Contributions to Protection from *Streptococcus pneumoniae* Infection Using the Monovalent Recombinant Protein Vaccine Candidates PcpA, PhtD, and PlyD1 in an Infant Murine Model during Challenge

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A vaccine consisting of several conserved proteins with different functions directing the pathogenesis of pneumonia and sepsis would be preferred for protection against infection by *Streptococcus pneumoniae*. Infants will be the major population targeted for next-generation pneumococcal vaccines. Here, we investigated the potential efficacy provided by three recombinant pneumococcal vaccine candidate proteins—pneumococcal histidine triad D (PhtD), detoxified pneumolysin derivative (PlyD1), and pneumococcal choline-binding protein A (PcpA)—for reducing pneumonia and sepsis in an infant mouse vaccine model. We found vaccination with PhtD and PcpA provided high IgG antibody titers after vaccination in infant mice, similar to adult mice comparators. PlyD1-specific total IgG was significantly lower in infant mice, with minimal boosting with the second and third vaccinations. Similar isotypes of IgG for PhtD and PlyD1 were generated in infant compared to adult mice. Although lower total specific IgG to all three proteins was elicited in infant than in adult mice, the infant mice were protected from bacteremic pneumonia and sepsis mortality (PlyD1) and had lower lung bacterial burdens (PcpA and PhtD) after challenge. The observed immune responses coupled with bacterial reductions elicited by each of the monovalent proteins support further testing in human infant clinical trials.

Infants will be the major population targeted for next-generation pneumococcal vaccines. Therefore, using an infant animal model to study vaccine candidates provides value for determining the efficacy of potential candidate pediatric vaccines for protection from disease. A vaccine that elicits antibodies to several conserved proteins with different functions during pathogenesis of *Streptococcus pneumoniae* would be desirable to mitigate against vaccine-induced selection of strains expressing variations in the selected proteins (1, 2). Here, we studied three recombinant pneumococcal vaccine candidate proteins, pneumococcal histidine triad D (PhtD), detoxified pneumolysin derivative (PlyD1), and pneumococcal choline-binding protein A (PcpA), to determine their roles in protection against pneumococcal pneumonia and sepsis in an infant mouse vaccine model. We sought to further define the individual contributions to the observed protection. By including parallel studies in adult mice, we also sought to determine similarities and differences in immune responses elicited in infant and adult mice after vaccination.

PhtD is a well-conserved surface protein and a member of the Pht protein family characterized as having a histidine triad motif, and it is regulated by the extracellular zinc concentration (1–3). In adult animal models, PhtD has been studied extensively against sepsis, pneumonia, and colonization, with protection levels that are highly bacterial-strain dependent and in many cases did not correlate with antibody titers (1, 2, 4–6). A two-subunit (PhtD and dPly [detoxified pneumolysin]) vaccine protected rhesus macaques from pneumonia and also led to better survival after challenge (7). Human antibodies to PhtD were reported to be functional in an adult murine passive-protection sepsis model, and a phase I exploratory study of PhtD vaccine showed it to be safe and immunogenic in human adults (8). Natural colonization, as well as infection, by *S. pneumoniae* can lead to antibodies directed against PhtD, but antibody levels to PhtD have not correlated with protection against disease (4, 9–11). A study of natural plasma antibodies against PhtD showed reduced adhesion of *S. pneumoniae* to lung epithelial cells *in vitro* (12), but it is not known whether a PhtD protein vaccination would produce similar protective antibodies in the lungs.

Pneumolysin (Ply) is a highly conserved, membrane pore-forming protein located in the cytoplasm but released into the medium during autolysis (13). Ply is a major virulence factor that exerts cytotoxic effects on epithelial cells and immune cells (13). Human antibodies to Ply can be detected in colonized or convalescent humans, and these antibodies can provide passive protection in challenged adult mice (14). However, due to its hemolytic activity, Ply needs to be detoxified, either genetically or chemically, for vaccination studies. Vaccines using Ply chemically modified to inactivate its hemolytic function have shown some level of protection in animal studies (5, 15–18), thus demonstrating that neutralization of Ply by antibodies may provide some protection against pneumonia and bacteremia. Recent development of a highly detoxified genetic mutant of Ply (PlyD1) has shown limited protection in mice against challenge with *S. pneumoniae* and lung injury (17). Phase I studies have demonstrated that PlyD1 is safe and immunogenic in adults (19). Natural *S. pneumoniae* colonization leads to lower Ply-specific plasma IgG levels in infants and young children than other *S. pneumoniae* proteins or in older
children (9). Therefore, the concentration of total specific IgG generated in response to Ply and the function after vaccination in infants are important to study further to better understand the efficacy of a potential trivalent vaccine containing the component.

