

Cholesterol as Treatment for Pneumococcal Keratitis: Cholesterol-Specific Inhibition of Pneumolysin in the Cornea

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PURPOSE. The purpose of this study was to determine whether cholesterol, the host cell receptor for pneumolysin of *Streptococcus pneumoniae*, could effectively treat pneumococcal keratitis.

METHODS. New Zealand White rabbits were intrastromally injected with 10^5 colony-forming units (CFUs) of *S. pneumoniae* D39. Corneas were treated with topical drops of 1% cholesterol every 2 hours beginning 25 hours after infection and were examined by slit lamp microscopy 24, 36, and 48 hours after infection. Rabbits were killed, and CFUs were recovered from the corneas after the final slit lamp examination (SLE). Minimal inhibitory concentration (MIC) assays of cholesterol against bacteria were performed. Specific inhibition of pneumolysin by cholesterol in the rabbit cornea was tested by intrastromal injection of pneumolysin with or without cholesterol and was compared with cholesterol inhibition of pneumolysin in vitro using hemolysis assays with rabbit erythrocytes.

RESULTS. Corneas treated with cholesterol had significantly lower SLE scores 48 hours after infection than corneas treated with vehicle ($P = 0.0015$). Treated corneas also had significantly less \log_{10} CFUs than vehicle-treated corneas ($P = 0.0006$). Cholesterol at a 1% concentration was bactericidal to bacteria in vitro, and lower concentrations of cholesterol were partially inhibitory in a concentration-dependent manner. Cholesterol also specifically inhibited 1 μ g pneumolysin in vivo and up to 50 ng pneumolysin in vitro.

CONCLUSIONS. Topical cholesterol is an effective treatment for *S. pneumoniae* keratitis. Cholesterol not only inhibits pneumolysin, it is also bactericidal. (*Invest Ophthalmol Vis Sci.* 2007; 48:2661-2666) DOI:10.1167/iov.07-0017

Bacterial infections of the cornea occur in approximately 25,000 people in the United States each year and in a variable number of people worldwide. *Streptococcus pneumoniae* often occurs as one of the top three species causing bacterial keratitis.¹⁻¹⁶ Antibiotics are routinely used to treat

ocular pneumococcal infections; however, this bacterium is developing resistance to antibiotics.^{17,18} Identification of the ocular virulence factors of this organism and development of new therapies to treat pneumococcal keratitis could prove useful for the prevention of loss of vision.

One of the key virulence factors of *S. pneumoniae* is the toxin pneumolysin, which belongs to the family of pore-forming cholesterol-dependent cytolysins (CDCs) including perfringolysin, streptolysin, and listeriolysin.¹⁹ The binding of cholesterol and the ability to form pores in host cell membranes are major characteristics of the members of this group of toxins.²⁰⁻²³ In addition to its cytolytic function, pneumolysin activates host complement, resulting in inflammation mediated by polymorphonuclear leukocytes (PMNs). The cytolytic and complement-activating functions of pneumolysin are associated with different domains of the toxin.²⁴⁻²⁷

Pneumolysin is important for the inflammation associated with pneumococcal keratitis. Johnson et al.²⁸ produced a pneumolysin-deficient strain of *S. pneumoniae* and showed that this strain was defective in causing the full effects of keratitis compared with the parent strain. Restoration of the gene for pneumolysin allowed this strain to recover its full virulence.²⁹ The major clinical difference between the parent or rescued strain and the pneumolysin-deficient strain was the degree of inflammation, especially polymorphonuclear leukocyte (PMN) infiltration into the cornea. When the complement activation domain of the pneumolysin gene was deleted from *S. pneumoniae*, corneal virulence was also significantly reduced, but to a lesser extent than when the entire gene was deleted.³⁰ These results suggested that the complement activation domain of pneumolysin contributed to corneal virulence, though this domain was not fully responsible for the damage caused by the toxin.

