Protection from *Streptococcus pneumoniae* Keratitis by Passive Immunization with Pneumolysin Antiserum


**PURPOSE.** To determine whether passive immunization with pneumolysin antiserum can reduce corneal damage associated with pneumococcal keratitis.

**METHODS.** New Zealand White rabbits were intrastromally infected with *Streptococcus pneumoniae* and then passively immunized with control serum, antiserum against heat-inactivated pneumolysin (HI-PLY), or antiserum against cytotoxin-negative pneumolysin (d-Ply). Slit lamp examinations (SLEs) were performed at 24, 36, and 48 hours after infection. An additional four corneas from rabbits passively immunized with antiserum against d-Ply were examined up to 14 days after infection. Colony forming units (CFUs) were quantitated from corneas extracted at 20 and 48 hours after infection. Histopathology of rabbit eyes was performed at 48 hours after infection.

**RESULTS.** SLE scores at 36 and 48 hours after infection were significantly lower in rabbits passively immunized with HI-PLY antiserum than in control rabbits (P ≤ 0.043). SLE scores at 24, 36, and 48 hours after infection were significantly lower in rabbits passively immunized with d-Ply antiserum than in control rabbits (P ≤ 0.010). The corneas of passively immunized rabbits that were examined up to 14 days after infection exhibited a sequential decrease in keratitis, with an SLE score average of 2.000 ± 1.586 at 14 days. CFUs recovered from infected corneas were not significantly different between each experimental group and the respective control group at 20 or 48 hours after infection (P ≥ 0.335). Histologic sections showed more corneal edema and polymorphonuclear leukocyte (PMN) infiltration in control rabbits compared with passively immunized rabbits.

**CONCLUSIONS.** HI-PLY and d-Ply both elicit antibodies that provide passive protection against *S. pneumoniae* keratitis. (*Invest Ophthal Vis Sci. 2008;49:290–294*) DOI:10.1167/ iovs.07-0492

Pneumolysin (PLY) is a pore-forming toxin produced by *Streptococcus pneumoniae*, a common cause of bacterial keratitis, conjunctivitis, and endophthalmitis.1–3 This toxin causes both direct cellular damage by forming pores in host cell membranes and immune-derived damage by activating complement and inducing inflammation. It has also been found that PLY reduces the opsonic activity for *S. pneumoniae*, which could allow for more bacterial replication and more toxin release from the bacteria.4–5 The inflammation induced by PLY in pneumococcal keratitis is a significant problem because the dense infiltration of leukocytes can result in corneal opacity and loss of vision. Prior studies have been undertaken to investigate the effects that purified recombinant PLY has on rabbit corneal tissue.6–8 The reduced ability of PLY-negative mutant strains of *S. pneumoniae* to cause corneal tissue damage and extensive polymorphonuclear leukocyte (PMN) recruitment when compared with wild-type strains confirms that PLY plays a major role in the pathogenicity of pneumococcal keratitis.9 Several factors have been identified as to why PLY is such a destructive virulence factor in ocular infections. Leukocyte-deficient rabbits showed significantly less corneal epithelial damage when challenged with wild-type pneumococcal bacteria, further suggesting that the recruitment of leukocytes and other immune cells is responsible for corneal opacity.6–8 Findings also show that even at concentrations too low to cause lysis, PLY retains chemotactic activity while reducing PMN viability.10,11 Antibiotics, if effective, can kill the bacteria present, but cannot eliminate the PLY that has already been produced.

Pneumococcal pneumonia has primarily been the focus of immunization strategies against *S. pneumoniae*. Although antibody to the capsule has been the main focus of vaccine development, several studies have been devoted to developing antibodies to PLY to elicit protection against this well-known virulence factor of *S. pneumoniae*.12–14 Anti-PLY antibodies have been predominantly used to help protect against nonocular pneumococcal infections, more commonly pneumonia.12,13 Despite the information from studies using PLY as an immunogenic substance to protect against a wide range of pneumococcal infections, no attention has been given to immunization with PLY to prevent or treat pneumococcal keratitis. Adjunct therapies to neutralize the toxic and inflammatory effects of PLY would be beneficial in treating *S. pneumoniae* ocular infections. In this study, we focused on the role that passively administered antiserum to PLY plays in reducing corneal epithelial tissue damage and massive recruitment of specific host immune defenses that are characteristic of pneumococcal keratitis.

**METHODS**

**Bacterial Strains and Growth Conditions**

*Streptococcus pneumoniae* WU2,15 a serotype 3 strain, was routinely grown on a blood agar base containing 5% defibrinated sheep erythrocytes. Colonies were selected from the agar and grown in Todd Hewitt broth with 5% yeast extract (THY) at 37°C in 5% CO2 overnight. The overnight culture was diluted 1:100 in THY and incubated at 37°C.
in 5% CO₂ until the optical density at 600 nm reached 0.3, which corresponded to 10⁶ CFU/mL, as determined by growth curve analysis.

