Mucosal Immunization with Polyamine Transport Protein D (PotD) Protects Mice Against Nasopharyngeal Colonization with *Streptococcus pneumoniae*

Pratik Shah, David E. Briles, Janice King, Yvette Hale and Edwin Swiatlo

Exp Biol Med (Maywood) 2009 234: 403
DOI: 10.3181/0809-RM-269

Mucosal Immunization with Polyamine Transport Protein D (PotD) Protects Mice Against Nasopharyngeal Colonization with *Streptococcus pneumoniae*

PRATIK SHAH,* DAVID E. BRILES,†,‡ JANICE KING,† YVETTE HALE,† AND EDWIN SWIATLO*,§,||,[1]

*Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi 39216; †Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294; ‡Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama 35233; §Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi 39216; and ||Research Service, Veterans Affairs Medical Center, Jackson, Mississippi 39216

Streptococcus pneumoniae **is an encapsulated pathogen that can cause invasive disease following colonization of the nasopharynx. Targeting colonization of mucosal surfaces may, therefore, be the best approach for vaccination to prevent pneumococcal invasive disease. Previous studies in our laboratory have shown that immunization with recombinant polyamine transport protein D (PotD) protects mice against systemic pneumococcal infections. In this study we investigated the efficacy of mucosal immunization with rPotD to protect against pneumococcal carriage and invasion in a murine model. Mice were intranasally immunized with either rPotD and cholera toxin B subunit (CTB) or CTB alone. Significantly less pneumococci were recovered from the nasopharynx of immunized mice compared to the control animals following intranasal challenge with either EF3030 (serotype 19F) ($P < 0.05$) or an invasive serotype 4 isolate (TIGR4) ($P < 0.05$). PotD immunized mice also had lesser bacteria in their sinus tissues ($P < 0.05$), brains ($P < 0.05$), lungs and olfactory bulbs following intranasal challenge with TIGR4. ELISA analysis demonstrated the presence of IgG antibodies to PotD in the serum and IgA antibodies in the saliva. These results indicate that mucosal immunization with PotD generates both mucosal and systemic immune responses and prevents establishment of nasopharyngeal carriage by multiple pneumococcal serotypes. Thus, PotD is a potentially important antigen for development of a pneumococcal protein vaccine.**
Exp Biol Med 234:403–409, 2009

Key words: *Streptococcus pneumoniae*; PotD; vaccine

Introduction

Streptococcus pneumoniae (pneumococcus) is a gram positive encapsulated human pathogen which has over 90 different capsular serotypes. Colonization of the upper respiratory tract is an indispensable first step in the pathogenesis of pneumococcal disease. In certain conditions pneumococci may extend to cause pneumonia, bacteremia, otitis media and meningitis (1). Pneumococcal infections annually cause more than a million deaths worldwide and pose a significant health issue in both the developing and industrialized nations (2). Although there are two currently licensed pneumococcal vaccines, neither of them offer comprehensive protection. The polysaccharide vaccine incorporates capsular polysaccharides from 23 different serotypes, but is poorly immunogenic in children, the elderly, or immuno-compromised individuals (3). A 7-valent protein-polysaccharide conjugate vaccine has been subsequently licensed for use in infants and children (4). The conjugate vaccine, however, has limited serotype coverage and is too expensive for use in most developing countries, where most pneumococcal deaths occur. Thus, capsule-based vaccines are sub-optimal due to high cost and limited serotype coverage. Additionally, concomitant in-

The authors declare no conflict of interest.

Presented at the 108th American Society for Microbiology general meeting on June 4, 2008 in Boston, MA.

This research was funded by the Veterans Administration Office of Research.

[1] To whom correspondence should be addressed at Research Service (151), Veterans Affairs Medical Center, 1500 Woodrow Wilson Drive, Jackson, MS 39216. E-mail: edwin.swiatlo@va.gov

Received September 6, 2008.
Accepted January 13, 2009.

DOI: 10.3181/0809-RM-269
1535-3702/09/2344-0403$15.00

crease in carriage, and subsequently disease, with non-vaccine serotypes has made treatment of pneumococcal infections even more difficult (5).

