Short Communication

Ophthalmic Research

Ophthalmic Res 2009;42:141–146
DOI: 10.1159/000229028

Received: February 10, 2009
Accepted after revision: April 16, 2009
Published online: July 23, 2009

Development of a *Streptococcus pneumoniae* Keratitis Model in Mice

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Key Words
Streptococcus pneumoniae · Keratitis · Cornea · *Pneumococcus* · Mouse

Abstract
Background: *Streptococcus pneumoniae* is a common cause of bacterial keratitis, and models to examine the ocular pathogenesis of this bacterium would aid in efforts to treat pneumococcal keratitis. The aim of this study was to establish a murine model of pneumococcal keratitis. ***Methods:*** The corneas of A/J, BALB/c or C57BL/6 mice were scratched and topically infected with a clinical strain of *S. pneumoniae*. Slitlamp examination (SLE), enumeration of bacteria in the corneas and histology were performed. ***Results:*** Bacteria were recovered from the eyes of A/J mice on postinfection (PI) days 1 [1.96 ± 0.61 $\log_{10}$ colony-forming units (CFU)] and 3 (1.41 ± 0.71 $\log_{10}$ CFU). SLE scores were significantly higher in the infected A/J mice as compared to the BALB/c or C57BL/6 mice on PI day 3 ($p < 0.0001$) and steadily increased over time, reaching a maximal value of 3.00 ± 0.35 on PI day 10. Histopathology revealed stromal edema and the influx of polymorphonuclear leukocytes on PI days 7 and 10, and corneal disruption on PI day 7. ***Conclusions:*** *S. pneumoniae* keratitis was established in A/J mice, but not BALB/c or C57BL/6 mice.

Introduction

Streptococcus pneumoniae is an opportunistic pathogen that causes bacterial keratitis [1–10], which can result in irreversible corneal scarring that could potentially lead to blindness [11]. *S. pneumoniae* is one of the most common bacterial pathogens associated with infection of compromised corneas [12, 13]. The corneal damage observed in pneumococcal keratitis has been attributed mainly to pneumococcal virulence factors, such as the toxin pneumolysin, that initiate a robust immune response [14–16]. Previous studies have reported pneumococcal keratitis in the rabbit model [14, 17–19]. The rabbit has served as an appropriate model to study bacterial virulence factors, host effects such as inflammation, antibiotic treatments and the testing of novel therapies.

Drawbacks of the rabbit model of pneumococcal keratitis are the lack of availability of immunological reagents and genetically altered animal strains.

The establishment of a pneumococcal keratitis model in the mouse would provide the examination of the pathogen-host interaction. The role of *S. pneumoniae* virulence factors can be determined in the mouse eye which would provide new insights into corneal pathogenesis. Whereas the rabbit eye remains an appropriate model for the investigation of therapies designed to target bacteria

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and bacterial virulence factors, the mouse model would give a better understanding of the effects that bacteria and their virulence factors have on the host immune system and would thus provide possible host targets for the refinement of therapies to treat these infections. Mouse models of *Staphylococcus aureus* keratitis have been established and have proven useful for the study of the host response to infection [20–22]. The mouse model of *Pseudomonas aeruginosa* has been studied extensively and has provided insight into the host innate and adaptive immune responses to keratitis infection [23]. However, a mouse model of pneumococcal keratitis has not been previously demonstrated. The purpose of the current study was to establish the mouse model of pneumococcal keratitis which will allow future studies of the elucidation of the host factors directly involved in the host response, especially with regard to innate immunity.

Materials and Methods

S. pneumoniae clinical keratitis strain K1263 (capsule type 35F) was provided by the Charles T. Campbell Ophthalmic Eye Microbiology Laboratory at the University of Pittsburgh Medical Center. Bacteria for infection were grown overnight in Todd-Hewitt broth with 0.5% yeast extract at 37°C and then subcultured to log phase under the same conditions until the optical density at 600 nm was 0.3, which corresponded to 10^8 colony-forming units (CFU) per milliliter.

