Ocular Virulence of Capsule-Deficient Streptococcus pneumoniae in a Rabbit Keratitis Model

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PURPOSE. Determine the ocular virulence of noncapsular Streptococcus pneumoniae in a rabbit keratitis model.

METHODS. Mice were infected intraperitoneally with 10⁵ colony-forming units (CFUs) of Avery’s strain (capsular type 2) or strain R6 (a noncapsular derivative of type 2), and mortality was monitored daily. In addition, 10⁵ CFU of each strain was injected into rabbit corneas. Bacterial loads in rabbit corneas were determined at 20 or 48 hours after infection. Slit lamp examination (SLE) of rabbit eyes was performed at 24, 36, and 48 hours after infection. Controls included corneas inoculated with bacterial suspension medium and UV-killed bacteria.

RESULTS. One hundred percent mortality was observed in mice infected intraperitoneally with the encapsulated strain at 2 days after infection, whereas all mice infected with the nonencapsulated strain survived for 21 days. The nonencapsulated strain caused the same pathologic effects in the rabbit cornea as the encapsulated strain at 24, 36, and 48 hours after infection (P ≥ 0.080). Control corneas showed no pathologic effects and had significantly lower SLE scores than corneas infected with live bacteria (P ≤ 0.001). Mean bacteria log CFU ± SEM recovered at 20 hours after infection were 7.069 ± 0.094 for the encapsulated and 6.533 ± 0.116 for the nonencapsulated strain (P = 0.001). Bacteria recovered from the corneas at 48 hours after infection were 6.712 ± 0.549 and 1.807 ± 0.462 for the encapsulated and nonencapsulated strains, respectively (P < 0.001).

CONCLUSIONS. The S. pneumoniae noncapsular strain was as virulent in the rabbit cornea as was the encapsulated strain, but unlike the encapsulated strain, was avirulent in the mouse peritoneum. (Invest Ophthalmol Vis Sci. 2005;46:604 – 608) DOI:10.1167/iovs.04-0889

A major ocular pathogen capable of causing keratitis, conjunctivitis, and endophthalmitis, Streptococcus pneumoniae is one of the top three causes of bacterial keratitis both in the United States and worldwide.1–10 In one study, pneumococcal keratitis accounted for 33.3% of bacterial keratitis.11 Clinical presentation of pneumococcal keratitis typically includes a central yellowish or grayish-white ulcer associated with infiltrates, folds in Descemet’s membrane, and hypopyon.8 S. pneumoniae keratitis is commonly treated with benzylpenicillin or cefazolin,11 however, effective treatment for keratitis is challenged by the increasing resistance of S. pneumoniae to antibiotics.12–16

Most studies of pneumococcal virulence have been conducted in models of pneumonia,17 meningitis,18 peritoneal infections,19 sepsis,20 or otitis media.21,22 But there have been few studies of pneumococcus as an agent of keratitis. Pneumococcus produces a variety of virulence factors,23 most of which are located in or on the membrane or cell wall. The capsule of pneumococcus has been implicated as a major virulence factor in infection models of the lung24 and the systemic model of intraperitoneal infection of mice.19,25,26 The current vaccine used to treat S. pneumoniae infections comprises purified capsule components.27,28

Pneumococcus can exist in a noncapsular form or in a capsular form, with 90 known capsular types.29 The proposed function of the capsule is to help the bacterium evade phagocytosis by polymorphonuclear leukocytes.24,30–33 However, the role of the capsule in corneal virulence has not been investigated.

The purpose of the present study was to determine the role of the capsule in the rabbit keratitis model using different forms of type 2 S. pneumoniae: an encapsulated form and a nonencapsulated form derived from the encapsulated strain.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. pneumoniae strains included: Avery’s capsular type 2 strain (6302; American Type Culture Collection [ATCC], Manassas, VA) and its capsule-deficient derivative strain R6 (ATCC BAA-255). Strain R6 was chosen as the noncapsular strain because it is well characterized. Isolated colonies were grown overnight at 37°C in Todd-Hewitt broth (BD Diagnostics, Sparks, MD). Each culture was diluted (1:100) in fresh Todd-Hewitt broth and grown to an optical density (A₆₀₀) of 0.25 (~10⁷ CFU/mL). Dilutions of bacteria grown to an optical density of 0.25 were plated in triplicate on Chocolate II agar (BD Diagnostics) to determine the exact CFUs used for inoculation in each experiment. In cases in which killed bacteria were used, the bacteria were centrifuged at 5000g for 10 minutes and suspended in sterile PBS, and the suspension was subjected to UV irradiation for 15 minutes. The killed bacteria were centrifuged again and suspended in Todd-Hewitt broth. Plating on Chocolate II agar verified the sterility of the UV-irradiated bacteria.