Much effort is now focused on identifying pneumococcal proteins that elicit a protective immune response. Protein antigens induce T-helper cells and lead to long-term memory responses and can be produced in large quantities relatively inexpensively, since chemical conjugation is not required. Protein antigens are also generally well-conserved among multiple serotypes, thereby overcoming one of the shortcomings of capsular vaccines (6). Pneumococcal surface protein A, C (PspA, PspC) and pneumolysin toxoid are well characterized protein antigens that are capable of protecting immunized animals (6–8). In the last few years, additional pneumococcal proteins have emerged as promising vaccine candidates (9–14). Several of these proteins have been tested in murine models of pneumococcal colonization, and are capable of generating protective immune responses in both mucosal secretions and serum of immunized animals after intranasal immunization with mucosal adjuvants. Previous studies also indicate that mixtures of proteins offer greater levels of protection than individual proteins by themselves (15–17). A successful pneumococcal protein vaccine would most likely incorporate several immunogenic proteins.

Polyamines are small organic compounds with a hydrocarbon backbone and multiple positively charged amino groups spaced at regular intervals. Putrescine, spermidine and cadaverine represent the most widely distributed microbial polyamines. Polyamine uptake, synthesis, and degradation in bacteria are coordinated processes, and intracellular polyamine levels are stringently regulated (18). Most prokaryotes have a *de novo* synthesis pathway in which polyamines are synthesized via enzymatic modification of precursor amino acids. Polyamine acquisition and biosynthesis plays a key role in virulence and pathogenesis of many human bacterial pathogens (18). Putrescine is involved in regulating the swarming-motility in *Proteus* spp. and biofilm formation in *Yersinia pestis* and *Vibrio choleae*. *Helicobacter pylori* increase polyamine biosyntheses during infection, leading to host cell apoptosis and cancer while *E. coli* colicin-colE7 production is regulated by polyamine biosynthesis and transport (18). *E. coli* has a four-gene polyamine uptake ABC transport operon identified as polyamine transport operon (*pot*) that has been well characterized. PotD is the periplasmic substrate binding protein that binds putrescine and spermidine and facilitates their uptake via transmembrane proteins PotB and PotC, while cytosolic PotA is an ATPase that supplies energy for this process (18, 19).

S. pneumoniae has an operon designated *potABCD* with a high degree of similarity to one found in *E. coli* (20). PotD in *S. pneumoniae* is a membrane-associated protein expressed by diverse capsular serotypes and is antigenically conserved at a constant molecular weight across all capsular serotypes included in the currently licensed vaccines (21, 24). All four sequenced pneumococcal genomes have genes coding for a *pot* operon. Additionally, analyses of 19 pneumococcal isolates, which are currently being sequenced (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), show that they also have genes homologous to the *potABCD* system. Pneumococcal mutants lacking *potD* are significantly attenuated in murine models of bacteremia and pneumonia, suggesting that uptake of environmental polyamines is crucial for full expression of virulence *in vivo* (22, 23). Furthermore, increase in pneumococcal *potD* transcription is observed in response to oxidative and temperature stress, choline limitation and during murine bacteremia (24). Previous work in our laboratory has shown that systemic immunization with rPotD results in a vigorous antibody response in mice that protects against invasive pneumococcal infections (12). These data suggest an important role for PotD in pneumococcal pathogenesis and immunity.

An effective protein antigen should ideally be able to protect against both nasopharyngeal carriage as well as systemic infections. This would reduce carriage of pneumococci in populations and reduce subsequent person-to-person transmission. Reduced carriage should also reduce invasive disease in both immunized and unimmunized individuals in a population. In this work, we examined the ability of mucosal immunization with rPotD to protect against pneumococcal colonization of mucosal surfaces in mice by two different serotypes capable of causing disease in humans.