Six- to 7-week-old A/J, BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me., USA). All animals were maintained according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse eyes were infected using a protocol modified from Girgis et al. [21]. Briefly, groups of mice were anesthetized with an appropriate dose of xylazine (Butler Company, Columbus, Ohio, USA) and ketamine (Butler Company). A 30.5-gauge needle was used to scratch the corneal surface in 4 places without penetrating the superficial stroma. A 5-μl aliquot containing 1×10^5 CFU of log phase K1263 was applied to each scratched cornea. Scratch-only controls and unscratched controls were included.

The mice (n = 3 mice/group, repeated twice) were monitored by slitlamp examination (SLE) on postinfection (PI) days 1, 3, 5, 7 and 10. Microscopic evaluation was performed using a slitlamp biomicroscope (Topcon, Kogaku Kikai KK, Tokyo, Japan). The parameters were as follows: 0 = clear and normal; +1 = readily detectable opacity; +2 = dense opacity or opacity partially covering the entire corneal surface over the pupil; +3 = dense opacity covering the entire corneal surface over the pupil; +4 = moderate to dense opacity covering the entire corneal surface with corneal erosion [21]. Scores were expressed as the means ± standard errors of the mean.

Mice were sacrificed at each time point, and eyes were enucleated to enumerate the viable bacteria present (n = 4 eyes/group) or to examine histopathology (n = 2/group). Enucleated eyes were homogenized in 1.0 ml sterile phosphate-buffered saline. The bacteria were quantified by plating of serial dilutions (1:10) on blood agar (5%) in triplicate and incubation at 37°C for 24 h. The colonies were counted and CFU per cornea were expressed as log base 10 values. For histopathology and immunohistochemistry analysis, enucleated eyes were fixed in 10% neutral buffered formalin and were processed by Excalibur Pathology (Moore, Okla., USA). Immunohistochemistry was performed using rabbit anti-CD11b/c polyclonal antibody (Thermo Fisher Scientific, Rockford, Ill., USA) according to the manufacturer's instructions.

Data were analyzed using the Statistical Analysis System program for computers (Cary, N.C., USA). Clinical SLE scores were analyzed using a nonparametric one-way analysis of variance. Bacterial CFU were analyzed using the general linear models procedure of least squares means. All experiments except corneal CFU quantification at time points up to 24 h after infection were performed twice, yielding similar results. $p < 0.05$ was considered significant. Data were expressed as means ± the standard errors of the mean.

Results

The total number of viable bacteria recovered from each eye was quantified on PI days 1, 3 and 5 for each strain of mouse. The CFU from the infected A/J mouse eyes yielded no statistical differences in the bacterial recovery on PI day 1 compared to the BALB/c and C57BL/6 mouse eyes ($p = 0.0879$ and $p = 1.0000$, respectively; fig. 1a). It was noted that only 1 out of 4 eyes from the BALB/c mice yielded viable pneumococci whereas all of the eyes from the other strains yielded viable bacteria. However, by PI day 3, the infected eyes of A/J mice ($p = 0.0485$) continued to yield viable bacteria whereas the eyes of BALB/c and C57BL/6 mice did not yield any viable bacteria. The eyes of the BALB/c and C57BL/6 mice were deemed sterile after PI day 3. A/J mouse eyes, however, were not deemed sterile until PI day 5. Further analysis of the pneumococcal viability was examined in a separate experiment in the A/J mouse for multiple time points (4, 8, 12 and 24 h after infection; fig. 1b). The data demonstrate that the bacterial load declined by 3 $\log_{10}$ units within the first 12 h after infection and continued to decline to about 1.5 $\log_{10}$ units by 24 h after infection.

Each group of mice was examined by SLE at each time point (fig. 2). The three strains of infected mice exhibited no pathological changes, and the corneas were clear and normal on PI day 1. However, by PI day 3, slight corneal opacity was observed in the A/J mice and BALB/c mice. In contrast, no corneal opacity was observed in the C57BL/6 mice. The eyes of the infected A/J mice had significantly higher SLE scores as compared to the eyes of

