The Round Window Membrane in Otitis Media

Effect of Pneumococcal Proteins

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Objective: To determine whether mutants of Streptococcus pneumoniae that are deficient in pneumococcal surface protein A (PspA), pneumococcal surface antigen A (PsaA), or pneumolysin (Ply) are less virulent and less likely to penetrate the round window membrane (RWM).


Setting: Otopathology Laboratory, Department of Otolaryngology, University of Minnesota Medical School, Minneapolis.

Participants: Forty young chinchillas (weight, 250-350 g) with normal external auditory canals and tympanic membranes.

Intervention: Animals were divided into 3 groups and bullae inoculated with wild-type S pneumoniae serotype 2, strain D39, or its mutants deficient in PspA, PsaA, or Ply. Two days after inoculation, bullae were processed for light microscopy and transmission electron microscopy.

Main Outcome Measures: Comparison of inflammatory cell infiltration and penetration of bacteria into the round window membrane and adjacent scala tympani.

Results: Histopathologic findings using wild-type S pneumoniae and Ply− mutant were similar and included otitis media and the presence of inflammatory cells and damage to and passage of bacteria through the RWM. Although otitis media was seen with the PspA− and PsaA− mutants, we observed no passage of bacteria through the RWM.

Conclusions: Both PspA and PsaA affect the ability of S pneumoniae to penetrate the RWM. Understanding the role of S pneumoniae virulence proteins in the pathogenesis of the middle ear, RWM, and inner ear will provide new strategies for the prevention and treatment of otitis media and its complications.


Bacterial otitis media (OM) is one of the most common diseases of childhood throughout the world. Otitis media can lead to inner ear disease and acquired hearing loss. Functional studies1,3 of children and adults have demonstrated a high-frequency hearing loss secondary to OM, and histopathologic evidence4 of inner ear damage in patients with chronic OM has been reported. Although the incidence of pneumococcal OM has decreased since the introduction of pneumococcal conjugate vaccines, Streptococcus pneumoniae remains 1 of the major pathogens in OM. Animal experiments have demonstrated that inflammatory mediators,5 bacterial products,6 and whole bacteria7 pass from the middle ear through the round window membrane (RWM) into the inner ears, resulting in inner ear damage.

Several proteins have been shown to contribute to the pathogenesis of S pneumoniae in different systemic and invasive diseases, including OM. The function of these proteins seems to facilitate significant aspects of pneumococcal colonization and invasion; among these are pneumococcal surface protein A (PspA), pneumococcal surface antigen A (PsaA), and pneumolysin (Ply). These and other proteins and combinations of these proteins are under investigation for use as vaccine candidates against pneumococcal infection.8 It has been recently shown that live attenuated strains of S pneumoniae that contain a combination of deletions in the Ply and PspA genes induce systemic and mucosal protection in mice from challenge with a high dose of the parent S pneumoniae strain.9 Pneumococcal surface protein A is a membrane-bound protein that...
can prevent activation of complement by pneumococci10,11 and is capable of binding to and preventing the killing of S pneumoniae by apolactoferrin.12 Pneumococcal surface protein A is a manganese and zinc transporter involved in growth and virulence.13 Pneumolysin is a cholesterol-binding pore-forming protein with cytotoxic and complement activation properties.14 Higher levels of antibodies to PsaA and Ply in children are associated with lower risk of bacterial carriage and OM.15

The pneumococcal strains used in this study were S pneumoniae serotype 2 strain D39 and its isogenic Ply−, PspA−, or PsaA− mutants deficient in Ply, PspA, or PsaA proteins, respectively. All mutant strains were derived from the National Collection of Type Cultures 7466 parent strain. The Ply− mutant had an insertionally inactivated Ply gene; the PspA− mutant was derived by insertional inactivation mutagenesis of the PspA gene and was from the American Type Collection Culture derivation (No. 55143); and the PsaA− mutant was an insertional duplication derivative of the National Collection of Type Cultures 7455 parent strain. Strains were grown in Todd-Hewitt broth (THB) (Becton Todd-Hewitt Broth; BD Diagnostics, Sparks, Maryland), which contained 0.5% yeast extract (Bacto Yeast Extract; BD Diagnostics), and plated on sheep blood agar plates. Erythromycin (0.3 and 0.2 µg/mL) was added to the THB and blood agar plates, respectively, for growth of the Ply−, PspA−, or PsaA− mutants. The bacterial strains were stored in a 10% glycercorived by insertional inactivation mutagenesis of the gene Ply−, PspA−, or PsaA− mutants deficient in Ply, PspA, or PsaA proteins, respectively.