Materials and Methods

Bacterial Strains, Media and Growth Conditions. Animal infections were performed with either TIGR4 (serotype 4) (25) or EF3030 (serotype 19F) (26). Pneumococci were grown overnight at 37°C in 5% CO_2 in THY medium (Todd-Hewitt broth supplemented with 0.5% yeast extract; Difco Laboratories, Detroit, MI). Cells were harvested by centrifugation and were resuspended in fresh medium containing 10% glycerol and stored at –80°C. One week prior to infection, cells were thawed, serially diluted in sterile Phosphate Buffered Saline (PBS) and plated on nutrient agar plates containing 5% sheep RBCs (BAP) to determine colony forming units (CFU) per milliliter. Previous studies in our laboratory have shown expression of PotD by both EF3030 and TIGR4 when grown under these laboratory conditions (21). To enumerate pneumococci recovered from nasal washes of mice, the nasal washes were serially diluted in sterile PBS and plated on BAPs with 4 μg/ml gentamicin.

Cloning, Expression and Purification of rPotD. The gene for PotD from capsule type 4 strain TIGR4 (Sp1386; http://www.tigr.org) was amplified with Pfu polymerase PCR using primers that exclude the coding region for the 31 amino acid peptide at the N-terminus (*potD* forward:-CACCATGTTAGATAGTAAAATCAAT-

Table 1. ELISA Titers Following Mucosal Immunizations with rPotD + CTB or CTB Alone

Challenge organism	Immunogen	Reciprocal endpoint titer (mean ± SEM)	
		IgG (serum)	IgA (saliva)
EF3030	10 μg rPotD	3125 ± 0	2291 ± 527
	PBS + CTB	<0.07	458 ± 166
TIGR4	10 μg rPotD	2708 ± 416	2236 ± 447
	PBS + CTB	<0.07	125 ± 0

30; *potD* reverse:CTTCCGATACATTTTAAACTGTA). Recombinant purified PotD was obtained by following previously described procedures (12). Purified protein was concentrated to 1 mg/ml in sterile PBS.

Mucosal Immunization. All animal studies were approved by the Institutional Animal Care and Use Committee. Mucosal immunizations were essentially performed as previously described (8, 26). Briefly, for both TIGR4 and EF3030, two groups of six- to eight-week-old non-anaesthetized CBA/CAHN-XID/J (CBA/N) mice (Jackson Laboratories, Bar Harbor, ME, USA) were immunized intranasally with 4 μg cholera toxin B-subunit (CTB; Sigma-Aldrich, St. Louis, MO) and 10 μg of rPotD resuspended in a total volume of 14 μl. Control animals in each experimental group received 4 μg of CTB alone in PBS. The immunization schedule was the same as previously described for other pneumococcal protein antigens (27). Briefly, the immunization schedule included two doses of either rPotD and CTB or CTB alone per week, for two consecutive weeks. In the third week, animals were immunized twice either with rPotD (immunized group) or PBS alone (control group). Mice were rested for an additional three weeks after the final immunization prior to infection. The data presented include the pooled results of two independent experiments.

Measurement of Antibody. One day prior to challenge with *S. pneumoniae*, saliva and serum were collected from all animals. Mice were injected intraperitoneally (i.p.) with 0.25 mg carbamylcholine chloride per kg body weight. (Sigma Aldrich, St. Louis, MO, USA) and approximately 50 μl of saliva was collected from each mouse with a sterile pipet tip. Saliva samples were stored at –80°C prior to ELISA analysis. For measurement of IgG, blood was collected by retro-orbital puncture. Serum was separated and stored at –80°C until analyzed. ELISA experiments with serum were done as previously described (12).

For measurement of IgA in saliva, 5 μg/ml of purified rPotD was coated on 96-well plates (Nunc, Chantilly, VA, USA) and incubated at 37°C overnight. Plates were washed three times with PBS-0.05% Tween 20 (PBST) and then blocked with 1% bovine serum albumin-PBS for 2 h at 37°C. A 1:5 dilution of the saliva samples were added to individual wells, serially diluted and incubated overnight at 4°C. Plates were washed as described above, and then biotin-labeled goat anti-mouse IgG (Southern Biotech, Birmingham, AL) diluted 1:2000 in PBST was added to each well and incubated for 2 h at 37°C. After three washes with PBST, a 1:4000 dilution of horseradish peroxidase-streptavidin (Southern Biotech, Birmingham, AL) in PBST was added to each well and incubated at room temperature for 1 h. Disodium p-nitrophenol phosphate (Sigma Aldrich, St. Louis, MO, USA) was used 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).