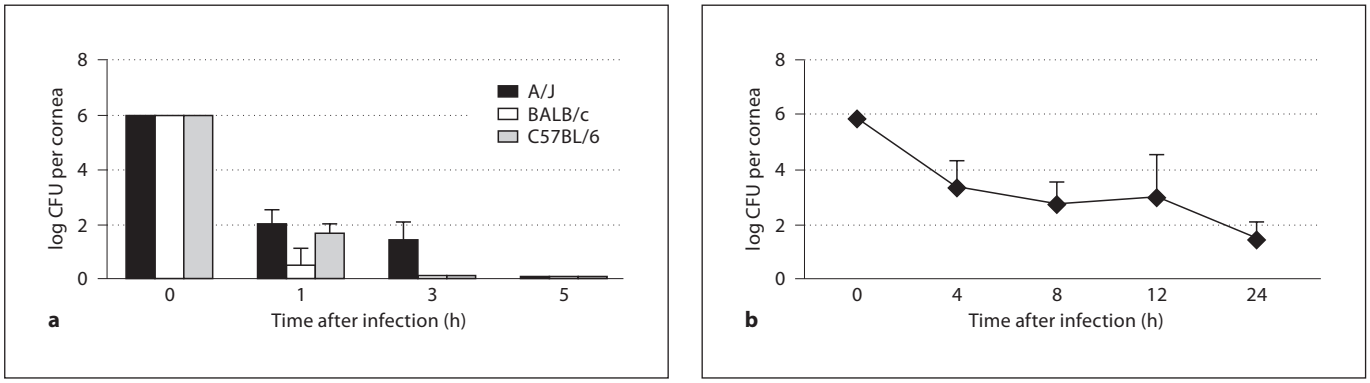

Fig. 1. a CFU of *S. pneumoniae* recovered from the eyes of A/J, BALB/c and C57BL/6 mice. p = 0.0879 for A/J versus BALB/c, p = 1.000 for A/J versus C57BL/6 and p = 0.0485 for A/J versus both BALB/c and C57BL/6. **b** Time course of the decline in the survival of *S. pneumoniae* in A/J mouse eyes.

the uninfected mice that had only been scratched ($p < 0.001$). The SLE scores were significantly higher for the infected corneas of the A/J mice as compared to the BALB/c ($p < 0.05$) and C57BL/6 ($p < 0.001$) mice by PI day 5 and steadily increased over time reaching a maximal value of 3.00 ± 0.35 on PI day 10 (fig. 2). The BALB/c mice exhibited SLE scores that slightly increased to 1.1 ± 0.10 by PI day 10.

The establishment of pneumococcal keratitis is evident in the macroscopic examination of the infected corneas of the A/J mice (fig. 3). The infection also led to the occurrence of corneal epithelial erosions which were observed in the A/J mice by PI day 5 (data not shown). A/J mice demonstrated severe gross pathology by PI day 5 (fig. 3a), which worsened by PI day 10 (fig. 3c, d). In contrast, the BALB/c mice (fig. 3e) showed mild pathology, and the C57BL/6 mice (fig. 3f) exhibited no signs of infection. Overall, the A/J mice had more obvious corneal infiltrate, corneal opacity and corneal ulceration.

Whole eyes were extracted on PI days 1, 3, 5, 7 and 10 which were then sectioned and stained with hematoxylin and eosin. The A/J mice demonstrated severe stromal edema and influx of polymorphonuclear leukocytes (PMNs) on PI days 5, 7 and 10 (fig. 4a–c). These findings correlated with the progression of the SLE scores. Histological analysis also exhibited disruption of corneal epithelial cells and stromal edema by PI day 7 (fig. 4d). Histopathological analysis of the infected C57BL/6 and BALB/c eyes revealed minimal signs at most of keratitis. The results of the C57BL/6 (fig. 4e) and BALB/c (fig. 4f) infected mice exhibited slight stromal edema and the minimal presence of PMN activity. To determine whether the scratch led to the inflammatory response, a naïve A/J mouse cornea was scratched but no bacteria were applied. The histology analysis shows slight stromal edema and few PMNs (fig. 4g). Thus, the results further demonstrate that BALB/c and C57BL/6 strains of mice seem to be resistant to pneumococcal keratitis as compared to the A/J mouse strain.

