Viability and Virulence of Pneumolysin, Pneumococcal Surface Protein A, and Pneumolysin/Pneumococcal Surface Protein A Mutants in the Ear

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IMPORTANCE Understanding how pneumococcal proteins affect the pathology of the middle ear and inner ear is important for the development of new approaches to prevent otitis media and its complications.

OBJECTIVES To determine the viability and virulence of Streptococcus pneumoniae mutants deficient in pneumolysin (Ply−) and pneumococcal surface protein A (PspA−) in the chinchilla middle ear.

DESIGN Bullae of chinchillas were inoculated bilaterally with wild-type (Wt), Ply−, PspA−, and Ply−/PspA− strains. Bacterial colony-forming units (CFUs) in middle ear effusions were counted at 48 hours. The CFUs of the PspA− group were also counted at 6 to 36 hours after inoculation. Temporal bone histopathological results were compared.

SETTING AND PARTICIPANTS Twenty-seven chinchillas in an academic research laboratory.

EXPOSURE Chinchilla middle ears were inoculated with S pneumoniae to produce sufficient volumes of effusions and noticeable histopathological changes in the ears.

MAIN OUTCOMES AND MEASURES The CFU counts in the middle ear effusions and histopathological changes were compared to determine the effect of pneumococcal protein mutations on chinchilla ears.

RESULTS At 48 hours, CFUs in middle ears were increased for the Wt and Ply−/PspA− strains, but Ply− remained near inoculum level. No bacteria were detected in the PspA− group. The CFUs of PspA− decreased over time to a low level at 30 to 36 hours. In vitro, PspA− in Todd-Hewitt broth showed an increase in bacterial growth of 2 logs at 43 hours, indicating PspA− susceptibility to host defenses in vivo. The PspA− and Ply− groups had fewer pathologic findings than the Wt or Ply−/PspA− groups. Histopathological analysis showed significant differences in the number of bacteria in the scala tympani in the Wt group compared with the Ply−, PspA−, and Ply−/PspA− groups. The PspA− strain was the least virulent.

CONCLUSIONS AND RELEVANCE The PspA− mutant was much less viable and less virulent in the ear than the Wt, Ply−, and Ply−/PspA− strains. There was no significant attenuation in the viability and virulence of the Ply−/PspA− mutant compared with the Wt or single mutants. The viability and virulence of pneumococcal mutants seemed to be protein and organ specific.

titis media is the most common indication for the pre-
scription of antibiotics in the United States, and pneu-
mococci are responsible for 30% to 50% of otitis me-
dia cases in patients. Bacterial resistance to antibiotics has
necessitated the search for alternative methods for the
prevention and treatment of acute otitis media. Although pneu-
mococcal capsular polysaccharide vaccines are available, the
current pneumococcal vaccine for children elicits antibody
against a limited number of common serotypes. Furthermore,
serotype replacement has become a serious problem. In otitis
media vaccine trials, the vaccine groups had 33% more
episodes of otitis media caused by serotypes not included in
the vaccine. Newer vaccine approaches have been devel-
oped that are based on the use of conserved external surface
proteins, such as pneumococcal surface protein A (PspA) and
PspC. Pneumococcal proteins, alone or in combination, are
being investigated as potential vaccine candidates. Pneu-
ccoccal surface protein A is a surface-bound protein that
can bind to apolactoferrin to prevent its killing. It has been shown
to interfere with complement deposition on pneumococci, re-
ducing opsonization and clearance of bacteria by the host im-
mune system. Pneumolysin (Ply) is a pore-forming protein that
is cytotoxic and cytolytic. Pneumolysin can activate the classic complement
pathway and prevent complement deposition on Streptococ-
cus pneumoniae. Other activities of Ply include promotion of
inflammation, impairment of leukocyte function, inhibition
of epithelial ciliary movement, and disruption of respiratory
epithelial tight junctions. Both PspA and Ply are expressed in
most pneumococcal serotypes, making them good vaccine
candidates.

It was previously demonstrated that the pneumococcal
mutant deficient in PsPA was less virulent in the middle ear
than a mutant deficient in Ply. In this study, we compared the
viability and virulence of the Ply-deficient (Ply−) and PsPA-
deficient (PsPA−) single mutants, the Ply−/PsPA− double mu-
tant, and their wild-type (Wt) parent strain D39 (serotype 2)
in the middle ear and inner ear following inoculation in the
chinchilla middle ear, as well as in vivo bacterial growth to as-

Methods

The Institutional Animal Care and Use Committee of the Uni-
versity of Minnesota approved the care and use of the ani-

mals in these experiments. Streptococcus pneumoniae sero-
type 2 strain D39 (National Collection of Type Cultures 7460) was the Wt used in our experiments and the parent strain for our
mutants. This strain and its isogenic Ply−, PsPA−, and Ply−/
PsPA− mutants have been described previously.

