MAJOR ARTICLE

Pneumococcal Surface Protein A Contributes to Secondary *Streptococcus pneumoniae* Infection after Influenza Virus Infection

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We compared the growth of *Streptococcus pneumoniae* mutants with a disruption in the gene for either pneumococcal surface protein A ($PspA^-$), neuraminidase A ($NanA^-$), or hyaluronidase (Hyl^-) to that of the parental strain D39 by means of a competitive growth model in mice with and those without prior influenza virus infection. The numbers of total bacteria recovered from mice with prior influenza virus infection were significantly greater than those recovered from mice without prior influenza virus infection. Although the Hyl^- and $NanA^-$ mutants did not display attenuation in mice with or without prior influenza virus infection, the $PspA^-$ mutant exhibited attenuation both in mice with and in mice without prior influenza virus infection. This defect was severe in influenza virus–infected mice, for which growth of the $PspA^-$ mutant was 1800-fold lower than that of the parental strain D39. Furthermore, PspA immunization significantly reduced secondary bacterial lung burdens and concentrations of specific markers of lung damage in mice receiving serotypes 2, 3, and 4 pneumococci. Our findings indicate that PspA contributes to secondary *S. pneumoniae* infection after influenza virus infection and that PspA immunization mitigates early secondary pneumococcal lung infections.

Influenza A virus infection dramatically increases susceptibility to secondary *Streptococcus pneumoniae* infection, resulting in significantly greater morbidity and mortality [1]. Viral effects that have been implicated include disruption of innate effector responses [2, 3] and modification of respiratory mucosa [4, 5]. Although viral contributions to secondary pneumococcal infection have received considerable attention, the contributions of *S. pneumoniae* virulence factors remain unclear. Much effort has focused on pneumococcal virulence factors as targets for vaccine development in primary infection [6–8]. Among those that have been explored are hyaluronidase (Hyl), neuraminidase A (NanA), and pneumococcal surface protein A (PspA). Hyl targets hyaluronic acid in host connective tissues and the extracellular matrix, enhancing access for tissue invasion and colonization [8, 9]. Like influenza virus neuraminidase, pneumococcal NanA cleaves terminal sialic acid residues on respiratory surface glycoconjugates, promoting adhesion and colonization of bacteria [10, 11]. PspA is a choline-binding surface protein, which inhibits complement-mediated phagocytosis and binds to and prevents killing by lactoferrin [12, 13]. Numerous studies have documented attenuation in mutants lacking PspA [12, 14–16], and PspA protein is protective in a variety of delivery and challenge models [17–22].

Current pneumococcal vaccines have been effective in decreasing the incidence of invasive pneumococcal disease [23, 24]; however, protecting the elderly and selection-driven shifts in colonizing and disease-caus-

Received 29 August 2008; accepted 11 March 2009; electronically published 8 July 2009.

Potential conflicts of interest: none reported.

Presented in part: Northern Rocky Mountain Conference on Infectious Disease and Environmental Health, Big Sky, Montana, 27–29 September 2007.

Financial support: IDeA Network of Biomedical Research Excellence–Biomedical Research Infrastructure Network (grant RR16455); National Institutes of Health (Centers of Biomedical Research Excellence grant RR020185).

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The Journal of Infectious Diseases 2009;200:537–45

0022-1899/2009/20004-0009$15.00

DOI: 10.1086/600871

ing serotypes remain prominent concerns [23, 25, 26]. Because *S. pneumoniae* virulence factors are attractive candidates for vaccine development, further examination of their roles in influenza virus–infected lungs will help identify targets that are critical for secondary pneumococcal infection. The present study examined the relative contributions of Hyl, NanA, and PspA to *S. pneumoniae* virulence in healthy and influenza virus–infected mice. Additionally, the ability of immunization with PspA to reduce serotype 2, 3, and 4 primary and secondary pneumococcal growth and lung damage was assessed.

METHODS

Mice. Healthy female C57BL/6 mice (6–8 weeks old) were obtained from the National Cancer Institute–Frederick Animal Production Area. All procedures were approved by the Montana State University Institutional Animal Care and Use Committee.