Pneumococcal choline-binding protein A (PcpA) is distinct from another pneumococcal choline-binding protein named CbpA or PspC (20). The pcpA gene is conserved among different S. pneumoniae strains examined (21), and the PcpA protein is surface exposed (21) under the control of extracellular manganese concentrations (22). PcpA is not required for colonization of the murine nasopharynx (21, 22). Human antibodies to PcpA have been detected in infants and children with pneumococcal bacteremia or pneumonia (23). In monovalent vaccines containing PcpA, some level of protection from pneumonia was found, and a delay in morbidity after sepsis challenge was detected in an adult mouse model (21). The mechanism of this protection may be antibody mediated, as interference with adhesion by antibodies derived from colonized human hosts can block binding to lung-derived cell lines (12). A phase I study of PcpA in combination with PhtD showed that the bivalent vaccine was safe and immunogenic in human adults (24).

In the current study, we examined the protection provided by monovalent vaccination with recombinant PcpA, PhtD, and PlyD1 (designed from serotype 6B) from challenge with serotype 6A. This study is novel, as it is the first to describe the efficacy of the proteins in an infant model of vaccination and to provide direct comparisons of immune responses between infant and adult mice in a model system. Moreover, determining protection for each of these components in an anticipated trivalent vaccine is critical for future efficacy clinical trials in infants and children.

MATERIALS AND METHODS

Animals. Six-week-old male and female C57BL/6 mice were purchased from the NCI and housed in a specific-pathogen-free (SPF) biosafety level 2 (BSL2) murine facility at Rochester General Hospital Research Institute (RGHRI) using microisolator housing. C57BL/6 infant mice were obtained by breeding at RGHRI. All procedures were approved by the IACUC at RGH. Vaccination experiments were performed using five mice per group, with two or three separate replicates vaccinated at different times, as stated in the figure legends.

Vaccinations. Recombinant PcpA, PhtD, and PlyD1 proteins, constructed from a serotype 6B S. pneumoniae strain, were obtained from Sanofi Pasteur. Protein doses previously described as optimal in BALB/c mice (25) and determined to be optimal in our preliminary work in C57BL/6 mice were combined with aluminum hydroxide (alum) as an adjuvant. Unvaccinated controls received alum alone. Injections (25 μl) into both caudal muscles of the hind legs were performed, with three vaccinations on an accelerated weekly schedule, due to the rapid aging of infant mice (Fig. 1).

S. pneumoniae challenge. S. pneumoniae BG3722, a serotype 6A strain, was obtained from Sanofi Pasteur and expanded from streaked plates of Todd-Hewitt broth (THB) with 1% yeast extract (Difco). Expanded S. pneumoniae (optical density at 600 nm [OD600] = 0.6) was centrifuged, resuspended to 1 × 10⁶ CFU/ml in THB with 10% glycerol, and frozen at −80°C. On the day of the challenge, S. pneumoniae was thawed and grown in THB with 1% yeast extract at 37°C with 5% CO₂ until mid-log phase (OD600 = 0.6). The bacteria were then centrifuged, washed twice with phosphate-buffered saline (PBS), and resuspended at 25 × 10⁸ CFU/ml in PBS with dilution plating to confirm the number of CFU. Mice were anesthetized with isoflurane, and 40 μl of bacterial challenge stock (1 × 10⁶ CFU; 2 times the 50% lethal dose [LD₅₀]) was instilled into the nasal passages of each mouse.

Antibody assays. Serum was obtained after each vaccination by tail bleeding (Fig. 1) and after S. pneumoniae challenge by cardiac puncture following euthanasia. Recombinant proteins, obtained from Sanofi Pasteur, were plated on Immulon II enzyme-linked immunosorbent assay (ELISA) plates (ThermoFisher, Hampton, NH) at 0.5 μg per well overnight at 4°C. The plates were then blocked with nonfat milk, and sera, as well as reference sera (obtained from vaccinated mice with known antibody concentrations), were assayed with a secondary rabbit anti-mouse alkaline phosphatase (AP) (Jackson ImmunoResearch, West Grove, PA) at 1:10,000 dilution. The total IgG for each serum was calculated from a standardized curve and a reference serum. Endpoint titers for total IgG or IgG1, IgG2a, IgG2b, IgG2c, and IgG3 were performed for each antigen with specific donkey secondary antibodies AP (Jackson ImmunoResearch) at 1:10,000 dilution.

