Contribution of Mucoviscosity-Associated Gene A (magA) to Virulence in Experimental Klebsiella pneumoniae Endophthalmitis

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PURPOSE. Endogenous endophthalmitis secondary to Klebsiella pneumoniae liver abscess is a blinding infection that is being reported more frequently in the literature. The K1 capsule and magA contribute to virulence of systemic infection in mice; however, little is known about the role of magA in secondary ocular infections.

METHODS. To assess the role of K. pneumoniae capsule in endophthalmitis, the authors induced experimental endophthalmitis by direct inoculation of 100 colony-forming unit wild-type, magA-deficient, or magA-complemented K. pneumoniae into the posterior segments of mouse eyes. Eyes were analyzed by quantitation of viable bacteria, retinal function, and inflammatory cell influx as well as by histology.

RESULTS. Wild-type K1 K. pneumoniae caused significant ocular disease. At the end point of 24 hours postinfection, eyes infected with wild-type K. pneumoniae retained significantly less retinal A-wave function than eyes infected with an isogenic magA-mutant strain. B-wave function retention was also greater in eyes infected with the magA mutant than with wild-type K. pneumoniae. Additionally, intracocular growth of the magA-deficient strain was less than it was in the wild-type strain. The amount of myeloperoxidase elicited was also significantly higher for wild-type-infected eyes at 24 hours.

CONCLUSIONS. These results indicate that in the eye, the K1 capsule of invasive K. pneumoniae significantly contributes to the ability of the bacteria to disrupt retinal function, to grow to high density, and to persist despite immune cell recruitment. (Invest Ophthalmol Vis Sci. 2011;52:6860–6866) DOI:10.1167/iovs.11-7798

Klebsiella pneumoniae has become an important pathogen in recent years. Invasive strains causing pyogenic liver abscesses and other soft tissue abscesses have been reported with increasing frequency. K. pneumoniae was recognized as the etiologic agent of community-acquired pyogenic liver abscess in the Far East1 and has surpassed Escherichia coli as the primary etiologic agent of Gram-negative pyogenic liver abscesses in Taiwan.2 Recently, infections have been reported in the United States, Europe, Middle East, and Australia.3–7 Of importance was the discovery of the mucoviscosity-associated gene A (magA and later designated wzy_K1) in screens of invasive liver isolates.8 In general, magA-containing strains produce copious amounts of polysaccharide capsule and are resistant to serum killing and phagocytosis. The gene encoding MagA is a marker specific to the K1 serotype and is designated as the capsule polymerase, presumably responsible for creating higher order polymers from precursor lipitated polymers.9 Although not all invasive hypermucoviscous (HMV) K. pneumoniae encode MagA, a significant proportion of invasive strains do, making magA an attractive therapeutic target. MagA contributed significantly to the LD50 in a mouse peritoneal infection model. The LD50 for magA-positive strains was <100 colony-forming units (CFUs) and was approximately 4 to 5 logs lower than isogenic mutants, which were deficient for magA.9

Invasive Klebsiella are epidemiologically important. Many reports retrospectively summarize the clinical presentation, risk factors, sequelae, and outcomes of patients diagnosed with community-acquired K. pneumoniae liver abscesses. More than half these patients were diabetic. From these soft tissue abscesses, K. pneumoniae can spread to the eye or meninges, causing metastatic endophthalmitis, meningoit(s), or both. The risk for metastatic spread to the meninges or eye with underlying K. pneumoniae primary liver abscess is approximately 3% to 10%.10–11 When examined alone, approximately two-thirds of patients presenting with K. pneumoniae endogenous endophthalmitis (KPEE) had an underlying liver abscess caused by the same bacterium.12 Despite treatment, most patients with K. pneumoniae endophthalmitis lose useful vision.13–15 The visual prognosis for these patients is uniformly poor.

Because many patients may not seek medical attention for an underlying liver abscess until they experience ocular pain or vision loss, coupled with the poor prognosis of KPEE, it was important to understand the contribution of the HMV phenotype, specifically magA, to disease in the eye. To this end, we used an experimental model of K. pneumoniae endophthalmitis16 to compare infections with wild-type, isogenic magA-deficient, and trans-complemented magA K. pneumoniae strains. The results clearly demonstrate that magA is a virulence factor in experimental endophthalmitis.
**METHODS**

**Animals**

Eight- to 10-week-old C57BL/6j mice were acquired from the Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with an intramuscular injection of ketamine (85 mg/kg) and xylazine (14 mg/kg). Eyes were topically anesthetized with 0.5% proparacaine before infection. All procedures were carried out in strict accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, institutional guidelines, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Bacteria**

*K. pneumoniae* clinical isolate NTUHK2044 and the isogenic Δ*magA* mutant are described in Fang et al.8 For trans-complementation of *magA*, a 1.7-kb fragment containing *magA* was amplified by PCR from strain NTUHK2044 using primers *magA*-Agel (121) 5′-GAGACCCGTAACGGTGATCAGAATTTCAACGAGGC and *magA*-BamHI (1591) 5′-GATCGGATCCCTCCAGAGGTGGTGGTTAAATGCG, which annealed at −121 and +1591 nucleotides from the start codon and contained Agel and BamHI restriction sites, respectively. Plasmid pBR328 was digested with Agel and BamHI, and the larger 3.9-kb fragment was gel purified. The 3.9-kb fragment was blunted with T4 polymerase. After digesting the 1.7-kb *magA*-containing fragment with Agel and BamHI, the fragment was ligated to the 3.9-kb fragment from pBR328 to generate pBR328Δ::*magA*, which was introduced into NTUHK2044Δ*magA* and selected on chloramphenicol to generate NTUHK2044Δ*magA* pBR328Δ::*magA* or "*magA* complement." The presence of *magA* transcript was verified by semiquantitative RT-PCR. Briefly, 2 μg RNA was reverse transcribed using a reverse transcription system (GoScript; Promega, Madison, WI) and the cDNA was amplified using primers specific to *magA* (magnA +1115 (forward) 5′-CAGATCCTCGCCGACGTCCGAAAGTGAACG and magnA +1755 (reverse) 5′-GAGGAGGTCGCGCAATGTCGCAATTTTGCTGTTAG) and the 16S rRNA gene (16S forward) 5′-GCGGTAATACGGAGGCTTGAGACGCA and 16S (reverse) 5′-CACATCGCACTGCAACAGCC) *Klebsiella*. A strain containing only the empty vector (pBR328Δ) was constructed by self-ligating the 3.9-kb fragment of pBR328, previously generated, after using T4 polymerase to fill in 5· overhangs.

