

Hypermucoviscosity as a Virulence Factor in Experimental Klebsiella pneumoniae Endophthalmitis

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PURPOSE. Klebsiella pneumoniae is a common cause of endogenous bacterial endophthalmitis, a disease that frequently results in a poor visual outcome. Hypermucoviscosity has been identified as a virulence factor among clinical bacteremia isolates of K. pneumoniae. In this study, an experimental murine model of K. pneumoniae endophthalmitis was established, and the role of hypermucoviscosity in its pathogenesis was analyzed.

METHODS. C57BL/6J mice were intravitreally injected with 100 CFU of hypermucoviscous (HMV+) or nonhypermucoviscous (HMV−) K. pneumoniae. Intraocular bacterial growth, retinal function, gross pathology, and inflammatory responses were monitored every 3 hours until the eyes lost significant (>90%) retinal function, or the infection appeared to clear.

RESULTS. The HMV+ strain grew logarithmically in eyes until approximately 15 hours postinfection (PI), reaching a stationary phase of growth at approximately 8.0 log10 CFU/eye. The HMV− strain grew logarithmically to approximately 7.6 log10 CFU/eye by 18 hours, but bacterial count declined to approximately 6.4 log10 CFU/eye by 21 hours PI. Eyes infected with the HMV+ strain retained approximately 35% a-wave and <10% b-wave function by 18 hours PI. These eyes also had a cumulative clinical score of 14+ by 18 hours and underwent phthisis between 21 and 24 hours. Eyes infected with the HMV− strain had a cumulative clinical score of <6 and retained >60% a-wave and >50% b-wave function throughout 21 hours. Five of 7 eyes had <100 CFU HMV− K. pneumoniae at 27 hours PI.

CONCLUSIONS. The findings demonstrate the site-threatening consequences of K. pneumoniae endophthalmitis and the importance of the hypermucoviscosity phenotype in the pathogenesis of experimental K. pneumoniae endophthalmitis. (Invest Ophthalmol Vis Sci. 2008;49:4931–4938) DOI:10.1167/iovs.08-2276

Bacterial endophthalmitis is an infection in the posterior segment of the eye that frequently results in significant vision loss, if not loss of the eye itself. Bacteria can infect the eye after intraocular surgery or a penetrating eye injury, with infection probabilities ranging between 0.1% and 0.2% and 3% and 17%, respectively.1–5 Endogenous endophthalmitis results from bacterial spread from a distant focus of infection into the eye and accounts for approximately 2% to 8% of all endophthalmitis cases.6–8 Although endogenous endophthalmitis is rare, the potential for vision loss, including bilateral blindness, makes this type of infection a potential threat to patients with underlying systemic bacterial infections.6,9

Within the past 20 years, Klebsiella pneumoniae has become the most commonly reported bacterial species responsible for endogenous endophthalmitis.6,10 K. pneumoniae is an opportunistic, Gram-negative bacterium responsible for both communal and hospital-acquired infections.6,11,12 Mortality rates of nosocomial K. pneumoniae bacteremia range from 20% to 50%, with no improvement in outcomes within the past decade.11–13 K. pneumoniae is known worldwide for its rapid acquisition of resistance to traditional and new antimicrobial drugs, including resistance to third-generation cephalosporins, fluoroquinolones, carbapenem, and extended-spectrum β-lactamases.12–19 K. pneumoniae endophthalmitis has been strongly associated with the presence of primary liver abscesses and an underlying diabetic condition.6,20 Despite surgical and/or therapeutic intervention, K. pneumoniae endophthalmitis commonly results in a poor visual outcome and often the need for evisceration or enucleation of the eye.6,10 As the number of communal and hospital acquired K. pneumoniae infections continues to rise with the drastic increase in the incidence of diabetes worldwide, the number of cases of endogenous K. pneumoniae endophthalmitis may also increase.