Lung bacterial counts. CFU were determined by dilution plating from clarified lung extracts (48 h postchallenge) processed with a mortar and pestle and resuspended in 1 ml of PBS onto TSA II plates containing gentamicin (BD Biosciences, San Jose, CA). The plates were incubated overnight at 37°C.

Antibodies and reagents. Fluorochrome-conjugated anti-CD3, CD11b, CD11c, GR-1, CD45, F4/80, and major histocompatibility class I/II (MHC-1/II) were obtained from BioLegend (San Diego, CA).

Flow cytometry. Lungs (perfused by PBS) from 48-h postchallenge infected mice underwent collagenase digestion (1 h at 37°C in CDTI medium containing 1 mg/ml collagenase, 1 mg/ml DNase, 1 mg/ml trypsin inhibitor) and were passed through a 100-μm cell strainer and washed 2 times with PBS. This was followed by lysis with ammonium-chloride-potassium (ACK) solution (5 min at room temperature) to remove red blood cells (RBCs). The cells were then washed 2 times with PBS. The remaining cells were surface stained with fluorochrome-conjugated antibodies, fixed, and acquired using an LSRII flow cytometer (BD Biosciences) with a minimum acquisition of 300,000 events. Analysis of acquisition events was accomplished using FlowJo software (Tree Star, Portland, OR) with gating using live amine dye (Invitrogen, Carlsbad, CA) and double discrimination with CD3⁺ dump gating.

Histology. Lungs were obtained from mice 48 h postinfection after vascular perfusion with PBS. The lobes were clamped at the bronchioles and perfused with 4% buffered formalin. Sections (5 μm) were cut and stained with H&E. Images were processed with Adobe Photoshop (San Jose, CA) with auto levels selected to automatically adjust brightness, color balance, and contrast for all images. Influenza virus infection leads to significant infection of the large left lobes (data not shown), and therefore, we assumed equal distribution of S. pneumoniae into the large left lobes, since inoculation techniques were similar.

Absolute counts. Lungs were processed as previously stated using CDTI and processed over a Ficoll gradient. Cell pellets containing macrophages and granulocytes were lysed with ACK as described above. Cell counts were obtained using a cell counter (Bio-Rad, Hercules, CA), and absolute numbers were determined by calculation from data obtained by flow cytometric analysis of macrophage (GR1⁻ F4/80⁻) percentages.

FIG 1 Infant mice were injected at the times shown with monovalent vaccines containing PcpA, PhID, or PlyD1. Injections were into both hind leg caudal muscles (25 μl per muscle). Four weeks (wks) after the third vaccination, the mice were challenged by intranasal instillation with S. pneumoniae.

Vaccination and Challenge Schedule

| Infant Mice | Adult Mice |
|-------------|------------|
| 7 days old  | 6wks       |
| 14 days     | 7 wks      |
| 21 days     | 8 wks      |
| 7 wks       | 12 wks     |

Infect | Inject | Bleed | Inject | Bleed | Inject | Bleed | Infect |

| Infant Mice | Adult Mice |
|-------------|------------|
| 7 days old  | 6wks       |
| 14 days     | 7 wks      |
| 21 days     | 8 wks      |
| 7 wks       | 12 wks     |

