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Pathogenesis of A Clinical Ocular Strain of *Streptococcus pneumoniae* and the Interaction of Pneumolysin with Corneal Cells

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Abstract

Streptococcus pneumoniae is an important cause of bacterial keratitis, an infectious disease of the cornea. This study aimed to determine the importance of pneumolysin (PLY), a pneumococcal virulence factor, in keratitis using a clinical keratitis isolate (K1263) and its isogenic mutant deficient in PLY (K1263ΔPLY) and determine the effect of these strains on primary rabbit corneal epithelial (RCE) cells. Each strain was injected into the corneal stromas of rabbits, clinical examinations were performed, and the recovered bacterial loads were determined. Bacterial extracts were exposed to RCE cells, and morphology and viability were assessed. The mutant strain deficient in PLY, K1263ΔPLY, caused significantly lower ocular disease scores than the parent strain (K1263), although a higher bacterial load was recovered from corneas infected with the mutant strain. Histological examination showed increased inflammatory cells in the anterior chamber and increased edema in eyes infected with the parent strain. RCE cells exposed to the parent strain had significantly decreased cell viability and showed increased evidence of cellular damage. This study confirms that in a strain that can cause clinical keratitis, PLY is a significant cause of the damage associated with pneumococcal keratitis. It also shows for the first time that the results from an *in vitro* model using RCE cells correlates with *in vivo* results thereby establishing a less invasive way to study the mechanisms of pneumococcal keratitis.

Keywords: Pneumolysin; Keratitis; *Streptococcus pneumoniae*

Introduction

Bacterial keratitis is a devastating infection of the cornea that can lead to corneal scarring and vision loss [1-4]. *Streptococcus pneumoniae* keratitis frequently follows surgery or trauma to the eye and is more common in patients with coexisting ocular disease [4-7]. Approximately 30,000 cases of bacterial keratitis are reported each year in the United States alone [8]. The top causes of bacterial keratitis include *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *S. pneumoniae* [2,5,6,9-14]. *Streptococcus pneumoniae*, or pneumococcus, is an important worldwide pathogen capable of causing a variety of diseases including pneumonia, meningitis, otitis media and ocular infections [11,14-20]. Traditionally, pneumococcal infections affect primarily elderly individuals or young children due to their weakened immune systems [21]; however, pneumococcal eye disease can affect people of all ages. Pneumococci possess a variety of virulence factors including a polysaccharide capsule, pneumolysin (PLY), and neuraminidases (Nan) that enable this pathogen to cause disease.

Pneumolysin is a member of the family of bacterial cholesterol dependent cytolysins which also includes perfringolysin and listeriolysin [22]. It is highly conserved among clinical isolates and capable of causing many different toxic effects depending on its concentration and the cell type affected [23-27]. PLY, a 53 kDa thiol-activated cytolytic protein, has two functional domains, a pore forming (cytolytic) domain and a complement activating domain. At high concentrations, PLY binds cholesterol within the host cell and inserts into the lipid bilayer where it oligomerizes and forms a transmembrane pore that results in cell lysis [23]. At subcytolytic concentrations, PLY causes immunological damage by activating the classical complement pathway and the inflammatory response [24]. PLY activates this pathway extracellularly by binding antibody via the F_c region, though PLY can also activate complement in the absence of antibodies [24,28,29]. By activating complement, pneumococci are able to enhance the inflammatory response and thereby increase host tissue destruction via the host's own immune response. Additionally, the domain of PLY that recognizes the F_c region is homologous to the region of C-reactive protein (CRP) that activates the classical complement cascade by directly binding C1q. It is possible that PLY may act as a CRP analogue and compete with CRP possibly reducing potential protective effects of CRP [30]. The importance of this homology *in vivo* remains unclear. PLY has also been shown to stimulate human monocytes to produce inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) [25] as well as cause an increase in the surface expression of intercellular adhesion molecule 1 (ICAM-1) [26]. PLY can also induce apoptosis of dendritic cells [27].