Binding of pneumolysin by exogenous cholesterol, the target site for pneumolysin binding, has been shown to inhibit the cytolytic function of the toxin.^{31,32} If the activity of pneumolysin is important in keratitis, then cholesterol could have therapeutic value by providing an excess of toxin-binding sites and inhibiting the binding of pneumolysin to host cells. In this study, soluble cholesterol was used as a topical treatment for *S. pneumoniae* keratitis to determine whether this treatment could inhibit the effects of pneumolysin in the cornea and thus could ameliorate the damage associated with pneumococcal keratitis.

METHODS

Bacterial Strains and Growth Conditions

S. pneumoniae D39 (Item 6302; American Type Culture Collection, Manassas, VA) was cultured on blood agar base (Oxoid; Remel, Inc., Lenexa, KS) containing 5% sheep blood (Hemostat Laboratories, Dixon, CA) at 37°C and routinely grown in Todd Hewitt broth supplemented with 0.5% yeast extract (THY; BD Biosciences, Sparks, MD) at

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37°C, unless otherwise indicated. Subcultures of bacteria were grown to concentrations of 10^8 colony-forming units (CFUs)/mL and were diluted in THY broth to achieve an inoculum of 10^5 CFUs/ $10\ \mu\text{L}$ for intrastromal injections. Tenfold dilutions of the inoculum were cultured in triplicate on 5% sheep blood agar to verify the number of CFUs.

Rabbit Corneal Infections and Treatment with Cholesterol

New Zealand White rabbits were used in these studies and were maintained in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of the University of Mississippi Medical Center approved the animal protocol. Rabbits were anesthetized by subcutaneous injections 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 (Falcon Pharmaceuticals, Fort Worth, TX) was topically applied to each eye before intrastromal injection. *S. pneumoniae* D39 (10^5 CFUs/ $10\ \mu\text{L}$) was injected intrastromally. Topical treatment of rabbit eyes was initiated at 25 hours and continued at 2-hour intervals up to 47 hours after infection. Treatment consisted of one drop of a solution of 1% soluble cholesterol (40 mg pure cholesterol/g, as determined by HPLC, complexed with methyl- β -cyclodextrin; Sigma-Aldrich, St. Louis, MO) and 20% glycerol in PBS in each of 11 eyes and one drop of a solution of 20% glycerol in PBS (control solution) in each of 10 eyes.

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 examination (SLE) of infected rabbit corneas 24, 36, and 48 hours after infection. Seven ocular parameters were graded: injection, chemosis, iritis, fibrin, hypopyon, corneal edema, and corneal infiltrate.²⁸ Each parameter was given a grade from 0 (normal) to 4 (very severe). Grades were totaled for each eye, and an average was calculated for the two observers, resulting in an overall score for each eye ranging from 0 (normal) to a theoretical maximum of 28. Scores were expressed as the mean \pm SEM.

Forty-eight hours after infection, the rabbits were killed by intravenous overdose of sodium pentobarbital (100 mg/mL; Sigma-Aldrich). Corneas were removed, dissected, and homogenized in sterile PBS using a tissue homogenizer (IKA Works, Inc., Wilmington, NC). Aliquots of corneal homogenates were serially diluted and plated in triplicate on blood agar for 48 hours at 37°C. Colonies were counted, and bacterial CFUs for the corneas were determined and expressed in base 10 logarithm \pm SEM. These experiments were performed twice, yielding similar results, and the data from the two experiments were combined ($n = 11$ corneas for cholesterol-treated rabbits; $n = 10$ corneas for PBS-treated control rabbits).

Cholesterol Inhibition of *S. pneumoniae* In Vitro

Cholesterol was tested for possible inhibition of *S. pneumoniae* D39 using the broth macrodilution method of the Clinical and Laboratory Standards Institute.³³ *S. pneumoniae* colonies were isolated on 5% sheep blood agar and resuspended in cation-adjusted Mueller-Hinton broth containing 5% lysed horse blood to a McFarland turbidity standard of 0.5. Bacteria were adjusted to 1×10^6 CFU/mL and were incubated for 20 hours at 37°C with an equal volume of PBS, PBS containing 20% glycerol, or 2-fold dilutions of cholesterol in PBS containing 20% glycerol. Absorbance at 625 nm was determined for each sample, and 10-fold dilutions of each sample were plated in triplicate on 5% sheep blood agar. Three separate experiments yielded similar results.