Bacteria were grown in Todd-Hewitt broth (BD Diagnos-
tics) containing 0.5% added yeast extract (BD Diagnostics)
(hereinafter THB) plated on sheep blood agar plates and stored
in 10% glycerin solution at −80°C. Mutants were grown on
sheep blood agar plates and in THB, both containing 0.3 μg/mL
of erythromycin. Bacteria were grown until they were in log
phase. Their optical densities were measured at 660 nm on a
spectrophotometer, and they were diluted to the desired con-
centration in phosphate-buffered saline. Ten-fold dilutions
were plated, and viable cells were counted to confirm the ac-
tual concentration. The time-dependent viability of the PsPA−
mutant was also studied in THB at room temperature at 0 to
43 hours after inoculation. Strains were confirmed as S pneu-
mococci by optochin sensitivity and production of serotype 2
capsule using specific antisera as described previously. The presence of Ply and PsPA in all strains was analyzed from ly-
sate preparations by Western blot using specific antisera as pre-
viously described.

All animals were anesthetized before bacterial inocula-
tion with 0.25 mL of a combination of ketamine hydrochlo-
ride (100 mg/kg) and acepromazine maleate (10 mg/kg). Chin-
chilla middle ears were inoculated with 0.5 mL of bacteria as
follows: 7 chinchillas with 1 × 10^6 colony-forming units
(CFU)/mL of Wt, 7 chinchillas with 6.6 × 10^5 CFUs/mL of Ply−,
6 chinchillas with 3.5 × 10^5 CFUs/mL of PsPA−, and 7 chinchil-
las with 1.3 × 10^6 CFUs/mL of Ply−/PsPA−. We chose high in-
ocula and intrabulbar inoculation based on previous studies. We
knew that this method and inoculum would result in suf-
ficient volumes of middle ear effusions (MEEs) and notice-
able histopathological changes in most ears in the Wt strain
and the protein-deficient mutants.

Bullae were removed, and MEEs were harvested for bac-
terial counts. Cochleae from all groups were embedded in ep-
oxy resin, sectioned at a thickness of 1 μm, and stained with
toluidine blue. The round window membranes (RWMs) were
biseected, and one side was randomly selected for histopatho-
logical evaluation. Images of the RWMs were obtained at ×1000
magnification at the center of the sample and at 1 mm to the
right and left of the center. Images of the scala tympani (ST)
were obtained immediately under adjacent areas of the RWMs.
Analyses of the RWMs included RWM thickness and the num-
ber of inflammatory cells. Evaluation of the adjacent ST in-
cluded inflammatory cell infiltration and measurement of the
number of bacteria, the number of bacteria within inflamma-
tory cells, and the number of bacteria free in the perilymph (per
area). The measurements of these 3 selected areas of RWMs
and the ST from each animal were averaged. The means of these
numbers for Wt and mutant groups were then used for statis-
tical analysis to compare all animal groups infected with the
Wt or its isogenic mutant strains. All results are expressed as the
mean (SE). Differences between the groups were analy-
ized with 1-way analysis of variance using statistical soft-
ware (SPSS, version 18; SPSS Inc). Differences were consid-
ered significant at P < .05.

The analysis of viability in the chinchilla middle ear in-
cluded 30 animals inoculated bilaterally with 0.5 mL of 2.7 × 10^6
CFUs/mL of PsPA−. Animals were killed at 6, 12, 18, 24, 30, and
36 hours after inoculation for bacterial counts of MEEs and his-

topathological analysis.

Results

At 48 hours after inoculation, CFU counts in MEEs (Figure 1A)
were lower than the inoculum level for the PsPA− mutants and

Figure 1 A)
Viability and Virulence of Ear Protein Mutants

Figure 1. Effects of Mutations in Pneumococcal Surface Protein A (PspA) and Pneumolysin (Ply) on Bacterial Growth

A, At 48 hours after inoculation, colony-forming units (CFUs) in middle ear effusion (MEE) were significantly lower (P < .001) for the Ply-deficient (Ply−) and PspA-deficient (PspA−) mutants compared with the wild-type (WT) and Ply−/PspA− strains. Significant differences (P < .001) in CFU counts were seen between the Ply− and PspA− mutants. Counts for the WT and Ply−/PspA− strains were increased, while those for Ply− remained near the initial inoculum levels. No CFUs were detected in the PspA− group. B, In MEE, CFUs of the PspA− mutant decreased steadily over time, with few remaining at 30 hours after inoculation. However, after 43 hours in Todd-Hewitt broth (THB), there was an increase of about 2 logs.