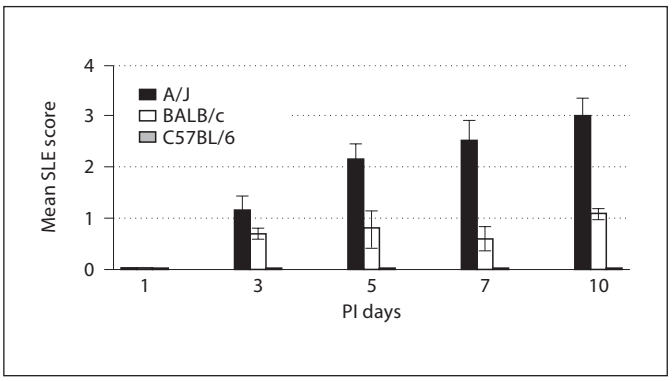

Fig. 2. Mean SLE scores of infected A/J, BALB/c and C57BL/6 mice. $p < 0.05$ for A/J versus C57BL/6, $p < 0.05$ for A/J versus BALB/c, $p < 0.001$ for A/J versus C57BL/6 and $p < 0.001$ for A/J versus both BALB/c and C57BL/6.

Immunohistochemistry was used to determine the presence of immune cells in the eye. The results further demonstrate monocytes, macrophages and granulocytes localized in the stroma and aqueous of A/J mice (fig. 4h, i). BALB/c (fig. 4j) and C57BL/6 (fig. 4k) mice were shown to be clear of PMNs or other immune cells in the eye.

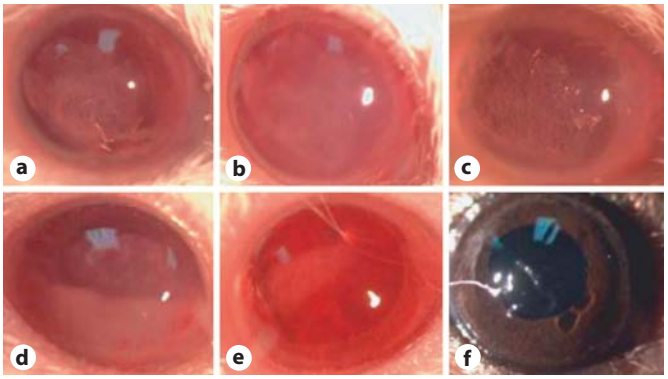

Fig. 3. Photomicrographs of ocular disease in A/J (**a–d**), BALB/c (**e**) and C57BL/6 (**f**) mice infected with *S. pneumoniae*. Photomicrographs were taken on PI days 5 (**a**), 7 (**b**) and 10 (**c–f**). The infected A/J mouse eye exhibited a partial dense opacity covering the corneal surface (**a**), which progressed to a dense opacity covering the entire corneal surface (**b**), and then corneal ulceration (**c**) and hypopyon (**d**). The infected BALB/c (**e**) and C57BL/6 mice (**f**) showed few signs of pathological damage on PI day 10. Magnification ×25.

