Active Immunization with Pneumolysin versus 23-Valent Polysaccharide Vaccine for *Streptococcus pneumoniae* Keratitis

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PURPOSE. The purpose of this study was to determine whether active immunization against pneumolysin (PLY), or polysaccharide capsule, protects against the corneal damage associated with *Streptococcus pneumoniae* keratitis.

METHODS. New Zealand White rabbits were actively immunized with Freund's adjuvant mixed with pneumolysin toxoid (ψ PLY), Pneumovax 23 (PPSV23; Merck, Whitehouse Station, NJ), or phosphate-buffered saline (PBS), before corneal infection with 10^5 colony-forming units (CFU) of *S. pneumoniae*. Serotype-specific rabbit polyclonal antisera or mock antisera were passively administered to rabbits before either intravenous infection with 10^{11} CFU *S. pneumoniae* or corneal infection with 10^5 CFU of *S. pneumoniae*.

RESULTS. After active immunization, clinical scores of corneas of the rabbits immunized with ψ PLY and Freund's adjuvant were significantly lower than scores of the rabbits that were mock immunized with PBS and Freund's adjuvant or with PPSV23 and Freund's adjuvant at 48 hours after infection ($P \leq 0.0010$), whereas rabbits immunized with PPSV23 and Freund's adjuvant failed to show differences in clinical scores compared with those in mock-immunized rabbits ($P = 1.00$) at 24 and 48 hours after infection. Antisera from rabbits actively immunized with PPSV23 and Freund's adjuvant were nonopsonizing. Bacterial loads recovered from infected corneas were higher for the ψ PLY- and PPSV23-immunized rabbits after infection with WU2, when compared with the mock-immunized rabbits ($P \leq 0.007$). Conversely, after infection with K1443, the ψ PLY-immunized rabbits had lower bacterial loads than the control rabbits ($P = 0.0008$). Quantitation of IgG, IgA, and IgM in the sera of ψ PLY-immunized rabbits showed high concen-

trations of PLY-specific IgG. Furthermore, anti-PLY IgG purified from ψ PLY-immunized rabbits neutralized the cytolytic effects of PLY on human corneal epithelial cells. Passive administration of serotype-specific antisera capable of opsonizing and killing *S. pneumoniae* protected against pneumococcal bacteremia ($P \leq 0.05$), but not against keratitis ($P \geq 0.476$).

CONCLUSIONS. Active immunization with pneumococcal capsular polysaccharide and Freund's adjuvant fails to produce opsonizing antibodies, and passive administration of serotype specific opsonizing antibodies offers no protection against pneumococcal keratitis in the rabbit, whereas active immunization with the conserved protein virulence factor PLY and Freund's adjuvant is able to reduce corneal inflammation associated with pneumococcal keratitis, but has variable effects on bacterial loads in the cornea. (*Invest Ophthalmol Vis Sci.* 2011; 52:9232–9243) DOI:10.1167/iov.10-6968

The pathogen *Streptococcus pneumoniae* (pneumococcus) is a major cause of a variety of infections worldwide, including pneumonia, bacteremia, meningitis, and otitis media.¹ In addition, it is one of the primary ocular pathogens capable of causing keratitis, conjunctivitis, and endophthalmitis.^{2–9} There are approximately 30,000 cases of bacterial keratitis in the United States each year.¹⁰ Although keratitis infections rarely occur in normal eyes, predisposing conditions such as contact lens use, trauma, corneal surgery, and diseases of the ocular surface, allow bacteria to penetrate the cornea's natural resistance and establish a sight-threatening infection. Pneumococcus is often isolated as one of the top causes of bacterial keratitis.^{6,11–16}

Bacterial keratitis is a devastating disease that can lead to permanent scarring of the cornea and loss of vision.^{15,17–20} For most cases of bacterial keratitis, the standard of care involves antibiotic therapy. However, due to the increasing resistance of bacterial isolates to antibiotics and the damage that may still occur due to inflammation once the pathogen has been eradicated, it is imperative that new therapies be investigated. Vaccines or immunization regimens based on pathology-causing proteins and polysaccharides have been shown to provide protection for all major pathogens causing bacterial keratitis, including *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*.^{21–24} Pneumococcus produces a wide variety of cell-associated and released virulence factors,²⁵ many of which have been studied as vaccine candidates for *S. pneumoniae* systemic infections.^{26–29} Pneumococcal keratitis studies have focused on the polysaccharide capsule or the cytotoxin pneumolysin (PLY) and their roles in pathogenesis.^{30–32}

In nearly all models of pneumococcal infection, including pneumonia, meningitis, and otitis media, the most significant

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virulence factors are those associated with the cell envelope.¹ In fact, the polysaccharide capsule has been shown to be an important virulence factor helping the bacteria evade phagocytosis by inhibiting opsonization by complement proteins, therefore preventing the bacteria from being killed by macrophages.¹ The capsule is such an important virulence factor in most types of diseases that current vaccination strategies to protect against pneumococcal infections rely solely on targeting the capsular serotypes most associated with infection.^{33,34} Pneumovax 23 (PPSV23; Merck, Whitehouse Station, NJ) and Prevnar 13 (Pfizer, New York, NY), the currently approved pneumococcal vaccines, protect against the 23 and 13 serotypes, respectively, that are responsible for most invasive pneumococcal disease.^{35,36} The role of the capsule in corneal infections, however, is less significant. The ocular pathology of rabbits infected with *S. pneumoniae* D39 (Avery's strain; capsule type 2) was not significantly different from that of rabbits infected with its capsule-deficient derivative, R6.³¹ Furthermore, a pneumococcal strain isolated from a clinical case of keratitis showed no difference in the clinical symptoms when compared to the strain's nonencapsulated isogenic mutant in a rabbit model of keratitis,³⁷ indicating that factors other than the capsule are involved in the pathology of pneumococcal keratitis.

