Immunization with Pneumolysin Protects Against Both Retinal and Global Damage Caused by *Streptococcus pneumoniae* Endophthalmitis

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Abstract

Purpose: To determine whether immunization with pneumolysin (PLY) protects against pneumococcal endophthalmitis.

Methods: New Zealand white rabbits were immunized with a mutant form of PLY that retains only 1% of its cytolytic activity until serum IgG titers were $\geq 51,200$. For a negative control, rabbits were immunized with phosphate-buffered saline (mock). Each vitreous was injected with 10^2 colony-forming units of a clinical endophthalmitis isolate of *Streptococcus pneumoniae*. Severity of endophthalmitis was graded by slit lamp examination at 24 and 48 h postinfection (PI). Serial dilutions of vitreous were plated for bacterial colony-forming units quantitation, eyes were extracted for histology, and a whole blood survival assay was performed.

Results: Immunized rabbits had a significantly lower mean slit lamp examination score at 24 and 48 h PI when compared to mock immunized rabbits ($P \leq 0.002$). There was not a significant difference in bacterial load in the vitreous at 24 or 48 h PI. Histological sections showed that retinas of mock immunized rabbits appeared to be destroyed, whereas those of PLY immunized rabbits remained largely intact. Damage spread to the aqueous humor, stroma, and conjunctiva of mock immunized rabbits by 48 h PI. Minimal damage was observed in the vitreous of PLY immunized rabbits and did not spread to other parts of the eye. Whole blood from immunized rabbits inhibited the growth of bacteria better than whole blood from mock immunized rabbits.

Conclusion: Immunization with PLY helps protect the eye from damage caused by pneumococcal endophthalmitis.

Introduction

BACTERIAL ENDOPHTHALMITIS is a devastating infection of the vitreous leading to destruction of the retina and vision loss.^{1,2} This disease is caused by trauma, ocular surgery, or an endogenous infection.² The clinical severity of infection is dependent upon the bacteria causing infection. *Streptococcus pneumoniae* has been shown to cause blindness more rapidly than other bacteria during endophthalmitis.³ This is due in part to pneumolysin (PLY), a toxin produced by *S. pneumoniae*. PLY belongs to the family of cholesterol-dependent cytolytic toxins.⁴ This toxin binds to cholesterol in host cell membranes and forms pores, often resulting in lysis of the host cell.⁵⁻⁷ Subcytolytic concentrations of PLY have been shown to activate host cell complement.^{8,9} Complement activation can exacerbate pneumococcal infections due to inflammation.¹⁰⁻¹³

PLY has been reported to be important in the pathogenesis of pneumococcal endophthalmitis. PLY alone causes the same clinical severity as *S. pneumoniae* in a rabbit endophthalmitis model.¹⁴ When vitreous of rabbits were infected with a PLY-deficient strain of *S. pneumoniae*, clinical scores were significantly lower than those of its respective parent strain at 24 h postinfection (PI).¹⁵

The aim of the current study was to determine whether immunization with PLY toxoid could provide protection against pneumococcal endophthalmitis. New Zealand white rabbits were immunized with either PLY toxoid or phosphate-buffered saline (PBS) in adjuvant then challenged intravitreally with a clinical ocular strain. Differences in clinical severity, bacterial clearance in the eye, and bacterial clearance by whole blood were compared for the 2 immunization groups.

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Methods

Active immunization

Specific pathogen-free New Zealand white rabbits (Harlan Sprague Dawley, Inc., Oxford, MI) were used in these studies and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. For primary immunizations, rabbits were subcutaneously injected at 4 sites along the dorsal side with a total of 0.1 mg Ψ PLY (a form of recombinant PLY with a Trp433Phe substitution that results in retention of only 1% cytolytic activity¹⁶) with Freund's Complete Adjuvant (Sigma-Aldrich, St. Louis, MO) in a 1:1 (vol:vol) ratio. The expression plasmid harboring Ψ PLY was provided by Dr. Larry S. McDaniel (University of Mississippi Medical Center, Jackson, MS). The rabbits were injected 2 additional times with 0.05 mg of Ψ PLY with Freund's Incomplete Adjuvant (Sigma-Aldrich) at a 1:1 ratio (vol:vol) 1 month apart. Control rabbits were injected with a mixture of Freund's Complete Adjuvant and PBS and Freund's Incomplete Adjuvant and PBS (mock-immunized) using the same method as described above. Blood was collected from each rabbit before the first immunization and 1 week after each boost for the isolation of serum. Serum anti-PLY IgG titers were determined by ELISA.¹⁷ As a control for antibodies against the histidine tag of Ψ PLY, ELISA plates were coated with an unrelated protein containing the same type of histidine tag to determine IgG titers to the histidine tag in the rabbit serum. Titers were defined as the highest dilution that was double the background absorbance (A_{410}).