Numerous studies have identified virulence determinants of K. pneumoniae in clinical isolates. Of importance are clinical isolates that possess serotype K1 or K2 capsules and/or the presence of genes associated with a capsule-associated mucopolysaccharide web, also known as the hypermucoviscous (HMV) phenotype.21–24 When a standard bacteriologic loop is passed through a colony and forms a mucoviscous string greater than 5 mm, the strain is characterized as having the HMV phenotype.21 Capsular serotypes K1 and K2 have been shown to perturb phagocytosis resistance to neutrophils obtained from patients with type II diabetes25,27 and increased lethality in mice intraperitoneally injected with K. pneumoniae.25 K. pneumoniae clinical isolates with the HMV phenotype are commonly associated with bacteremia and distinct invasive syndromes such as primary liver abscesses, meningitis, and endophthalmitis.28,29 Both magA (mucoviscosity-associated gene A) and rmpA (regulator of the mucoid phenotype) have been associated with K. pneumoniae strains with the HMV phenotype.21,22,24,25,26,28–30 The magA gene has been found to be restricted to K1 isolates, whereas the rmpA gene is found in K1, K2, and other serotypes.7,24–26,29,30 The specific role of these genes in association with endophthalmitis has not been experimentally analyzed.

The purpose of this study was twofold: to develop an experimental K. pneumoniae endophthalmitis murine model...
amenable to the analysis of pathogenesis and to test the hypothesis that the HMV phenotype contributes to the intraocular virulence of experimental *K. pneumoniae* endophthalmitis. Using the experimental murine endophthalmitis model, we compared the intraocular virulence of hypermucoviscous and nonhypermucoviscous strains of *K. pneumoniae* and determined that the HMV phenotype plays a role in the pathogenesis of infection.

**METHODS**

**Bacteria and Growth Conditions**

The nonhypermucoviscous (HMV−) *K. pneumoniae* strain was a clinical isolate from the vitreous of an endophthalmitis patient and a generous gift from Darlene Miller (Buscom Palmer Eye Institute, Miami, FL). The hypermucoviscous (HMV+) strain was a clinical isolate from a patient with a primary liver abscess and a generous gift from Steve Libby (University of Washington, Seattle, WA). Single colonies of each *K. pneumoniae* strain were cultured for 18 hours in brain–heart infusion media (BHI; EMD/Merck KGaA, Darmstadt, Germany) at 37°C with aeration. Overnight cultures were subcultured into BHI, grown to logarithmic phase and serially diluted in BHI to approximately 10^6 CFU/mL for intravitreous injection.

**Preparation of Supernatants and Metabolically Inactive *K. pneumoniae***

Single colonies of each *K. pneumoniae* strain were cultured for 18 hours in BHI at 37°C with aeration. For preparation of supernatants, the cultures were centrifuged, filter-sterilized with a 0.22-μm filter unit (Millex GV; Carrigtwahil Co., Cork, Ireland), and kept on ice until used. The supernatants were inoculated on BHI plates and incubated overnight at 37°C. A standard bacteriologic loop was used to harvest a single colony of each strain. The overnight culture of bacteria was harvested by centrifugation. The supernatant was extracted with an equal volume of chloroform, ethanol precipitated, and washed with 70% ethanol before the pellet was resuspended in 50 μL of water. Each PCR reaction consisted of 0.6 mM of each forward and reverse primer, 0.05 mM of each dNTP, 2 mM MgCl2, 1 U Taq polymerase, and buffer (GoTaq; Promega, Madison, WI). Fragments were amplified with primers specific for *magA*, *rmpA*, or a 16s ribosomal subunit specific for *K. pneumoniae* (Table 1). PCR was performed in the following conditions: 94°C initial denaturation for 3 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 74°C for 1 minute 30 seconds, and a final extension at 74°C for 5 minutes. DNA fragments were separated and visualized on a 1.2% agarose gel with sodium borate buffer containing 0.2 μg/mL ethidium bromide.

**Experimental *K. pneumoniae* Endophthalmitis**

C57BL/6j mice (male, 6–8 weeks old; Jackson Laboratories, Bar Harbor, ME) were obtained and maintained in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were anesthetized with a cocktail of ketamine (85 mg/kg of body weight; Phoenix Scientific, Inc., St. Joseph, MO) and xylazine (14 mg/kg of body weight; Bayer Corp., Shawnee Mission, KS). A single drop of topical anesthetic (0.5% proparacaine HCl; Allergan, Horningueros, Puerto Rico) was placed on each eye before injection. The eyes were intravitreously injected as previously described.

