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

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Abstract

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

Methods—New Zealand White rabbits were intrastromally injected with *Streptococcus pneumoniae* and then passively immunized with control serum, antiserum against heat-inactivated pneumolysin (HI-PLY), or antiserum against cytotoxin-negative pneumolysin (ψ 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 ψ 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 ($P \leq 0.043$). SLE scores at 24, 36, and 48 hours after infection were significantly lower in rabbits passively immunized with ψ PLY antiserum than in control rabbits ($P \leq 0.010$). 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 ($P \geq 0.335$). Histologic sections showed more corneal edema and polymorphonuclear leukocyte (PMN) infiltration in control rabbits compared with passively immunized rabbits.

Conclusions—HI-PLY and ψ PLY both elicit antibodies that provide passive protection against *S. pneumoniae* keratitis.

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

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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 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 10⁸ 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 previously¹⁶ and was heated at 100°C for 30 minutes. Recombinant mutant PLY (ψ PLY), which retained only 1% of its cytolytic property, was purified as previously described.¹⁶ Antiserum to HI-PLY and ψ PLY were produced by three monthly subcutaneous injections of either HI-PLY or ψ PLY. For primary immunizations, Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) was mixed with 0.1 mg of either HI-PLY or ψ PLY 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 ψ PLY 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.¹⁷ Titers were defined as the inverse of the highest dilution at which the A₄₁₀ 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 (10^5 CFU in 10 μ L) 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 ψ PLY 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.¹⁰ 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 $\pm$ SEM. These experiments were performed twice, yielding similar results, and the data from the two experiments were combined.

In a separate experiment, naïve rabbit corneas were intrastromally injected with 10^5 CFU of WU2 and immediately afterward intravenously injected with 1 mL of antiserum to HI-PLY (titer = 102,400), antiserum to ψ PLY (titer = 409,600), serum from mock-immunized rabbits, or PBS ($n = 4, 4, 8$, and 4, respectively). The rabbits were killed at 20 hours after infection, and the corneas were surgically removed, dissected, and homogenized in sterile PBS. The corneal homogenates were treated as previously described and bacterial CFUs were determined.

Another experiment was performed to determine whether passively immunized rabbits could recover from keratitis. The corneas ($n = 4$) were injected with bacteria as just described, the rabbits were immediately administered antiserum to ψ PLY intravenously (titer = 102,400), and SLE was performed daily through 7 days after infection and then again at 14 days after infection. Control rabbits were infected and given mock serum. However, these rabbits were not allowed to survive after 48 hours after infection for humane reasons.

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 by

using the Kolmogorov-Smirnov Test because numerous corneas were sterile by this time point. $P < 0.05$ was considered significant.

Results

Active Immunizations and ELISA Titers

Active immunization of rabbits using either HI-PLY or ψ PLY 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 ψ PLY-immunized rabbits that had titers of 409,600 were chosen for the initial passive immunization experiments. Sera from ψ PLY-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 cornea¹⁰ 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 ψ PLY 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 ψ PLY 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 R6.¹ 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 10^5 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 ψ 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 ψ 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.

Discussion

This study demonstrates that passive immunization with antiserum to PLY can significantly minimize the initial corneal damage commonly observed with pneumococcal keratitis and promote full recovery from keratitis. These findings are noteworthy in that there is no prior literature with respect to the use of antiserum to PLY as a means for protecting against pneumococcal keratitis in the rabbit model. The most significant aspect of this study is its potential in creating a possible means for combating pneumococcal keratitis by specifically targeting PLY as the primary target for therapy. Previous studies have shown that PLY is a key virulence factor in pneumococcal keratitis^{6–10}; however, the use of PLY as a target for therapy was not addressed.

The model of keratitis used in this study was an intrastromal injection model because *S. pneumoniae* keratitis will not occur in the rabbit after corneal scratching and subsequent topical application of bacteria (unpublished findings). It is unknown why humans can develop pneumococcal keratitis after the cornea is scratched but rabbits do not. Use of passive immunization to treat pneumococcal keratitis after a corneal scratch should be helpful because the major reduction in damage observed in this study was due to decreased PMN infiltration into the cornea from the anterior chamber, not from decreased topical corneal damage.

Both the HI-PLY and ψ PLY forms of the immunogen were effective in producing protective antisera. Others have chosen ψ PLY as the immunogen in mouse models of systemic and intranasal infection, stating that native PLY should not be used due to its toxicity.^{13,14} It is recommended, therefore, that ψ PLY be used as an immunogen in future studies of ocular pneumococcal infections. Another reason for using ψ PLY as the immunogen of choice for future studies was shown by the regression of pneumococcal keratitis observed in rabbits passively immunized with antisera against ψ PLY.

Antiserum to either HI-PLY or ψ PLY 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,¹⁸ 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, a 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 afforded 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,^{6,9,10} 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.²³ The use of cholesterol as an adjunct to antibody therapy may facilitate recovery from pneumococcal keratitis.

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Figure 1.

Treatment of *S. pneumoniae* keratitis with passively administered antiserum to (A) HI-PLY or (B) ψ 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 ψ PLY, respectively. (E, F) A cornea from a rabbit passively immunized with antiserum to ψ 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.

Figure 3.

Ocular histology from a rabbit passively immunized with antiserum to ψ PLY (**A**) or control serum (**B**). Whole globes were sectioned for the visualization of the anterior chamber in addition to the cornea. The cornea of the immunized rabbit (**A**) had PMNs at the endothelial surface and in the anterior chamber (*white arrow*). The cornea of the control rabbit (**B**) exhibited massive stromal edema below a thinning epithelium (*black arrow*) and the influx of a large amount of PMNs at the endothelium and in the anterior chamber (*white arrows*). Magnification, $\times 400$.

Table 1
CFU Recovered from Corneas after Infection

	Log CFU $\pm$ SEM	<i>n</i>	<i>P</i>
20 h after infection			
Immunized	6.500 $\pm$ 0.184	8	0.604
Mock	6.300 $\pm$ 0.248	8	
48 h after infection			
Passive immunization set 1			
HI-PLY	1.308 $\pm$ 0.670	12	0.335
Mock	2.440 $\pm$ 0.656	11	
Passive immunization set 2			
ψ PLY	1.675 $\pm$ 0.822	10	0.759
Mock	0.585 $\pm$ 0.318	10	