Bacterial growth

S. pneumoniae E353, a clinical endophthalmitis strain, was kindly provided by Regis Kowalski (Charles T. Campbell Eye Microbiology Lab, Pittsburgh, PA). Bacterial colonies were isolated on 5% sheep blood agar and incubated overnight at 37°C and 5% CO₂. Todd Hewitt broth containing 0.5% yeast extract (THY) was inoculated with one colony and incubated at 37°C in 5% CO₂ overnight. The overnight culture was inoculated into fresh THY at a 1:100 dilution. The bacteria were grown to an optical density at A_{600} that corresponded to $\sim 10^8$ colony-forming units (CFU) per mL as previously determined by growth curve. Accuracy of the bacterial CFU was verified by plate counts of serial dilutions.

Infection

Each rabbit was anesthetized by an intramuscular injection of a mixture of ketamine hydrochloride (100 mg/mL; Butler Company, Columbus, OH) and xylazine (100 mg/mL; Butler Company). Proparacaine hydrochloride drops were applied to each eye before injection. Bacterial cultures were diluted such that each vitreous humor was infected with $\sim 10^2$ CFU in a volume of 10 μ L. A 30-gauge needle was used to inoculate the bacteria into the vitreous humor of each eye.

Slit lamp examination

Slit lamp examination (SLE) for endophthalmitis was performed as previously described.¹⁸ In short, 8 parameters were used for determining the severity of endophthalmitis:

injection, chemosis, corneal inflammation, anterior chamber cell, anterior chamber flare, red reflex, vitreal clarity, and retinal clarity. Each parameter was given a grade from 0 (no pathogenesis) to 4 (maximal pathogenesis), resulting in a total score with a theoretical maximum of 32. Each eye was scored by 2 examiners who were blind to the experiment, and the 2 scores were averaged.

CFU recovery

Vitreous (0.3 mL, nonemulsified) was removed from each eye at 24 and 48 h PI using a 22-gauge needle. Each vitreous was serially diluted, cultured in triplicate on blood agar, and incubated in 5% CO₂ at 37°C overnight for quantitation of log₁₀ CFU recovered.

Whole blood survival assay

Whole blood was removed from rabbits at 48 h PI. A bacterial survival assay was performed as previously described.¹⁹ In short, 1 mL of whole blood from Ψ PLY or mock immunized rabbits was mixed with 100 CFU of *S. pneumoniae* in THY. Each sample was placed in a shaker incubator at 37°C for 3 h, serially diluted, plated in triplicate, and incubated overnight to determine bacterial survival.

Neutralization assays

IgG was isolated and purified using protein-A coupled to Sepharose beads²⁰ from tertiary sera collected from rabbits immunized with Ψ PLY and Freund's Complete/Incomplete Adjuvants. Fifty microliters of several concentrations of fully cytolytic recombinant PLY were purified and incubated with 50 μ L of several concentrations of purified anti-PLY IgG or control IgG purified from sera of mock-immunized rabbits in 96-well round-bottom microplates in triplicate at 37°C for 30 min. A 5% solution of rabbit red blood cells (RBCs) were added to each well and incubated in triplicate at 37°C for 30 min. The plates were centrifuged at 3,200 rpm for 5 min to pellet nonlysed RBCs. The supernatants were measured for hemoglobin content by spectrophotometry at A_{450} . Saponin (0.5%) was used as the positive control that represented 100% hemolysis. Percent inhibition of hemolysis was determined by averaging triplicate values of each concentration and comparing these values to positive controls.

Histopathology

Whole eyes were removed and histology was performed by Excalibur Pathology, Inc. (Moore, OK). Hematoxylin and eosin staining was used to stain the eye sections. Polymorphonuclear cells were determined by morphology.

Statistics

Clinical SLE scores and bacterial CFU data were analyzed as dependent variables using a 2-level factorial model in the analysis of variance. The main factors were treatment (mock or Ψ PLY) and time (24 or 48 h). The model was analyzed as a repeated measures model where within subject correlation (eyes within rabbit) was taken into account.²¹ The error term was based on 2 replications of each of the experiments. Mean values for the interaction of time by treatment were separated in *post-hoc* testing using a method of simulation for

alpha level adjustment for multiple comparisons.²² Whole blood survival data were analyzed using the nonparametric Mann-Whitney U test.²³ The alpha level for all hypothesis tests in all statistical procedures was preset at 0.05. All data analysis and manipulation was carried out using the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Immunization

Rabbits immunized with Ψ PLY had PLY-specific IgG titers $\geq 51,200$. No anti-PLY IgG titer was observed for mock immunized rabbits. A low titer (~ 800) was observed for immune or mock serum when plates were coated with the unrelated histidine-tagged protein.