Bacterial strains. *S. pneumoniae* strains D39 (serotype 2), WU2 (serotype 3), and TIGR4 (serotype 4) were cultured at 37°C and 5% CO_2 in Todd-Hewitt broth with 0.5% yeast extract (THY broth). Insertion-duplication mutants of D39 lacking PspA ($PspA^-$), Hyl (Hyl^-), and NanA ($NanA^-$) were provided by J. C. Paton. These mutants have been described elsewhere [15], and, although these and other insertion-duplication mutants have been routinely used [14, 27, 28], the possibility of polar effects exists. Bacteria were cultured in THY broth supplemented with 0.2 μg/mL erythromycin when appropriate and harvested in mid- to late-log phase, and aliquots containing 20% glycerol were snap-frozen and stored at −80°C. To determine stock colony-forming unit (CFU) concentrations, 10-fold serial dilutions of aliquots were plated on 5% sheep blood agar plates containing 25 μg/mL neomycin, supplemented when appropriate with 0.2 μg/mL erythromycin, and incubated at 37°C and 5% CO_2 for 24 h. Aliquots were thawed, washed with sterile phosphate-buffered saline (PBS), and diluted to desired concentrations for inoculation.

Infection model. Groups of 5–7 mice were infected via oropharyngeal aspiration [29] with 400 plaque-forming units (PFUs) of the H1N1 mouse-adapted influenza A/Puerto Rico/8/34 (PR8) influenza virus in 50 μL of PBS or received sham treatment with PBS. On day 6 after influenza virus or control inoculation, mice were infected via oropharyngeal aspiration with a mixture of 5×10^5 CFUs of D39 and 5×10^5 CFUs of $PspA^-$, Hyl^-, or $NanA^-$ mutant in 50 μL of PBS. The numbers of mutant and total bacteria in each inoculum were determined by selective and nonselective plating, respectively, as described above. Twenty-four hours after the pneumococcal infection, mice were killed, and lungs were lavaged with 5 mL of PBS. Lungs were removed, placed in recovered bronchioalveolar lavage fluid (BALF), homogenized, snap-frozen, and stored at −80°C. The numbers of mutant and total bacteria present in lung homogenates were again determined by selective and nonselective plating, as described above. Competitive index (CI) values were calculated by dividing the ratio of the recovered mutants to total bacteria in lung homogenates by the ratio of the mutants to total bacteria present in inocula, as described elsewhere [30]. To quantify PFUs, lung homogenates were serially diluted, and plaque assays were performed as described elsewhere [31].

Because insertion-duplication mutants are subject to reversion, in vivo stability was assessed by infecting mice with the individual Hyl^-, $NanA^-$, and $PspA^-$ mutants. Homogenates of lungs recovered after 24 h were serially diluted and plated on both selective and nonselective blood agar plates. Bacteria numbers obtained from selective plating were divided by those recovered from nonselective plating to assess increases in nonselected populations due to revertants. Mean values calculated for the Hyl^- (0.9904), $NanA^-$ (1.018), and $PspA^-$ (1.025) mutants were not significantly different than the expected value of 1 when analyzed by 1-sample t test ($\alpha = .05$), indicating that reversion rates were not confounding in vivo growth through 24 h.

Intranasal immunization with PspA. To assess the inhibition of primary and secondary pneumococcal growth by PspA immunization, mice under light isoflurane anesthesia received intranasal immunizations twice weekly for 3 weeks. Groups of 10–14 mice received 1 μg of recombinant N-terminal His-tagged PspA protein (rPspA/Rx1), provided by J. C. Paton, along with 4 μg of cholera toxin B subunit (CTB) (List Biological Laboratories) in 20 μL of sterile saline. Control groups received PBS or adjuvant alone. Mice receiving PspA were inoculated with adjuvant for 2 weeks and received only protein in the third week, and PBS and adjuvant control mice received only saline in the third week. Twenty days after immunization, mice in the 3 groups were divided into subgroups (5–7 mice) receiving either 400 PFUs of PR8 influenza virus or sham treatment. Six days after influenza virus or sham infection, all mice received 1×10^6 CFUs of D39, WU2, or TIGR4 pneumococci via intratracheal instillation. Twenty-four hours after inoculation of bacteria, BALF and lungs were harvested. Viral and bacterial loads in lungs were determined as described above. Titers of PspA-specific immunoglobulin G (IgG) and immunoglobulin A (IgA) in serum were determined by enzyme-linked immunosorbent assay (ELISA).

For ELISA, high-binding 96-well plates were coated with 1 μg/mL PspA in 0.05 mol/L carbonate buffer (pH 9.6) for 3 h at 37°C and then at 4°C overnight. Plates were washed 3 times with wash buffer (0.005% Tween 20 in PBS), and nonspecific binding sites were blocked with 5% nonfat dried milk in PBS at 37°C for 1 h. After 3 washes, 2-fold serial dilutions of serum samples in wash buffer were added and incubated at 37°C for 1 h. After 3 washes, alkaline phosphatase–conjugated isotype-specific anti-mouse secondary antibody (IgG, Sigma-Aldrich;