The importance of PLY in ocular infections has been studied. Pneumococci lacking the *ply* gene showed attenuated virulence in a rabbit model of keratitis [31,32], however, this previous study used a non-ocular strain of *S. pneumoniae* that had been passaged annually in mice for 15 years to increase virulence [33]. Moreover, recent work has shown that pneumococcal pathogenicity in the cornea is different from other pneumococcal infections in the body in that the polysaccharide capsule of the bacterium is not necessary to cause disease [16,34]. Although PLY is known to be cytolytic to many types of cells [35-38], an effect of PLY on corneal cells has never before been reported. The study described herein aimed to determine the importance of PLY *in vivo* using a clinical keratitis isolate and a PLY-deficient isogenic mutant. Additionally, the *in vivo* importance of PLY was compared to an *in vitro* experimental system using primary rabbit corneal epithelial cells in an effort to correlate *in vivo* and *in vitro* findings. It is hypothesized that deletion of the gene encoding PLY in a clinical strain will cause

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significant reduction in corneal virulence *in vivo* and cytotoxicity of corneal epithelial cells *in vitro*.

Methods

Bacterial strains

S. pneumoniae clinical keratitis strain K1263 was obtained from Regis Kowalski (Charles T. Campbell Eye Microbiology Laboratory, University of Pittsburgh Medical Center, Pittsburgh, PA), for use in this experiment. Bacteria were routinely grown on blood agar plates containing 5% sheep erythrocytes. Individual colonies were selected from the plate and grown in Todd Hewitt Broth (BD Biosciences, Sparks, MD) supplemented with 0.5% yeast extract (THY) at 37°C and 5% CO_2 overnight. The overnight cultures were then diluted 1:100 in fresh THY and grown to an optical density corresponding to 10^8 colony forming units per mL (CFU/mL). Serial dilutions of each inoculum were cultured on 5% blood agar to verify the accuracy of the inoculum CFU.

For preparation of cell-free intracellular and extracellular components of *S. pneumoniae*, bacteria were grown for 18 hours to an optical density of 0.8 at 600 nm and 1 mL aliquots were collected by centrifugation at 5000 x g for 10 minutes at 25°C. The extracellular components were removed and retained, and the bacterial pellet was suspended in 1 mL of PBS. While on ice, the suspension was sonicated using a microtip and a Sonicator 3000 (QSonica, LLC., Newtown, CT). The sample was sonicated 5 times for 15 seconds with a 30-60 second rest periods between cycles. Intracellular and extracellular portions were passed through 0.22 µm filters to remove any remaining bacteria. Samples were prepared and used immediately in experiments to avoid possible degradation of virulence factor activity.

Creation of PLY-negative mutant

A PLY-negative isogenic mutant was created using homologous transformation of donor DNA from a mutant of *S. pneumoniae* strain D39, in which the pneumolysin gene was replaced with a cassette containing resistance to trimethoprim (Tmp) [26]. The parent strain, K1263, was grown in competence medium (CM) to A_{600} of 0.6. The cultures were diluted 1:50 in fresh CM. After 200 ng of competence-stimulating peptides 1 and 2 [39] were added, the cultures were incubated at 37°C and 5% CO_2 for twelve minutes. One microgram of donor DNA was added to the mixture. A double-crossover event occurred in which the *ply* gene was replaced with the Tmp-resistance cassette of the donor DNA. Transformants were selected on 5% sheep blood agar plates containing 50 µg/mL trimethoprim and incubated overnight at 37°C and 5% CO_2. Colonies that grew were further screened using PCR and hemolysis assays. The PCR reaction used primers PlymutF (5'-CCCGGTACCGGAGAAGGATTATATTGTCAAGGTTA-3') and PlymutR (5'-CCCTCTAGACTCCAGACATATCATAGTTCAAGTAAAT-3'). These primers were designed to amplify approximately 500 bp on either side of *ply* in the pneumococcal chromosome. A successful deletion mutant was named K1263ΔPLY.