**Electroretinography**

Retinal function was analyzed as previously described. The mice were dark-adapted for a minimum of 6 hours and then anesthetized, and the eyes were topically anesthetized as just described. Pupils were dilated with topical phenylephrine (10%; Acorn, Inc., Buffalo Grove, IL). Gold-wire electrodes (99.95%, 0.25 mm; Alfa Aesar, Ward Hill, MA) were placed on each cornea, along with a small drop of a hypromellose ophthalmic demulcent (Solution Gonioscopic Prism Solution 2.5%; Wilson Ophthalmic Corp., Mustang, OK) between the cornea and electrode to facilitate maximum conductance.

Full-field scotopic ERGs were recorded in each eye (UTAS-E 5000; LKC Technologies, Inc., Gaithersburg, MD). The amplitude of the a-wave (a measure of photoreceptor cell function) was measured from the prestimulus baseline to the a-wave trough. The amplitude of the b-wave (a measure of Müller, bipolar, and amacrine cell function) was measured from the a-wave trough to the b-wave peak. The percentage of retinal function retained was calculated by using the following equations:

\[
100 - \left[\left(1 - \frac{\text{experimental a-wave amplitude}}{\text{absolute control a-wave amplitude}}\right) \times 100\right]\quad (1)
\]

or

**Table 1. PCR Primers**

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<tr>
<th>Primer</th>
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<td>5′-GAGAGGATCCGGTGTCTTACATCTTCGCTG-3′</td>
<td>21</td>
</tr>
<tr>
<td><em>rmpA</em> forward</td>
<td>5′-ACGGGGTCTACCTCCTGCTCA-3′</td>
<td>26</td>
</tr>
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<td><em>rmpA</em> reverse</td>
<td>5′-GGGTAACGGAGGGTGC-3′</td>
<td>26</td>
</tr>
<tr>
<td>16s RNA forward</td>
<td>5′-GGGTAATACGGAGGGTGCG-3′</td>
<td>22</td>
</tr>
<tr>
<td>16s RNA reverse</td>
<td>5′-CACATCGACTTGAGAGACC-3′</td>
<td>22</td>
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the eyes were enucleated, placed into 400 μL of sterile PBS, and homogenized (60 seconds, 5000 rpm; Mini-BeadBeater, Biospec Products, Inc., Bartlesville, OK) by using sterile glass beads (1.0 mm; Bio-Beads, Bio-Rad Laboratories, Hercules, CA). The tissue was washed in 400 μL of sterile PBS, and the resulting slurry was homogenized for a second time. Homogenized samples were then centrifuged for 10 minutes at 10,000 g to concentrate the pellets. The supernatants were discarded, and 400 μL of 50% trichloroacetic acid (TCA) was added to precipitate the proteins. The samples were then treated at 4°C for 30 minutes and then centrifuged again for 10 minutes at 10,000 g to remove the precipitated proteins. The supernatants were discarded and 400 μL of PBS was added to each respective sample. The samples were then vortexed vigorously and then left to precipitate for 30 minutes at 4°C. Following this, the samples were centrifuged for 10 minutes at 10,000 g and the supernatants were discarded. The pellets were then resuspended in 100 μL of 0.5% acetic acid (pH 5.0) and stored at −80°C until ready for analysis.

Analysis of Intraocular Inflammation

Myeloperoxidase (MPO) concentrations of infiltrating polymorphonuclear leukocytes (PMNs) were assayed in whole eyes, as previously described. The eyes were removed and homogenized with glass beads in 400 μL lysis buffer (20 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol (vol/vol), 1 mM PMSF, 1 μg/ml leupeptin, and 28 μg/ml aprotinin). Absolute controls were included. Supernatants were further diluted 1:4 and analyzed for MPO by ELISA according to the manufacturer’s instructions (mouse MPO ELISA Test Kit; Cell Sciences, Canton, MA). The results are reported as nanograms MPO per eye.

Results

Phenotypic and Genetic Analysis of K. pneumoniae Strains

The hypermucoviscous phenotype of the strains used in this study was determined by a modified string test (Fig. 1). The HMV+ mucoid string was approximately 12 mm, indicating a hypermucoviscous phenotype. The HMV− string was approximately 1 mm, indicating a nonhypermucoviscous phenotype. The presence of the magA and rmpA genes was verified by PCR (Fig. 2, 1282- and 585-bp fragments, respectively). PCR analysis verified that the HMV+ strain used in this study possessed magA and rmpA, whereas the HMV− strain did not. Both strains were PCR positive for the K. pneumoniae 16S rRNA fragment.