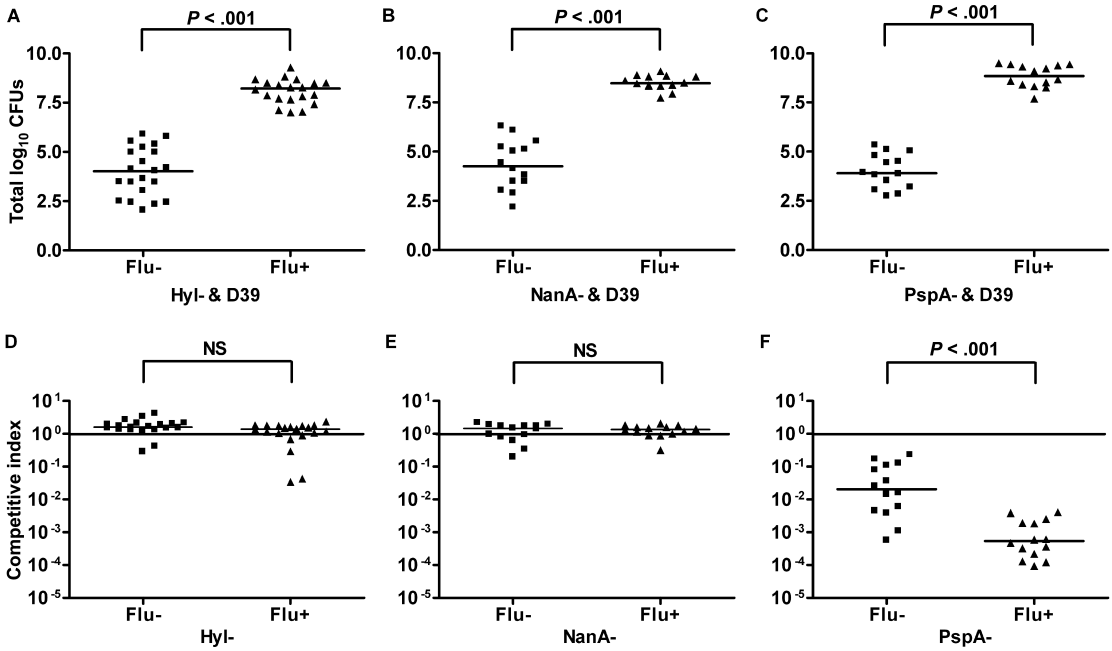

Figure 1. *A–C*, Total number of colony-forming units (CFUs) recovered from mouse lungs 24 h after infection with a hyaluronidase (Hyl^-) mutant and the parental strain D39 (*A*), a neuraminidase A ($NanA^-$) mutant and D39 (*B*), or a pneumococcal surface protein A ($PspA^-$) mutant and D39 (*C*). Mice received ~5×10^5 CFUs of D39 concurrently with ~5×10^5 CFUs of the Hyl^-, $NanA^-$, or $PspA^-$ mutant (1×10^6 CFUs in total) on day 6 after influenza virus infection (▲; Flu^+) or sham treatment (■; Flu^-). *D–F*, Competitive indices for the growth of the Hyl^- (*D*), $NanA^-$ (*E*), and $PspA^-$ (*F*) mutants from panels A, B, and C, respectively, in mice with (▲; Flu^+) or without (■; Flu^-) prior influenza virus infection. Horizontal bars represent group medians. Data presented were pooled from at least 2 independent replicates and were analyzed by the 2-tailed Mann-Whitney *U* test.

IgA, Serotec) was added and incubated at 37°C for 2 h. After 5 washes, *p*-nitrophenyl phosphate in diethanolamine buffer was added and incubated at 37°C for 30 min. End-point titers were defined as the highest reciprocal dilution exhibiting absorbance (read at 405 nm) >0.100 optical-density units above that for negative controls [32].

To evaluate the effects of PspA immunization on lung pathology, levels of albumin and lactate dehydrogenase (LDH) in recovered BALF were determined using commercially available kits (631-2, Sigma Diagnostics; CytoTox 96, Promega).

Statistical analyses. The 1-sample *t* test, the 2-tailed Mann-Whitney *U* test, 1-way analysis of variance (ANOVA) with the Bonferroni posttest, and 2-way ANOVA with the Bonferroni posttest were performed using GraphPad Prism software (version 4.00; GraphPad Software).

RESULTS

Competitive primary and secondary growth of the $PspA^-$, $NanA^-$, and Hyl^- mutants. Before administration of the mutant and D39 mixtures on day 6 after influenza virus and control inoculations, mice having previously received influenza virus exhibited symptoms and behavior consistent with influenza, including clustering, ruffled fur, and a wasted appearance, whereas control mice appeared to be symptom free. Influenza virus–infected mice had 1×10^6 to 1×10^7 PFUs in their lungs on day 7 after viral inoculation, whereas control mice revealed no detectable virus (data not shown). Median numbers of total bacteria recovered from mice with prior influenza virus infection were significantly greater than those recovered from mice without prior influenza virus infection for all 3 mixed mutant and wild-type inocula (figure 1*A*, 1*B*, and 1*C*), with increases ranging from 15,000-fold (Hyl^- and D39) to 29,000-fold ($PspA^-$ and D39).