Challenge Experiments. For EF3030 experiments, approximately 5×10^6 cells were resuspended in 20 μl of sterile PBS and administered intranasally to mice using a sterile pipet tip. For TIGR4, approximately 4×10^5 cells were used for the challenge experiments. This inoculum results in colonization as well as invasion of the adjacent mucosal sites and organs by TIGR4 without bacteremia (28). Five days after intranasal challenge all animals were euthanized and nasal cavities were washed with 150 μl of sterile PBS as previously described (26). All collected samples were serially diluted and plated on BAPs with 4 μg/ml gentamicin and incubated at 37°C for 24 h. For animals infected with TIGR4, in addition to nasal washes, sinus tissues, lungs, olfactory bulbs and brains were also aseptically harvested as previously described (28). Each isolated tissue was homogenized in 1 ml of PBS, serially diluted and plated on BAPs. Blood was collected from all animals prior to nasal lavage and harvest of tissues in both groups to assess for bacteremia.

Statistical Analyses. All pneumococcal CFU are reported as Log_{10} values. Statistical analysis was performed using the GraphPad software program (GraphPad Software, San Diego, CA, USA). The Mann-Whitney two-sample rank test was used to compare CFU from the nasal wash and tissues. *P* values less than 0.05 were considered to represent significant differences between groups.

Results and Discussion

Mucosal Immunization with rPotD Results in IgA and IgG Responses. Active immunization with rPotD resulted in high-titer and specific immune responses in both serum and saliva of all immunized animals (Table 1). Conversely, no detectable antibody titers were observed following immunization with CTB in control animals. Thus, rPotD seems to be a potentially effective immunogenic

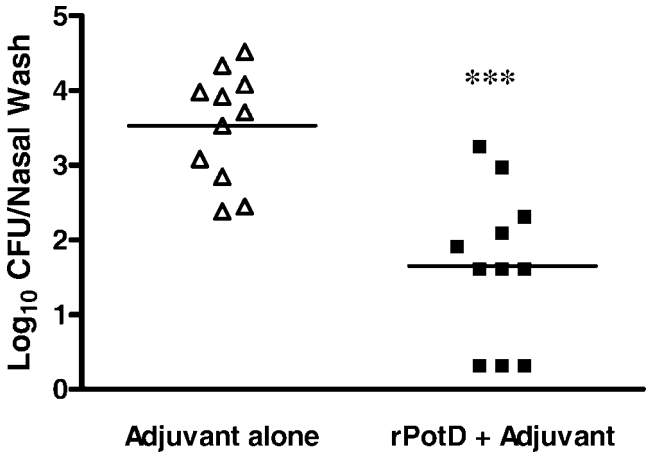

Figure 1. Protection conferred by mucosal immunizations with rPotD in a murine model of colonization with serotype 19F isolate-EF3030. CBA/N mice were immunized intranasally with rPotD mixed with cholera toxin B or with cholera toxin alone. Mice were challenged intranasally with 5×10^6 CFU of EF3030. Five days post-infection, mice were sacrificed and bacterial counts were determined from nasal washes. The horizontal line denotes median Log10CFU (*** $P = 0.0005$, Mann-Whitney U test).

antigen capable of inducing mucosal as well as systemic immunity (12) following intranasal immunization.

Protection Against Mucosal Colonization. *S. pneumoniae* serotype 19F-EF3030 strain readily colonizes the nasopharynx and does not progress to bacteremia, making it an ideal strain to test for protection against mucosal colonization (29). Intranasal infection of mice with approximately 10^6 cells suspended in 20 μl results in transient colonization of the nasopharynx without bacteremia. While, the same dose resuspended in 40 μl and given to anesthetized mice causes pneumococcal pneumonia (30). Ten- to 100-fold fewer pneumococci were recovered from nasal washes of animals immunized with rPotD compared to the control animals ($P = 0.0005$) (Fig. 1). These results indicate that immunization with rPotD results in immunity capable of significantly reducing *S. pneumoniae* colonization in the nasopharynx.