Hemolysis assay

The pneumolysin activity of the pneumococcal strains used in this experiment was assessed by hemolysis assay. The bacterial strains were grown overnight in THY to A_{600} of 0.8. The bacteria were collected by centrifugation and incubated with 100 µL lysis buffer (0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15M sodium citrate) for 30 minutes at 37°C, after which 100 µL of PBS was added. Using a 96-well round bottom plate, 50 µL of sample cell lysate containing containing a protein concentration of 300 µg/mL was serially diluted into 100 µL of dithiothreitol (DTT) buffer (10 mL PBS, 0.01 g BSA, 0.015 g dithiothreitol). After the addition of 50 µL of 1% sheep red blood cells, the plate was incubated for 30 minutes at 37°C and 5% CO_2, and centrifuged at 1000 x g for 10 minutes to collect non-lysed red blood cells. Lysis was measured by spectrophotometry of the resulting supernatants containing released hemoglobin at A_{450}.

Rabbit corneal infections

New Zealand White rabbits (Harlan Rabbitry, Indianapolis, IN) were used in this study. They were maintained according to the guidelines of the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC) and the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by subcutaneous injection of a mixture of xylazine (50 mg/kg; Lloyd Laboratories, Shenandoah, IA) and ketamine hydrochloride (10 mg/kg; Butler Animal Health Supply, Dublin, OH). Proparacaine hydrochloride (Akorn, Inc., Buffalo Grove, IL) was topically applied to each eye, and either *S. pneumoniae* K1263 or K1263ΔPLY (10^5 CFU in 10 µL) was injected intrastromally. Two observers, masked to the identity of the rabbit groups, used a biomicroscope to perform clinical examinations of infected rabbit corneas using seven ocular parameters: injection, chemosis, iritis, fibrin in the anterior chamber, hypopyon, corneal edema, and corneal infiltrate (infiltrate being defined as host cells, usually neutrophils, that infiltrate the eye in response to an infection) [40]. Each parameter 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). At 48 hours post-infection, rabbits were killed by an intravenous overdose of pentobarbital sodium (100 mg/mL; Sigma-Aldrich, St. Louis, MO). Corneas were removed, dissected, and homogenized in sterile PBS. Corneal homogenates were serially diluted and plated in triplicate on 5% sheep blood agar. Plates were incubated at 37°C for 24 hours. Colonies were counted and bacterial CFUs were determined and expressed in mean logarithmic units ± standard error of the means (SEM).

Histology of rabbit eyes

Whole rabbit eyes were dissected at 48 hours post-infection and placed in 4% paraformaldehyde. Histologic sectioning and hemotoxylin and eosin staining of the rabbit eyes were performed by Excalibur Pathology, Inc. (Moore, OK). Briefly, fixed eyes were processed to paraffin. Five micron sections were deparaffinized through xylene and rehydrated gradually in water containing decreasing concentrations of alcohol. The sections were stained with Gill III hematoxylin for 10 minutes, rinsed in water, dipped three times in acid alcohol, again rinsed in running water followed by ammonia water and 95% alcohol for one minute each. The sections were then treated with treosin for 15 seconds prior to three changes in 100% alcohold and three changes of xylene. The sections were finally coverslipped with resin.

Primary rabbit corneal epithelial cell line

Primary rabbit corneal epithelial cells were obtained as previously described [41]. Briefly, rabbits were anesthetized and euthanized as described above. The corneas were then excised from New Zealand white rabbit eyes, placed in 100-mm plates filled with 1% dispase in Keratinocyte Serum-Free Medium (KSFM; Invitrogen, Carlsbad, CA) and incubated at 4°C overnight. The following morning, a scalpel was used to gently remove the epithelial cells from the cornea. The rabbit