Competitive growth is a common method for identifying fitness defects between coadministered bacterial strains [30, 33, 34]. To better resolve the interactions between pneumococcal virulence factors and prior influenza virus infection, we chose this method to normalize the lung deposition of mutant and wild-type strains in influenza virus–infected mice, given that the synergistic nature of this superinfection can amplify a small initial variation in numbers of bacteria. We calculated CI values as a measure of mutant fitness; thus, a CI value of 1 indicates that both strains grew equally well, and a CI value of 0.1 indicates a 10-fold reduction in the growth and/or survival of the mutant relative to that of the wild-type strain.

Median CI values for the Hyl^- mutant in mice with and those without prior influenza virus infection at 24 h were 1.39 and 1.6, respectively, indicating that the fitness of the mutant

was not attenuated relative to that of the wild-type strain (figure 1*D*). The difference between these CI values was not statistically significant ($P = .057$), suggesting that prior influenza virus infection did not affect the role played by this virulence factor. Similarly, the NanA$^-$ mutant exhibited median CI values of 1.37 and 1.43 in mice with and those without prior influenza virus infection, respectively, and thereby failed to exhibit attenuation (figure 1*E*). Again, the difference in the CI values for the NanA$^-$ mutant in mice with and those without prior influenza virus infection was not significant ($P = .982$). Contrary to the Hyl$^-$ and NanA$^-$ mutants, the PspA$^-$ mutant displayed attenuation in both mice with and those without prior influenza virus infection (figure 1*F*). The median CI value for the PspA$^-$ mutant in mice without prior influenza virus infection was 0.021, representing a 47-fold reduction in fitness relative to that of the D39 strain. The median CI value for the PspA$^-$ mutant in mice with prior influenza virus infection was 0.00053, representing a >1800-fold reduction in the relative growth of the PspA$^-$ mutant, which was highly significant compared with the growth of the PspA$^-$ mutant in mice without prior influenza virus infection.

Reduction in primary and secondary pneumococcal infections with the D39, WU2, and TIGR4 strains by PspA immunization. Mice receiving influenza virus again displayed visible symptoms on day 6 after infection and had 1×10^5 to 1×10^6 PFUs in their lungs, whereas control mice appeared healthy and did not have detectable virus (data not shown). PspA-specific IgG and IgA were detected in the serum of PspA-immunized mice but were not detected in the serum of control mice treated with PBS or adjuvant only (data not shown).

Twenty-four hours after primary D39 challenge, PspA-immunized mice exhibited statistically significant 15- and 14-fold reductions in mean bacterial titers compared with PBS- and CTB-treated control mice, respectively (figure 2*A*). In influenza virus–infected groups receiving secondary D39 challenges, PspA-immunized mice exhibited significant 35- and 53-fold reductions compared with PBS- and CTB-treated control mice, respectively (figure 2*A*). No significant differences were detected between PBS- and CTB-treated groups for either primary or secondary D39 challenges. Despite a significant reduction in secondary D39 infection in the PspA-immunized group, mean titers of bacteria for this group remained significantly higher than those for PBS-treated, CTB-treated, and PspA-immunized mice receiving primary D39 infection when analyzed by 1-way ANOVA with the Bonferroni posttest ($P < .001$ for all) (figure 2*A*).

Because albumin and LDH levels in BALF have been shown to be specific markers of lung pathology [35], they were measured to assess the mitigation of lung damage by PspA immunization 24 h after primary and secondary pneumococcal infection. Albumin concentrations and LDH activity in BALF