PLY, another key virulence factor in a variety of infection models, is a member of the family of bacterial cholesterol-dependent cytotoxins that also includes perfringolysin O and listeriolysin O.³⁸ It is a pore-forming cytolytic protein that binds cholesterol within the host cell and inserts into the lipid bilayer where it oligomerizes and forms a transmembrane pore.³⁹ In addition, PLY causes immunologic damage by activating the classic complement pathway and the inflammatory response.⁴⁰ Both of these actions lead to significant host tissue damage. Pneumococci lacking the *ply* gene show attenuated virulence in a rabbit model of keratitis, indicating PLY's role in corneal infections.⁴¹ Passive immunization with serum from rabbits immunized with heat-inactivated recombinant PLY and recombinant mutant PLY have shown significant protection in a rabbit model of keratitis. Corneas of rabbits passively immunized exhibited significantly reduced pathology during the course of the infection and showed protection for as long as 14 days after infection.³⁰

The purpose of the present study was to determine the extent of protection conferred to rabbits during pneumococcal keratitis when actively immunized against *S. pneumoniae* polysaccharide capsule (PPSV23) or PLY and to determine the protective value of anti-PLY antibody on human corneal epithelial cells. We determined that active immunization against PLY mixed with Freund's adjuvant provided significant protection against corneal inflammation associated with pneumococcal keratitis. The effect of PLY immunization on bacterial loads in the cornea, however, was different depending on the challenge strain of *S. pneumoniae*. In addition, anti-PLY IgG protected corneal epithelial cells from damage caused by PLY, whereas control IgG had no effect on the cytolytic ability of PLY. As a comparison, immunization with PPSV23 mixed with Freund's adjuvant was tested for keratitis. The rabbits were unable to produce opsonizing antibodies, cornea disease severity was not improved, and passive transfer of commercially available opsonizing antibodies was unable to decrease corneal damage.

METHODS

Bacterial Strains

S. pneumoniae WU2 and clinical strain K1443 were used in this study. WU2, a serotype 3 strain, was originally obtained by passage of a

human clinical strain in mice.⁴² K1443, a clinical keratitis strain, was obtained from Regis Kowalski (Charles T. Campbell Laboratory, University of Pittsburgh, Pittsburgh, PA) and was determined to be serotype 19A by the multiplex PCR method of Pai et al.⁴³ Capsule production was confirmed in strain K1443 with an *S. pneumoniae* test kit (Pneumoslide; BD Biosciences, Franklin Lakes, NJ). Both strains were routinely grown on blood agar base containing 5% sheep erythrocytes. Individual colonies were selected from the blood agar and grown in Todd Hewitt Broth (BD Biosciences) supplemented with 0.5% yeast extract (THY) at 37°C and 5% CO₂ overnight. Each overnight culture was then diluted 1:100 into fresh THY and grown to an optical density corresponding to 10⁸ colony forming units (CFU) per milliliter for inoculations. Serial dilutions of each inoculum were cultured on 5% blood agar to verify the accuracy of the inoculum CFU. For Western blot analysis, bacteria were grown overnight in THY as described above to equivalent optical densities and were then centrifuged at 4°C and 5000 rpm for 5 minutes to remove live cells. The supernatant was then concentrated 10-fold before analysis.

PLY Purification

Recombinant PLY or ψ PLY⁴⁴ containing a 6x His tag was purified with metal-affinity resin (Talon; BD Biosciences). The purity of the protein was confirmed by SDS-PAGE. Fractions were pooled and dialyzed against PBS overnight at 4°C. A bicinchoninic acid assay (BCA) was performed to determine the PLY concentration. The hemolytic activity of PLY was confirmed by a microplate assay in which 50 μ L of PLY was serially diluted into 100 μ L of DTT buffer (10 mL PBS, 0.01 g BSA, and 0.015 g dithiothreitol) and then co-incubated with 50 μ L of 1% sheep red blood cells for 30 minutes at 37°C. Lysis of red blood cells indicated an active protein.

Active Immunizations and Corneal Challenge

New Zealand White rabbits (Harlan Rabbity, Indianapolis, IN) were used in the study and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Antiserum was produced by a minimum of three subcutaneous injections of recombinant mutant pneumolysin (ψ PLY) or PPSV23 (Merck) administered 1 month apart. PPSV23 is composed of 23 pneumococcal serotypes, including types 3 and 19A. Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) was mixed with 0.1 mg ψ PLY or 0.5 mL PPSV23 in a 1:1 (vol/vol) ratio for primary immunization and injected subcutaneously at four locations along the dorsal side of each rabbit. Subsequent immunizations consisted of a mixture of 0.05 mg ψ PLY or 0.5 mL PPSV23 and Freund's incomplete adjuvant in a 1:1 (vol/vol) ratio administered in the same manner as primary immunizations. Control rabbits were mock immunized through an injection with a mixture of Freund's complete adjuvant and PBS or Freund's incomplete adjuvant and PBS.

Blood was collected from the rabbits before the first immunization and 1 week after each subsequent immunization for serum studies. ELISAs were performed on the serum to determine IgG titers against PLY or PPSV23,⁴⁵ in which ψ PLY and PPSV23 were used to coat the plates, respectively. Additional ELISAs were performed on sera isolated from PPSV23-immunized rabbits in which the sera were adsorbed with cell wall polysaccharide (Statens Serum Institut, Copenhagen, Denmark), and the plates were coated with purified pneumococcal capsular polysaccharide types 3 or 19A.⁴⁶ The ELISA plates were also coated with a nonrelated protein containing the same 6x His tag as the ψ PLY, to determine the IgG titers to only the histidine tag in sera of rabbits immunized with ψ PLY. Titers were defined as the inverse of the highest dilution at which the A₄₀₅ was at least double the background absorbance. A Western blot analysis was performed with one of the polyclonal antisera to PLY (1:200 dilution) to determine specificity to cell-free extracts of WU2 and K1443.

Rabbits were anesthetized by subcutaneous injection of a mixture of xylazine (5–10 mg/kg; Lloyd Laboratories, Shenandoah, IA) and

ketamine hydrochloride (50 mg/kg; Butler Animal Health Supply, Dublin, OH). Proparacaine hydrochloride (Akorn, Inc., Buffalo Grove, IL) was topically applied to each eye, and *S. pneumoniae* WU2 or K1443 (10^5 CFU in $10\ \mu\text{L}$) was injected intrastromally into immunized and control rabbits. A biomicroscope was used by two observers who were masked to the identity of the rabbit groups to perform slit lamp examinations (SLEs) of infected rabbit corneas using seven ocular clinical disease parameters: injection, chemosis, iritis, fibrin, hypopyon, corneal edema, and corneal infiltrate.³² Each factor was given a grade from 0 (normal) to 4 (most severe). The grades were totaled and averaged for the two observers resulting in an overall score ranging from 0 (normal) to a maximum of 28 (most severe). Rabbits were euthanized at 48 hours after infection by an intravenous overdose of pentobarbital sodium (100 mg/mL; Sigma-Aldrich). Corneas were removed, dissected, and homogenized in sterile PBS. Corneal homogenates were then serially diluted and plated in triplicate on 5% blood agar. Plates were incubated at 37°C and 5% CO_2 for 24 hours. Colonies were counted, and bacterial CFUs were determined and expressed in mean logarithmic units per milliliter $\pm$ SEM.