Rabbit endophthalmitis model

The eyes of Ψ PLY immunized rabbits infected with the *S. pneumoniae* endophthalmitis clinical strain had a significantly lower mean SLE score than mock immunized rabbits at 24 and 48 h PI ($N=14$ eyes per time point) with an average SLE score $\pm$ standard error of the mean of 9.02 ± 1.06 and 17.21 ± 1.64 , respectively. Mock immunized rabbits scores at 24 and 48 h PI were 13.43 ± 1.72 and 24.06 ± 1.52 , respectively ($P \leq 0.002$; Fig. 1A). More inflammation was observed in the eyes of mock immunized rabbits as com-

FIG. 1. (A) Clinical scores of PLY or mock immunized rabbits infected with *Streptococcus pneumoniae* at 24 and 48 h PI. (B) Representative pictures of infected eyes of PLY immunized or mock immunized rabbits at 24 and 48 h PI. PLY, pneumolysin; PI, postinfection. *Significant difference in SLE score.

TABLE 1. Log_{10} COLONY-FORMING UNITS RECOVERED FROM VITREOUS INFECTED WITH *STREPTOCOCCUS PNEUMONIAE*

	Log_{10} CFU/mL $\pm$ SEM (N) ^a	
	24 h	48 h
Immune	10.93 $\pm$ 0.28 (8)	8.01 $\pm$ 0.42 (7)
Mock	10.32 $\pm$ 0.62 (7)	6.20 $\pm$ 0.29 (7)

^a $P=0.46$ at 24 h PI. $P=0.07$ at 48 h PI.

CFU, colony-forming units; PI, postinfection; SEM, standard error of the mean.

pared to the eyes of Ψ PLY immunized rabbits at both timepoints (Fig. 1B).

CFU recovery

At 24 and 48 h PI, there was not a significant difference in log_{10} CFU recovered from the vitreous of Ψ PLY immunized ($N=8$ and 7 eyes, respectively) compared to that of mock immunized ($N=7$ and 7 eyes, respectively) rabbits ($P \geq 0.07$; Table 1).

Whole blood survival assay

Whole blood collected from Ψ PLY immunized rabbits significantly lowered the ability of *S. pneumoniae* to grow as compared to whole blood collected from mock immunized rabbits. The mean CFU/mL $\pm$ standard error of the mean recovered from whole blood samples of Ψ PLY immunized rabbits was 129 ± 51.2 . For mock immunized rabbits, a mean of 1267 ± 208.9 was recovered from whole blood samples ($P < 0.0001$).

Neutralization assays

Neutralization assays were performed to characterize whether there was a significant difference in the neutralizing ability of anti-PLY IgG on the hemolytic activity of PLY compared to that of control IgG isolated from normal serum. At a concentration of 200 μ g, anti-PLY IgG was able to prevent lysis of RBCs by PLY at concentrations of 10 and 50 ng. Control IgG at 200 μ g had little to no neutralizing effects on PLY. Lower concentrations of anti-PLY IgG were used but were unable to neutralize the activity of the PLY (data not shown).

Histopathology

All eyes were removed in whole, sectioned, and stained with hematoxylin and eosin. The infection became so severe in mock immunized rabbits that it caused panophthalmitis involving massive inflammation of the vitreous, aqueous humor, cornea, and conjunctiva (Figs. 2A–C and 3A–C). A representative retina from a mock immunized rabbit was destroyed by the pneumococcal endophthalmitis infection (Fig. 2B). The pathology was less severe in Ψ PLY immunized rabbits. Fewer immune cells and less damage to the whole eye were observed (Figs. 2D–F and 3D–F).

Discussion

A recent study examined 497 patients with endophthalmitis admitted to the Wills Eye Institute from 1989 to 2000.

FIG. 2. Histology of infected eyes of mock immunized (A–C) or PLY immunized (D–F) rabbits at 48 h PI. (A, D) Retina 200 $\times$. (B, E) Vitreous 40 $\times$. (C, F) Retinal cells (blue arrow) and PMNs (black arrow) 1,000 $\times$.