Efficacy of mucosal immunization with rPotD to reduce colonization with a more virulent serotype 4 isolate was also evaluated. TIGR4 is highly virulent but colonizes the nasopharynx without causing bacteremia when given in a reduced inoculum (28). All rPotD immunized mice showed significant reduction of nasopharyngeal colonization with TIGR4 ($P = 0.04$) (Fig. 2A) in comparison to mice immunized with adjuvant alone. Significant reduction in TIGR4 colonization of sinus tissue ($P = 0.01$) was also seen in the immunized mice compared to the controls (Fig. 2B).

Protection Against Locally Invasive Pneumococcal Infection. Previous studies in mice have shown that intranasal infection with TIGR4 results not only in carriage, but also in infection of the olfactory bulbs, brains and lungs (28). We wanted to test the efficacy of mucosal immunization with rPotD in preventing pneumococcal invasion in these organs. Five days after intranasal infection with TIGR4 olfactory bulbs, brains and lungs were aseptically harvested and pneumococci enumerated. Significantly fewer pneumococci were recovered from brains ($P = 0.005$) of immunized animals compared to the controls (Fig. 2C). The immunized animals also had fewer pneumococci in their lungs (Fig. 2E) and olfactory bulbs (Fig. 2D) in comparison to the control group, but the differences did not reach statistical significance. Overall, mucosal immunization with rPotD seems to significantly reduce pneumococcal infection of the brain and reduces the bacterial burden in the lungs and the olfactory bulbs of mice. The reasons for observing less protection in some anatomic sites versus another are not clear. It is possible that mucosal immunization with rPotD does not induce sufficient immunity to protect against lower pulmonary or olfactory bulb infections.

Asymptomatic colonization with *S. pneumoniae* can be followed by invasive disease in susceptible individuals. Although, the exact factors that govern the progression to systemic infections is not yet clear, old age, chronic alcoholism, immune suppression and many viral infections increase the risk of pneumococcal disease. The currently licensed polysaccharide vaccines are not highly efficacious at preventing pneumococcal nasal colonization. Moreover, colonization of the nasopharynx is considered a prerequisite to the spread of pneumococci to the lower respiratory tract and the middle ear (31–33). Preventing carriage of pneumococci on mucosal surfaces should ideally result in reduction in invasive pneumococcal infections.

Surface proteins of bacterial pathogens are important for interactions with the host during infection, and often play a significant role in microbial virulence and pathogenesis. Gram positive pathogens express multiple membrane transporters which have surface exposed proteins and are potential targets for vaccine design (34). Components of *S. pneumoniae* ABC transporters involved in transport of iron, manganese and oligopeptides have previously been shown to induce protective immunity following immunizations by various routes (9, 35, 36). Additionally, signature-tagged mutagenesis screens have identified numerous *S. pneumoniae* ABC-transporters which seem to be important for expression of complete virulence in murine models of infections (22, 37). Polyamine ABC transporter proteins also seem to be promising vaccine antigens. PotD has been reported to be involved in pneumococcal virulence in animal models of sepsis and pneumonia (22, 23). Similarly, immunizations with recombinant PotF protein (part of the putative *potFHGI* ABC transport system involved in *Burkholderia pseudomallei* putrescine transport) protect mice against lethal challenge with *B. pseudomallei* (38).

These studies were undertaken to investigate the efficacy of pneumococcal rPotD in inducing protective immunity on mucosal surfaces. Our results indicate that animals immunized with rPotD have significant reduction in nasopharyngeal colonization and invasive disease by two