Assessment of Ocular Inflammation

Whole rabbit eyes were removed at 48 hours after infection and placed in 4% buffered formalin. Histologic sectioning and hematoxylin and eosin staining of the rabbit eyes were performed by Excalibur Pathology, Inc. (Moore, OK). Relative myeloperoxidase concentration in infected corneas, a measure of neutrophil presence, was determined by ELISA and was performed in triplicate for each group ($n = 3$ per group).

Passive Immunizations and Corneal or Systemic Challenge

In a separate experiment, corneas were infected with *S. pneumoniae* WU2 or K1443 (10^5 CFU in $10\ \mu\text{L}$). Immediately after infection, 1 mL of rabbit polyclonal antisera specific for serotype 3 or serogroup 19 from the Statens Serum Institut (SSI, Copenhagen, Denmark) or antisera from mock-immunized rabbits was injected into each rabbit's ear vein. The experiment then proceeded with clinical examinations and euthanasia as described above. Opsonophagocytosis assays were performed on antisera specific for serotype 3 or serogroup 19, as previously described.⁴⁷ To confirm that 1 mL of polyclonal antisera was protective in extraocular settings, the rabbits were infected intravenously with 10^{11} CFU *S. pneumoniae* WU2. Immediately after infection, the rabbits were immunized with 1 mL of rabbit polyclonal antisera specific for serotype 3 or antisera from mock-immunized rabbits. Blood was collected from each rabbit at 1, 3, 12, and 24 hours after infection and bacterial load was determined.

Quantification of PLY-Specific Immunoglobulins

ELISAs were performed on serum collected from rabbits, to determine the titers of PLY-specific IgG, IgM, and IgA antibodies in the blood of ψ PLY and mock-immunized control rabbits, with 6x His tagged recombinant PLY used as the antigen. In addition, ELISAs were performed on the homogenates of corneas removed 48 hours after infection of the ψ PLY and control rabbits.⁴⁵ Corneal homogenates from naive rabbits were used as negative controls. An additional control to account for antibodies produced against the histidine tag included an ELISA in which a nonrelated protein containing a 6x His tag was used to coat the plate. Titers were defined as the inverse of the highest dilution at which the A_{405} was at least double the background absorbance.

Cell Culture

Immortalized human corneal epithelial (HCE) cells were a kind gift from Haydee Bazan (Department of Ophthalmology, Louisiana State University Health Sciences Center, New Orleans, LA). They were originally established by Roger W. Beuerman (Department of Ophthalmology, Louisiana State University Health Sciences Center) and maintained

in serum-free keratinocyte growth medium (KGM; Clonetics BioWhittaker Europe, Verviers, Belgium) supplemented with growth factors and antibiotics. The cells were passaged with trypsin and neutralized with KGM with 10% fetal bovine serum. The cells were subsequently centrifuged, suspended in KGM, and seeded in a 1:3 split ratio. For all experiments, HCE cells at passages 30 to 40 were seeded into 96-well plates at 1 to 4×10^5 cells per well.^{48,49}

Cytotoxicity and Neutralization Assays

Polyclonal IgG was isolated from the tertiary serum of one of the rabbits with high-titer antibodies to PLY and one rabbit that had been mock immunized with PBS using protein A coupled to Sepharose beads.⁵⁰ HCE cells were exposed to increasing concentrations of PLY alone, $4\ \mu\text{g}$ PLY with $100\ \mu\text{g}$ anti-PLY IgG, or $4\ \mu\text{g}$ PLY with $100\ \mu\text{g}$ of control IgG and incubated for 3 hours at 37°C and 5% CO_2 , in triplicate. The positive control was 1% saponin, and the negative control was medium alone. The PLY was preincubated with nothing, anti-PLY IgG, or control IgG for 15 minutes at 37°C before it was applied to the cell cultures. HCE cell viability was then determined (Live/Dead Viability/Cytotoxicity Kit; Invitrogen, Carlsbad, CA). Briefly, the cells were washed with Dulbecco's (D)PBS (DPBS) and then treated with $100\ \mu\text{L}$ DPBS and $100\ \mu\text{L}$ calcein AM for a final concentration of $2\ \mu\text{M}$ calcein AM. After a 30-minute incubation at room temperature, fluorescence was measured at 485/530 nm using a fluorescence microtiter plate reader. A separate experiment in which the incubation step was extended to 3 hours was performed, and the cells were visualized by fluorescence microscopy.

To examine possible neutralization in vivo, $1\ \mu\text{g}$ PLY was incubated in the presence or absence of $200\ \mu\text{g}$ anti-PLY IgG and then injected intrastromally into rabbit corneas. Anti-PLY IgG alone was also tested. A total volume of $20\ \mu\text{L}$ was injected into each cornea. Corneas were monitored 1, 2, 4, 5, 7.5, 24, and 48 hours after infection.

Statistics

Clinical SLE scores, bacterial CFU data, quantification of PLY-specific immunoglobulins, and neutralization data were analyzed as dependent variables using a two-level factorial model in the analysis of variance. The main factors were treatment (mock, PPSV23, or ψ PLY) and time (24 or 48 hours). The model was analyzed as a repeated-measures model in which within-subject correlation (eyes within rabbit) was taken into account.⁵¹ The error term was based on two replications of each of the experiments. Mean values for the interaction of time by treatment were separated in post hoc testing by using a method of simulation for α level adjustment for multiple comparisons.⁵² Myeloperoxidase assays and the bacteremia experiment were analyzed by Student's *t*-test. The α level for all hypothesis tests in all statistical procedures was preset at 0.05. (All data analysis and manipulation: Statistical Analysis System; SAS Institute, Cary, NC).