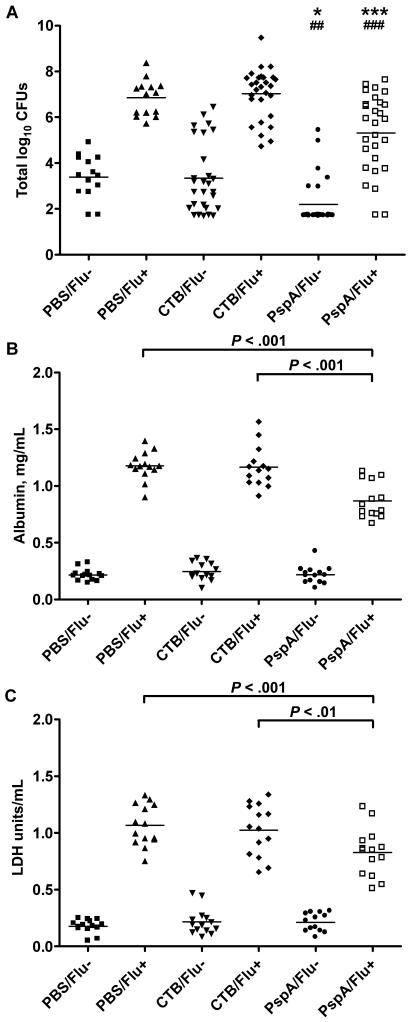

Figure 2. D39 bacteria titers (*A*) and concentrations of albumin (*B*) and lactate dehydrogenase (LDH) (*C*) recovered 24 h after pneumococcal challenge from the lungs of mice immunized intranasally with pneumococcal surface protein A (PspA). After immunization with phosphate-buffered saline (PBS) only, cholera toxin B subunit (CTB) only, or both CTB and PspA (indicated as PspA in the figure), mice received either 400 plaque-forming units of PR8 influenza virus (Flu$^+$) or sham treatment (Flu$^-$). Six days later, all mice received 1×10^6 colony-forming units (CFUs) of D39. Significance values for panel A are as follows: $^*P < .05$ for PspA/Flu$^-$ versus PBS/Flu$^-$; $^{***}P < .001$ for PspA/Flu$^+$ versus PBS/Flu$^+$; $^{\#\#}P < .01$ for PspA/Flu$^-$ versus CTB/Flu$^-$; $^{\#\#\#}P < .001$ for PspA/Flu$^+$ versus CTB/Flu$^+$. Values for CTB groups were not significantly different than those for PBS groups when compared between respective influenza virus treatments in panel A, B, or C. All Flu$^+$ groups yielded titers of bacteria and concentrations of lung-damage markers that were significantly higher at the $P < .001$ level than those of the Flu$^-$ groups within their respective immunization treatments in panels A, B, and C, with a single exception in panel C, where PspA/Flu$^+$ versus PspA/Flu$^-$ yielded a significance of $P < .01$. Horizontal bars represent group means. Data presented were pooled from at least 2 independent replicates and were analyzed by 2-way analysis of variance with the Bonferroni posttest.

recovered from mice receiving primary D39 infection were very low, suggesting that D39 infection alone does not cause extensive lung damage after 24 h. Thus, no significant differences were detected in albumin or LDH levels between groups receiving primary D39 infection (figure 2*B* and 2*C*). All mice challenged with D39 after influenza virus infection exhibited significantly increased levels of albumin compared with their respective immunization treatment group without prior influenza virus infection ($P < .001$ for all) (figure 2*B*). Similar results were observed for LDH levels (for PBS and CTB, $P < .001$; for PspA, $P < .01$) (figure 2*C*). Although levels of both lung-damage markers were pronounced during secondary D39 infection, PspA immunization significantly reduced levels of albumin (figure 2*B*) and LDH (figure 2*C*).

To determine whether PspA immunization reduces infections caused by strains of other serotypes, the immunization and challenge experiments were repeated for strains WU2 (serotype 3) and TIGR4 (serotype 4). PspA immunization significantly reduced titers of recovered bacteria 3100- and 2800-fold for primary WU2 infection compared with control treatment with PBS and CTB, respectively (figure 3*A*). PspA-immunized mice also exhibited significant 62- and 91-fold reductions in secondary WU2 titers relative to PBS- and CTB-treated control mice, respectively (figure 3*A*). No significant differences were detected between PBS- and CTB-treated control groups receiving either primary or secondary WU2 challenge. PspA-immunized mice receiving secondary WU2 challenge after influenza virus infection again exhibited significantly greater WU2 titers than did PBS-treated, CTB-treated, and PspA-immunized groups receiving WU2 infection alone when analyzed by 1-way ANOVA with the Bonferroni posttest ($P < .001$ for all) (figure 3*A*). Primary WU2 infections did not yield substantial albumin or LDH levels in recovered BALF, whereas secondary WU2 infections yielded significantly greater levels of both lung-damage markers ($P < .001$ for all) (figure 3*B* and 3*C*). PspA immunization resulted in a significant reduction in albumin concentration in mice receiving secondary WU2 challenge relative to both PBS- and CTB-treated control mice (figure 3*B*), with parallel results observed for LDH concentrations (figure 3*C*).