**METHODS**

**BACTERIAL STRAINS AND GROWTH CONDITIONS**

The pneumococcal strains used in this study were S pneumoniae serotype 2 strain D39 and its isogenic Ply−, PspA−, or PsaA− mutants deficient in Ply, PspA, or PsaA proteins, respectively. All mutant strains were derived from the National Collection of Type Cultures 7466 parent strain. The Ply− mutant had an insertionally inactivated Ply gene; the PspA− mutant was derived by insertional inactivation mutagenesis of the PspA gene and was from the American Type Collection Culture derivation (No. 55143); and the PsaA− mutant was an insertional duplication derivative of the National Collection of Type Cultures 7455 parent strain. Strains were grown in Todd-Hewitt broth (THB) (Becton Todd-Hewitt Broth; BD Diagnostics, Sparks, Maryland), which contained 0.5% yeast extract (Bacto Yeast Extract; BD Diagnostics), and plated on sheep blood agar plates. 

**RESULTS**

Actual concentrations of bacteria used for inoculation of the animals ranged from $8.0 \times 10^6$ to $3.4 \times 10^7$ for the wild-type strain, $3.0 \times 10^4$ to $8.0 \times 10^5$ for PsaA−, $6.0 \times 10^3$ to $5.0 \times 10^6$ for PspA−, and $2.0 \times 10^6$ to $6.0 \times 10^7$ for Ply− mutant strains. The wild-type strain of S pneumoniae was more virulent than mutant bacterial strains; of the 15 wild-type S pneumoniae–inoculated animals, 3 died and 3 were ill and had to be killed 1 day after inoculation. The Table lists the type of middle ear fluid, RWM inflammatory cell infiltration, and injury for all groups. In 7 of the 12 wild-type S pneumoniae–inoculated animals, bacteria penetrated the RWM and passed into the adjacent scala tympani (Figure 1 and Figure 2). The Ply-deficient mutant seemed to be highly virulent and produced pathologic effects on the structure and permeability of the RWM using the experimental rat model of S pneumoniae OM.16

**ANIMALS AND HISTOLOGIC ANALYSIS**

All animals were housed and fed under standard conditions at our institutional animal care facility. Experiments were performed on young chinchillas (weight, 250–350 g) with normal external auditory canals and tympanic membranes. The care and use of animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. All animals were anesthetized before intrabullar inoculations with a combination of ketamine hydrochloride (100 mg/kg) and acepromazine (10 mg/kg). Animals were killed by an overdose of sodium pentobarbital.

A total of 41 chinchillas were given bilateral intrabullar inoculations of 0.5 mL of approximately $10^6$, $10^7$, or $10^8$ colony-forming units of S pneumoniae serotype 2 strain D39 (n = 15) or its isogenic PsaA− (n = 10), PspA− (n = 7), or Ply− (n = 9) mutants (Table). Animals were killed 2 days after inoculation. Bulle were removed and the cochlea were perfused via the apex and oval window with 2% glutaraldehyde in 0.1-M phosphate buffer, pH 7.4. Fixation was continued by emersion for 2 hours. Samples were decalcified in 10% EDTA on a rotator for 3 days. EDTA was changed daily. Samples were washed in buffer and postfixed in 1% osmium tetroxide in buffer for 1 hour. They were washed again in buffer, dehydrated in a graded series of ethanol followed by propylene oxide, and embedded in epoxy resin. Samples were cut at a thickness of 1 µm and stained with toluidine blue for light microscopic assessment. For electron microscopy, samples were cut at a thickness of 20 nm, stained with uranyl acetate and lead citrate, and examined by electron microscopy.

**Table. Comparison of the Virulent Characteristics of Streptococcus pneumoniae and Its Isogenic Mutants**

<table>
<thead>
<tr>
<th>Type of Bacteria</th>
<th>Type of MEE</th>
<th>Died Before Killing</th>
<th>Early Killing</th>
<th>Sick Before Killing</th>
<th>Bacteria in RWM</th>
<th>Inflammatory Cells in RWM</th>
<th>Bacteria in ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n=15)</td>
<td>12 Thin-thick over RWM</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Ply (n=9)</td>
<td>1 Thick, 5 thin-thick over RWM, and 4 thin</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PspA (n=10)</td>
<td>1 Thick, 5 thin-thick over RWM, and 3 thin</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>PsaA (n=7)</td>
<td>3 Sticky and 4 thin</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: MEE, middle ear effusion; Ply, pneumolysin; PsaA, pneumococcal surface antigen A; PspA, pneumococcal surface protein antigen; RWM, round window membrane; ST, scala tympani.
changes similar to those of the wild-type strain. In the Ply−-inoculated animals, bacteria were observed in the RWM and adjacent scala tympani in most animals (7 of 9 animals) (Figure 3).