RESULTS

Active Immunizations and Corneal Challenge

Active immunizations of rabbits with Freund's adjuvant and ψ PLY or PPSV23 produced high-titered anti-PLY or anti-PPSV23 antibodies, respectively, as quantified by ELISA. Rabbits with anti-PLY or anti-PPSV23 IgG titers at or above 25,600 were used for the subsequent active immunization experiments. Control rabbits that were mock immunized with PBS produced negligible specific antibody titers. IgG titers to the 6x His tag were also negligible in the ψ PLY-immunized rabbits and comparable to titers of preimmune sera. After the initial immunization, two boosts were needed for the rabbits immunized with ψ PLY to reach high antibody titers, whereas at least four additional boosts were necessary for the animals immunized with PPSV23. The rabbits in the ψ PLY-immunized group had a mean anti-PLY serum IgG titer of $112,640 \pm 16,722$ compared with

FIGURE 1. Western blot of recombinant PLY (lane 1) and concentrated supernatants from overnight cultures of WU2 (lane 2) and K1443 (lane 3) *S. pneumoniae*. The blot was probed with polyclonal antiserum (1:200 dilution) from a ψ PLY-immunized rabbit.

the anti-PPSV23 serum IgG titer of $98,980 \pm 26,450$ for the rabbits in the PPSV23-immunized group. When the anti-PPSV23 serum was preadsorbed with cell wall polysaccharide and capsular serotype-specific IgG titers were measured, the anti-serotype 3 titers were 5120 ± 1760 and the anti-serotype 19A titers were 2250 ± 1220 . No correlation between serotype-specific titer and clinical scores were observed (data not shown). A Western blot performed on recombinant PLY (rPLY) and concentrated cell-free extracellular extracts from overnight cultures of WU2 and K1443 showed that the polyclonal rabbit antiserum from a ψ PLY-immunized rabbit was specific for PLY and no other detectable pneumococcal protein (Fig. 1). The antiserum recognized a protein for rPLY that was slightly larger than the proteins from the bacterial cultures because of the 6x His tag.

By 48 hours after infection, the rabbits actively immunized with ψ PLY had less corneal damage than control and PPSV23 actively immunized rabbits (Fig. 2). Clinical SLE scores were used to analyze possible differences in protection between the ψ PLY-immunized, PPSV23-immunized, and control rabbit groups after intrastromal challenge with *S. pneumoniae*. Statistical analysis of the infected corneas showed that the rabbits immunized against ψ PLY had significantly lower SLE scores at 48 hours after infection than did the control rabbits, regardless of which bacterial strain was used for the infection (Fig. 3). The means of SLE scores between the ψ PLY- and mock-immunized control groups at 48 hours after infection with WU2 and K1443 were significantly different ($P = 0.0006$ and $P = 0.0002$, respectively) for pair-wise comparison of these means. P values were derived as described in the Statistics section. In addition, after corneal infections with either WU2 or K1443, the ψ PLY-immunized rabbits had significantly lower SLE scores than did the PPSV23-immunized rabbits at 48 hours after infection ($P = 0.0010$ and $P < 0.0001$, respectively). Conversely, the rabbits immunized with PPSV23 failed to show any reduction in SLE scores when compared with the rabbits in the control group. The lack of protection from corneal damage by PPSV23 active immunization occurred at both 24 and 48 hours after infection and after infections with both *S. pneumoniae* strains ($P = 1.00$ and 1.00 for WU2 and $P = 1.00$ and $P = 1.00$ for K1443 at 24 and 48 hours after infection, respectively).

FIGURE 2. Eyes of rabbits infected with *S. pneumoniae* WU2 or K1443 after immunization with ψ PLY, PPSV23, or PBS (control). At 48 hours after infection with *S. pneumoniae* WU2, the eye from the ψ PLY-immunized rabbit had no discernable anterior chamber inflammation. The eye of the PPSV23-immunized rabbit, however, did have anterior chamber inflammation as evidenced by the haziness behind the cornea and the beginnings of a hypopyon. With *S. pneumoniae* K1443 at 48 hours after infection, the corneal infiltrate in the eye of the PPSV23-immunized rabbit was much larger, covering the pupil and part of the surrounding area, than the infiltrate in the eye of the ψ PLY-immunized rabbit. Eyes were scored and photographed 24 and 48 hours after infection.

CFUs recovered at 48 hours from infected corneas were significantly different between the ψ PLY-immunized and control groups. Interestingly, more bacteria were recovered from both the ψ PLY- and PPSV23-immunized groups after infection with *S. pneumoniae* strain WU2 than from the mock-immunized control group ($P = 0.0007$ and $P = 0.0002$, respectively). In contrast, after infection with *S. pneumoniae* K1443, significantly less bacteria were recovered from the corneas of the ψ PLY-immunized rabbits compared with the corneas of the mock-immunized control rabbits ($P = 0.0008$). There was, however, no statistical significance between the PPSV23-immunized and control groups after infection with K1443. Likewise,

FIGURE 3. Clinical scores of mock-, ψ PLY-, and PPSV23-immunized rabbits after infection with either *S. pneumoniae* strain WU2 or *S. pneumoniae* strain K1443. Scores are shown at 24 and 48 hours after infection. **Significant difference between the clinical scores ($P < 0.05$). $n = 14$, 11, and 9 for the ψ PLY, PPSV23, and control groups, respectively, for strain WU2. $n = 10$, 12, and 10 for ψ PLY, PPSV23, and control groups, respectively, for strain K1443.

FIGURE 4. Log₁₀CFU/mL recovered from corneal homogenates of mock-immunized, ψ PLY-immunized, and PPSV23-immunized rabbits after infection with *S. pneumoniae* strain WU2 and *S. pneumoniae* strain K1443. **Significant difference in recovered bacterial load between the two strains ($P < 0.05$). $n = 13, 10$, and 8 for ψ PLY, PPSV23, and control groups, respectively, for strain WU2. $n = 9, 11$, and 8 for ψ PLY, PPSV23, and control groups, respectively, for strain K1443.

there was no difference in the recovered CFU between the ψ PLY and PPSV23-immunized groups, regardless of the strain used for infection (Fig. 4).

Histopathology and Myeloperoxidase Concentration

At 48 hours after infection, whole rabbit eyes were dissected, fixed, sectioned, and stained with hematoxylin and eosin (Excalibur Pathology, Inc.). After infection with *S. pneumoniae* WU2, the corneas of the rabbits immunized against PLY contained less infiltrate than the corneas of control rabbits (Fig. 5). In addition, the rabbits immunized with PPSV23 and infected with WU2 appeared to have reduced infiltrate in the corneal

stroma and aqueous humor than control rabbits. Comparison of the ψ PLY- and PPSV23-immunized corneas show that the rabbits immunized with ψ PLY and infected with WU2 had reduced infiltrate in the corneal stroma, though slightly more infiltrate in the aqueous humor. After infection with *S. pneumoniae* K1443, there was more fibrin and immune cells within the aqueous humor of the control and PPSV23-immunized rabbits when compared to the ψ PLY-immunized rabbits. The corneas of rabbits immunized against capsule showed slight histologic differences from the control rabbits in the same experiment group (Fig. 5). In fact, the corneas of the PPSV23-immunized rabbits appeared to have more infiltrate than the corneas of the mock-immunized control rabbits after infection with K1443 (Fig. 5).