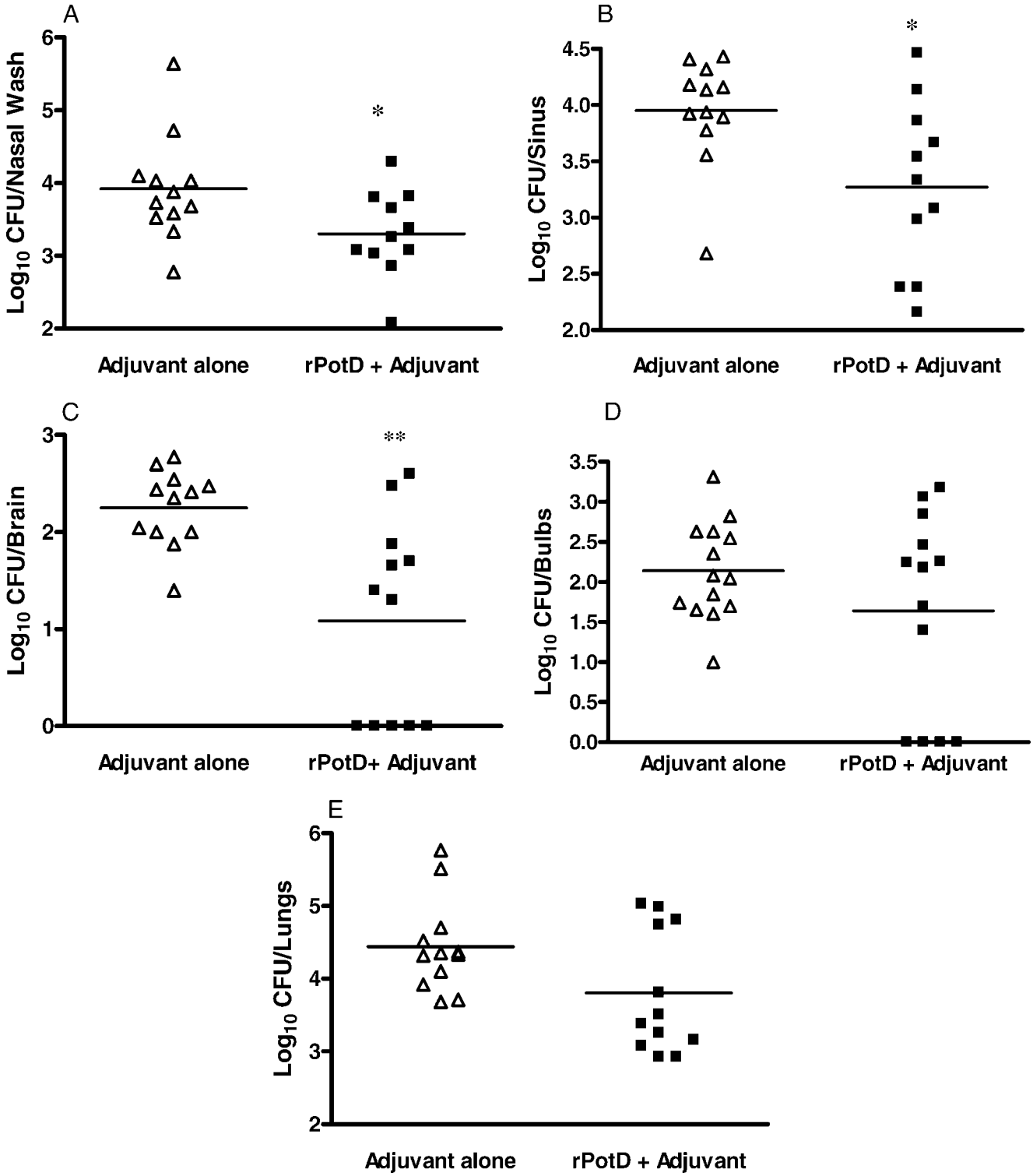

Figure 2. Protection conferred by mucosal immunizations with rPotD in a murine model of colonization and invasion with serotype 4 isolate-TIGR4. CBA/N mice were immunized intranasally with rPotD mixed with cholera toxin B or with cholera toxin B alone. Mice were challenged intranasally with 4×10^5 CFU of TIGR4. Five days post-infection, mice were sacrificed and bacterial counts were determined from (**A**) nasal washes (* $P = 0.04$); (**B**) sinus tissue (* $P = 0.01$); (**C**) brains (** $P = 0.005$, Mann-Whitney U test); (**D**) olfactory bulbs and (**E**) lungs. The horizontal line denotes median Log10CFU.