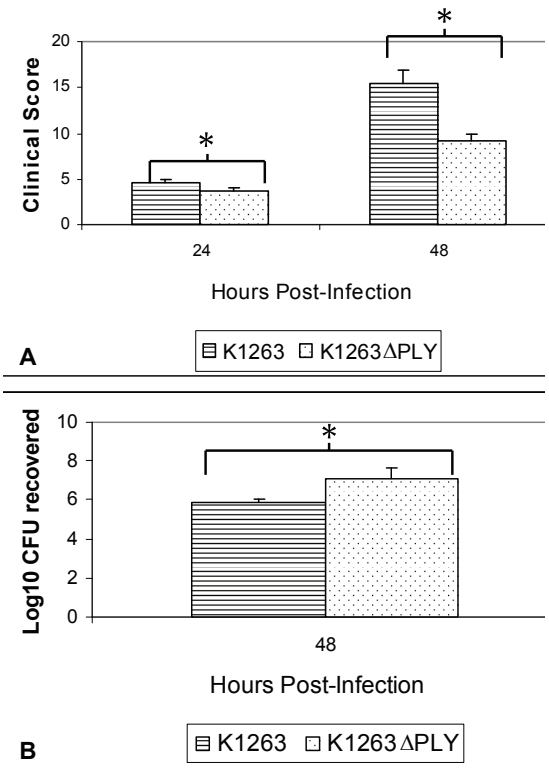

Figure 1: (A) Average clinical scores of keratitis at 24 and 48 hours post-infection caused by parent (n = 11) or PLY-deficient isogenic mutant strain (n = 17). Pathology of the corneas was evaluated with slit lamp examination and the average clinical scores are provided. P = 0.023 at 24 hours post-infection and p = 0.0006 at 48 hours post-infection. (B) Average $\log_{10}$ CFU recovered from the corneas infected with the parent strain (n = 10) or the mutant strain (n = 16) at 48 hours post-infection. P = 0.007. Parent = K1263, Mutant = K1263ΔPLY. (*) indicates a significant difference between the scores. Scores are reported as the mean scores ± standard errors of the means.

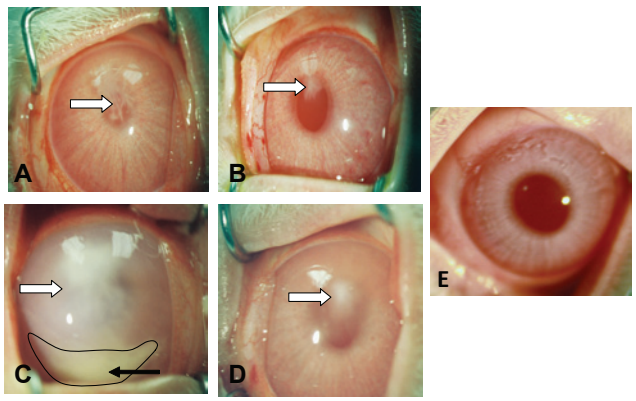

Figure 2: Representative eyes showing keratitis caused by either the parent strain (A,C), the PLY-deficient isogenic mutant strain (B,D), or an uninfected control (E). At 24 hours post-infection, there was more infiltration of host PMNs in eyes infected with the parent strain (A) compared to eyes infected with the mutant strain (B). The clinical symptoms worsened by 48 hours post-infection and appeared more severe in eyes infected with the parent strain (C) than those infected with the mutant strain (D). Parent = K1263, Mutant = K1263ΔPLY. White arrows indicates corneal infiltrate. Hypopyon is indicated by the black arrow and outlined in black.

corneal epithelial (RCE) cells were suspended in 0.05% trypsin-EDTA (Invitrogen) and incubated for 30 minutes at 37°C. Cells were then suspended in KSFM supplemented with 1% penicillin-streptomycin, 0.1% amphotericin B and 10% fetal bovine serum. The RCE cells were maintained at 37°C and 5% CO_2.