An ELISA performed on corneal homogenates of rabbits infected with *S. pneumoniae* WU2 showed that the rabbits actively immunized with ψ PLY had reduced myeloperoxidase ($A_{405} = 0.376 \pm 0.018$) compared with the rabbits that were either immunized with PPSV23 or mock immunized ($A_{405} = 0.499 \pm 0.074$ and 0.501 ± 0.041 , respectively), though the only significant difference was between the ψ PLY- and mock-immunized groups ($P < 0.05$). After infection with *S. pneumoniae* K1443, the rabbits actively immunized with PPSV23 had a mean relative absorbance (A_{405}) of 0.647 ± 0.082 , higher than that of the rabbits actively immunized with ψ PLY ($A_{405} = 0.448 \pm 0.056$) or mock-immunized ($A_{405} = 0.478 \pm 0.043$), though the difference did not meet the level of significance.

Passive Immunizations and Corneal or Systemic Challenge

Whole-blood survival and opsonophagocytosis assays performed on blood from the rabbits actively immunized against PPSV23 showed that, although the rabbits produced high titers of anti-capsular antibodies, these antibodies were not capable of opsonizing or killing pneumococci in vitro (data not shown). To determine whether the lack of opsonization by anticapsular antisera accounted for the lack of protection against corneal challenge with *S. pneumoniae* in the actively immunized rabbits, we obtained anticapsular antibodies specific for serotype 3 or serogroup 19 from SSI. Opsonophago-

FIGURE 5. Ocular histology from rabbits infected with *S. pneumoniae* WU2 or K1443 after immunization with ψ PLY, PPSV23, or PBS (control). Corneas and aqueous humor from rabbits previously immunized with ψ PLY show reduced infiltrate when compared with the mock-immunized control and PPSV23-immunized rabbits. Corneas from rabbits immunized with PPSV23 and then infected with *S. pneumoniae* show similar, and possibly more, amounts of infiltrating immune cells compared with corneas from mock-immunized control rabbits infected with the same pneumococcal strain. Arrows: infiltrate.

FIGURE 6. (A) Clinical scores of rabbits passively immunized with antisera to PBS (mock), serotype 3, and serogroup 19 immediately after infection with *S. pneumoniae* WU2 or K1443. Scores are shown at 24 and 48 hours after infection. **Significant difference between the clinical scores ($P < 0.05$). $n = 6$ for all groups. (B) Representative eyes photographed 24 and 48 hours after infection.

cytosis assays performed on the commercially available serum showed a high killing (98%–100%) rate of pneumococci when the target bacterium was matched with serotype-specific antisera (data not shown). These sera, capable of opsonophagocytosis, were then passively administered to the rabbits immediately after infection with *S. pneumoniae* WU2 or K1443.

The rabbits passively immunized with the commercial rabbit polyclonal antiserum to pneumococcal serotype 3 or serogroup 19 immediately after infection with *S. pneumoniae* WU2 or K1443 had clinical scores that were either not significantly different from or worse than the scores of rabbits passively immunized with mock serum and infected with the same strain at 24 or 48 hours after infection (Fig. 6A). There were no significant differences between the scores of any immunization groups at 24 or 48 hours after infection with *S. pneumoniae* WU2 ($P \geq 0.125$; Fig. 6A). After infection with *S. pneumoniae* K1443, the rabbits passively immunized with serogroup 19 antisera had significantly higher clinical scores at 24 hours after infection than did both the rabbits passively immunized with mock antisera ($P = 0.0169$) and those passively immunized with antisera to serotype 3 (0.0072), although there were no significant differences between any other groups. By 48 hours after infection, there were no significant differences between any groups ($P \geq 0.305$; Fig. 6A).

For the passively immunized rabbits infected with *S. pneumoniae* WU2, there were no significant differences in the bacterial load recovered from the corneas, regardless of the

immunization group ($P \geq 0.476$; Fig. 7). This lack of significance was also true of the rabbits infected with *S. pneumoniae* K1443 ($P = 0.99$; Fig. 7), although those infected with K1443 tended to have lower quantities of bacteria recovered than those infected with WU2.

FIGURE 7. Log₁₀CFU/mL recovered from corneal homogenates of rabbits passively immunized with antisera to PBS (mock), serotype 3, and serogroup 19 immediately after infection with either *S. pneumoniae* WU2 or *S. pneumoniae* K1443. $n = 5$ for all groups.

Finally, to confirm that the dose of passively administered antibodies was capable of protecting against pneumococcal disease in nonocular settings, rabbits were injected intravenously with 10^{11} CFU *S. pneumoniae* WU2 immediately before immunization with 1 mL serotype 3-specific antisera or 1 mL mock antisera. At 1 hour after infection, there was a significant difference between the blood bacterial load of the immunized rabbits compared with the mock-immunized rabbits (5.57 ± 0.950 vs. $7.79 \pm 0.477 \log_{10}$ CFU/mL; $P = 0.04$, $n = 4$ per group). At 3 hours after infection, the difference in bacterial load remained statistically significant (5.56 ± 1.02 vs. $8.73 \pm 0.081 \log_{10}$ CFU/mL, $P = 0.023$, $n = 4$ per group). By 6 hours after infection, all mock-immunized rabbits had died. One rabbit immunized with serotype-specific antisera died at 12 hours after infection ($4.20 \pm 1.09 \log_{10}$ CFU/mL, $n = 3$). At 24 hours after infection, the bacterial load recovered from the blood of the immunized rabbits was $2.88 \pm 1.29 \log_{10}$ CFU/mL ($n = 3$). Two of the passively immunized rabbits cleared the infection and survived for more than 1 week after infection.