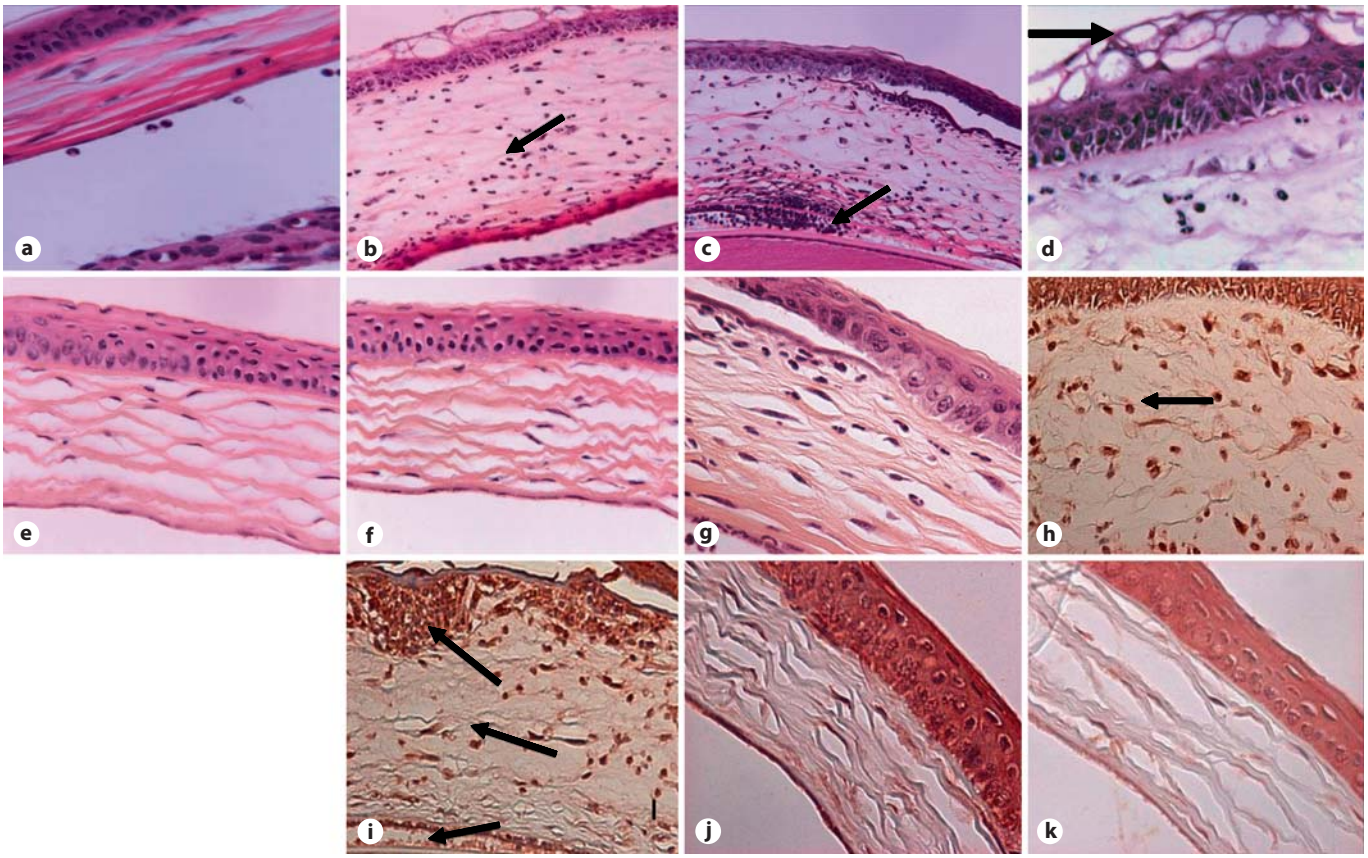

Fig. 4. Ocular histology from A/J (**a–d**, **h**, **i**), BALB/c (**e**, **j**) and C57BL/6 (**f**, **k**) mice infected with *S. pneumoniae*, and a scratch-only control eye from an A/J mouse (**g**). A/J corneas exhibited signs of stromal edema on PI day 5 (**a**), increased edema and PMN infiltration (arrow) on PI day 7 (**b**), and further increases in stromal edema, PMN infiltration and loss of endothelial integrity (arrow) on PI day 10 (**c**). Disruption of corneal epithelium was also observed on PI day 7 (**d**, arrow). **e** The BALB/c mouse eye on PI day 10 demonstrated slight edema with few PMNs dispersed in the stroma. **f** The C57BL/6 mouse eye on PI day 10 illustrated slight edema. **g** A scratched A/J mouse eye (no bacteria added) on PI day 10 exhibited slight edema and the presence of PMNs. For immunohistochemistry of A/J (**h**, **i**), BALB/c (**j**) and C57BL/6 (**k**) mice, rabbit anti-CD11b/c polyclonal antibody was used to stain for the presence of monocytes, macrophages and granulocytes. A/J mice on PI days 7 (**h**) and 10 (**i**) demonstrate the presence of PMNs (arrows) in the stromal region of the cornea. BALB/c (**j**) and C57BL/6 (**k**) mice had no detectable immune cells. **a–g** HE staining. Magnification ×400. **j**, **k** Immunohistochemistry. Magnification ×400. **h**, **i** Immunohistochemistry. Magnification ×200.

Discussion

The focus of this study was to establish pneumococcal keratitis in the mouse model. Three strains of mice were analyzed; however, only the A/J mouse strain was fully susceptible to pneumococcal keratitis. It has been determined based on SLE scores and histopathology that BALB/c and C57BL/6 mice were not susceptible to infection under the experimental conditions that were employed.