That study determined that *S. pneumoniae* was one of the top 3 causative bacteria in patients who had bacterial endophthalmitis diagnosed in the first 3 days after infection.²⁴ Moreover, the visual prognosis for patients with pneumococcal endophthalmitis is poor.² Many of the cases of pneumococcal endophthalmitis occur after ocular surgery²⁵; therefore, preventive measures to protect against this disease would be beneficial to patients undergoing these types of surgeries. Currently, the only pre-emptive defenses against *S. pneumoniae* are polyvalent capsular vaccines, which are usually administered for the prevention of pneumonia. Since

PLY is an important virulence factor of *S. pneumoniae*, and has been shown to cause damage in endophthalmitis, it would be useful to use this toxin in a vaccine to help protect against pneumococcal endophthalmitis.

PLY is a destructive toxin produced by *S. pneumoniae*. It has been shown that PLY protein alone injected into the vitreous causes the same damage as pneumococcal endophthalmitis.¹⁴ In a later study by the same group, rabbits were challenged intravitreally with a parent strain and a PLY-negative mutant strain of *S. pneumoniae*. At 24 h PI, the severity of infection caused by the mutant strain was significantly

FIG. 3. Histology of infected eyes of mock immunized (A–C) or PLY immunized (D–F) rabbits at 48 h PI. (A, D) Whole eyes; no magnification. (B, E) Cornea 40 $\times$. (C, F) Conjunctiva 40 $\times$.

lower than that caused by the parent strain. However, by 48 h PI, there was not a significant difference in clinical severity between the 2 strains,¹⁵ suggesting that there are other factors involved in the course of infection of pneumococcal endophthalmitis. The results presented herein offer some support of these previous findings in that the rabbits immunized with Ψ PLY were not 100% protected; however, the severity of the disease was reduced by immunization. Adjunct prophylaxes or therapies, such as administration of antibiotics in addition to immunization with Ψ PLY, would be warranted for more complete protection. Since the pa-

thology was not completely abrogated, other virulence factors of *S. pneumoniae* may cause the pathology observed in PLY immunized rabbits. Other pneumococcal antigens, such as PspA, PspC, and capsular polysaccharide, have been shown to protect against non-eye-related diseases.^{26–30} Perhaps using these antigens in conjunction with PLY could lead to a more effective vaccine.

A previous study by this laboratory showed that passive immunization with PLY antiserum protected rabbits against pneumococcal keratitis, but did not lower the log₁₀ CFU recovered from the corneas at 24, 36, or 48 h PI when compared

to rabbits passively immunized with nonimmune serum.³¹ The current study had similar findings, with no significant difference observed in log₁₀ CFU recovered from the vitreous of PLY immunized or mock immunized rabbits at 24 or 48 h PI.

Whole blood from Ψ PLY immunized rabbits was significantly more proficient at preventing the growth of bacteria than whole blood from mock immunized rabbits. However, the whole blood from Ψ PLY immunized rabbits did not eradicate the bacteria. Obviously fewer immune cells entered the eye (as observed by lower SLE scores, visual evidence in Fig. 1B, and fewer immune cells in the histological section of vitreous), so perhaps less clearing of bacteria occurred in the vitreous.

Since antibiotic eye drops are commonly used after surgery, the lack of bacterial clearing observed in this study for immunized rabbits would not likely be a major concern for postsurgery patients who would use a PLY-based vaccine. Also, since the PLY toxoid vaccine helps slow the progression of the ocular pathology, drugs could be administered after detection of the infection to aid in killing of the bacteria for people who have been vaccinated with PLY toxoid and suffer from eye trauma leading to pneumococcal endophthalmitis. Some antibiotics have been shown to eliminate *S. pneumoniae* in patients with pneumococcal endophthalmitis³²; however, the antibiotics are not capable of protecting the eye against global damage.^{32,33} It may be of great value to use a combination of antibiotics and immunization to both eradicate the bacteria and to protect against damage, since use of one or the other alone appears to be insufficient in accomplishing both tasks.

This laboratory has employed the rabbit model of pneumococcal endophthalmitis to determine the efficacy of topical fluoroquinolones in the prophylaxis and treatment of this disease and determined that these antibiotics can effectively reduce bacterial loads in the aqueous humor.³⁴ Since surgery is one of the leading causes of bacterial endophthalmitis, and pneumococcal endophthalmitis can rapidly lead to blindness, a combination of prophylactic antibiotics and the protective vaccine described herein may be clinically useful. The practicality of using vaccination to protect humans before eye surgery remains to be determined. Perhaps a combination vaccine that targets the most common causes of bacterial endophthalmitis in addition to *S. pneumoniae*, such as *Staphylococcus* species, may be more practical due to a broader spectrum of coverage.

Author Disclosure Statement

No competing financial interests exist for any author.

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