Quantification of PLY-Specific Immunoglobulins

Because active immunization with ψ PLY, but not PPSV23, appeared to protect corneas from damage after pneumococcal challenge, ELISAs were used to quantify the specific anti-PLY immunoglobulins in the sera collected from the rabbits actively immunized with ψ PLY and control rabbits 48 hours after infection. The control rabbits had no or little anti-PLY antibody response, whereas those actively immunized with ψ PLY showed increased average titers of IgG and IgM of 112,640 and 1,300, respectively, but no significant IgA titer. Infected corneas from these rabbits were removed 48 hours after infection, homogenized, and used to determine anti-PLY antibody levels in the cornea. Corneal homogenates of the rabbits actively immunized with ψ PLY had no PLY-specific IgM antibodies but did have titers for IgG and IgA (Table 1). Corneal homogenates from the mock-immunized and naive rabbits had little or no anti-PLY titers for the antibody isotypes tested. ELISAs performed to determine the anti-His tag antibody response showed that serum and corneal homogenates of immunized rabbits had negligible titers (data not shown).

Cytotoxicity and Neutralization Assays

PLY was cytotoxic to HCE cells (1×10^5 cells/well) in a concentration-dependent manner (Fig. 8A) such that 51.42%, 36.23%, and 20.85% of the cells were alive after treatment with 1, 2, and 4 μ g PLY, respectively. The negative control was medium alone, which produced 7.69% cytotoxicity, and the positive control was 1% saponin, which produced 98.8% cytotoxicity. The ability of antibody to PLY to neutralize cytotoxicity was then tested by incubating PLY with anti-PLY IgG purified from immune serum because IgG was the isotype determined to be predominant (Table 1). Co-incubating PLY with purified rabbit polyclonal anti-PLY IgG inhibited the ability of PLY to kill HCE cells (4×10^5 cells/well; $P = 0.006$), whereas incubation with IgG from a mock-immunized rabbit

had no effect on the cytotoxic capability of PLY ($P = 0.132$; Fig. 8B). Immunoglobulin alone had no effect on the corneal cells (data not shown). Fluorescence microscopy of treated HCE cells supported the cell viability data (Fig. 8C). Live cells stained green when the cell-permeable calcein AM dye was converted to an intensely fluorescent dye by the intracellular esterase activity of the cells. EthD-1 stained the nucleic acids of dead HCE cells red on entering the damaged membranes of the cells.

Injection of purified recombinant or native PLY into rabbit corneas is described in other publications.^{53,54} Recombinant PLY at a concentration of 1 μ g was previously determined to cause corneal epithelial erosions⁵⁴; therefore, 1 μ g of PLY was chosen for the in vivo neutralization assay. A concentration of 200 μ g anti-PLY IgG was chosen to induce amplified neutralization of PLY, since 100 μ g was sufficient to neutralize PLY in vitro (Fig. 8B). Intrastromal injection of IgG alone caused corneal defects as early as 2 hours (data not shown), but the effects dissipated by 48 hours. PLY was previously shown to cause corneal erosions as early as 2 hours, with the erosions healing by 48 hours.⁵⁴ The effects of the IgG in the cornea masked any possible inhibition of PLY in vivo, as corneas injected with a mixture of PLY and IgG appeared similar to those injected with IgG alone at all time points (data not shown).

DISCUSSION

This study demonstrated that active immunization with ψ PLY and Freund's adjuvant significantly decreased the corneal damage associated with pneumococcal keratitis, whereas immunization (active or passive) against the polysaccharide capsule using the commercially available adult vaccine (PPSV23) did not. The method of active immunizations using bacterial virulence factors in the rabbit model of bacterial keratitis has been successfully used with other organisms.²¹⁻²⁴ Active immunization with noncytolytic forms of pneumolysin has also been successfully used in other nonkeratitis models of infection, and immunization of mice with pneumolysin toxoid conferred a significant degree of protection when the mice were challenged intraperitoneally or intranasally.^{55,56} Before this study, the protective value of actively immunizing with PPSV23 or ψ PLY had not been evaluated for the cornea.

Our results show that PPSV23 served as a poor immunogen in this model system. It required twice as many immunization boosts to reach titers considered high enough to proceed with the challenge compared with using ψ PLY as an immunogen. In addition, serotype-specific antibody titers performed on serum preadsorbed with cell wall polysaccharide showed that PPSV23 immunization produced inconsistent IgG titers with anti-serotype 19A titers ranging from 0 to 12,800 and anti-serotype 3 titers ranging from 800 to 12,800. The higher titers also failed to correlate with better protection during the course of the infection (data not shown). The protective value of the PPSV23 vaccine remains highly controversial, with numerous clinical trials showing a widely varying degree of antibody

TABLE 1. Specific Anti-PLY Antibody Isotype Titers from Immunized Rabbits

	Serum		Corneal Homogenates	
	Immune ($n = 10$)	Control ($n = 4$)	Immune ($n = 15$)	Control ($n = 7$)
IgG	112,640 $\pm$ 16,722	0	767 $\pm$ 280	0
IgM	1300 $\pm$ 3.16	125 $\pm$ 25	0	0
IgA	0	75 $\pm$ 48	6760 $\pm$ 2269	57 $\pm$ 30

FIGURE 8. (A) Percentage of live human corneal cells after 3-hour incubation at 37°C and 5% CO₂ with increasing quantities of PLY. Not shown are the positive and negative controls, which caused 98.8% and 7.69% cytotoxicity, respectively. Bars indicate SE. (B) Neutralization of PLY by polyclonal anti-PLY IgG. **Significant difference between the two groups ($P < 0.05$). (C) Cellular viability of HCE cells treated with (Ca–Cc) 4 μ g PLY alone, (Cd–Cf) 4 μ g PLY and 100 μ g anti-PLY IgG, or (Cg–Ci) 4 μ g PLY and 100 μ g control IgG for 3 hours at 37°C and 5% CO₂. Live cells were stained with 2 μ M calcein AM (Ca, Cd, Cg), and dead cells were stained with 4 μ M EthD-1 (Cb, Ce, Ch). Micrographs of live and dead cells were then overlaid (Cc, Cf, Ci). Original magnification, $\times 100$.

production,^{57,58} as well as variation in the opsonic capacity and ability of these antibodies to protect against infection.^{59–66} It is also important to note that all clinical and observational trials regarding the protection afforded by PPSV23 immunization correspond to systemic pneumococcal infections. Our study is the first to evaluate the efficacy of PPSV23 immunization and corneal infections.