The A/J mouse exhibited signs of corneal disease beginning on PI day 3 and continuing to PI day 10. The diseased corneas exhibited the presence of PMNs in the stroma, opacity and disruption of the epithelium. Bacterial viability declined after PI day 3, which suggests that pneumococcal clearance might be attributed to the host innate immune response. Furthermore, the pneumococcus faces unfavorable growth conditions in the cornea, which would cause the bacteria to release autolysin and die [24]. This occurrence of pneumococcal death is not a new phenomenon, according to Johnson et al. [16] and Green et al. [17], who demonstrated pneumococcal keratitis in rabbits. Both studies found that by 48 h after infection, bacteria were cleared from most of the corneas. Although no bacteria were recovered, the pathological damage associated with the pneumococcal infection was quite evident as shown by SLE and histological analysis. The findings from the present study indicate that bacterial colonization alone only accounts for part of the pathogenesis of pneumococcal keratitis. Other important aspects of infection include bacterial virulence factors and host cellular responses that lead to a damaging inflammatory response.

The C57BL/6 and BALB/c mice apparently possess immune functions that render their corneas resistant to pneumococcal infection. Girgis et al. [20] and Hume et al. [21] established mild to moderate staphylococcal infection in C57BL/6 mice. The C57BL/6 and BALB/c mouse strains might require a highly virulent pneumococcal strain or a higher inoculum to establish infection.

The present study demonstrates the establishment of pneumococcal keratitis in a murine model which to our knowledge has not been shown to date. Questions regarding the bacteria-host interactions and their subsequent contributions to disease could be addressed using the mouse model due to the availability of transgenic and knockout mice. Murine studies with *P. aeruginosa* have led to a better understanding of regulatory mechanisms in the cornea following bacterial challenge [25–27]. The *Staphylococcus* keratitis mouse model has been used to study the innate host defenses of the eye, mechanisms of host response and the action of toxin [20, 28, 29]. Likewise, the development of a mouse model of pneumococcal keratitis can also lead to new therapies that specifically target the host signaling cascades involved in pneumococcal keratitis.

Acknowledgements

The authors would like to thank Regis Kowalski for providing the clinical isolate used in the study. The authors also thank Dr. Ed Swiatlo for his suggestions and comments in the preparation of the paper. This work was supported by Public Health Services Grant R01EY016195 and Research Supplement R01EY016195-S1, National Eye Institute, National Institutes of Health.

References

1 Parmar P, Salman A, Kalavathy CM, Jesudasan CA, Thomas PA: Pneumococcal keratitis: a clinical profile. Clin Exp Ophthalmol 2003;31:44–47.
2 Bhave P, Chamie G: *Streptococcus pneumoniae* keratitis. J Hosp Med 2008;3:353.
3 Wagoner MD, Al-Ghamdi AH, Al-Rajhi AA: Bacterial keratitis after primary pediatric penetrating keratoplasty. Am J Ophthalmol 2007;143:1045–1047.
4 Hooi SH, Hooi ST: Culture-proven bacterial keratitis in a Malaysian general hospital. Med J Malaysia 2005;60:614–623.
5 Schaefer F, Bruttin O, Zografos L, Guex-Crosier Y: Bacterial keratitis: a prospective clinical and microbiological study. Br J Ophthalmol 2001;85:842–847.
6 Asbell P, Stenson S: Ulcerative keratitis: survey of 30 years' laboratory experience. Arch Ophthalmol 1982;100:77–80.
7 Dunlop AA, Wright ED, Howlader SA, Nazrul I, Husain R, McClellan K, Billson FA: Suppurative corneal ulceration in Bangladesh: a study of 142 cases examining the microbiological diagnosis, clinical and epidemiological features of bacterial and fungal keratitis. Aust NZ J Ophthalmol 1994;22:105–110.
8 Dada T, Sharma N, Dada VK, Vajpayee RB: Pneumococcal keratitis after laser in situ keratomileusis. J Cataract Refract Surg 2000;26:460–461.
9 Kunimoto DY, Sharma S, Reddy MK, Gopinathan U, Jyothi J, Miller D, Rao GN: Microbial keratitis in children. Ophthalmology 1998;105:252–257.
10 Green MD, Apel AJ, Naduvilath T, Stapleton FJ: Clinical outcomes of keratitis. Clin Exp Ophthalmol 2007;35:421–426.
11 Liesgang TJ: Bacterial and fungal keratitis; in Kaufman HE, Barron BA, McDonald MB (eds): The Cornea. Boston, Butterworth-Heinemann, 1998, pp 159–219.