Intraperitoneal Infection of Mice

Male A/J mice (6–7 weeks old) were purchased from the National Cancer Institute (Frederick, MD) and housed and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice (10 per strain) were injected intraperitoneally with either Avery’s strain or strain R6 (10⁵ CFU in 100 µL). Mice were monitored for mortality on a daily basis. The peritoneal cavities of dead mice were lavaged with 0.5 mL sterile phosphate-buffered saline (PBS) and lavage dilutions were plated on Chocolate II agar to isolate pneumococci.

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Infection of Rabbit Corneas

New Zealand White rabbits were used in these studies and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized by subcutaneous injection 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 was topically applied to each eye before intrastromal injection. Five groups of rabbits were used, and the corneas of these rabbits were injected intrastromally with 10 μL of the following: (1) Todd-Hewitt broth (the suspending solution), (2) capsular S. pneumoniae Avery’s strain (approximately 10⁸ CFU), (3) noncapsular S. pneumoniae strain R6 (approximately 10⁵ CFU), (4) UV-killed Avery’s strain (equivalent to approximately 10⁹ CFU), and (5) UV-killed strain R6 (equivalent to approximately 10⁶ CFU). The number of corneas for groups 1 to 5, were 6, 13, 11, 6, and 6, respectively.

Slit Lamp Examinations

At least two observers performed the slit lamp examination (SLE) of rabbit corneas and were masked as to which strains were used to infect a particular rabbit. The observers were previously trained in SLE according to the method of Johnson et al. SLE of infected rabbit corneas at 24, 36, and 48 hours after inoculation were determined with a biomicroscope (Topcon; Koakiki KK, Tokyo, Japan). Seven ocular parameters were graded: injection, chemosis, iritis, fibrin, hypopyon, corneal edema, and corneal infiltrate. Each parameter was given a grade from 0 (normal) to 4 (very severe). The grades were totaled for each eye, giving an overall score for each eye ranging from 0 to 28. The scores of the observers for each cornea were averaged. The mean ± SEM for each experimental group was calculated according to the averaged scores of all the corneas in that group.

Quantification of Bacteria

At 20 or 48 hours after infection, infected rabbits were anesthetized and killed by an intravenous injection of a pentobarbital sodium solution (100 mg/mL; Sigma-Aldrich, St. Louis, MO). The 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 Chocolate II agar. The plates were incubated for 48 hours at 37°C. Colonies were counted and bacterial CFUs for the corneas were determined and expressed in logarithmic units. At 20 hours after infection, the corneas of six rabbits (n = 12) were used for the quantification of Avery’s strain, and the corneas of six rabbits (n = 12) were used for the quantification of strain R6. At 48 hours after infection, seven rabbits (n = 13) were used for Avery’s strain and six rabbits (n = 11) were used for strain R6. Rabbits inoculated with broth or UV-killed bacteria were not killed.

Statistical Analysis

Data were analyzed on computer (Statistical Analysis System; SAS, Cary, NC). Analysis of variance and Student’s t-tests between least-squares means from each group showing statistical variances were performed for CFU and SLE determinations. P < 0.05 was considered significant.

RESULTS

Virulence in Mouse Peritoneal Cavity

Growth curves determined that an optical density of approximately 0.25 was equal to 10⁶ CFU/mL for both Avery’s strain and strain R6. The actual inoculum for each experimental group of mice was determined by dilution plating and ranged from 3.9 × 10⁴ to 1.9 × 10⁵ CFU.

The peritoneal cavities of A/j mice were inoculated with the encapsulated S. pneumoniae (Avery’s strain; n = 10 mice) or the nonencapsulated strain (R6; n = 10 mice), and mouse survival was monitored daily. Ninety percent of the mice infected intraperitoneally with Avery’s strain were dead by day 1, and 100% were dead by day 2. Lavage and plating of the intraperitoneal cavities confirmed the presence of S. pneumoniae. All mice infected with the nonencapsulated strain (R6) survived for at least 5 weeks (Fig. 1), at which time the experiment was terminated. Pneumococci were not recovered from the intraperitoneal lavages of mice inoculated with the noncapsulated R6 strain.