Cell culture exposure

Prior to exposure to bacterial extracts, RCE cells were suspended in 6-well or 96-well tissue culture plates in serum-free media in order to eliminate the inhibitory effect of serum cholesterol against pneumolysin. The tissue cultures were exposed to either medium alone or the intracellular or extracellular components of *S. pneumoniae*

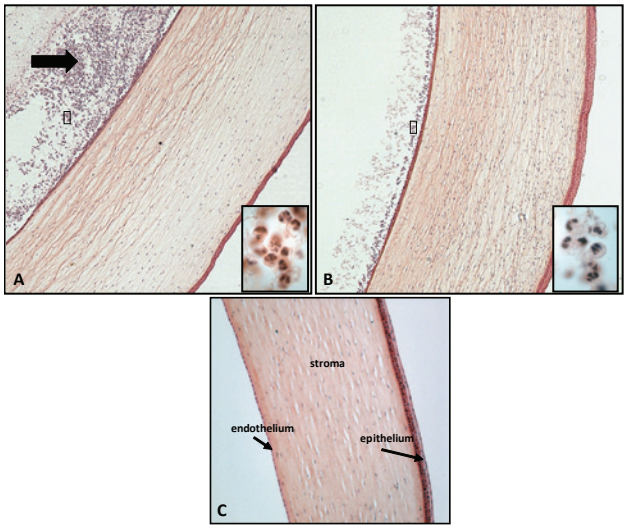

Figure 3: Ocular histology from representative eyes infected with either the (A) parent, (B) the mutant strain, or (C) uninfected control. Boxed inserts (A & B) show higher magnification of PMNs in the anterior chambers of infected eyes. The corneas of the rabbits infected with the parent strain showed an influx of cells at the endothelium and in the anterior chamber (black arrow). Original magnification, 40X. Boxed insert magnification, 1000X.

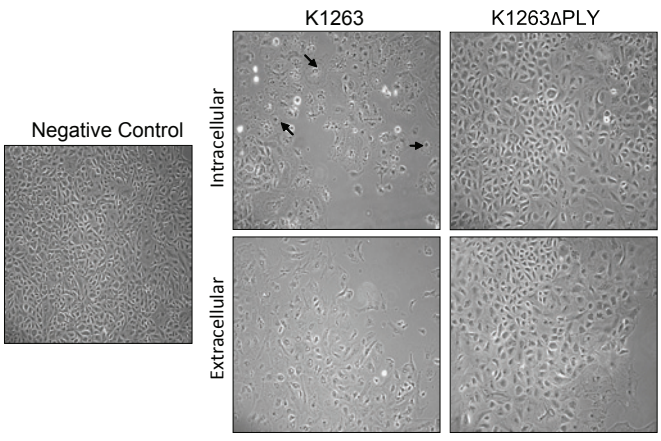

Figure 4: Morphology of RCE cells treated with intracellular and extracellular components of K1263 and K1263ΔPLY for 3 hours at 37°C and 5% CO_2. Black arrows indicate round, abnormal cells. Original magnification 100x.

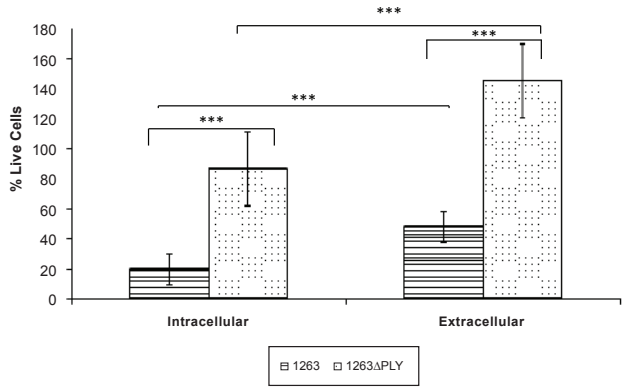

Figure 5: Percent live RCE cells following treatment with intracellular and extracellular components of K1263 and K1263ΔPLY for 3 hours at 37°C and 5% CO2. RCE cells exposed to media alone were considered 100% viable. Following incubation, cells were treated with 2 μM calcein AM and the absorbance measured at 485/530 nm. (*) indicates a significant difference between cell viability. Scores are reported as the mean scores ± standard errors of the means. N equals 3 for all groups.

strains K1263 and K1263ΔPLY. Cells were co-incubated at 37ºC in 5% CO_2 for 1,3,6, or 24 hours. The bacteria-to-corneal epithelial cell ratio was approximately 100:1 for all of the experiments. After exposure to bacterial extracts, RCE cells were photographed and morphology was observed. Assays were performed in triplicate.