**Production of PLY Antiserum**

Specific pathogen-free (SPF) New Zealand White rabbits (Myrtle’s Rabbitry, Thompson Station, TN) were used in these studies and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wild-type recombinant PLY (WT-PLY) was purified as described previously and was heated at 100°C for 30 minutes. Recombinant mutant PLY (ϕPLY), which retained only 1% of its cytolytic property, was purified as previously described. Antiserum to HI-PLY and ϕPLY were produced by three monthly subcutaneous injections of either HI-PLY or ϕPLY. For primary immunization, Freund’s complete adjuvant (Sigma-Aldrich, St. Louis, MO) was mixed with 0.1 mg of either HI-PLY or ϕPLY in a 1:1 (vol:vol) ratio and subcutaneously injected into four sites along the dorsal side of each rabbit. For subsequent immunizations, additional injections of 0.05 mg of either HI-PLY or ϕPLY with Freund’s incomplete adjuvant (Sigma-Aldrich) at a 1:1 ratio (vol:vol) were also administered in the same manner. Six control rabbits were injected with a mixture of Freund’s complete adjuvant and PBS and Freund’s incomplete adjuvant and PBS (mock-immunized) in the same manner as the immunized rabbits. Blood was collected from the rabbits before the first immunization and 1 week after each of the three immunizations for the isolation of serum. Serum IgG titers against PLY were determined by ELISA. Titers were defined as the inverse of the highest dilution at which the A₄₅₀ was at least double the background absorbance.

**Rabbit Corneal Infections and Passive Immunizations**

Naïve rabbits were anesthetized by subcutaneous injection of a mixture of xylazine (100 mg/mL; Butler Company, Columbus, OH) and ketamine hydrochloride (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine hydrochloride was topically applied to each eye, and S. pneumoniae WU2 (10⁵ CFU in 1 μL) was injected intrastromally. Immediately after intrastromal injection of bacteria, each rabbit received an ear vein injection of 1 mL of antiserum to HI-PLY that had an anti-PLY IgG titer of 102,400 (n = 6 rabbits), antiserum to ϕPLY that had an anti-PLY IgG titer of 409,600 (n = 6 rabbits), or serum from a mock-immunized rabbit (n = 12 rabbits).

Two observers, masked as to the identity of the rabbit groups, used a biomicroscope (Topcon; Koaku Kikai K.K., Tokyo, Japan) to perform slit lamp examinations (SLEs) of infected rabbit corneas. Seven ocular parameters were graded: injection, chemosis, iritis, fibrin, hypopyon, corneal edema, and corneal infiltrate. Each parameter was assigned a grade from 0 (normal) to 4 (most severe). The grades were totaled for each eye and an average calculated for the two observers, resulting in an overall score for each eye ranging from 0 (normal) to a theoretical maximum of 28.

At 48 hours after infection, the rabbits were killed by an intravenous overdose of pentobarbital sodium (100 mg/mL; Sigma-Aldrich). The corneas were removed, dissected, and homogenized in sterile PBS with a tissue homogenizer (IKA Works, Inc., Wilmington, NC). Corneal homogenates were serially diluted and plated in triplicate on 5% sheep blood agar. The plates were incubated for 48 hours at 37°C. The colonies were counted, and bacterial CFUs for the corneas were determined and expressed in mean logarithmic units.

**Histology of Rabbit Eyes**

Whole rabbit eyes were dissected at 48 hours after infection and were placed in 10% buffered formalin. Histologic sectioning and hematoxylin and eosin staining of the rabbit eyes were performed by Excalibur Pathology, Inc. (Moore, OK).

**Statistics**

Data were analyzed using the Statistical Analysis System (SAS) program for computers (Cary, NC). Clinical SLE scores were analyzed with a nonparametric one-way analysis of variance. Bacterial CFUs at 20 hours after infection were analyzed by using the general linear models procedure of least-squares means. Bacterial CFUs at 48 hours after infection were analyzed with a nonparametric one-way analysis of variance. Variance.

**Results**

**Active Immunizations and ELISA Titers**

Active immunization of rabbits using either HI-PLY or ϕPLY produced high anti-PLY antibody titers as quantified by ELISA. Sera from HI-PLY-immunized rabbits that had titers of 102,400 and sera from ϕPLY-immunized rabbits that had titers of 409,600 were chosen for the initial passive immunization experiments. Sera from ϕPLY-immunized rabbits that had titers of 102,400 were used for the long-term passive immunization experiment. Sera from mock-immunized rabbits that had negligible titers were chosen for the control.