Conical Virulence of Capsular and Noncapsular S. pneumoniae

The actual inoculum for each experimental group of rabbits was determined by dilution plating and ranged from 3.8 × 10⁴ to 1.4 × 10⁵ CFU. Bacteria that were UV-killed were also quantified before killing, to ensure similar CFUs.

Rabbit corneas infected with Avery’s capsular type 2 strain and noncapsular strain R6 were examined by SLE at 24, 36, and 48 hours after infection. Eyes infected with either strain showed mild signs of keratitis at 24 hours after infection (Fig. 2A, 2B). At 36 hours after infection, eyes infected with either strain demonstrated inflammation and a corneal infiltrate (Fig. 2C, 2D), and these pathologic effects increased in severity by 48 hours after infection (Fig. 2E, 2F). In contrast, corneas inoculated with Todd-Hewitt broth, UV-killed capsular bacteria, or UV-killed noncapsular bacteria showed virtually no signs of keratitis (Fig. 5).

SLE scores for Avery’s strain (n = 13 corneas) and the noncapsular strain R6 (n = 11 corneas) at 24, 36, and 48 hours after infection were not significantly different (Table 1). The conjunctiva of the eyes in these control groups initially showed signs of slight redness, as indicated by low SLE scores at 24 hours after inoculation (Table 1). This mild redness resolved by 48 hours after inoculation (Table 1). The SLE scores of these three control groups were not significantly different from each other at 24, 36, or 48 hours after inoculation (P ≥ 0.104). However, the SLE scores of rabbit corneas infected with live Avery’s capsular strain or live noncapsular strain R6 were significantly higher than any of the SLE scores of the control groups at all time points (P ≤ 0.001). Rabbits inoculated with broth or UV-killed bacteria were not killed, since they were healthy and their eyes were normal.

Bacterial CFUs in the corneas were determined at 20 hours after infection. The bacteria in corneas at 20 hours after infection were 7.069 ± 0.094 log CFU for Avery’s strain (n = 12 corneas) and 6.533 ± 0.116 log CFU for strain R6 (n = 12 corneas; P = 0.001). The number of viable bacteria in corneas at 48 hours after infection differed significantly between the
The noncapsular strain of \textit{S. pneumoniae} was as virulent in the rabbit cornea as its capsular counterpart. However, the noncapsular strain injected intraperitoneally into mice failed to kill any mice by 21 days, whereas the capsular strain killed all infected mice within 2 days. One important implication of these findings is that individuals immunized against pneumococcal capsular carbohydrate may not be protected against corneal infection by noncapsular strains. This observation also implies that the pathogenic mechanisms of pneumococcus for the cornea are possibly distinct from those of other body sites and as such are not well understood.

One difference between the capsular and noncapsular strain was that the CFUs recovered from the corneas at 48 hours after infection were different by approximately 5 logs. High bacterial loads (>6 log CFU) were recovered from eyes infected with the capsular strain, whereas low bacterial loads (<2 log CFU) were recovered from eyes infected with the noncapsular strain. A possible explanation for this difference is that the host was able to clear the noncapsular bacteria from the cornea, but not before toxic or inflammatory factors released by the bacteria mediated their damaging effects. To clarify whether strain R6 was able to grow to high CFUs in the cornea before this 48-hour time point, rabbit corneas were infected with Avery’s strain or strain R6, and CFUs were determined at 20 hours after infection. Although there was a statistical difference between the strains, both grew to high bacterial loads and there was <1 log difference between the strains (approximately 7 log CFU for Avery’s strain and approximately 6.5 log CFU for strain R6). The ability of strain R6 to grow to 6.5 log CFU by 20 hours after infection indicates that its decrease to <2 log CFU in the cornea at 48 hours after infection is not due to a defect in growth. Presumably, the infiltration of polymorphonuclear leukocytes into the corneas between 20 and 48 hours after infection reduces the number of viable nonencapsulated bacteria at a rate far greater than that of the encapsulated bacteria. The induction of inflammation by toxic factors such as pneumolysin could explain how ocular pathologic effects are observed to be most severe after noncapsular bacteria are cleared from the cornea. Further studies are needed to test whether toxic factors are involved.