Primary infection with the TIGR4 pneumococcal strain was also found to be significantly reduced in PspA-immunized mice 135- and 424-fold compared with PBS-treated and CTB-treated control mice, respectively (figure 4*A*). PspA immunization significantly reduced lung burdens 12- and 6-fold in mice receiving secondary TIGR4 infection after influenza virus infection, compared with PBS-treated and CTB-treated control mice (figure 4*A*). No significant differences were detected between PBS- and CTB-treated control groups for either primary or secondary TIGR4 challenge. Again, 1-way ANOVA with the Bonferroni posttest revealed that PspA-immunized mice with prior influenza virus infection receiving secondary TIGR4 infection ex-

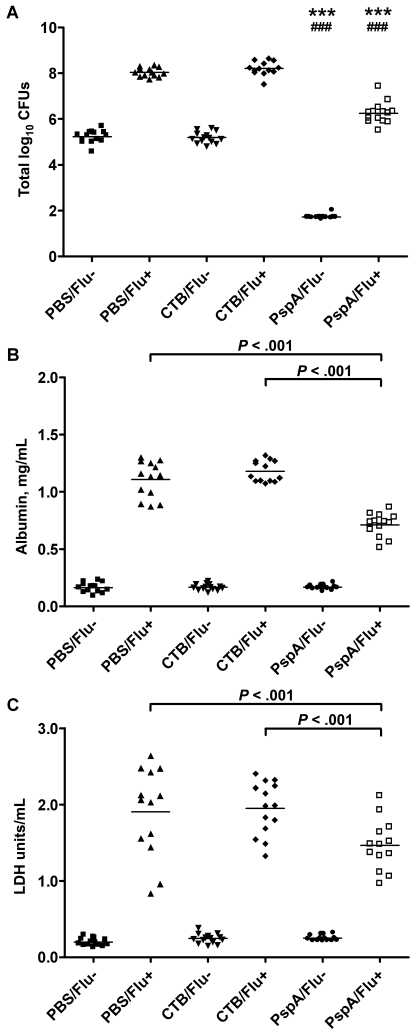

Figure 3. WU2 bacteria titers (*A*) and concentrations of albumin (*B*) and lactate dehydrogenase (LDH) (*C*) recovered 24 h after pneumococcal challenge from the lungs of mice immunized intranasally with pneumococcal surface protein A (PspA). After immunization with phosphate-buffered saline (PBS) only, cholera toxin B subunit (CTB) only, or both CTB and PspA (indicated as PspA in the figure), mice received either 400 plaque-forming units of PR8 influenza virus (Flu^+) or sham treatment (Flu^-). Six days later, all mice received 1×10^6 colony-forming units (CFUs) of WU2. Significance values for panel A are as follows: $^{***}P < .001$ for PspA/Flu^- versus PBS/Flu^- and for PspA/Flu^+ versus PBS/Flu^+; $^{\#\#\#}P < .001$ for PspA/Flu^- versus CTB/Flu^- and for PspA/Flu^+ versus CTB/Flu^+. Values for CTB groups were not significantly different than those for PBS groups when compared between respective influenza treatments in panel A, B, or C. All Flu^+ groups yielded titers of bacteria and concentrations of lung-damage markers that were significantly higher ($P < .001$) than those of the Flu^- groups within their respective immunization treatments in panels A, B, and C. Horizontal bars represent group means. Data presented were pooled from 2 independent replicates and were analyzed by 2-way analysis of variance with the Bonferroni posttest.