Cell viability

RCE cell viability was determined using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen). Cell viability was determined on cells grown in a 96-well tissue culture plate, exposed to cell-free components and controls as described above, washed with Dulbecco's PBS (D-PBS) and then treated with 100 μL D-PBS and 100 μL calcein AM for a final concentration of 2 μM calcein AM. A non-florescent calcein AM diffuses into intact live cells where it is hydrolyzed by intracellular esterases into calcein, a fluorescent compound that remains in the cell cytoplasm. Fluorescence was measured at 485/530 nm using a fluorescence microtiter plate spectrophotometer following a 30 minute incubation at room temperature. Values determined from RCE cells exposed to media alone were considered 100% live. Assays were performed in triplicate.

Statistics

Data were analyzed using the Statistical Analysis System (SAS) program for computers (Cary, NC). Clinical scores and cell viability data were analyzed using Non-parametric One-way ANOVA and CFU data were analyzed using General Linear Model of Least Square Means. A p value < 0.05 was considered significant.

Results

Creation of PLY-deficient mutant

A mutant deficient in PLY was successfully created in clinical keratitis strain K1263. Amplification of genomic DNA using primers PlymutF and PlymutR confirmed the presence of the Tmp resistance cassette with the flanking regions (1860 bp) in the mutant strain K1263ΔPLY and the presence of *ply* with flanking region (2605 bp) in parent strain K1263 (data not shown). The PLY-deficient mutant was confirmed with a hemolysis assay in which cell-free K1263 extract was able to lyse rabbit red blood cells while K1263ΔPLY was not (data not shown).

Rabbit challenge

In a keratitis model of infection, the ocular pathology of the PLY-deficient mutant strain was significantly reduced when compared to the pathology caused by the parent strain. Clinical scores of rabbit corneas (n = 11) infected with K1263 (4.66 ± 0.37) were significantly higher than corneas (n = 17) infected with K1263ΔPLY (3.60 ± 0.26) at 24 hours post-infection (p = 0.023; Figure 1A). This difference increased at 48 hours post-infection such that the clinical scores of corneas infected with K1263 were 15.41 ± 1.42, significantly higher than those infected with K1263ΔPLY at 9.21 ± 0.78 (p = 0.0006; Figure 1). All seven parameters used for scoring keratitis were higher for the rabbits infected with parent strain K1263. Increased infiltration of immune cells (which are usually neutrophils in *S. pneumoniae* keratitis; unpublished findings) into the cornea was observed as early as 24 hours post-infection for corneas infected with K1263 when compared to those infected with K1263ΔPLY (Figure 2 A-B). By 48 hours post-infection, there were considerably more infiltrating host cells in the eyes infected with K1263. Hypopyon, which is a collection of infiltrating host cells in the anterior chamber, was also present in the eyes of rabbits infected with K1263 but not with K1263ΔPLY (Figure 2 C-D). Additionally, the $\log_{10}$ CFU recovered from corneas at 48 hours post-infection were significantly different between corneas such that those infected with the parent strain had less CFU recovered (5.86 ± 0.50, n = 10) than those infected with the mutant strain (7.13 ± 0.14, n = 16; p = 0.007, Figure 1B).

Histopathology

Histological examination of infected eyes show slight differences within the corneas such that the eye infected with the parent strain appeared slightly more edematous while the cornea infected with the PLY-deficient mutant strain had a slightly thicker epithelium layer (Figure 3). Athough, it is important to note, these observations may be due to small differences between the eyes of rabbits and may not be significant to the course of the infection. The striking difference between the two eyes is the considerably larger influx of immune cells into the aqueous humor of the eye infected with the parent strain compared to the eye infected with the mutant strain (Figure 3). Under higher magnification, the immune cells have the typical appearance of polymorphonuclear neutrophils (PMNs).