Injection of Pneumolysin into Rabbit Corneas

Recombinant pneumolysin was purified as described previously,³⁴ and its purity was confirmed by SDS-PAGE. After systemic anesthesia with ketamine and xylazine and topical anesthesia with proparacaine, pneumolysin in PBS containing 20% glycerol (1 μg , 500 ng, 100 ng, 50 ng,

10 ng, 5 ng, and 0 ng) or pneumolysin mixed 1:1 (vol/vol) with 2% cholesterol in PBS containing 20% glycerol (final concentration of 1% cholesterol) was injected into rabbit corneas. Corneas were examined periodically for 48 hours by two observers, corneal erosions were stained with fluorescein and measured, and photographs were taken with the aid of a slit lamp microscope.

Neutralization of Pneumolysin In Vitro

Neutralization of pneumolysin by cholesterol was determined by a hemolysis assay performed in triplicate. Fifty microliters of pneumolysin in PBS at a concentration of 500 ng, 100 ng, 50 ng, 10 ng, 5 ng, or 0 ng were mixed and incubated with 100 μL PBS containing 20% glycerol or 100 μL of 2% cholesterol in PBS containing 20% glycerol in round-bottom wells of 96-well plates. Rabbit erythrocytes were washed and suspended to 5% with PBS, and then 50 μL suspension was added to each well. The plates were incubated for 30 minutes at 37°C and then placed at 4°C until intact erythrocytes settled into the bottoms of the wells. Supernatants were placed into wells of flat-bottom, 96-well plates, and the absorbance was taken at 450 nm. Erythrocytes incubated with PBS containing 20% glycerol and erythrocytes incubated with 2% cholesterol in PBS containing 20% glycerol served as negative controls and as background subtraction for the appropriate wells. Erythrocytes incubated with 0.5% saponin and PBS containing 20% glycerol served as a 100% lysis control.

Statistical Analysis

Data were analyzed using the statistical analysis system (SAS) for computers (SAS Institute, Cary, NC). Clinical scores were analyzed using nonparametric one-way analysis of variance. Bacterial loads were analyzed using the general linear models procedure of least squares means. $P < 0.05$ was considered significant.

RESULTS

Treatment of Experimental Pneumococcal Keratitis

Cholesterol was tested as a treatment for experimental pneumococcal keratitis. Rabbit eyes that received topical cholesterol drops after intrastromal injections of *S. pneumoniae* had significantly lower SLE scores 48 hours after infection than eyes that received drops of the control solution (Fig. 1; $P = 0.0015$).

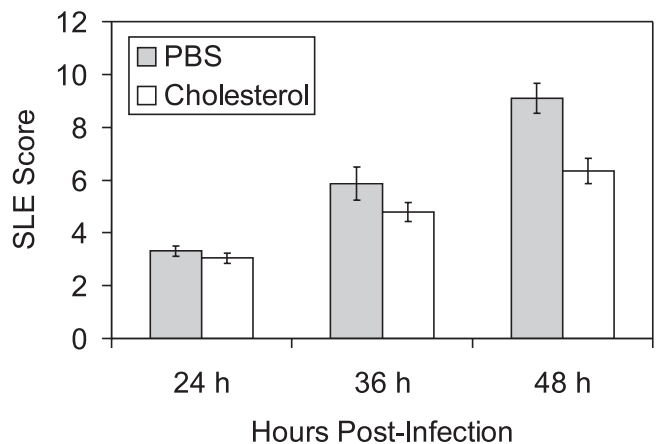


FIGURE 1. SLE scores of infected corneas treated with cholesterol or placebo. Rabbit corneas, infected with *S. pneumoniae* and then treated with either PBS (control) or 1% cholesterol in PBS, were examined by SLE. Corneas were examined 24, 36, and 48 hours after infection. Corneas treated with cholesterol had significantly lower SLE scores than control corneas 48 hours after infection. Data are mean \pm SEM.