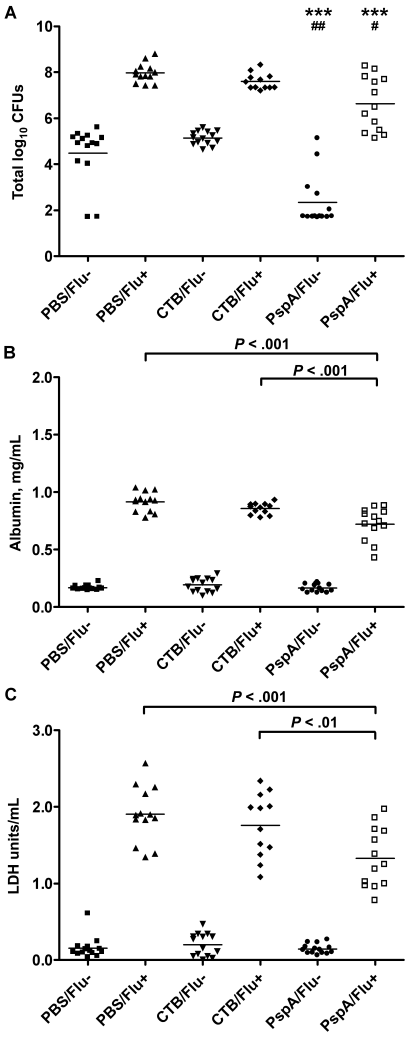

Figure 4. TIGR4 bacteria titers (*A*) and concentrations of albumin (*B*) and lactate dehydrogenase (LDH) (*C*) recovered 24 h after pneumococcal challenge from the lungs of mice immunized intranasally with pneumococcal surface protein A (PspA). After immunization with phosphate-buffered saline (PBS) only, cholera toxin B subunit (CTB) only, or both CTB and PspA (indicated as PspA in the figure), mice received either 400 plaque-forming units of PR8 influenza virus (Flu^+) or sham treatment (Flu^-). Six days later, all mice received 1×10^6 colony-forming units (CFUs) of TIGR4. Significance values for panel A are as follows: $^{***}P < .001$ for PspA/Flu^- versus PBS/Flu^- and for PspA/Flu^+ versus PBS/Flu^+; $^{\#\#}P < .01$ for PspA/Flu^- versus CTB/Flu^-; $^{\#}P < .05$ for PspA/Flu^+ versus CTB/Flu^+. Values for CTB groups were not significantly different than values for PBS groups when compared between respective influenza treatments in panel A, B, or C. All Flu^+ groups yielded titers of bacteria and concentrations of lung-damage markers that were significantly higher at the $P < .001$ level than those of the Flu^- groups within their respective immunization treatments in panels A, B, and C, with a single exception in panel C, where PspA/Flu^+ versus PspA/Flu^- yielded a significance of $P < .01$. Horizontal bars represent group means. Data presented were pooled from 2 independent replicates and were analyzed by 2-way analysis of variance with the Bonferroni posttest.

hibited significantly greater bacterial lung burdens than did PBS-treated ($P < .001$), CTB-treated ($P < .01$), and PspA-immunized ($P < .001$) mice receiving primary TIGR4 challenges (figure 4*A*). As with D39 and WU2, primary infection with TIGR4 did not induce substantial levels of albumin or LDH in recovered BALF; however, significant increases were observed when comparing influenza virus–infected mice receiving TIGR4 to mice receiving primary TIGR4 infection (for albumin, $P <$ .001 for all; for LDH, $P < .001$ for PBS and CTB and $P < .01$ for PspA) (figure 4*B* and 4*C*). Albumin levels in PspA-immunized mice were significantly reduced compared with those for PBS- and CTB-treated control mice receiving secondary TIGR4 infection (figure 4*B*), with similar results observed for LDH levels (figure 4*C*).

DISCUSSION

Influenza virus infection is known to denude respiratory epithelium, exposing pneumococcal ligands on the basement membrane [5], whereas bacterial hyaluronidases are believed to facilitate invasion through the targeting of hyaluronic acid, a constituent of the extracellular matrix [9, 36]. We hypothesized that herein may lay an opportunity for enhanced application of pneumococcal Hyl. A strong correlation exists between clinical isolates and Hyl production, specifically in pneumococcal meningitis [37]. A serotype 19F Hyl^- mutant is attenuated in a pneumonia model [38], and, although a serotype 6 Hyl^- mutant is attenuated in intraperitoneal infection [39], a D39 mutant was not [15]. Our results show that growth of the Hyl^- mutant of D39 in the lungs is not attenuated in mice with or without prior influenza virus infection, and, although these results do not indicate that Hyl contributes to secondary infection, they may support suggestions that the role that Hyl plays in virulence is serotype specific [38, 39].

The relative fitness of the $NanA^-$ mutant was not compromised in either primary or secondary pneumococcal infection. A lack of attenuation in the primary challenge was a surprise to us, given that NanA cleaves terminal sialic acid from host glycoconjugates decorating respiratory epithelium, where this action enhances colonization [27, 40]. We suspected that the contribution of pneumococcal NanA to colonization would be superseded by the influenza virus neuraminidase in secondary bacterial challenge but that this virulence factor might then reveal some novel application; however, secondary competitive growth of the $NanA^-$ mutant failed to support this hypothesis. $NanA^-$ mutants of D39 demonstrated little to no attenuation in intraperitoneal challenges [6, 15], in which colonization is not critical for infection. In intranasal challenges, NanA of serotype 2 strains is important for virulence in 2 reports [27, 28] but not in another [6]. It has been suggested that, among the methodological differences, the susceptibilities of the mouse

strains used might explain dissimilar results [6]. This may be the case in our use of C57BL/6 mice, rather than the BALB/c mice used by others. Furthermore, our results might also reflect our competitive growth model, in which the NanA$^-$ mutant and D39 strains were coadministered in a mixed inoculum. NanA of the wild-type D39 strain might modify the surface of the respiratory tract for both D39 and NanA$^-$ bacteria. If this is the case, then the competitive growth method may serve to better identify those *cis* virulence factors and potential vaccine targets, such as PspA, required of each individual bacterium.