Clinical Progression of Experimental Murine K. pneumoniae Endophthalmitis

Photographs and clinical scores of eyes infected with K. pneumoniae are summarized in Figure 3. Preoperative clinical scores were similar to those in absolute control eyes scored throughout the experiment (data not shown). Clinical scores of absolute control eyes were similar to those of infected eyes until 12 hours postinfection (PI). At that time point, eyes infected with either strain had cumulative clinical scores of ≤3. By 15 hours PI, a mild vitreous haze and slightly reduced red reflex were observed in eyes infected with either HMV− or HMV+ strains, resulting in clinical scores of 4.25 ± 0.06 or 6.75 ± 1.88, respectively (P < 0.05). Eyes infected with the HMV− strain maintained a clinical score of ≤6 throughout 21 hours PI. In contrast, eyes infected with the HMV+ strain presented with a clinical score of 15.75 ± 0.06 (P < 0.001) at 21 hours PI. At this time, red reflexes were absent, and a dense vitreous haze was present in eyes infected with the HMV+ strain.

Retinal Function

Retinal function analysis of K. pneumoniae-infected eyes is summarized in Figure 4. Retinal function of absolute control eyes was similar to that of preoperative eyes measured throughout the experiment (data not shown). In eyes infected with the HMV+ strain, significant a-wave function declines began at 15 hours PI, decreasing to approximately 35% by 18 hours PI. Cumulative b-wave function declines also began at 15 hours PI, decreasing to approximately 37% by 18 hours PI. In eyes infected with the HMV− strain, retinal function remained similar to those of absolute control eyes, although significant declines were observed in b-wave function at 21 hours PI, decreasing to approximately 40%.

Analysis of Intraocular Inflammation

Myeloperoxidase (MPO) concentrations of infiltrating polymorphonuclear leukocytes (PMNs) were assayed in whole eyes, as previously described. The eyes were removed and homogenized with glass beads in 400 μL lysis buffer (20 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol (vol/vol), 1 mM PMSF, 1 μg/ml leupeptin, and 28 μg/ml aprotinin). Absolute controls were included. Supernatants were further diluted 1:4 and analyzed for MPO by ELISA according to the manufacturer’s instructions (mouse MPO ELISA Test Kit; Cell Sciences, Canton, MA). The results are reported as nanograms MPO per eye.

Statistics

All values represent the mean ± SEM for four or more eyes per time point unless otherwise specified. Descriptive statistics and two-tailed, two-sample t-tests were used for statistical comparisons between groups. P ≤ 0.05 was considered significant.

Bacterial Quantitation

Quantitation of bacteria in ocular tissues has been described. Briefly, the eyes were enucleated, placed into 400 μL of sterile PBS, and homogenized (60 seconds, 5000 rpm; Mini-BeadBeater, Biospec Products Inc., Bartlesville, OK) by using sterile glass beads (1.0 mm; Bio-Beads, Bio-Rad Laboratories, Hercules, CA). Intraocular bacteria were quantified by plating serial 10-fold dilutions in triplicate on BHI agar.

Biomicroscopy and Histology

The eyes were photographed with an operating biomicroscope (model S7; Carl Zeiss Meditec, Inc., Thornwood, NY) before harvest. A rating scale was used to score the degree of change in anterior and posterior segment inflammation and retinal architecture, based on a scale from 0 (no change) to 4+ (significant inflammation and retinal architectural damage). The eyes used for histology were enucleated and fixed (4% formaldehyde, 0.2% glutaraldehyde) in 0.1 M phosphate buffer (pH 7.4) for 24 hours, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin by standard procedures.

Analysis of Intraocular Inflammation

Myeloperoxidase (MPO) concentrations of infiltrating polymorphonuclear leukocytes (PMNs) were assayed in whole eyes, as previously described. The eyes were removed and homogenized with glass beads in 400 μL lysis buffer (20 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol (vol/vol), 1 mM PMSF, 1 μg/ml leupeptin, and 28 μg/ml aprotinin). Absolute controls were included. Supernatants were further diluted 1:4 and analyzed for MPO by ELISA according to the manufacturer’s instructions (mouse MPO ELISA Test Kit; Cell Sciences, Canton, MA). The results are reported as nanograms MPO per eye.