Control eyes exhibited a larger amount of corneal infiltrate and opacity than treated eyes (Fig. 2). The parameters contributing to the significant difference in SLE scores between control eyes and treated eyes were presence of hypopyon (average 3-fold difference), fibrin (2.3-fold difference), stromal edema (1.9-fold difference), iritis (1.5-fold difference), and stromal infiltrate (1.5-fold difference). Control eyes had an average SLE score of approximately 9 at 48 hours after infection, though the theoretical maximum is 28; this result was attributed to some of the parameters being graded on a percentage coverage scale. For example, the hypopyon shown in Figure 2B (left panel) covers approximately 1/16 of the eye, so the parameter grade for the hypopyon was assigned 0.25 out of a possible 4.0. Cholesterol-treated corneas also had significantly lower log CFU compared with control eyes (2.64 ± 0.50 vs. 5.35 ± 0.42 ; $P = 0.0006$). Administration of topical drops of cholesterol to uninfected eyes had no effect (not shown).

Cholesterol Inhibition of *S. pneumoniae* In Vitro

The possible growth inhibition of *S. pneumoniae* by cholesterol was examined to determine whether the significant difference in CFU recovered from infected rabbit corneas could have resulted from the inhibition of bacterial growth by the topical cholesterol treatments (Table 1). Optical density measurements after minimal inhibitory concentration assay were confusing in that the optical densities of the bacteria incubated with the two lowest concentrations of cholesterol were lower than the optical densities of bacteria incubated with higher concentrations of cholesterol or with no cholesterol. These optical density measurements suggested that small amounts of cholesterol inhibited bacterial growth, whereas high concentrations of cholesterol had little or no effect. To resolve these unexpected results, the minimal inhibitory concentration assay was repeated, and the bacteria were serially diluted and plated in triplicate. Colony counts of the resultant CFUs showed that the cholesterol was bactericidal in a concentration-dependent manner; that is, as the concentration of cholesterol increased, the CFUs of the bacteria decreased (Table 1). Glycerol was also tested for possible effects because it was present in all the

TABLE 1. Effect of Cholesterol on *S. pneumoniae* In Vitro

Sample Incubated with Bacteria	CFU/mL*
PBS	2.86×10^7
PBS + 20% glycerol	4.99×10^7
2% cholesterol	140
1% cholesterol	0
0.5% cholesterol	6.93×10^2
0.25% cholesterol	1.96×10^3
0.125% cholesterol	1.29×10^4
0.063% cholesterol	3.33×10^4

Cholesterol was prepared in PBS containing 20% glycerol.

* CFU/mL data are from 1 of 3 representative experiments.

cholesterol formulations for this study. The CFUs of the bacteria incubated in broth containing 20% glycerol in PBS were similar to the CFUs of bacteria incubated in broth containing PBS alone, indicating that glycerol had no effect on bacterial growth.

Specific Inhibition of Pneumolysin by Cholesterol In Vivo

To test whether the ability of topical cholesterol to significantly reduce the clinical symptoms of pneumococcal keratitis could be attributed to specific inhibition of pneumolysin, purified pneumolysin in the presence or absence of cholesterol was intrastromally injected into rabbit corneas. At concentrations of 500 ng or lower, pneumolysin had no effect on the corneas (data not shown). At a concentration of 1 μ g, however, pneumolysin caused corneal epithelial erosions beginning between 2 and 5 hours and continuing until 24 hours after infection (Figs. 3A, 3B). The erosions in the corneas receiving pneumolysin began to heal 24 hours after injection, as evidenced by a re-epithelialization of the cornea and diffuse fluorescein staining (Figs. 3A, 3B). Pneumolysin mixed with cholesterol did not cause erosions until 24 hours after injection (Figs. 3C, 3D). Pneumolysin alone caused corneal erosions that were 6 mm in diameter, whereas pneumolysin mixed with cholesterol caused corneal erosions 4 mm in diameter. All corneas were fully healed by 48 hours after injection. Corneas injected with cholesterol alone became slightly hazy at the site of injection; this haze faded with time (48 hours), and no other changes in the corneas were observed.