PspA is well established as both a crucial virulence factor [12, 14–16] and a broadly protective antigen in a variety of immunization and challenge models [17–22]. Given the lower respiratory tract inflammatory response that accompanies influenza virus infection, we hypothesized that the function of this virulence factor in inhibiting complement-mediated clearance would become critical in a secondary pneumococcal infection. Fitness of the PspA$^-$ mutant was reduced 47-fold relative to D39 in the absence of influenza virus infection, in agreement with observations that PspA is required for pulmonary *S. pneumoniae* infection [14]. Additionally, in support of the notion that PspA makes a significant contribution to secondary pneumococcal infection, we observed an 1800-fold reduction in the relative growth of the PspA$^-$ mutant in mice with prior influenza virus infection.

PspA immunization experiments further supported the concept that PspA makes a significant contribution to secondary infection. Intranasal PspA immunization significantly reduced numbers of D39, WU2, and TIGR4 in the lungs of both mice with and those without prior influenza virus infection compared with control mice. Consistent with these results and a unique role for PspA in secondary infections, BALF levels of albumin and LDH were significantly reduced in PspA-immunized groups but not in PBS- or CTB-treated control mice infected with D39, WU2, or TIGR4 after influenza virus infection. Despite substantial sequence variability, both cross-reactivity and protection have been observed between immunized serum and heterologous PspA proteins [17, 41]. The rPspA/Rx1 (family 1, clade 2, and type 25) PspA antigen used in the present study has been shown to induce immune mouse serum that is cross-reactive with PspA proteins present on D39, WU2, and EF3296 pneumococcal strains [17], where the N-terminal region of the EF3296 PspA protein is identical to that of TIGR4 [42]. Additionally, it has been shown that immunization with the D39 type 25 PspA can provide protection against lethal challenges with the D39 and WU2 strains and perhaps some (albeit not statistically significant) protection against EF3296 [43]. Although this earlier work may support our observation that rPspA/Rx1 immunization reduces 24-h lung titers in primary and secondary infections with the D39, WU2, and TIGR4 strains, another study has specifically assessed this antigen with respect to protection against WU2 and TIGR4 [42]. In this prior study, the JAS218 PspA/Rx1 fragment elicited protection against lethal challenge with the family 1–bearing WU2 strain but not the family 2–bearing TIGR4 strain. The observations we present here may again reflect the different methodologies and end points used in that, although our model examines the ability of a PspA antigen to reduce pneumococcal infection in the respiratory compartment at a defined end point early in primary and secondary infections, the aforementioned study assessed this PspA antigen in its capacity to protect against a complex systemic disease outcome. Thus, although informative within the context of the narrow aim of our model, our observations may not be easily compared to those of studies examining protection against pneumococcal disease resulting from established infections, given the different temporal, spatial, virulence, and pathogenic scales under consideration. Current vaccines and vaccine candidates are assessed for their ability to protect against disease; however, our focus on a 24-h end point in this study reflects our belief that a vaccine developed to effectively prevent pneumococcal infection would ultimately serve to limit pneumococcal disease but may additionally serve to counteract the influenza virus–induced susceptibility to secondary pneumococcal colonization and infection.

PspA immunization was effective in limiting early primary and secondary infection, relative to that in control mice; however, immunization with this antigen was not sufficient to overcome influenza virus–induced susceptibility when comparing primary and secondary D39, WU2, and TIGR4 lung titers between PspA-immunized groups. Despite these findings, that pneumococcal growth in influenza virus–infected lungs can be significantly reduced by a targeted immune response regardless of virus-induced suppression of host defenses is encouraging. Furthermore, given previous observations [18, 20, 44], it is probable that combinations of different PspA families along with other pneumococcal virulence factors into a protein vaccine could, while protecting against a broad range of invasive serotypes, significantly increase protection against secondary pneumococcal infection after influenza virus infection.

Analyses of the 1918 Spanish influenza pandemic indicated that the high mortality associated with this pandemic was likely the result of increased susceptibility to secondary bacterial pathogens [45, 46]. We strongly feel that identification of pneumococcal vaccine candidates that offer protection against both pneumococcal infection alone and the significantly greater threat of pneumococcal infection after influenza virus infection may serve to reduce expected increases in morbidity and mortality attributable to secondary pneumococcal infection during what many believe to be inevitable future influenza pandemic events.

Acknowledgments

We sincerely thank Dr James C. Paton for providing the insertion-duplication *S. pneumoniae* mutants and recombinant PspA protein. We also thank Drs Susan Hollingshead, Edwin Swiatlo, and Elaine Tuomanen for sharing pneumococcal strains used in this research. We additionally thank all the members of the Harmsen laboratory who assisted in this work and Dr Jovanka Voyich-Kane for assistance in developing the manuscript.

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