12 Callegan MC, O'Callaghan RJ, Hill JM: Pharmacokinetic considerations in the treatment of bacterial keratitis. Clin Pharmacokinet 1994;27:129–149.
13 Liesgang TJ: Bacterial and fungal keratitis; in Kaufman HE, et al (eds): The Cornea. New York, Churchill Livingstone, 1988, pp 217–270.
14 Johnson MK, Hobden JA, Hagenah M, O'Callaghan RJ, Hill JM, Chen S: The role of pneumolysin in ocular infections with *Streptococcus pneumoniae*. Curr Eye Res 1990;9:1107–1114.
15 Harrison JC, Karcioglu ZA, Johnson MK: Response of leukopenic rabbits to pneumococcal toxin. Curr Eye Res 1982;2:705–710.
16 Johnson MK, Callegan MC, Engel LS, O'Callaghan RJ, Hill JM, Hobden JA, Boulnois GJ, Andrew PW, Mitchell TJ: Growth and virulence of a complement-activation-negative mutant of *Streptococcus pneumoniae* in the rabbit cornea. Curr Eye Res 1995;14:281–284.
17 Green SN, Sanders M, Moore QC III, Norcross EW, Monds KS, Caballero AR, McDaniel LS, Robinson SA, Onwubiko C, O'Callaghan RJ, Marquart ME: Protection from *Streptococcus pneumoniae* keratitis by passive immunization with pneumolysin antiserum. Invest Ophthalmol Vis Sci 2008;49:290–294.
18 Marquart ME, Monds KS, McCormick CC, Dixon SN, Sanders ME, Reed JM, McDaniel LS, Caballero AR, O'Callaghan RJ: Cholesterol as treatment for pneumococcal keratitis: cholesterol-specific inhibition of pneumolysin in the cornea. Invest Ophthalmol Vis Sci 2007;48:2661–2666.
19 Reed JM, O'Callaghan RJ, Girgis DO, McCormick CC, Caballero AR, Marquart ME: Ocular virulence of capsule-deficient *Streptococcus pneumoniae* in a rabbit keratitis model. Invest Ophthalmol Vis Sci 2005;46:604–608.
20 Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ: A new topical model of *Staphylococcus* corneal infection in the mouse. Invest Ophthalmol Vis Sci 2003;44:1591–1597.
21 Hume EB, Cole N, Khan S, Garthwaite LL, Aliwarga Y, Schubert TL, Willcox MD: A *Staphylococcus aureus* mouse keratitis topical infection model: cytokine balance in different strains of mice. Immunol Cell Biol 2005;83:294–300.
22 Sun Y, Hise AG, Kalsow CM, Pearlman E: *Staphylococcus aureus*-induced corneal inflammation is dependent on Toll-like receptor 2 and myeloid differentiation factor 88. Infect Immun 2006;74:5325–5332.
23 Hazlett LD: Role of innate and adaptive immunity in the pathogenesis of keratitis. Ocul Immunol Inflamm 2005;13:133–138.
24 Koch AL: Autolysis control hypotheses for tolerance to wall antibiotics. Antimicrob Agents Chemother 2001;45:2671–2675.
25 Hazlett LD: Inflammatory response to *Pseudomonas aeruginosa* keratitis. Ocul Surf 2005;3:S139–S141.
26 Hazlett LD: Corneal response to *Pseudomonas aeruginosa* infection. Prog Retin Eye Res 2004;23:1–30.
27 Huang X, Hazlett LD, Du W, Barrett RP: SIGIRR promotes resistance against *Pseudomonas aeruginosa* keratitis by down-regulating type-1 immunity and IL-1R1 and TLR4 signaling. J Immunol 2006;177:548–556.
28 Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ: Effects of toxin production in a murine model of *Staphylococcus aureus* keratitis. Invest Ophthalmol Vis Sci 2005;46:2064–2070.
29 Hume EB, Cole N, Garthwaite LL, Khan S, Willcox MD: A protective role for IL-6 in staphylococcal microbial keratitis. Invest Ophthalmol Vis Sci 2006;47:4926–4930.