pneumococcal strain WU2 and observed for 10 days.

RESULTS

Recombinant purified PotD was analyzed by SDS-PAGE and immunoblotting using an anti-His6 tag monoclonal antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:3,000 to confirm the molecular weight and purity (Fig. 1). Recombinant
PotD was purified to homogeneity, as determined by total protein staining of an SDS-PAGE gel containing column eluate (Fig. 1A). The recombinant protein, which did not include the N-terminal signal peptide, had an apparent molecular mass of 38 kDa. This is the expected size for the recombinant protein, compared with the full-length, native PotD protein, which has a predicted molecular mass of 41 kDa. Removal of the leader sequence from the recombinant protein increased its solubility in the *E. coli* expression host, facilitating purification from the cytoplasmic fraction. Active immunization with recombinant PotD resulted in very-high-titer antibody responses in mice as measured by ELISA endpoint dilution (Table 1). Unimmunized mice had relatively low titers of cross-reactive antibody. This naturally occurring cross-reactive antibody may arise from exposure to polyamine transporter components of commensal bacteria. Polyamine transporters are highly conserved across bacterial species which colonize and infect mammalian hosts (33). Immunoblotting with serum from immunized mice confirmed that the immune response was specifically directed against PotD when preparations were examined with either recombinant protein or whole-cell lysates of WU2 (Fig. 2B). Immunized mice had 90% survival against bacteremia with a virulent type 3 pneumococcal strain, compared with control mice that received either *E. coli* lysate or PBS alone, which had 100% mortality within 72 h (Fig. 3). The infectious challenge dose used in these experiments was approximately 100 times the 50% lethal dose of the strain, and the mortality for control groups was 100% within 72 h.

Rabbit antiserum to PotD was tested for specificity using lysates of WU2 and recombinant PotD. The rabbit antiserum reacted with a single protein that was the expected molecular mass from WU2 and also reacted with recombinant PotD expressed in *E. coli* (Fig. 2A). Preimmune rabbit serum had a reciprocal endpoint dilution titer of 9,000, while postimmune serum had a titer of 243,000. In passive protection studies two groups of six mice each were given rabbit antiserum diluted 1:100 and 1:1,000, and a third group was given preimmune rabbit serum diluted 1:100 as a control. All mice receiving antiserum diluted 1:100 survived challenge with 100 times the 50% lethal dose of WU2, and five of six animals which received the 1:1,000 dilution survived the challenge. Five of six mice which received preimmune serum succumbed within 72 h of infection. Comparison of all the mice which received postimmune serum with the mice which received preimmune serum demonstrated the significant protective effect of passive immunization (*P* < 0.004).

TABLE 1. ELISA endpoint dilution titers for mice immunized with recombinant PotD

<table>
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<tr>
<th>Group</th>
<th>Reciprocal endpoint titer (mean ± SEM)</th>
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<tr>
<td>Preimmune</td>
<td>235 ± 25</td>
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<tr>
<td>Immunized</td>
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<tr>
<td>PotD</td>
<td>18,749 ± 3,068</td>
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<tr>
<td>BL21 lysate</td>
<td>1,562 ± 625</td>
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<td>PBS</td>
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FIG. 2. Immunoblots of recombinant PotD and of whole-cell lysates of WU2. (A) Polyclonal rabbit anti-PotD serum was used to detect recombinant His$_6$-tagged PotD (38 kDa) (lanes 1 and 2) and full-length PotD (41 kDa) (lanes 3 and 4) from whole-cell lysates of WU2. (B) Mouse immune serum following immunization with PotD. Lane 1, full-length PotD (41 kDa) from whole-cell lysate of WU2; lanes 2 and 3, recombinant PotD.

FIG. 3. Survival of mice after intravenous challenge with WU2. Groups of 12 mice were immunized with PotD, and 6 mice each received *E. coli* BL21 lysate or PBS. All immunized mice were challenged 1 week after the last immunization with $10^6$ CFU of WU2. Mice immunized with recombinant PotD showed significantly increased survival compared to the control mice (*P* < 0.001).
**DISCUSSION**

Bacterial pathogens express multiple ABC transporters which include surface-exposed proteins. Numerous ABC transporter proteins in pneumococci have been shown to be required for full expression of virulence (22, 24). Because of their cellular location ABC transporters are potential immunogens which may elicit protective immune responses. Two different ABC transporters for iron contain surface-exposed proteins that can elicit protective immunity in a pneumococcal sepsis model (8, 19, 34). An ABC transporter for manganese can induce a protective antibody response in a mouse model (30). A putative pneumococcal polyamine transporter contains a lipoprotein, PotD, which is surface exposed and accessible to antibodies. PotD has been reported to be involved in virulence in both an animal model of sepsis and pneumonia (24, 32). These studies were undertaken to investigate the role of PotD in protective immunity and to assess its potential as a vaccine candidate.

Immunization with PotD induces a vigorous antibody response in mice. Antibodies to PotD do not appear to cross-react with other pneumococcal proteins when PotD antiserum is used to probe pneumococcal lysates by immunoblotting. Preexisting antibodies to PotD induced by immunization with recombinant protein provide a significant degree of protection against intravenous challenge with an otherwise lethal inoculum of a virulent type 3 pneumococcal strain. Passive transfer of PotD immune serum effectively protects against pneumococcal bacteremia, suggesting that protection results specifically from a humoral immune response.

Antibodies directed against PotD may protect against pneumococcal infection by at least two different, although not necessarily mutually exclusive, mechanisms. Polymamines are essential to cell growth and can be acquired by either de novo synthesis or uptake from the environment. The pneumococcal genomes which have been sequenced contain homologues of known genes encoding enzymes which synthesize polymamines from ornithine and arginine. However, uptake of polymamines from the environment appears to be necessary for full expression of pneumococcal virulence (24, 32). PotD is a putative polyamine-binding surface protein, and inhibition of its function with antibodies may alter intracellular polyamine levels to an extent which attenuates virulence. Alternatively, PotD antibodies may promote phagocytosis of pneumococci by opsonization and activation of complement. In future studies we will look at in vitro opsonophagocytosis of pneumococci with purified PotD immunoglobulin G.

Pneumococcal capsular polysaccharides are immunodominant antigens, and antibodies to capsule protect against invasive pneumococcal infections; however, many persons at high risk for pneumococcal infection do not respond to certain capsular polysaccharides with an adequate immune response. In addition, there are more than 90 different pneumococcal serotypes, which precludes a comprehensive polysaccharide-based vaccine. Protein antigens offer the advantages of inducing strong memory immune responses and being conserved across all serotypes. PotD represents a potential protein-based vaccine candidate which deserves further study for its ability to induce protective immunity. Studies of mucosal and pulmonary immune responses against PotD are currently under way and may suggest an improved means to protect against both invasive pneumococcal infection and carriage on mucosal surfaces.

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**REFERENCES**


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ERRATUM

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Volume 74, no. 10, p. 5888–5892, 2006. Page 5890: Figure 3 should appear as shown below.

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- ▲ 5 μg PotD
- ■ PBS
- □ BL21
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