near the inoculum level for the Ply− mutants. Counts for the WT and the Ply−/PspA− strains were increased compared with the inoculum. Significant differences (P < .001) in CFU counts were seen between the Ply− and PspA− mutants and between each of the single mutants compared with the WT or Ply−/PspA− strains. No significant difference was found between the WT and Ply−/PspA− strains. The PspA− mutant had no detectable bacteria.

Because the PspA− mutant was not viable in vivo at 48 hours, a time course study was performed using 30 chinchillas inoculated with 2.7 × 10⁶ CFUs/mL of PspA−. Animals were killed, and MEEs were harvested at 6, 12, 18, 24, 30, and 36 hours after bacterial inoculation (Figure 1B). In MEEs, CFUs of the PspA− mutant decreased steadily over time, with few CFUs remaining by 30 hours of inoculation. In vitro, CFU counts for the PspA− strain grown in THB were performed at 0, 2, 24, 30, and 43 hours to study the effect of host defenses on bacterial growth. However, after 43 hours in THB, there was an increase of about 2 logs, indicating that the loss of PspA− viability in vivo was probably due to the host immune system.

At 48 hours after inoculation, histopathological data from the bullae of the WT, Ply−, PspA−, and Ply−/PspA− groups were examined to compare RWM thickness, the number of inflammatory cells in the RWMs, and the number of inflammatory cells and bacteria in the ST (Figure 2). Although not significant, the RWMs of the PspA− and Ply− mutant groups tended to be less thick than those of the WT or double-mutant groups (Figure 2A). No significant difference was found among the groups in the number of inflammatory cells in the RWMs (Figure 2B); however, the PspA− group tended to have less inflammation. No significant difference was observed in the number of inflammatory cells in the ST of the inner ear (Figure 2C) among the groups, but the PspA− mutant had the fewest cells. A significant difference was noted in the number of free-floating bacteria in the ST (Figure 2D) between the WT and Ply−
groups (P = .009), the WT and PspA− groups (P = .006), and the WT and Ply−/PspA− groups (P = .03). No bacteria were seen in the PspA− group. No significant difference was observed in the number of phagocytized bacteria in inflammatory cells in the ST (Figure 2E), but fewer bacteria were found in the PspA− group. The absence of free-floating bacteria in the ST is consistent with no detectable CFUs in MEEs for the PspA− group at 48 hours after inoculation (Figure 2F).

There was variability between animals in each infected group. Figure 3 shows the most severe pathologic RWMs and adjacent areas in each group. This RWM from a chinchilla inoculated with the WT strain (Figure 3A) was thickened from inflammatory cell infiltration. Bacteria were observed in the ST, both free floating and within inflammatory cells. After inoculation with the Ply− mutant, the histopathological findings of the RWMs (Figure 3B) were similar to those of the WT strain, with infiltration of inflammatory cells and bacteria in the RWMs and the ST. This RWM from the animal inoculated with the PspA− mutant (Figure 3C) was not as thick as in the other groups, and there was no penetration of bacteria into the ST. No pathologic attenuation was observed in the Ply−/PspA− strain compared with that in the WT or single-mutant strains (Figure 3D).

Discussion

Streptococcus pneumoniae continues to be one of the most commonly cultured organisms from middle ears of children with otitis media. Because of the increased antibiotic resistance of pneumococci, interest has focused on a vaccine to prevent pneumococcal otitis media. The current polysaccharide-based vaccines are successful in some populations against invasive pneumococcal diseases (bacteremia and meningitis); however, the vaccines are serotype specific and have poor re-
response in children for protection against otitis media, especially in those younger than 2 years. 20 Furthermore, Eskola et al 3 found a reduction in the rate of otitis media due to these serotypes in the vaccine and those that cross-react with them, but an increase of 33% in the rate of acute otitis media was attributed to other pneumococcal serotypes.

This suggests the need for a different vaccine approach. There is an interest in producing a vaccine based on 1 or more of the pneumococcal proteins. 21 This requires a thorough understanding of the effect of these proteins on the viability and virulence of pneumococci within the middle ear and inner ear to select the most suitable vaccine candidates. Two potential candidates are Ply and PspA. Both proteins are expressed in virtually all pneumococcal serotypes, and immunization with these antigens can provide serotype-independent protection against pneumococci.