Statistics

All values represent the mean ± SEM for four or more eyes per time point unless otherwise specified. Descriptive statistics and two-tailed, two-sample t-tests were used for statistical comparisons between groups. P ≤ 0.05 was considered significant.
hours PI. b-Wave function declines began at 12 hours PI, decreasing to <10% function by 18 hours PI. Eyes infected with HMV− K. pneumoniae had significantly greater a-wave (P ≤ 0.03) and b-wave (P ≤ 0.001) function at 18 and 21 hours PI than eyes infected with the HMV+ strain. When compared with eyes infected with the HMV+ strain, eyes infected with the HMV− strain experienced slower declines in a- and b-wave function until 21 hours PI. Retinal function of eyes infected with the HMV− strain was also measured at 27 hours PI. At this time, a- and b-wave amplitudes had recovered to approximately 100% and 80%, respectively.

**Intraocular Bacterial Growth**

The intravitreous growth rates of HMV+ and HMV− strains are summarized in Figure 5. Both strains grew at similar rates (P ≥ 0.168) until 15 hours PI. A stationary phase of growth was reached by 15 hours PI at approximately 8.5 log_{10} CFU/eye for all eyes infected with the HMV+ strain, and intraocular counts remained constant throughout 21 hours PI. Eyes infected with HMV− strain maintained logarithmic growth until 18 hours PI (~7.6 log_{10} CFU/eye), but declined to approximately 6.4 log_{10} CFU/eye by 21 hours PI. Bacterial quantities of HMV− infected eyes were also measured at 27 hours PI. In this group, no bacteria were recovered from two of seven eyes, <100 bacteria were recovered from three of seven eyes, and the remaining two eyes had 1.05 × 10^6 CFU and 1.13 × 10^5 CFU.

**MPO Assay**

MPO analysis of eyes infected with K. pneumoniae is summarized in Figure 6. MPO was not detected in control eyes. MPO concentrations in eyes infected with the HMV+ strain steadily increased between 9 and 21 hours PI (P ≤ 0.005). MPO concentrations in eyes infected with the HMV− strain steadily increased until 15 hours PI, with no significant change in MPO concentrations in these eyes between 15 and 21 hours PI (P > 0.05). Significantly greater concentrations of MPO were detected in eyes infected with the HMV+ strain compared with that of eyes infected with HMV− K. pneumoniae, at all times except 15 hours PI (P < 0.05).

**Histologic Analysis**

Histology sections of evolving K. pneumoniae endophthalmitis are presented in Figure 7. Eyes infected with the HMV− strain contained inflammatory cells in the posterior chamber, minimal amounts of fibrin, and no disruption of retinal layers at 15 or 21 hours PI. In contrast, eyes infected with the HMV+ strain had inflammatory cells in both anterior and posterior segments, fibrin in the anterior chamber, and retinal folds in <25% of the retinal area in all eyes at 15 hours PI. At 21 hours PI, eyes infected with the HMV+ strain presented with inflammatory cells and fibrin in the anterior and posterior segments, and retinal folds and detachments were observed in 50% to 75% of the retinal area in all eyes.
Hypermucoviscosity and K. pneumoniae Endophthalmitis