Specific Inhibition of Pneumolysin by Cholesterol In Vitro

The potency of the specific cholesterol formulation used in this study was quantified in vitro. Pneumolysin preparations at concentrations of 10 ng or lower did not lyse rabbit erythrocytes, but concentrations of 50 ng and greater caused at least 81% lysis (Fig. 4). Cholesterol at a concentration of 1% inhibited the hemolytic activity of 50 ng pneumolysin by 92% (6.43% hemolysis vs. 81.17% hemolysis) and 100 ng pneumolysin by 42% (47.50% hemolysis vs. 82.13% hemolysis). Cholesterol did not inhibit 500 ng pneumolysin at this concentration (Fig. 4).

DISCUSSION

A novel treatment for *S. pneumoniae* keratitis, based on bactericidal activity and the specific inhibition of the major ocular toxin pneumolysin by its cellular receptor (cholesterol), has been introduced in the present study. The binding of pneumolysin to cholesterol³⁵ and the ability of exogenous cholesterol to inhibit the hemolytic activity of pneumolysin^{31,32} have long been known. The actual application of this knowledge in the

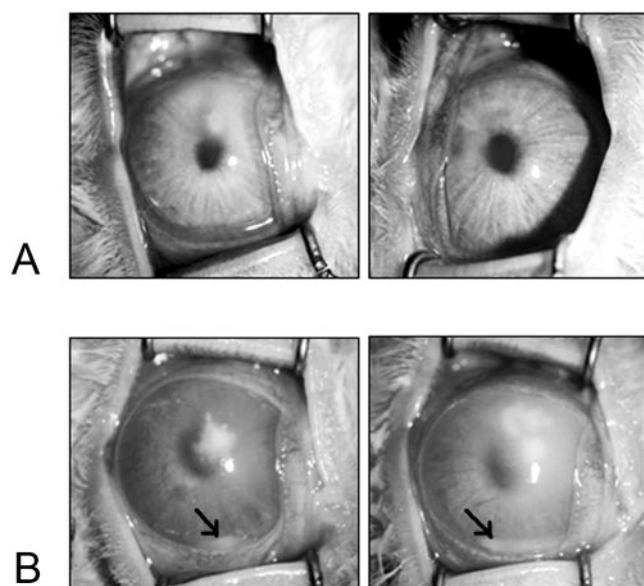


FIGURE 2. Infected eyes after treatment with cholesterol or placebo. Rabbit corneas were photographed 48 hours after infection. Corneas were treated with 1% cholesterol in PBS (A) or PBS alone as a control (B). Control corneas had more infiltrate and showed hypopyon formation (arrows).