Effects of PLY on corneal cells *in vitro*

RCE cells exposed to K1263ΔPLY had reduced membrane damage and monolayer disruption, and higher percentages of live cells compared to cells exposed to the parent strain K1263 (Figures 4,5). These results were observed when cells were exposed to both the cell-free intracellular and extracellular components of bacteria. RCE cells exposed to either the intracellular or extracellular components of K1263 for three hours showed signs of cellular damage and death, whereas cells exposed to mutant bacteria showed reduced damage (Figure 4). Corneal cells exposed to the intracellular components of K1263 showed the most apparent disruption of the monolayer.

Cell viability assays performed under identical conditions confirmed the morphology results. RCE cells had a significantly lower percentage of live cells following exposure to the intracellular components of the PLY-producing parent strain, K1263, than those exposed to the intracellular components of the non-PLY producing mutant strain, K1263ΔPLY (19.56% ± 5.99, n = 3 vs. 86.23% ± 14.16, n = 3; p = 0.013).

Additionally, there was a significant difference between the viability of RCE cells exposed to the extracellular components of K1263 and K1263ΔPLY such that RCE cells exposed to the PLY-producing parent strain, K1263, had a reduced cellular viability compared to those exposed to the non-PLY producing mutant strain (47.83% ± 7.33, n = 3 vs. 145.26% ± 15.11, n = 3; p = 0.0044; Figure 5). Interestingly, there was also a significant difference between the cell viability of RCE cells exposed to the intracellular and extracellular components of the same bacteria. Exposure to the intracellular components of either K1263 or K1263ΔPLY resulted in increased cellular toxicity than exposure to the extracellular components of the same strain, with the p values being 0.040 and 0.046, respectively (Figure 5).

Discussion

This study demonstrated the importance of PLY in causing much of the damage associated with pneumococcal keratitis using a rabbit model of infection. Additionally, the *in vivo* results successfully correlated with an *in vitro* model using primary rabbit corneal epithelial cells. The ability to use cultured corneal epithelial cells will allow for increased experimentation and determination of more precise mechanistic details involved with pneumococcal infections of the eye.

Previous research with pneumococcal keratitis has shown that PLY may be the predominant virulence factor in the cornea [40] since other major factors, such as capsule, do not seem to play a role [16,34]. Because earlier research on the role of PLY in pneumococcal keratitis used a strain of *S. pneumoniae* that had been passaged systemically in mice for 15 years [33], we wished to examine the role of PLY using a clinical keratitis isolate. Examination of the role of PLY in keratitis with a clinical ocular strain was important to test because another virulence factor that has long been deemed to be essential for pathogenesis in non-ocular infections – the polysaccharide capsule – has been shown to be non-essential for keratitis [16,34]. The observation that pneumococcal virulence factors are not necessarily the same for corneas and other anatomical sites underscores the possibility that clinical ocular strains may be genetically different than other strains. Considering that pneumococci are readily transformable and have been shown to exchange genetic information with other bacteria [42-45], it is possible that certain strains are adapted for the eye and certain strains are adapted for other areas of the host. Our results corroborate with the earlier study such that eyes infected with the PLY-deficient strain developed significantly less pathology than the eyes infected with the parent strain (Figures 2,3). Although significantly reduced, the pathology was not completely eliminated upon deletion of PLY. It is possible that other, as yet unknown, virulence factors play a role within the cornea or that the deletion of PLY allows for an upregulation of a known virulence factor that normally would not be a major cause of corneal pathology. It is also possible that pneumococcal cell wall, which is known to induce similar levels of inflammation as whole pneumococci in various models of disease [46-48], is responsible for at least a portion of the pathology still observed in eyes infected with the mutant PLY-deficient strain. Though beyond the scope of this study, the testing of purified cell wall and other pneumococcal virulence factors such as autolysin and zinc metalloproteases, which also stimulate inflammation [49,50], with this rabbit model of keratitis would lead to a better understanding of pneumococcal corneal infections.