posterior chamber at 24 and 48 hours, but no retinal folding or histologic analysis showed minimal inflammatory cell influx into the eyes infected with the two strains at either time point (Fig. 8C). A-wave), with no significant differences observed between the two strains used in this study contained magA (a part of the capsular polysaccharide gene cluster of serotype K1), rmpA (a gene associated with the hypermucoviscous phenotype), and tested positive for the hypermucoviscous phenotype on a string test. The HMV− strain used in this study lacked magA and rmpA and was not hypermucoviscous. The two strains used in these studies were clinical isolates from invasive infections, but were not isogenic. Important genetic and phenotypic differences in these two strains involved factors associated with the hypermucoviscous phenotype, but may also have involved differences in capsule production. In addition to the lack of rmpA and magA in the HMV− K. pneumoniae strain, two genes (age and wabG) involved in capsular biosynthesis were also not detected by PCR (data not shown). The HMV− strain did produce a capsule, but perhaps not the same capsular type as did the HMV+ strain. The bacterial capsule is known to be an important virulence factor in numerous infection models. The importance of the K. pneumoniae capsule in intraocular infection is being carefully analyzed, since the contribution of K. pneumoniae capsules to infection virulence has been shown to be model dependent.29 Aerobactin, a siderophore, has also been reported to be a virulence factor for K. pneumoniae isolates.30–36 The presence of aerobactin was not tested in the strains used in this study. In clinical isolates, the presence of rmpA correlated closely with the production of aerobactin,7 and so our HMV+ isolate may have also produced aerobactin. Although K. pneumoniae is the most common Gram-negative species responsible for endogenous endophthalmitis,8–10 the intracocular mechanisms involved in infection and vision loss caused by this pathogen have not been experimentally determined. Rabbit and mouse models have been used to analyze the pathogenesis of endophthalmitis caused by many common types of bacteria.34–36–38 The K. pneumoniae model in the present study was initiated by direct injection of bacteria into the vitreous, which does not mimic the route by which K. pneumoniae infects the eye during endogenous endophthalmitis. The model used herein bypasses the bloodstream-to-eye route of endogenous infection, but is relevant in that pathogenesis can be analyzed once the organisms reach the eye. To our knowledge, neither the mechanisms of bacterial spread into the eye from the bloodstream, nor the pathogenic mechanisms of K. pneumoniae in the eye have been addressed.

In this model, most of the eyes injected with 100 CFU of the HMV− K. pneumoniae strain appeared to clear the infection

**K. pneumoniae Supernatant and Metabolically Inactive K. pneumoniae**

To determine whether differences during infection were due to products secreted by K. pneumoniae or by K. pneumoniae cells themselves, we analyzed ocular changes after intravitreal injection of supernatants or metabolically inactive K. pneumoniae of each phenotype, respectively (Fig. 8). Filter-sterilized supernatants from HMV− or HMV+ cultures were intravitreally injected, and the eyes were analyzed at 24 and 48 hours PI. Eyes injected with HMV− or HMV+ supernatants retained approximately 70% retinal function at 24 hours (P ≥ 0.056, a- and b-wave) and >80% retinal function at 48 hours (P ≥ 0.083, a- and b-wave). At 48 hours, eyes injected with the HMV− supernatant had higher levels of retinal response than did eyes injected with the HMV+ supernatant, but this difference was not significant (Fig. 8A). Histologic analysis of eyes injected with either supernatant demonstrated minimal inflammatory cell influx into the posterior segment and no retinal folding after 24 or 48 hours (Fig. 8B).

Metabolically inactive HMV+ or HMV− K. pneumoniae (10⁸ CFU equivalents) were intravitreally injected, and the eyes were analyzed at 24 and 48 hours PI. Eyes injected with metabolically inactive HMV− or HMV+ retained >70% retinal function at 24 hours (P ≥ 0.30, a- and b-wave) and approximately 80% retinal function at 48 hours (P ≥ 0.46, a- and b-wave), with no significant differences observed between eyes injected with the two strains at either time point (Fig. 8C). Histology showed minimal inflammatory cell influx into the posterior chamber at 24 and 48 hours, but no retinal folding or detachment in any eyes (Fig. 8D).

**DISCUSSION**

Numerous reports have attributed the virulence of K. pneumoniae infections to capsular K1/K2 serotypes,24,25,27,29,30 the presence of a putative virulence gene magA,31,32 and/or a hypermucoviscous phenotype.36–38 The HMV+ strain used in this study contained magA (a part of the capsular polysaccharide gene cluster of serotype K1), rmpA (a gene associated with the hypermucoviscous phenotype), and tested positive for the hypermucoviscous phenotype on a string test. The HMV− strain used in this study lacked magA and rmpA and was not hypermucoviscous. The two strains used in these studies were clinical isolates from invasive infections, but were not isogenic. Important genetic and phenotypic differences in these two strains involved factors associated with the hypermucoviscous phenotype, but may also have involved differences in capsule production. In addition to the lack of rmpA and magA in the HMV− K. pneumoniae strain, two genes (age and wabG) involved in capsular biosynthesis were also not detected by PCR (data not shown). The HMV− strain did produce a capsule, but perhaps not the same capsular type as did the HMV+ strain. The bacterial capsule is known to be an important virulence factor in numerous infection models. The importance of the K. pneumoniae capsule in intraocular infection is being carefully analyzed, since the contribution of K. pneumoniae capsules to infection virulence has been shown to be model dependent.29 Aerobactin, a siderophore, has also been reported to be a virulence factor for K. pneumoniae isolates.30–36 The presence of aerobactin was not tested in the strains used in this study. In clinical isolates, the presence of rmpA correlated closely with the production of aerobactin,7 and so our HMV+ isolate may have also produced aerobactin. Although K. pneumoniae is the most common Gram-negative species responsible for endogenous endophthalmitis,8–10 the intracocular mechanisms involved in infection and vision loss caused by this pathogen have not been experimentally determined. Rabbit and mouse models have been used to analyze the pathogenesis of endophthalmitis caused by many common types of bacteria.34–36–38 The K. pneumoniae model in the present study was initiated by direct injection of bacteria into the vitreous, which does not mimic the route by which K. pneumoniae infects the eye during endogenous endophthalmitis. The model used herein bypasses the bloodstream-to-eye route of endogenous infection, but is relevant in that pathogenesis can be analyzed once the organisms reach the eye. To our knowledge, neither the mechanisms of bacterial spread into the eye from the bloodstream, nor the pathogenic mechanisms of K. pneumoniae in the eye have been addressed.