Infect | Inject | Bleed | Inject | Bleed | Inject | Bleed | Infect |

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Bacterial adhesion assay. For monovalent PlyD1 studies, *S. pneumoniae* BG3722 was grown in Todd-Hewitt broth–1% yeast extract. For PhtD and PcpA studies, *S. pneumoniae* was grown in Todd-Hewitt broth–1% yeast extract that was previously chelated with Chelex beads (Sigma) overnight with shaking and then supplemented with 1/1000 M MgCl₂, 1/1000 M MgSO₄, and 1/1000 M CaCl₂. In all studies, bacteria were grown to an OD₆₀₀ of 0.6, washed extensively with PBS followed by staining of 10⁸ bacteria in 1 mg/ml fluorescein isothiocyanate (FITC) (Sigma) at 4°C for 1 h, and then washed again with PBS. Approximately, 10⁶ *S. pneumoniae* cells were incubated with antibodies from vaccinated mice that were diluted 1:10 in 10% Dulbecco's modified Eagle's medium (DMEM), with or without prior chelation and supplementation (as described above), for 1 h at 37°C in the presence of 1 μg/ml guinea pig complement (Fisher). Bacteria were transferred onto primary lung epithelial (type II) or endothelial cells (Cell Biosystems), previously grown to 95% confluence in 8-well chamber slides, and incubated for 5 h. The cells were washed extensively 3 times with PBS and then fixed with PBS, and then washed again with PBS. Approximately, 10⁶ *S. pneumoniae* cells were incubated with antibodies that were generated in infant and adult mice vaccinated with PlyD1 vaccine. *S. pneumoniae* was then added to primary epithelial and primary endothelial lung cells and incubated at 37°C for 5 h. The cells were washed 3 times with PBS, and Sytox Orange (Invitrogen) was added at 0.1 μl/ml in PBS and incubated for 5 min, followed by washing 3 times with PBS and then fixation, mounting, and imaging as described above. Quantification was performed similarly to that for the adherence assay described above.

Statistics. Data were analyzed by two-tailed Student t tests or analysis of variance (ANOVA). Results with a P value of <0.05 were considered significant. Data were analyzed with Prism software (Graph Pad, La Jolla, CA).

RESULTS

Vaccination dosage optimization. In preliminary experiments, an optimal vaccine dose of each individual protein, PcpA (0.2 μg), PhtD (0.9 μg), and PlyD1 (5 μg), in infant and adult C57BL/6 mice was established by measuring antibody responses at 4 weeks post-tertiary vaccination (data not shown). We investigated a range of antigen dosages around those previously determined to be optimal in CB mice (personal communication from Sanofi Pasteur) and found the optimal doses (maximal IgG titers) to be similar, but not identical, in infant and adult mice for all three proteins. Alum was included as an adjuvant in all experiments described here because it is approved for use in humans and clinical trials and is included in current clinical trials with these proteins (19, 24).

Discordant antibody generation in infant and adult mice. Since protection from *S. pneumoniae* bacteremia has been correlated with seroconversion to surface proteins PcpA and PhtD (11), we assessed the total specific serum IgG concentrations for PcpA,
PhtD, and PlyD1 in monovalent injections at 7 days post-primary and post-secondary vaccinations and 4 weeks post-tertiary vaccination (Fig. 2A). We found comparable levels of IgG in infant and adult mice vaccinated with PcpA and PhtD, and they showed similar increase trends after each vaccination. PlyD1-specific total IgG was significantly lower in infant mice, with minimal boosting with the second and third vaccinations, a finding similar to that seen in young children, who elicit only low IgG responses to Ply (the native form of pneumolysin) during natural exposure (26).

We next assessed the IgG isotype responses (endpoint titers) in the mice, since isotype antibodies direct different functions (27). We used IgG2a as a negative control, as C57BL/6 mice do not make this isotype and no IgG2a antibody was detected for any of the antigens (Fig. 2B). Both infant and adult mice showed higher levels of IgG1 than of other IgG isotypes (Fig. 2B) for all antigens, and the levels were higher in adult than in infant mice. The antibody responses to PcpA in adult mice were significantly higher for IgG3 and IgG1 than in infant mice. Similar titers of IgG2b but no detectable titers of IgG2c were measured. PhtD-vaccinated infant and adult mice had nonsignificant differences in IgG2b, IgG2c, and IgG3 titers, but adult mice showed significantly higher IgG1 titers. Antibody responses to PlyD1 were similar in both infant and adult mice for IgG2b and IgG2c but showed a trend of higher IgG1 and IgG3 titers in adults. Given that IgG1 endpoint titers were the highest IgG isotype among all the S. pneumoniae antigens, suggesting that IgG1 was the major induced antibody, there were greater differences between infant and adult mice vaccinated with PcpA or PhtD than between those vaccinated with PlyD1.