A distinction between the extent of pneumococcal replication and virulence was noted previously by Johnson et al.\textsuperscript{34} The number of CFUs in the cornea of a serotype 3 strain of pneumococcus at 48 hours after infection averaged 3 log CFU less than its corresponding pneumolysin mutant strain, yet the mutant strain caused significantly less disease in the cornea than the parent strain.\textsuperscript{34} It is uncertain what factor(s) could be involved in the ability of the cornea to clear the bacteria; however, ocular pathologic effects appear to be severe, regardless of the bacterial counts at 48 hours after infection.

The \textit{S. pneumoniae} capsule has been implicated as a major virulence factor in lung infections, the peritoneal cavity, and bacteremia by allowing the bacteria to escape phagocytosis by polymorphonuclear leukocytes.\textsuperscript{24,30–33,35–37} The current finding is that the noncapsular strain R6, reportedly avirulent in nonocular models of infection,\textsuperscript{38,39} causes the same inflammatory effects characteristic of \textit{S. pneumoniae} keratitis as the capsular strain. This finding implies that the capsule is not needed to establish a severe corneal infection. In fact, a recent outbreak of pneumococcal conjunctivitis due to a nonencapsulated strain further suggests that the capsule is not necessary in ocular infections.\textsuperscript{40}

Previous studies have shown that pneumolysin, a toxin released by \textit{S. pneumoniae}, is involved in the inflammation associated with keratitis.\textsuperscript{34,41–42} Pneumolysin consists of two functional domains, a pore-forming (cytolytic) domain and a complement activation domain.\textsuperscript{17,43–45} The complement activation domain is involved in polymorphonuclear leukocyte recruitment.\textsuperscript{46} However, deletion of this domain decreases but does not eradicate \textit{S. pneumoniae} corneal virulence,\textsuperscript{47} indicating that other factors are also necessary for full corneal disease. Further studies are needed to elucidate the mechanisms of pneumococcal ocular virulence, and to determine the differences between \textit{S. pneumoniae} keratitis and other \textit{S. pneumoniae} infections. Such information could be important in developing new therapies for this form of bacterial keratitis.

**FIGURE 2.** Rabbit corneas at 24 (A, B), 36 (C, D), and 48 (E, F) hours after intrastromal inoculation with \textit{S. pneumoniae} Avery’s strain (A, C, E) and strain R6 (B, D, F). Photographs are representatives of the respective groups.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>C</td>
<td>D</td>
</tr>
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<td>E</td>
<td>F</td>
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**FIGURE 3.** Rabbit corneas at 48 hours after intrastromal inoculation with Todd-Hewitt broth (A), UV-killed \textit{S. pneumoniae} Avery’s strain (B), and UV-killed \textit{S. pneumoniae} strain R6 (C). Photographs shown are representatives of the respective groups.
**Table 1. SLE Scores of Corneas at 24, 36, and 48 hours After Infection**

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>n*</th>
<th>24 hours</th>
<th>36 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Todd Hewitt broth†</td>
<td>6</td>
<td>0.917 ± 0.493</td>
<td>0.354 ± 0.139</td>
<td>0.063 ± 0.028</td>
</tr>
<tr>
<td>2</td>
<td>Avery’s strain (capsular)‡</td>
<td>13</td>
<td>4.606 ± 0.264</td>
<td>6.894 ± 0.348</td>
<td>9.038 ± 0.747</td>
</tr>
<tr>
<td>3</td>
<td>Strain R6 (noncapsular)</td>
<td>11</td>
<td>4.727 ± 0.225</td>
<td>7.682 ± 0.498</td>
<td>10.170 ± 1.319</td>
</tr>
<tr>
<td>4</td>
<td>Killed Avery’s strain†</td>
<td>6</td>
<td>0.853 ± 0.243</td>
<td>0.354 ± 0.094</td>
<td>0.042 ± 0.027</td>
</tr>
<tr>
<td>5</td>
<td>Killed strain R6†</td>
<td>6</td>
<td>0.313 ± 0.043</td>
<td>0.208 ± 0.053</td>
<td>0.063 ± 0.043</td>
</tr>
</tbody>
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SLE scores are expressed as the mean ± SEM.
* Number of corneas.
† SLE scores at all time points for groups 1, 4, and 5 were not significantly different from each other (P ≥ 0.104), but were all significantly less than those in groups 2 and 3 (P < 0.001).
‡ SLE scores at all time points were not significantly different from those in group 3 (P ≥ 0.080).

**References**