**Passive Immunizations**

SLE scores were recorded for the seven parameters outlined for analyzing the progression of S. pneumoniae infection in the cornea as a means of detecting differences in protection between the antisera with HI-PLY and the serum of a mock-immunized rabbit. The rabbit corneas were examined at 24, 36, and 48 hours after infection (Fig. 1A). Statistical analysis of SLE scores confirmed a significant decrease in the progression of S. pneumoniae destruction of the corneas of rabbits injected with HI-PLY antisera (n = 12 corneas) relative to rabbits receiving sera from a mock-immunized rabbit (n = 11 corneas) at 36 and 48 hours after infection. Probabilities generated from SLE scores for the HI-PLY experimental group at 24, 36, and 48 hours after infection were 0.490, <0.001, and 0.043, respectively.

The rabbits were passively immunized with antisera to ϕPLY or with sera from mock-immunized rabbits. The rabbits receiving the antisera (n = 10 corneas) had SLE scores significantly lower than those of the rabbits receiving the control sera (n = 10 corneas; Fig. 1B). Probabilities generated from SLE scores for the ϕPLY experimental group at 24, 36, and 48 hours after infection were <0.001, 0.003, and 0.010, respectively.

Antisera from immunized rabbits elicited protection against severe corneal opacity and massive infiltration of PMNs in comparison to rabbits who received mock serum (Fig. 2A–D). With the exception of hypopyon, all other parameters used to
calculate SLE scores were more severe in the groups receiving mock sera than in the groups receiving anti-PLY sera.

CFUs were not significantly different at 48 hours after infection between each immunization group and its respective control group (Table 1). Most of the corneas extracted at 48 hours after infection did not contain any viable bacteria to calculate CFUs, which was observed previously for *S. pneumoniae* strain R6. Data presented are the average obtained as a result of two experiments per toxoid antiserum group that yielded similar results.

To demonstrate that the bacteria grew above inoculum and to comparable log CFUs in each immunization group before the clearance observed at 48 hours after infection, corneas of the rabbits in each experimental group were intrastromally injected with 10^6 CFU of strain WU2 and cultured at 20 hours after infection. An equivalent number of bacterial CFUs was recovered from all corneas (Table 1), averaging 6.5 ± 0.184 log CFU recovered from immunized rabbits (HI-PLY and PLY combined) and 6.3 ± 0.248 log CFU from control rabbits (*P* = 0.604).

Four infected corneas from additional rabbits that were passively immunized with antiserum to PLY were examined by slit lamp up to 14 days after infection. By 7 days after infection, the SLE score average for these corneas was 4.406 ± 3.340 and the corneas were showing reduced opacity (Fig. 2E). By 14 days after infection, the score average was 2.000 ± 1.586 and the corneas were almost devoid of keratitis (Fig. 2F). These corneas were observed again at 3 weeks after infection, and all appeared completely healthy and clear (not shown).

**Histology of Rabbit Eyes**

Whole eyes were dissected at 48 hours after infection and were sectioned and stained with hematoxylin and eosin. The corneas of the control rabbits contained massive amounts of PMNs, especially at the endothelium and in the anterior chamber, and the stroma were extremely edematous underneath a thinning epithelial layer (Fig. 3). In contrast, the corneas of passively immunized rabbits contained few PMNs, were much less edematous, and had healthy-looking epithelia.

**TABLE 1.** CFU Recovered from Corneas after Infection

<table>
<thead>
<tr>
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<th>Log CFU ± SEM</th>
<th>n</th>
<th>P</th>
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<tr>
<td>20 h after infection</td>
<td></td>
<td></td>
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<tr>
<td>Immunized</td>
<td>6.500 ± 0.184</td>
<td>8</td>
<td>0.604</td>
</tr>
<tr>
<td>Mock</td>
<td>6.300 ± 0.248</td>
<td>8</td>
<td></td>
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<tr>
<td>48 h after infection</td>
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<tr>
<td>Passive immunization set 1</td>
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<tr>
<td>HI-PLY</td>
<td>1.308 ± 0.670</td>
<td>12</td>
<td>0.335</td>
</tr>
<tr>
<td>Mock</td>
<td>2.440 ± 0.656</td>
<td>11</td>
<td></td>
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<tr>
<td>Passive immunization set 2</td>
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<tr>
<td>PLY</td>
<td>1.675 ± 0.822</td>
<td>10</td>
<td>0.759</td>
</tr>
<tr>
<td>Mock</td>
<td>0.585 ± 0.318</td>
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**FIGURE 1.** Treatment of *S. pneumoniae* keratitis with passively administered antiserum to (A) HI-PLY or (B) PLY. Mock, control rabbits that received serum generated from control immunizations with PBS and adjuvant; Immune, rabbits that received PLY antiserum produced by another rabbit. (**) Significant differences in SLE scores between immunized and mock groups.