In this model, most of the eyes injected with 100 CFU of the HMV− K. pneumoniae strain appeared to clear the infection

**FIGURE 5.** Intraocular growth during experimental K. pneumoniae endophthalmitis. Approximately 100 CFU of either HMV+ or HMV− K. pneumoniae were injected into the mid vitreous. Bacteria were quantified from whole eyes at 6 hours PI and every 3 hours thereafter until HMV+ infected eyes underwent phthisis. The HMV+ and HMV− strains grew similarly until 12 hours PI. After that time, the HMV+ strain grew more rapidly than did the HMV− strain. All values represent the mean ± SEM of n ≥ 4 eyes per group.

**FIGURE 6.** MPO kinetics in whole eyes during experimental K. pneumoniae endophthalmitis. Approximately 100 CFU of either HMV+ or HMV− K. pneumoniae were injected into the mid vitreous. Whole eyes were homogenized and supernatants analyzed for MPO by ELISA. In general, the MPO concentrations of eyes infected with the HMV+ strain were greater than that of eyes infected with the HMV− strain, indicating greater inflammation in these eyes. Values represent the mean ± SEM of n ≥ 4 eyes per time point.
within 27 hours, but injection of 100 CFU of HMV+ K. pneumoniae resulted in phthisis within 24 hours PI. The clearance of HMV− K. pneumoniae from infected eyes after 18 hours PI is similar to that reported in an experimental Klebsiella oxytoca endophthalmitis rabbit model induced with intravitreous infection of 500 to 1000 CFU.40 In that study, reductions in viable K. oxytoca recovered from infected eyes began at 24 hours PI. In experimental Staphylococcus aureus endophthalmitis in C57BL/6 mice intravitreously infected with 500 to 1000 CFU, the infection cleared within 96 hours, but with 5000 CFU, the infection was not cleared, suggesting differences in outcome based on initial infection dose.37 In the present study, the infection did not clear in eyes injected with 100 CFU of the HMV+ strain, suggesting differences in infection outcome based on differences in the hypermucoviscous phenotype. Genotypic differences in capsular biosynthesis genes were also noted, which may have also contributed to differences in the virulence of these strains.

In this model, K. pneumoniae with the HMV+ phenotype caused rapid retinal function decline and a significantly greater cumulative clinical score after 15 hours PI when compared with eyes infected with the HMV− strain. The inflammatory changes observed between 15 and 21 hours PI in eyes infected with the HMV+ strain are comparable to those in experimental

![Image: Figure 7. Histology of experimental K. pneumoniae endophthalmitis. Approximately 100 CFU of either HMV+ or HMV− K. pneumoniae were injected into the mid vitreous and whole eyes harvested for histology. Eyes were sectioned and stained with hematoxylin and eosin by standard procedures. In general, eyes infected with the HMV+ K. pneumoniae strain had greater inflammation and retinal detachments than did eyes infected with the HMV− strain. Histology sections are representative of n = 3 eyes per group.](image1)