**Analysis of the bacterial lung load, bacteremia, and lung histopathology of mice vaccinated with monovalent PcpA, PhtD, or PlyD1 and then challenged.** We assessed the protective capacity of each protein by measuring the bacterial counts in the lungs of challenged infant and adult mice. Vaccination with PcpA led to a 2-log-unit reduction in the lung burden 48 h after challenge in infant mice and a 2.3-log-unit reduction in adult mice (Fig. 3A). PhT1 vaccination led to a smaller but significant reduction (0.83 log unit) in lung bacterial counts in both infant and adult mice (Fig. 3B). Vaccination with PlyD1 showed no reduction in lung bacteria in adult or infant mice (Fig. 3C). However, when we analyzed bacteria in the blood, only PlyD1-vaccinated mice demonstrated a statistically significant reduction in bacteremia in both infant and adult mice (Fig. 3D), suggesting that immune responses elicited by PlyD1 vaccination prevent transmigration of bacteria from the lungs into the bloodstream.

We compared the degrees of lung histopathology in vaccinated infant and adult mice 48 h postchallenge, as shown in Fig. 4. We found that vaccination with PcpA and PhT1 led to similar levels of lung histopathology in both infants (Fig. 4C and D) and adults (data not shown) compared to unvaccinated infected control mice (Fig. 4B). In agreement with a previous study (17), vaccination with PlyD1 led to a significant reduction in lung histopathology in infant (Fig. 4E) and adult (Fig. 4F) mice that appeared very similar to that in uninfected controls (Fig. 4A). Using a histopathologic scoring system with higher numbers reflecting greater lung tissue damage (17), we found that PlyD1-vaccinated infant and adult mice had the best histopathologic scores after challenge (Fig. 4G).

We tested survival after challenge and found that each vaccinated group had increased survival times that were better for PhT1- and PlyD1-vaccinated mice (Fig. 4H to J). For all vaccinees, adults had longer survival times than infants, except for PcpA-vaccinated mice. Interestingly, we found 30% survival of PlyD1-vaccinated adult mice.
Antibodies containing both PcpA and PhtD reduce \textit{S. pneumoniae} adherence to lung epithelial cells, while antibodies to PlyD1 neutralize epithelial damage. To assess a potential protective role in preventing \textit{S. pneumoniae} adherence to the lung epithelium, we performed an \textit{in vitro} adherence reduction assay using sera obtained from vaccinated mice (Fig. 5A to F). We found abundant adherence of \textit{S. pneumoniae} to lung epithelial cells in the absence of \textit{S. pneumoniae}-specific antibodies (Fig. 5B). We found moderately lower adherence with antibodies directed to PcpA (Fig. 5C and D). In contrast, we found much lower adherence with antibodies to PhtD (Fig. 5E and F) in vaccinated infant and adult mice that might correlate with better survival rates (Fig. 4I). Quantification of \textit{S. pneumoniae} in the presence or absence of antibodies raised in PlyD-vaccinated mice clearly demonstrated a significant reduction in cell damage (Fig. 4G).

To determine whether antibodies generated to PlyD1 could protect against epithelial and endothelial damage from the toxicity of native Ply and thus prevent potential translocation of \textit{S. pneumoniae} into the lung vasculature, we performed an \textit{in vitro} neutralization assay using primary lung cells (Fig. 5H and I). We found that primary lung cells incubated with antibodies derived from PlyD1-vaccinated infant and adult mice had significantly less cell damage after 5 h of incubation in the presence of \textit{S. pneumoniae}. Quantification of the damaged epithelial cells cultured with or without antibodies raised in PlyD-vaccinated mice in the presence of \textit{S. pneumoniae} clearly demonstrated a significant reduction in cell damage (Fig. 5J).

Increased macrophage infiltration after challenge. We examined the cell types infiltrating the lungs of postchallenge vaccinated mice by immunohistochemistry and found no difference in staining (number or distribution) between infant and adult mice (data not shown). We found no significant differences in the percentages of pulmonary macrophages (CD45\(^+\) GR-1\(^{lo/hi}\) F4/80\(^+\) MHC-I\(\alpha/\beta\) CD11b\(^+\)) or inflammatory monocytes (CD45\(^+\) GR-1\(^{hi}\) CD11b\(^{hi}\) MHC-IA/IE\(\alpha/\beta\)) for any of the postchallenge vaccinated mice compared to unvaccinated infected controls. Figure 6A shows a representative flow dot graph of pulmonary macrophages from the lungs of PhtD-vaccinated and challenged mice. Compared to uninfected controls, we observed increases in pulmonary macrophages and inflammatory monocytes in infected controls and in PcpA- and PhtD-vaccinated and challenged mice. PlyD1-challenged mice showed only a slight increase in macrophages compared to uninfected controls (Fig. 6B). Within each of the different groups, we observed similar numbers of infiltrating mac-
Trophages in both the infant and adult mice after challenge (Fig. 6B). We did not observe a difference between vaccinated and unvaccinated infected controls with respect to polymorphonuclear cells (GR1+/H11001 F4/80+/H11002 MHC-II+/H11002) at 48 h postinfection (data not shown).