The PspA− and PsaA− mutants were much less virulent compared with the wild-type and Ply− strains. Of the 10 animals inoculated with the PspA− mutant, only 1 died (9 were included for study), and none of the 7 animals died after inoculation with the PsaA− mutant. No bacteria were observed inside the RWM or scala tympani after inoculation of the PspA− (Figure 4A) or PsaA− (Figure 4B) mutants. Inflammatory cell infiltration in the RWM was observed in 2 of the PspA−-inoculated animals and in 2 animals inoculated with PsaA−.

**COMMENT**

The pathogenesis of OM is multifactorial with host defenses, virulence characteristics of bacteria, and environmental and genetic factors playing important roles. *Streptococcus pneumoniae* is a common pathogen in acute OM. Widespread use of oral antibiotics has resulted in an alarming increase of antibiotic-resistant bacterial strains, increasing the potential for labyrinthitis, tympanogenic meningitis, hearing loss, and other complications of OM. Pneumococcal proteins facilitate significant aspects of pneumococcal colonization and invasion and can, therefore, serve as potential components for new vaccines. In this article, we studied the effects of the Ply−, PspA−, and PsaA− mutants of *S pneumoniae* compared with the wild-type strain using a chinchilla model of OM. Several *S pneumoniae* proteins interact with complement components in vitro, including PspA, PsaA, and Ply.

Although Ply− mutants have been shown to be less virulent in various areas, including the lung and blood, we found the Ply− mutant, like its parent wild-type strain, able to colonize the middle ear and, contrary to PspA− and PsaA− mutants, penetrate and pass through the RWM into the scala tympani of the inner ear. Our findings support those of other investigators who showed the cell wall to be the most important contributor to pneumococcal pathogenicity in OM, with at most a modest additional effect of pneumolysin.17 However, our study involved high-dose bacterial infection for a short duration in the middle ear, which may affect the clearance and pathogenicity of the mutant strains.

We found PspA− and PsaA−-deficient strains to be much less virulent than the wild-type or Ply− strains. Furthermore, they were not observed to penetrate through the epithelial layer of the RWM. Pneumococcal surface protein A is involved in interactions with the host complement system, reducing complement-mediated clearance and phagocytosis.10,11 Another function of PspA in
pneumococcal virulence is the prevention of the bacterial effect of the host molecule, lactoferrin. Lactoferrin is an iron storage glycoprotein that is predominantly found in mucosal secretions, including middle ear effusions. The apo form of lactoferrin (apoL) is the form that is bactericidal against pneumococci, and PspA binding to apoL presumably protects pneumococci against its bactericidal effects. Because of the high concentrations of apoL in secretions, the process most likely takes place at the mucosal surface and facilitates colonization and carriage of S pneumoniae. It has been reported that resistance to pneumococcal carriage is dependent on mucosal rather than systemic immunity.

Immunization with the PspA protein prevented acute OM in an experimental model of S pneumoniae OM. Pneumococcal surface protein A is known to be a surface-binding protein; however, debate continues regarding its role as a pneumococcal adhesion molecule. The low virulence of PspA− strains has been attributed to their ability to transport manganese. PspA− mutants of S pneumoniae has been shown to be attenuated in the middle ears of gerbils. Pneumococcal surface protein A plays a significant role in pneumococcal carriage, and intranasal immunization with a cholera toxin B subunit–PsaA fusion protein was shown to protect mice against colonization with S pneumoniae. It has been shown that a low risk of nasal pharyngeal carriage progresses to acute OM in children who have a high titer of naturally developed serum and mucosal anti-PsaA antibodies. Immunizations with PspA and PsaA proteins have been shown to offer better protection against nasal carriage in mice than immunization with either protein alone. Although the roles of pneumococcal proteins PspA and PsaA are different, neither mutant penetrated the RWM, supporting their potential use as vaccine candidates for OM and making them extremely effective in preventing RWM and inner ear damage. Furthermore, their serotype independence and their theoretical advantage of inducing antibodies in children too young to mount effective antibody responses against polysaccharide antigens require further studies regarding these bacterial proteins and their possible use in prevention and treatment of OM and its complications.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Schachern, Tsuprun, Cureoglu, Briles, Paparella, and Juhn. Acquisition of data: Schachern, Tsuprun, Cureoglu, and Ferrieri. Analysis and interpretation of data: Schachern, Tsuprun, Cureoglu, and Paparella. Drafting of the manuscript: Schachern, Tsuprun, Cureoglu, and Paparella. Critical revision of the manuscript for important intellectual content: Schachern, Tsuprun, Cureoglu, Ferrieri, Briles, Paparella, and Juhn. Obtained funding: Juhn. Administrative, technical, and material support: Schachern, Tsuprun, Cureoglu, Ferrieri, Briles, Paparella, and Juhn. Study supervision: Ferrieri.

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REFERENCES