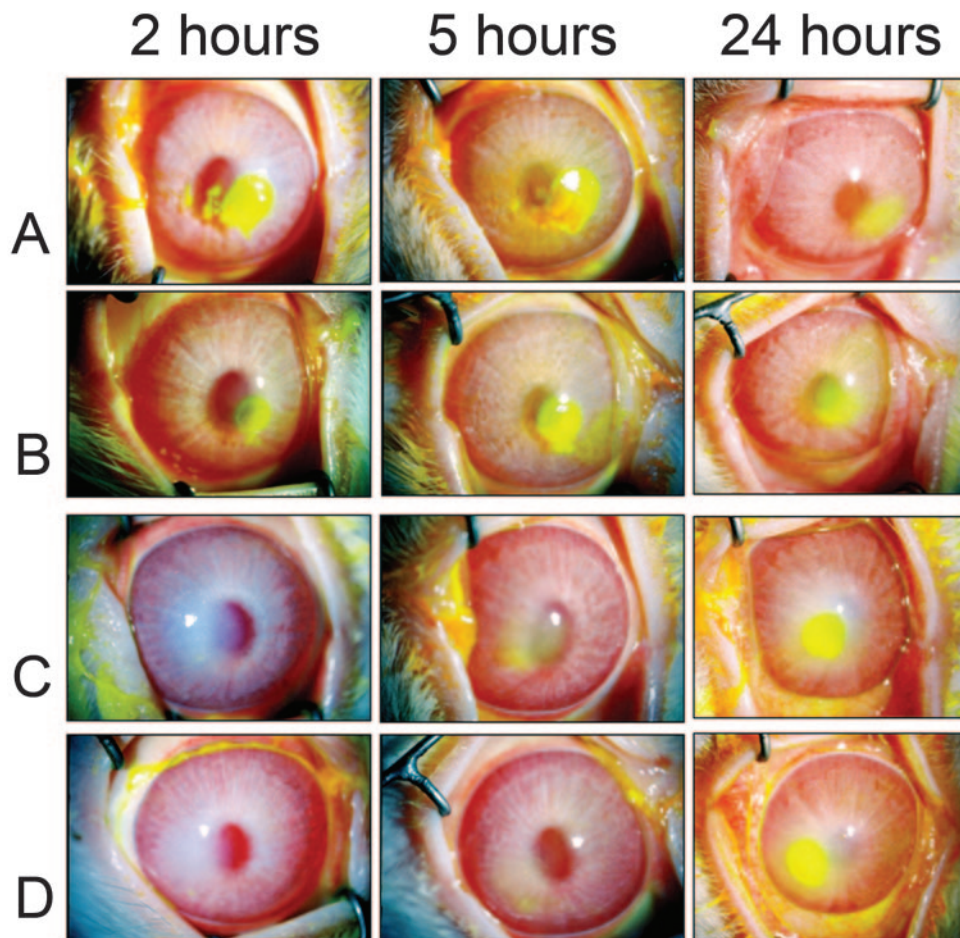


FIGURE 3. Cholesterol inhibition of erosions caused by pneumolysin. Rabbit corneas were injected with pneumolysin (A, B) or pneumolysin mixed with cholesterol (C, D). The presence of corneal erosions is highlighted by fluorescein staining, which is readily apparent 2 and 5 hours after injection in the corneas injected with pneumolysin (A, B) and 24 hours after injection in the corneas injected with pneumolysin mixed with cholesterol (C, D).

treatment of pneumococcal disease, however, has not been reported until now. Cholesterol was able to inhibit corneal epithelial erosion formation by pneumolysin, alleviate the clinical symptoms of *S. pneumoniae* keratitis, and kill *S. pneumoniae* in the cornea.

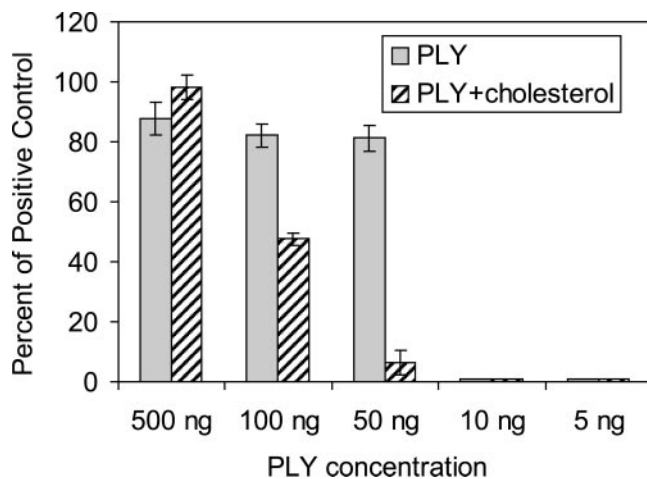


FIGURE 4. Cholesterol inhibition of hemolysis by pneumolysin. The hemolytic activity of pneumolysin (PLY) was inhibited by cholesterol. Different concentrations of pneumolysin, or pneumolysin mixed with 1% cholesterol, were incubated with rabbit erythrocytes. Lysis was measured by spectrophotometric analysis of supernatants after incubation and was compared with a saponin control that represented 100% lysis. Data are mean \pm SD.