DISCUSSION

A multicomponent protein-based vaccine that protects from all S. pneumoniae strains would be a significant advance (28). Here, we investigated the protective contributions of three S. pneumoniae proteins, PcpA, PhtD, and PlyD1, as candidate vaccine antigens in an infant murine challenge model, and we made direct comparisons of immune responses in infant and adult mice. We tested the protection afforded by the monovalent vaccines against a type 6A challenge strain, as the serotype was reproducibly lethal in C57BL/6 mice. The genetic background of the mice is important in studying pneumococcal pathogenesis, as CBA mice tend to be very susceptible to S. pneumoniae lethality, BALB/c mice are fairly resistant, and C57BL/6 mice are intermediate, and the degree of granulocyte activation by S. pneumoniae dictates the level of subsequent pathogenesis (29, 30). We utilized the challenge strain BG3722 due to its reproducible lethality in C57BL/6 mice. We considered testing serotype 6B in the animal model to initially explore immune responses to identical amino acids as the vaccine antigens, but the serotype is not lethal in C57BL/6 mice. Therefore, we chose a strain that has significant pathology in C57BL/6 mice and has been used by Sanofi Pasteur for their companion studies of these proteins.

Using monovalent vaccines, infant and adult mice showed similar increases in IgG antibody-specific titers to the vaccinated antigens, except for PlyD1, where infant mice elicited minimal titers. Subtyping of IgG-specific antibody responses demonstrated dominance of IgG1 antibodies. The dominance of IgG1 antibodies implies a strong Th2 or T follicular helper cell (TFH) CD4 T-cell memory response. An accelerated vaccine schedule of a primary and two boost vaccinations separated by 1 week was used in this study due to the rapid aging of the infant mice. Interestingly, the isotypes of IgG generated toward PcpA differed between infants and adults, where infants failed to generate IgG3 after vaccination and neither infant nor adult mice generated a detectable IgG2c response. The somewhat higher titers of IgG2c in PhtD- or PlyD1-vaccinated adult mice may indicate that Th1 memory development is more developed in adult mice, as suggested by other stud-
Th1 CD4 T-cell memory can influence the quality of the innate immune response, and therefore, the activity of innate cells could be altered by the higher potential memory in adult mice (31). Importantly, IgG1, IgG2b, and IgG3 have been shown to be equally protective against phosphocholine antigens, suggesting the broad class of isotypes elicited to these proteins could be additive for protection (32).

Lung bacterial loads were reduced with monovalent vaccines in both infant and adult mice. Mice given a lethal dose of *S. pneumoniae* 4 weeks after vaccination with monovalent PcpA vaccine showed ~2-log-unit reduction in the bacterial lung load, while PhtD-vaccinated mice showed ~0.5-log-unit reductions, and PlyD1-vaccinated mice showed no reduction. Our results are in agreement with previous studies that showed protection from *S. pneumoniae* challenge after vaccination with PcpA and PhtD in adult mice (2, 21, 33). Unlike the bacterial lung load, PlyD1 showed greater reduction in bacteremia than the other two monovalent vaccines. Ply expression is required for high levels of bacteremia (34), in part suggested by its ability to cooperate in degrading C3b on the bacterial surface (35), and we observed significantly lower levels of bacteremia in mice vaccinated with PlyD1. It has already been reported that PlyD1 vaccination prevents lung histopathology after lethal challenge by limiting lesions and inflammation in adult mice (14, 17, 36). We observed a reduction in bacteremia in PlyD1-vaccinated infant and adult mice that may result from better protection of the lung epithelium from the toxic effects of Ply and subsequent reduction of bacterial dissemination into the capillaries that line the epithelial barrier in the alveolar space. In fact, Ply is necessary for establishment of acute sepsis and high-titer replication in the blood (34, 37). Of interest, even with lower antibody titers directed to PlyD1 in infants than in adults, tissue histopathology was limited, suggesting that low titers of antibody could still neutralize the damage to lung epithelial cells caused by Ply. The difference in antibody titers between total specific antibody and isotypes most likely is due to the differences in precision between the two assays, with higher precision in total specific IgG assays.