different pneumococcal capsule types. Mucosal immunizations with rPotD also generate specific and high titer IgG and IgA in serum and saliva of immunized mice. The function of secretory immunoglobulins in protection against pneumococcal colonization is not clearly defined. Several reports suggest $CD4^+$ T cells seem to play an important role in protection against colonization of mucosal surfaces by pneumococci (39–43). Choline binding surface proteins PspA and PspC seem to be exceptions, as antibodies generated following mucosal immunizations seem to confer protection against pneumococcal disease (8, 9, 44–46). Anti-PotD antibodies produced after mucosal immunizations may block polyamine uptake by PotD resulting in deficiency of intracellular polyamines and decreased cell viability and virulence *in vivo* (23). Alternatively, anti-PotD IgG may lead to deposition of complement and subsequent opsonophagocytosis of pneumococci. Previous studies in our laboratory have shown that passive transfer of PotD immune serum generated following systemic immunizations with rPotD effectively protects mice against pneumococcal bacteremia (12). One or both of these mechanisms may be responsible for protection mediated by anti-PotD antibodies after mucosal immunizations. We intend to investigate the opsonophagocytosis of pneumococci using PotD antiserum to elucidate the mechanism of protection conferred by PotD antibodies. Overall, mucosal immunization with rPotD generates a vigorous and a highly specific immune response in saliva and serum of immunized animals and significantly reduces nasopharayngeal carriage and invasive disease by two different *S. pneumoniae* capsular serotypes. This protein shows promise as a component of an effective, next-generation pneumococcal vaccine.

1. Fedson DS, Scott JA. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 17 Suppl 1:S11–18, 1999.
2. WHO. Pneumococcal vaccines. World Health Organization position paper. Wkly Epidemiol Rec 74:177–183, 1999.
3. Fedson DS. The clinical effectiveness of pneumococcal vaccination: a brief review. Vaccine 17:S85–S90, 1999.
4. Eskola J. Immunogenicity of pneumococcal conjugate vaccines. Pediatr Infect Dis J 19:388–393, 2000.
5. Moore MR, Whitney CG. Emergence of nonvaccine serotypes following introduction of pneumococcal conjugate vaccine: cause and effect? Clin Infect Dis 46:183–185, 2008.
6. Swiatlo E, Ware D. Novel vaccine strategies with protein antigens of *Streptococcus pneumoniae*. FEMS Immunol Med Microbiol 38:1–7, 2003.
7. Alexander JE, Lock RA, Peeters CCAM, Poolman JT, Andrew PW, Mitchell TJ, Hansman D, Paton JC. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infect Immun 62: 5683–5688, 1994.
8. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun 70:2526–2534, 2002.
9. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, Virolainen A, Swiatlo E, Hollingshead SK. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 68:796–800, 2000.
10. Brown JS, Ogunniyi AD, Woodrow MC, Holden DW, Paton JC. Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. Infect Immun 69:6702–6706, 2001.
11. Green BA, Zhang Y, Masi AW, Barniak V, Wetherell M, Smith RP, Reddy MS, Zhu D. PppA, a surface-exposed protein of *Streptococcus pneumoniae*, elicits cross-reactive antibodies that reduce colonization in a murine intranasal immunization and challenge model. Infect Immun 73:981–989, 2005.
12. Shah P, Swiatlo E. Immunization with polyamine transport protein PotD protects mice against systemic infection with *Streptococcus pneumoniae*. Infect Immun 74:5888–5892, 2006.
13. Zhang Y, Masi AW, Barniak V, Mountzouros K, Hostetter MK, Green BA. Recombinant PhpA protein, a unique histidine motif-containing protein from *Streptococcus pneumoniae*, protects mice against intranasal pneumococcal challenge. Infect Immun 69:3827–3836, 2001.
14. Glover DT, Hollingshead SK, Briles DE. *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect Immun 76(6):2767–2776, 2008.
15. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun 75:350–357, 2007.
16. Briles DE, Hollingshead S, Brooks-Walter A, Nabors GS, Ferguson L, Schilling M, Gravenstein S, Braun P, King J, Swift A. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. Vaccine 18:1707–1711, 2000.
17. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, van Ginkel FW, Benjamin WH Jr. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. J Infect Dis 188:339–348, 2003.
18. Shah P, Swiatlo E. A multifaceted role for polyamines in bacterial pathogens. Molecular Microbiology 68:4–16, 2008.
19. Igarashi K, Kashiwagi K. Polyamine transport in bacteria and yeast. Biochem J 344 Pt 3:633–642, 1999.
20. Ware D, Watt J, Swiatlo E. Utilization of putrescine by Streptococcus pneumoniae during growth in choline-limited medium. J Microbiol 43: 398–405, 2005.
21. Shah P, Marquart M, Quin LR, Swiatlo E. Cellular location of polyamine transport protein PotD in *Streptococcus pneumoniae*. FEMS Microbiol Lett 261:235–237, 2006.
22. Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D. Large-scale identification of virulence genes from Streptococcus pneumoniae. Infect Immun 66:5620–5629, 1998.
23. Ware D, Jiang Y, Lin W, Swiatlo E. Involvement of *potD* in *Streptococcus pneumoniae* polyamine transport and pathogenesis. Infect Immun 74:352–361, 2006.
24. Shah P, Romero DG, Swiatlo E. Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia. Microb Pathog 45:167–172, 2008.
25. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, *et al*. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 293:498–506, 2001.
26. Wu H-Y, Virolainen A, Mathews B, King J, Russell MW, Briles DE. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. Microb Pathog 23:127–137, 1997.
27. Briles DE, Tart RC, Wu HY, Ralph BA, Russell MW, McDaniel LS. Systemic and mucosal protective immunity to pneumococcal surface protein A. Ann N Y Acad Sci 797:118–126, 1996.