Cholesterol not only targeted pneumolysin, it also targeted the bacteria by killing them. This effect was shown in vivo by a significant decrease in CFUs in the corneas that were treated with cholesterol and in vitro by a minimal inhibitory concentration assay. Minimal inhibitory concentration assays of cholesterol against *S. pneumoniae* should be performed with caution because the optical density measurements were not representative of actual CFUs (Table 1). It is recommended that cultures be plated for actual CFU counts after the assay.

The reduction in SLE scores by treatment with cholesterol was statistically significant. Considering the specificity of cholesterol for pneumolysin, however, the reduction would be expected to be more dramatic than it actually was. One explanation for the lack of dramatic reduction could be that the cholesterol did not inhibit the complement activation activity of pneumolysin. Alternatively, *S. pneumoniae* virulence factors other than pneumolysin could be responsible for some ocular disease parameters. Another possibility is that the cholesterol formulation used in this study was complexed with methyl- β -cyclodextrin, a cholesterol synthesis inhibitor, to make it soluble (Sigma-Aldrich). The presence of a cholesterol synthesis inhibitor could decrease the in vivo cellular production of cholesterol in the cornea, offsetting the benefits of treatment with exogenous cholesterol. It is expected that the use of a purer cholesterol formulation for treatment would cause a more dramatic reduction in SLE score, provided the cholesterol could be suspended in a feasible form for treatment purposes.

The inhibition of pneumolysin by cholesterol in the cornea (Fig. 3) was different from the in vitro inhibition (Fig. 4). First, nanogram concentrations of pneumolysin were not sufficient

to cause corneal epithelial erosions; 1 μg was the concentration shown to cause erosions. In the hemolysis assay, however, nanogram concentrations of the same pneumolysin preparation were able to cause hemolysis of rabbit red blood cells. Second, 1% cholesterol was able to inhibit erosion formation by 1 μg pneumolysin for up to 24 hours in vivo, whereas 1% cholesterol was incapable of inhibiting the hemolytic activity of 500 ng pneumolysin in vitro. The differences could have been the result of possible differences in the host cells (corneal cells vs. red blood cells). In vivo and in vitro results, however, showed that cholesterol specifically inhibited pneumolysin.

The two functions of pneumolysin, cytolysis and complement activation, have been reported to be localized to specific domains of the toxin.²⁴⁻²⁷ However, deletion of only the complement activation domain of the pneumolysin gene causes a reduction, but not a complete disappearance, of corneal virulence, and this reduction is not as extensive as when the entire gene is deleted.³⁰ These results suggest that the complement activation domain of pneumolysin contributes to corneal virulence, though this domain is not fully responsible for the damage caused by the toxin.

Pneumolysin activates the classical complement pathway,^{25,36-38} resulting in the production of proinflammatory cytokines. Interleukin (IL)-8 is a chemoattractant for neutrophils and has been shown to be upregulated in inflamed human corneas³⁹ and is upregulated in monocytes in response to pneumolysin.⁴⁰ The induction of IL-6 by pneumolysin in non-ocular model systems of *S. pneumoniae* infection has been observed.⁴¹ Interestingly, a mutant pneumolysin molecule with reduced cytolytic activity had decreased ability to induce IL-6, suggesting that the domain involved in cytolytic activity was involved in proinflammatory cytokine induction.⁴¹

The binding of pneumolysin to cholesterol causes a change in the secondary structure of pneumolysin²² rendering it unable to form pores in host cell membranes. Paton et al.²⁵ showed that preincubation of pneumolysin with cholesterol did not affect the ability of pneumolysin to convert complement protein C3. Based on this information, an ideal treatment for *S. pneumoniae* keratitis would be cholesterol combined with an inhibitor of pneumolysin complement activation activity. Blocking complement activation is expected to result in a decrease in the inflammation that is the hallmark of pneumococcal keratitis. Use of cholesterol to treat pneumococcal keratitis has been demonstrated herein as a viable alternative or an adjunct therapy and could offer a means to cope with the increasing frequency and complexity of antibiotic resistance demonstrated by *S. pneumoniae*.

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