We focused on early antibody-mediated protective responses. For our analysis of absolute counts, we took into account the total cellularity in the lungs of each mouse. However, we found no significant differences in absolute counts between PcpA- and PhtD-vaccinated mice and infected controls and infer that the lungs of these mice might have been equally inflamed, signaling strong polymorphonuclear and monomorphonuclear cell recruitment. What effects these vaccines have on activity is a subject for future studies if a reliable protein antigen opsonophagocytic killing assay (OPA) is developed. The lower recruitment of mononuclear cells in PlyD1-vaccinated mice may be due to significantly lower inflammation or protection by resident alveolar phagocytic function. Our assay conditions did not detect intracellular versus extracellular bacteria, and thus, it is possible that the bacteria we detected may derive in part from phagocytized *S. pneumoniae* bacteria. We intend to follow up these studies with additional characterization of these mechanisms.

We postulate that the levels of protection, albeit limited, afforded by each vaccine antigen may derive from two key factors. First, vaccination with PlyD1 leads to neutralization of Ply that in turn limits damage to the lung epithelium, which allows *S. pneumoniae*...
moniae entry into the vasculature and initiation of bacteremia and lethal sepsis, as stated above. Neutralizing Ply could also affect the level of virulence by limiting Ply-mediated complement depletion (38), prevention of phagocytosis by autolysis (39), and reduction in polymorphonuclear cell antibacterial properties (40). However, neutralizing Ply could reduce bacteremia (37), but the observed death in our PlyD1-vaccinated mice may indicate a reduction in bacteremia without a reduction in sepsis. Second, antibodies to PcpA and PhdT prevent binding of S. pneumoniae to airway epithelium, and here, we observed prevention of binding to primary epithelial cells for the first time. Previous studies have demonstrated a correlation between the levels of antibodies to PcpA (in conjunction with Ply) and reduction in pneumonia (23). Antibodies directed to PcpA and PhdT also appear to correlate with a reduction in bacteremia (11). We observed some reduction in bacteremia (trends) with vaccination with these two antigens. In contrast to another study (21), we did not observe complete protection with PcpA vaccination alone, indicating that a multicomponent vaccine may be necessary. Importantly, however, we contend that prevention of S. pneumoniae binding to epithelial cells may allow greater mucociliary clearance, an important protective response for S. pneumoniae (41), as observed with the reduced attachment and lower lung burdens. We did not observe an increase in macrophages/monocytes or polymorphonuclear cells in PcpA- or PhdT-vaccinated mice over infected controls. However, we did not determine whether vaccination with either of these antigens could affect their opsonophagocytic activity or if protection by the antigens is related more to mucociliary rates through reduction in attachment. PlyD1 vaccination to prevent epithelial cell death, along with vaccine antigens to prevent attachment, could protect the mucociliary potential of the lungs. Our data suggest that vaccines with PhdT or PcpA alone will not be able to prevent significant cellular recruitment to the lungs and pneumonia. Our data suggest that vaccines that also contain PlyD1 will protect against pneumonia by reducing the level of inflammation in the lungs in conjunction with reductions of bacteria due to antibodies to PhdT and PcpA. These two mechanisms are targets for future studies, especially if a reliable assay for protein antigen opsonophagocytosis can be developed.

In summary, we have shown that vaccination with monovalent protein-based vaccines containing PcpA, PhdT, and PlyD1 can contribute to the level of bacterial reduction in both infant and adult mice after a lethal-dose heterologous challenge. This study highlights the interplay necessary between vaccine-induced antibodies for direct neutralization of toxic virulence factors to prevent epithelial damage and bacteremia and recruitment of innate cells to the lungs. We are actively pursuing additional antibody-based assays to characterize the protection, and in future studies, we hope to isolate and transfer these antibodies and observe the effects on protection from challenge. Future studies to explore additional mechanisms of protection afforded by these vaccine proteins in infant mice are warranted.

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