28. van Ginkel FW, McGhee JR, Watt JM, Campos-Torres A, Parish LA, Briles DE. Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. Proc Natl Acad Sci U S A 100:14363–14367, 2003.
29. Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. Infect Immun 60:111–116, 1992.
30. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, van Ginkel FW, Benjamin WH. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. J Infect Dis 188:339–348, 2003.
31. Boulnois GJ. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. J Gen Microbiol 138:249–259, 1992.
32. Musher DM. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. Clin Infect Dis 14: 801–809, 1992.
33. Stenfors LE, Raisanen S. Abundant attachment of bacteria to nasopharyngeal epithelium in otitis-prone children. J Infect Dis 165: 1148–1150, 1992.
34. Garmory HS, Titball RW. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect Immun 72:6757–6763, 2004.
35. Jomaa M, Yuste J, Paton JC, Jones C, Dougan G, Brown JS. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 73:6852–6859, 2005.
36. Kerr AR, Adrian PV, Estevao S, de Groot R, Alloing G, Claverys JP, Mitchell TJ, Hermans PW. The Ami-AliA/AliB permease of *Streptococcus pneumoniae* is involved in nasopharyngeal colonization but not in invasive disease. Infect Immun 72:3902–3906, 2004.
37. Hava DL, Camilli A. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol Microbiol 45: 1389–1406, 2002.
38. Harland DN, Chu K, Haque A, Nelson M, Walker NJ, Sarkar-Tyson M, Atkins TP, Moore B, Brown KA, Bancroft G, Titball RW, Atkins HS. Identification of a LolC homologue in *Burkholderia pseudomallei*, a novel protective antigen for melioidosis. Infect Immun 75:4173–4180, 2007.
39. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc Natl Acad Sci U S A 102(13):4848–4853, 2005.
40. McCool TL, Weiser JN. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infect Immun 72:5807–5813, 2004.
41. van Rossum AM, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. Infect Immun 73:7718–7726, 2005.
42. Malley R, Srivastava A, Lipsitch M, Thompson CM, Watkins C, Tzianabos A, Anderson PW. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infect Immun 74:2187–2195, 2006.
43. Trzcinski K, Thompson CM, Srivastava A, Basset A, Malley R, Lipsitch M. Protection against nasopharyngeal colonization by *Streptococcus pneumoniae* is mediated by antigen-specific CD4+ T cells. Infect Immun 76(6):2678–2684, 2008.
44. Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. Infect Immun 69:6718–6724, 2001.
45. Pimenta FC, Miyaji EN, Areas AP, Oliveira ML, de Andrade AL, Ho PL, Hollingshead SK, Leite LC. Intranasal immunization with the cholera toxin B subunit-pneumococcal surface antigen A fusion protein induces protection against colonization with *Streptococcus pneumoniae* and has negligible impact on the nasopharyngeal and oral microbiota of mice. Infect Immun 74:4939–4944, 2006.
46. Wu HY, Nahm MH, Guo Y, Russell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis 175:839–846, 1997.