Moreover, whole-blood survival assays⁶⁷ and opsonophagocytosis assays⁴⁷ performed on blood and serum from PPSV23-immunized rabbits, respectively, showed that active immunization with PPSV23 failed to produce antibodies capable of opsonizing and killing either bacterial strain (data not shown). Previous studies have shown that pneumococcal polysaccharide-specific antibodies that are unable to promote opsonophagocytosis in vitro are still able to protect against pneumococcal infections in mice.^{68,69} Our study showed both a lack of bacterial killing in vitro and a lack of protection in vivo with PPSV23 active immunization. Antibodies capable of opsonizing serotype 3 (*S. pneumoniae* strain WU2) and serogroup 19 (*S. pneumoniae* strain K1443) were purchased and passively administered to rabbits immediately after infection, to confirm that the lack of protection observed after PPSV23 active immunization was not due to the rabbits' production of nonopsonizing antibodies. Our results indicate that even when

the antibodies were able to opsonize and kill serotype-matched pneumococci, there was no reduction in the severity of infection (Fig. 6), confirming that an immunization strategy other than PPSV23 immunization is necessary to protect against pneumococcal corneal disease. The dosage of passively administered antisera was proven to protect against bacteremia caused by *S. pneumoniae* WU2 in the rabbit model. Regardless, bacterial killing did not appear to be a factor in the corneal damage caused by pneumococcal keratitis, because bacterial recovery from corneas had no apparent correlation to clinical severity. This point is underscored by the observation that bacterial recovery from corneas of PPSV23-immunized rabbits mirrored the recovery from corneas of ψ PLY-immunized rabbits (Fig. 4), yet ψ PLY-immunized rabbits were more protected (Fig. 3). This unique observation could be due to the differences between the eye and other pneumococcal targets of the body that are vascular.

In contrast to the results obtained from PPSV23 immunizations, rabbits immunized against PLY and Freund's adjuvant showed a significant reduction in clinical scores when compared with control and PPSV23-immunized rabbits (Fig. 3). ψ PLY and Freund's adjuvant was also highly immunogenic. Furthermore, the protective value of antibodies generated after immunization with ψ PLY was confirmed by exposing HCE cells

to PLY in the presence and absence of purified polyclonal anti-PLY IgG. The anti-PLY IgG isolated from the serum of immunized rabbits protected HCE cells from death caused by PLY (Figs. 8B, 8C). It is important to note that the precise concentration of PLY used for the *in vitro* studies varied due to different preparations of recombinant PLY. Furthermore, the use of 4 μ g for the neutralization assay does not imply that it is the only dose at which anti-PLY IgG is protective. It is more likely that the ability of anti-PLY IgG to neutralize toxin is a dose-dependent phenomenon. Because previous studies suggest that corneal IgG levels are primarily due to the diffusion of serum antibodies into the cornea,⁷⁰ it is feasible to predict that these serum antibodies are capable of neutralizing PLY on diffusion into the cornea during an infection in a manner similar to the neutralization of PLY in cell culture. Unfortunately, determination of IgG-specific neutralization of PLY in the cornea in this study was hindered by the production of corneal defects after intrastromal injection of IgG. It is likely that a more specific ratio of PLY to antibody, as these factors are produced and delivered into the cornea in an *in vivo* situation, would have shown a neutralization effect. However, determination of a specific ratio would have required the use of numerous animals for an answer to a question (whether anti-PLY IgG neutralizes PLY toxicity) that had already been determined *in vitro* (Figs. 8B, 8C). Furthermore, it is likely that secretory IgA molecules found within the cornea are capable of neutralizing PLY. Corneal homogenates had high titers of PLY-specific IgA (Table 1). Although we were unable to purify detectable titers for use in a neutralization assay, it is feasible that the anti-PLY IgA acts locally to neutralize the toxin's effects in a manner much like serum IgG.

Serum antibody responses and corneal antibody responses were different after infection with *S. pneumoniae*, with serum being high in titers of PLY-specific IgG and relatively moderate in titers of IgM antibody isotypes, whereas corneal homogenates had high titers of PLY-specific IgA and relatively moderate titers of IgG. The lack of serum IgA response after a pneumococcal corneal infection is consistent with what has been seen in *P. aeruginosa* and *S. aureus* keratitis.^{71,72} Studies of serum and corneal antibody responses after *P. aeruginosa* keratitis in two mouse strains suggested that corneal IgA production is a local response, while corneal IgG levels may have primarily diffused from the serum with lower levels of local production.⁷⁰ The lack of serum IgA antibodies in ψ PLY-immunized rabbits before infection supports the idea that IgA antibodies found in the cornea after infection were made locally and did not diffuse from the serum.

It is interesting to note that bacterial CFUs did not appear to play a role in the clinical severity of the corneal infection in the groups in this study. For the active immunization experiments, the $\log_{10}$ CFUs recovered from ψ PLY-immunized rabbits were higher for WU2-infected corneas but significantly lower for K1443-infected corneas when compared with the corresponding controls (Fig. 4). PLY, which is released extracellularly, has recently been shown to also localize to the cell wall of pneumococci.⁷³ It is possible that antibodies were able to target PLY within the cell wall of strain K1443 but not WU2 because the polysaccharide capsule of K1443, although present, appears considerably less mucoid than that of WU2 as colonies on blood agar (Norcross EW, unpublished observation, 2008), and any cell-wall-associated PLY present in K1443 would be more exposed to the antibodies in the extracellular milieu. This antibody targeting of PLY within the cell wall may have resulted in a reduction of recovered CFUs from the corneas infected with *S. pneumoniae* K1443 by targeting the bacteria for killing within the macrophage. Alternatively, biochemical differences in capsule composition other than sheer mass, or differences in other components altogether, could account for

the differences in bacterial recovery. Differences in opsonization have been observed previously for types 6B and 19F,⁷⁴ but have not been specifically reported for type 3 versus type 19A. Although higher CFUs were obtained from WU2-infected corneas after immunization with ψ PLY, it is important to note that antibody to PLY has not been linked to an increase in bacterial counts for other types of infection. In fact, immunization with PLY has shown a reduction in recovered bacteria as well as an increase in survival after pneumonia and sepsis.^{55,56,75-82}