Both single mutants in our study, Ply − and PspA −, were less virulent (ie, they induced fewer histopathological changes to the RWMs compared with the Wt and double-mutant strains). The PspA − strain was the least virulent. At least in part, the de-
creased virulence of the PspA− mutant may be due to the decreased viability of PspA− in the middle ear. Berry and Paton 17 reported similar results that colonization of the nasopharynx decreased 30-fold from day 1 to day 4 in mice inoculated with the PspA− mutant. In our study, the Wt and other mutant strains were still viable at 48 hours after middle ear inoculation, although CFU counts of the PspA− mutant decreased over time, with low counts at 30 hours and no detectable levels at 48 hours. However, the in vitro experiment with the PspA− mutant in THB showed an expected increase of about 2 logs at 43 hours, suggesting that the PspA− mutant may be more susceptible to host factors in the ear. This is presumably because of killing of bacteria by apolactoferrin 5 or sensitivity to complement deposition. 22 In investigations of cobra venom–treated chinchillas, Sabharwal et al 23 found that strains limiting complement C3 proteindeposition on their surfaces more readily caused experimental otitis media.

An intriguing finding in our study is that the viability and virulence of the Ply−/PspA− double mutant were not attenuated compared with those of the single mutants but were similar to the Wt parent strain. When this was observed, we confirmed the serotype 2 capsule expression and the lack of expression of Ply and PspA by this strain. Comparison among studies of the streptococcal proteins is confounded by many factors, such as mode of infection, colonization vs survival, and different anatomic targets. Nevertheless, some variables can be compared. Berry and Paton 17 found an additive attenuation of the Ply−/PspA− double mutant compared with either of the single mutants based on survival of sepsis induced by intranasal challenge. Ogunniyi et al 24 also found increased survival of mice after infection with the Ply−/PspA− double mutant compared with the single mutants following intraperitoneal inoculation. However, after intranasal inoculation, they observed an increase in colonization of the nasopharynx with the Ply−/PspA− double mutant compared with the Ply− or PspA− mutants. The single mutant deficient in PspA decreased from day 1 to day 4, but levels of the Ply−/PspA− double mutant at day 4 were the same as those of day 1 and day 2, although CFU counts in the lungs were decreased. The increased colonization of the nasopharynx with the double mutant reported by Ogunniyi et al is similar to our findings in the middle ear with this strain.

Both the nasopharynx and the middle ear are composed of respiratory epithelium, which is a complex active epithelium involved in inflammation and host defense. It provides mucociliary clearance for the physical removal of bacteria, recognition of microbial exposure by pattern recognition receptors expressed on epithelial cells for detection of pathogen-
associated molecular patterns, and secretion of diverse proinflammatory and anti-inflammatory mediators and various antimicrobial substances, including antimicrobial peptides. It is not surprising that different organs and different epithelial mucosa might respond with immunologically diverse mechanisms to microbial infection with S pneumoniae. Oggioni et al observed 2 patterns of in vivo gene expression by S pneumoniae proteins. One pattern was characteristic of pneumococci in the brain and lungs and the other of pneumococci in the bloodstream. Based on the above findings, the expression of pneumococcal proteins seems to be organ specific.

A limitation of our study is that we used a pneumococcal strain that is rarely the cause of otitis media, and genetic background and variation can influence protection by anti-PspA antibodies. PsP proteins have been classified into 3 families and 6 clades. Ninety-eight percent of the PspAs are in family 1 (clades 1 and 2) and family 2 (clades 3-5). Although antibodies against PspA can be cross-protective, in some cases this may be limited to the same family or the same clade. In a study of the distribution of PspA families in MEEs from children, Melin et al found that most were distributed equally between families 1 and 2. Their findings suggest that a good vaccine candidate should include the 2 main PspA families. The PspA used in our study (serotype 2 strain D39) is from family 1 clade 2, should be protective against this family and clade, and may be cross-protective against other families and clades as well.

It was outside the scope of this study to determine the mechanism for the lack of attenuation of the Ply/PspA double mutant in the ear (ie, whether it could be related to complement-pathogen-associated molecular pattern recognition, a more active role of other pneumococcal virulence factors in the absence of Ply and PspA, or some other factor). However, it seems that pneumococcal proteins have diverse organ-specific effects on the viability and virulence of pneumococci. Further study is needed to better understand the potential of these proteins and their combinations as vaccine candidates against different diseases in diverse anatomic locations caused by pneumococci.

REFERENCES

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