**FIGURE 2.** Rabbit corneas. Corneas at 48 hours after infection from rabbits that were administered (A, C) control serum or (B, D) passively immunized with antiserum to HI-PLY or PLY, respectively. (E, F) A cornea from a rabbit passively immunized with antiserum to PLY at 7 and 14 days after infection, respectively. The SLE score of this cornea was 1.13 at 7 days after infection and 0.13 at 14 days after infection. Both photographs are of the same cornea. Control corneas could not be shown at 7 and 14 days after infection because the control rabbits were killed at 48 hours after infection.
Due to its toxicity,13,14 it is recommended, therefore, that the immunogen of choice for future studies was shown by the regression of pneumococcal keratitis observed in rabbits passively immunized with antiserum against ψPLY.

Antiserum to either HI-PLY or ψPLY was administered immediately after injection of corneas with S. pneumoniae in this study. This method was similar to other studies in which rabbits were passively immunized with Staphylococcus aureus α-toxin antiserum immediately after intrastromal injection of bacteria,19 or with antiserum against the Staphylococcus epidermidis immunogenic polysaccharide determinant of slime 1 day before intrastromal injection.19 Both studies showed that passive immunization is an effective method for decreasing the severity of keratitis.

A useful investigation for passive immunizations against S. pneumoniae, S. aureus, and other causes of bacterial keratitis would be to determine the length of time necessary for effective treatment with passively administered antibodies after the onset of infection. Zaidi et al.20 performed a study in which this length of time was determined in a mouse model of Pseudomonas aeruginosa keratitis using rabbit antiserum against a live attenuated strain of P. aeruginosa. Their study showed that passive immunization was effective up to 8 hours after infection if given as a single dose and up to 72 hours after infection if given in multiple doses starting as late as 24 hours after infection. Late time points for effective therapy by passive immunization with antiserum to PLY would be advantageous in a clinical situation, as the first effects of S. pneumoniae keratitis in the rabbit are observed at 20 hours after infection.

Another important finding was that bacterial CFUs from corneas extracted at 48 hours after infection were not significantly different between the immunized and control groups, suggesting that the bacterial load in vivo was not a contributing factor to the more severe progression of pneumococcal keratitis in control rabbits than in immunized rabbits. Moreover, this common occurrence observed in this laboratory is the clearance of bacteria by 48 to 72 hours after infection. This clearance is one key element accounting for the low CFUs recovered from the corneas at death. The subsequent experiment quantifying the CFUs in each group at 20 hours after infection verified that the bacteria were able to grow in the cornea before clearance and that the passive immunization had no effect on bacterial growth. One possibility for the clearance of the bacteria is that pneumococcus produces an autolysin that is thought to become active at an advanced stage of growth, accounting for the subsequent death of the infecting bacteria.21 Another possibility is that complement activation in the host could cause phagocytosis of the bacteria. The progression of the corneal inflammation observed in the control rabbits is due to the continued release of chemotactic factors from cells that were injured and/or destroyed via the lasting presence of PLY. Histology of eyes from control rabbits clearly showed the destructive effects of the prolonged existence of complement and other immune components, whereas eyes from immunized rabbits showed far less infiltration of PMNs, a significant reduction in damage of the outer epithelial layer, and relatively little stromal edema (Fig. 3).

The currently available 23- and 7-valent pneumococcal vaccines were designed to target specific capsule types of S. pneumoniae.22 These vaccines have been of limited effectiveness in preventing infections of the more common serotypes of S. pneumoniae encountered in pneumonia. Use of the 23- and 7-valent vaccines would not be highly effective in preventing keratitis, however, because the pneumococcal capsule is not necessary for the corneal infection or the damage associated with keratitis.1 Antibodies that are specific for PLY could bind to and inactivate the toxin, significantly reducing the release of chemokines from damaged cells and preventing a massive influx of PMNs and other immune cells. The protection af-
favored by the use of antibodies specific for PLY in this study is further evidence of the important role of PLY in pneumococcal keratitis. Since virulence studies have indicated that the pathogenicity of pneumococcal keratitis is mainly caused by the ability of PLY to induce a massive inflammatory response, antibodies to PLY or peptides constructed of antibody epitopes could be extremely beneficial when used in conjunction with antibiotics to prevent and treat pneumococcal keratitis. Recently, cholesterol has been suggested as a treatment for pneumococcal keratitis because exogenous cholesterol can neutralize PLY and kill the bacteria. The use of cholesterol as an adjunct to antibody therapy may facilitate recovery from pneumococcal keratitis.

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References