It is also important to note that we did not test extraocular tissues for the presence of bacteria to examine whether bacteria in the cornea could have spread to other sites. Although rabbits immunized with ψ PLY, PPSV23, or PBS did not exhibit any symptoms of pneumococcal spread such as lethargy, disorientation, or decreased appetite after intracorneal infection with *S. pneumoniae*, it is impossible to confirm that the spread of bacteria did not occur. Previous research has shown that, although treatments capable of suppressing the host immune system (neutropenia, MyD88 deficiency) may prevent pathology in ocular tissues, such treatments could also allow for bacterial spread to the brain and spleen.⁸³ Although it is possible that immunization with PLY toxoid could prevent disease within the cornea yet allow for extraocular spread of bacteria, the current literature does not provide information on PLY and possible immune suppression in the ocular setting. There are experimental findings showing that what occurs in the cornea is opposite to what occurs in other tissues. For instance, cystic fibrosis transmembrane conductance regulator (CFTR) in lung tissue increases the bacterial clearance of *P. aeruginosa*, whereas CFTR-mediated uptake in the cornea leads to pathogenesis.⁸⁴ Although it is possible that immunization with ψ PLY has negative impacts on bacterial spread into other organ tissues in our rabbit model, it seems unlikely, as immunization with PLY toxoid has not been shown to increase bacterial counts in other systems but rather leads to increased protection and survival.^{55,56,75,81}

It is unclear why immunization with either ψ PLY or PPSV23 led to significantly higher bacteria recovered from the cornea than in the mock-immunized rabbits after WU2 infection, but significantly lower bacteria after K1443 infection (Fig. 4). The presence of more bacteria is not a desirable effect for an immunization. Therefore, caution should be observed in extrapolating the findings to the clinical setting.

Although the precise reason for the variable bacterial clearance is unknown, a lack of correlation between reduced clinical scores and bacterial clearance from the cornea has been seen previously. After immunization with *S. aureus* α -toxin toxoid, there was no difference in bacterial killing between the immunized and mock-immunized rabbits, even though the SLE scores were lower for the immunized group.²⁴ In addition, when rabbits infected with *S. pneumoniae* WU2 were passively immunized with anti-PLY sera, there was no difference in recovered CFUs, although there was a decrease in the clinical scores of the passively immunized group.³⁰

It should be noted that Freund's complete adjuvant was used in the initial immunizations for all the groups regardless of immunogen. Freund's complete adjuvant contains multiple mycobacterial cell components and has been reported to elicit a variety of responses in host animal species ranging from the characteristic tubercle skin lesions to changes in delayed type hypersensitivity (for review, see Ref. 85). Moreover, it is possible that the components in this adjuvant cause production of antibodies that are cross-reactive with pneumococcal components, which would indicate that the effectiveness of active immunization reported herein is not all due to anti-PLY antibodies. However, the Western blot of anti-PLY serum produced by one of the rabbits in this study showed high specificity for PLY and no reactivity with other bacterial

components in pneumococcal extracts (Fig. 1). Freund's adjuvant was chosen for the present study based on its known ability to generate high titers, but cannot be used in the human situation. Alternate modes of immunization would have to be explored for possible use of PLY as an immunogen in humans.

PLY has been shown to play a significant role in pneumococcal keratitis. Studies showed that rabbits infected with a PLY-deficient *S. pneumoniae* strain had reduced pathology when compared with those infected with the wild-type strain.^{37,41} Much of the damage associated with pneumococcal keratitis is due to the inflammatory response caused by PLY.⁴¹ Antibodies directed against PLY could serve to bind to and inactivate the toxin, thereby preventing the release of chemokines and the invasion of PMNs and other immune cells. Histology of WU2-infected eyes supports this idea, in that the ψ PLY-immunized eyes showed reduced infiltration of immune cells when compared to mock- or PPSV23-immunized eyes (Fig. 5). The histologic evidence is supported by the relative amounts of myeloperoxidase in the corneal homogenates such that within each infection group, rabbits immunized with ψ PLY had reduced myeloperoxidase in their corneas compared with rabbits immunized with PPSV23 or PBS indicating that immunization with ψ PLY results in lower quantities of neutrophils infiltrating the cornea.

It is important that immunizing against *S. pneumoniae* provide protection against a broad range of pneumococcal serotypes. The currently available 23- and 13-valent pneumococcal vaccines were designed to target the most prevalent disease-causing capsule types of *S. pneumoniae* in the United States. Although the vaccines are successful against those serotypes, they have limited effectiveness in preventing infections overall due to the development of serotype replacement in which capsule types that are circulating in the community but are not included in the vaccine replace vaccine serotypes as the strains causing most disease.⁸⁶ To successfully immunize against *S. pneumoniae* using PLY as the immunogen, it is necessary that PLY immunization protect against a broad range of serotypes. The *ply* gene has limited variability across all pneumococcal strains regardless of serotype and thus is a promising immunogen.⁸⁷ Previous research showed that actively immunizing mice with PLY toxoid provided protection against at least nine pneumococcal serotypes in intraperitoneal and intranasal infection models,⁵⁶ and the use of protein-based immunizations, including those based on PLY-based vaccines, has been shown to be effective in other models of pneumococcal infections.^{27,88,89} In the present study, two different pneumococcal strains were used—one that has been characterized and one that is an uncharacterized clinical keratitis strain—to determine whether immunization could protect against more than one strain and type. Although it is beyond the scope of this study to test additional pneumococcal serotypes, it is likely that the generalized protection provided by immunization with PLY as reported in mouse intraperitoneal and intranasal infections can be extrapolated to corneal infections. Since the protection observed in this study was significant but not complete, the use of additional components such as conserved pneumococcal proteins other than PLY or specific peptides that target pneumococcal virulence factors might improve the immunization method described herein. To date, there have been no reports of any other pneumococcal virulence factors for keratitis, and PLY cannot be the only factor in pneumococcal keratitis, since disease still occurs in rabbit corneas infected with a PLY-negative strain, albeit significantly less severe than in those infected with a wild-type strain.³² Determination of these factors will aid in refining treatment strategies for pneumococcal keratitis.

In summary, ψ PLY served as an effective immunogen, and actively immunizing rabbits with ψ PLY and Freund's adjuvant

provided protection against corneal damage after challenge with *S. pneumoniae*, whereas active immunization with PPSV23 and Freund's adjuvant and passive immunization with serotype-specific anticapsular antisera did not. Furthermore, PPSV23 acted as a poor immunogen in our model system. In addition, anti-PLY IgG effectively neutralized the toxin in cell culture cytotoxicity assays. This study illustrates the importance of PLY in ocular infections and provides evidence that adding a protein-based immunogen to the current vaccination regimen may ameliorate pneumococcal ocular disease.

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