Bacterial recovery from infected corneas also corroborates with the earlier study such that the mean $\log_{10}$ CFU recovered from corneas infected with the parent strain was significantly less than that recovered from corneas infected with the mutant strain [40]. It is unknown as to why the bacterial load was higher in corneas infected with the mutant strain, but one explanation could be that the presence of PLY causes a more robust activation of the innate immune response causing more neutrophils to enter the eyes infected with the parent strain and therefore more bacteria phagocytosed in those eyes. By the same token, however, more influx of neutrophils into the anterior chamber resulted in more overall ocular damage due to the host inflammatory response. Our results show the anterior chamber of the eye infected with the parent strain obviously contained more neutrophils than the eye infected with the mutant strain. However, histopathology did not show more host cells present in the corneas infected with the parent strain; in fact, the representative cornea infected with the mutant strain appeared to possibly have more cells, although the inflammatory cells were not quantified (Figure 3). It is possible that determining the precise quantity and type of immune cells within the cornea and/or anterior chamber of infected eyes would better explain the strain-dependent variations in the recovered bacterial loads.

Our *in vitro* results provided complementary data to the results seen in the rabbit model of keratitis such that the RCE cells exposed to the PLY-deficient mutant strain had reduced cell damage and increased viability when compared to the RCE cells exposed to the parent strain (Figures 4, 5). Caution should be taken, however, in the correlation of these data because the cultured cells were epithelial cells whereas the *in vivo* model involves direct intrastromal injection, and the histology sections from the *in vivo* model did not show epithelial disruption. This difference is one of the drawbacks of using the rabbit as a model because topical infection of rabbit corneas cannot be achieved with *S. pneumoniae*. However, the mouse model of pneumococcal keratitis [51] uses a topical scratch method and demonstrates the disruption of the corneal epithelium. Interestingly, RCE cells exposed to the intracellular components of PLY-deficient mutant pneumococci still had increased pathology (approximately 14% cell death) compared to a negative control (Figure 5), showing that one or more additional virulence factors other than PLY may participate in causing corneal damage. One such factor could be hydrogen peroxide. Hirst, *et al*. (2000) showed that H_2O_2 released from a pneumococcal strain deficient in PLY was capable of causing ciliary stasis in ependymal cells in addition to H_2O_2-induced cellular toxicity [52]. The ability of H_2O_2 to cause toxicity in ocular epithelial cells, however, is still unknown.

It is interesting to note that both the intracellular and extracellular components of the parent strain caused substantial damage, with the intracellular component causing the most significant cell pathology. While it is likely that factors in addition to PLY are causing host cellular damage, it is also possible that the intracellular component of K1263 caused such significant damage due to an incomplete extracellular release of PLY. This PLY would have then been released from the bacterial cells during the process of lysis by sonication. It was previously thought that PLY is released extracellularly by the action of autolysin, a pneumococcal protein that causes the bacterium to self-lyse [15]. Newer evidence shows that this process is more ambiguous than previously believed and PLY release can occur in an autolysin independent manner [53,54]. Additionally, the time in which PLY is released varies between strains in which some strains release PLY early in the bacterial life cycle while other strains release PLY late in the life cycle [53,55]. The difference in PLY release is one possible explanation for the reduced RCE cell viability observed following exposure to the intracellular components.

In summary, we have established an *in vitro* experimental system that correlates to what is observed *in vivo*. This will allow for additional

data that would enhance and corroborate experiments performed *in vivo*. Additionally, PLY originating from a clinical keratitis isolate is a major virulence factor associated with pathology caused during pneumococcal keratitis. Since it is an important virulence factor in the cornea, PLY should be studied as a possible vaccine candidate for protection from pneumococcal keratitis. Better insight into the exact mechanisms used by PLY would be invaluable to our understanding and ultimately treatment of pneumococcal keratitis.

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