![Image: Figure 8. Histology and retinal function analysis of eyes injected with K. pneumoniae supernatant or metabolically inactive K. pneumoniae. (A, B) Eyes were intravitreously injected with 0.5 μL of sterile-filtered supernatant of cultures of HMV+ or HMV− K. pneumoniae. Minimal retinal function loss (A) and inflammation (B) were observed in both groups at 24 and 48 hours PI. (C, D) Eyes were intravitreously injected with 10⁶ CFU equivalents of HMV+ or HMV− K. pneumoniae. Again, minimal retinal function loss (C) and inflammation (D) were observed in both groups at 24 and 48 hours PI. Values represent the mean ± SEM for n ≥ 4 eyes per time point and sections are representative of n = 3 eyes per group.](image2)
**Bacillus** endophthalmitis at 6 and 8 hours PI. However, eyes infected with either **K. pneumoniae** strain failed to exhibit the significant retinal damage observed during experimental **Bacillus cereus**, **S. aureus**, or **Enterococcus faecalis** endophthalmitis. Retinal destruction in these infections has been attributed to toxins and other excreted factors in the eye during infection. Classic virulence factors, such as cytolsins, hemolysins, and enterotoxins, have been described frequently for **K. pneumoniae** and have not been analyzed in terms of their contributions to virulence. Both **K. pneumoniae** isolates were nonhemolytic on blood agar, nonproteolytic on skim milk agar, and noncytolytic when incubated with cultured retinal pigment epithelial cells (data not shown), indicating that classic lytic toxins or proteases were not produced by either strain. In the absence of toxin production by **K. pneumoniae**, the retinal function loss observed in eyes infected with the HMV+ strain may have been a consequence of bystander damage caused by the immune response. Compared with the intraocular response to infection with the HMV− strain, intraocular inflammation in response to infection with the HMV+ strain was quite robust, suggesting that factors associated with the hypermucoviscous phenotype were highly inflammmogenic.

Of interest was the recovery of retinal function in eyes injected with the HMV− strain. These eyes had less inflammation than did eyes infected with the HMV+ strain, and in most eyes the infection appeared to clear. A less robust inflammatory response may have resulted in less bystander damage to the retina. On the other hand, the mere presence of numerous inflammatory cells in the vitreous may have, in part, altered the light path to the retina, resulting in a muted retinal response. Once the infection began to clear in eyes infected with the HMV+ strain, inflammation subsided, and vitreous clarity was eventually restored.

Although the strains used in this study did not produce classic toxins, some strains of **K. pneumoniae** have been shown to produce proteases, toxins, and hemolysins. To confirm that the intraocular virulence associated with the HMV+ strain was not due to excreted factors, 18-hour supernatants were intravitreally injected, and the resulting ocular changes were compared by ERG and histology. Only minimal inflammation and retinal function declines were observed, indicating that secreted factors did not contribute significantly to these changes during infection. To analyze whether **K. pneumoniae** cells themselves contributed to inflammation and retinal function loss, 10⁶ CFU equivalents of metabolically inactive HMV+ or HMV− **K. pneumoniae** were intravitreally injected. Again, inflammation and retinal function loss were minimal. Surprisingly, differences in inflammation and retinal function loss between eyes injected with metabolically inactive HMV+ or HMV− strains were not observed. During intraocular infection, HMV+ **K. pneumoniae** was significantly more virulent. If the mucopolysaccharide web of the HMV+ strain contributed to virulence, one would expect that the presence of this phenotype would confer greater protection from the immune response, increasing the potential for bystander damage caused by a futile attempt by the host to clear the infection, whether the organism be alive or dead. However, these results suggested that factors involved in the hypermucoviscous phenotype were important to intraocular virulence, but these factors may only have been synthesized during active replication.

To our knowledge, this is the first study in which the intraocular pathogenicity of **K. pneumoniae**, an organism that causes nearly all blinding cases of endogenous endophthalmitis, has been examined. These results demonstrate the potentially devastating, sight-threatening consequences of **K. pneumoniae** endophthalmitis and establish the hypermucoviscous phenotype as important in intraocular virulence in this model. Several questions remain in terms of the mechanisms of migration of **K. pneumoniae** (and other organisms) into the eye from a distant site of infection, resulting in endogenous endophthalmitis. Future studies will focus on analyzing the mechanisms involved in metastatic spread of bacteria into the eye during systemic infection and therapies aimed at preventing the progression to endogenous endophthalmitis.

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