Plasmid stability of the *magA* complement was analyzed both in vitro and in vivo. For in vitro stability, bacteria were passaged overnight twice without selection. Total and chloramphenicol-resistant colonies were counted for each passage. For in vivo stability, approximately 100 CFUs of the *magA* complement strain were used to intracoacally infect mice, as described here. After 18 hours, eyes were removed and homogenized in PBS. Total and chloramphenicol-resistant (Col)7 colonies were counted. Data are shown as the mean of at least three replicate samples ± SD. Two-tailed, two-sample *t*-tests assuming equal variance were used to statistically compare groups.

**Quantification of Capsular Polysaccharide**

Bacteria were grown overnight in Luria-Bertani broth. Capsular polysaccharide was quantified by a uronic acid assay as previously reported,17 with adjustments made for smaller volumes. Briefly, cultures were mixed with one-fifth volumes of 1% Zwittergent 3–14 in 100 mM citric acid (pH 2). After incubation at 50°C, debris was removed by centrifugation, and 125 μL supernatant was precipitated with 0.5 mL ethanol. Precipitate was dissolved in 100 μL H2O and vigorously mixed with 600 μL of 20 mM sodium borate in concentrated H2SO4. Samples were boiled for 5 minutes and cooled in an ice H2O bath before the addition 10 μL of 0.15% 3-phenylphenol in 0.5% NaOH. Absorbance at 520 nm was measured, and a standard curve of glucuronic acid was used to calculate the concentration of uronic acids. Data are reported as the mean micrograms per 109 CFUs ± SD of triplicate samples.

**Transcription Electron Microscopy**

Eighteen-hour cultures were washed in PBS and fixed in 5% gluteraldehyde/4% paraformaldehyde in PBS. Cells were washed three times with PBS and once with distilled water, spotted on glow discharged 300 Mesh Cu Formvar-coated grids, stained for 10 seconds with 2% uranyl acetate, washed once with water, and allowed to dry at ambient conditions. Images were taken on a transmission electron microscope (H-7600; Hitachi, Tokyo, Japan) with a beam voltage of 80 kV.

**Experimental K. pneumoniae Endophthalmitis**

Approximately 100 CFUs of mid-logarithmic-phase bacteria in a volume of 0.5 μL were injected into the mid-vitreous of C57BL/6j mice as previously described.16 Eyes were analyzed by scotopic electrotetroretinography,16,18 bacterial quantitation,19 histology,18 and myeloperoxidase (MPO) abundance (HyCell Biotech, Uden, The Netherlands). Three replicate samples per time point were tested at 0, 3, and 6 hours (mean ± SEM), and six replicate samples per time point were tested for all other time points (mean ± SEM).

**Statistical Analysis**

For in vitro and in vivo analyses, two-tailed, two-sample *t*-tests assuming equal variance were used to statistically compare groups. *P* ≤ 0.05 was considered significant.

**RESULTS**

**Capsular Polysaccharide**

The capsule of *K. pneumoniae* was visualized by transmission electron microscopy. Wild-type NTUHK2044 had a fibrous halo corresponding to the HMV phenotype (Fig. 1A), similar to that described previously. This halo was absent in the *ΔmagA* mutant (Fig. 1B). However, the *magA*-complemented strain was similar in appearance to the wild-type strain, with extra-cellular material visible (Fig. 1C). After establishing that the wild-type and *magA*-complemented strains did not grow differently in vitro (data not shown), capsule production was assessed qualitatively by sedimentation. When equal CFUs were sedimented at 10,000g, the *ΔmagA* mutant cells packed well and formed a solid pellet (Fig. 1D). However, neither the wild-type nor the *magA*-complemented strain formed tight pellets, and the *magA* complement had a pellet more than twice the volume of the wild-type (Fig. 1D). The amount of capsule, as a function of uronic acid content, was quantified. There was no statistical difference in the amount of uronic acid detected in overnight cultures of the wild-type and *magA*-complement strains (7.25 ± 0.35 μg/107 CFU vs. 6.88 ± 0.68 μg/107 CFU [P = 0.44]), indicating the complement restored wild-type levels of capsule production. The amount of uronic acid produced by the *ΔmagA* strain was below the limit of detection for the assay. To verify the deletion and complementation of *magA*, we used semiquantitative RT-PCR to detect *magA* expression. The presence of the *magA* transcript was verified in wild-type and complemented strains but was not detected in the mutant strain (Fig. 1E). These data suggested that the complemented strain produced capsule equal to that of the wild-type *K. pneumoniae* and that the capsule from these strains inhibited tight packing of cells similar to what has been described previously.16

To ensure that none of the strains produced hemolytic or proteolytic toxins, cell-free supernatant from overnight cultures in LB were spotted onto agar plates containing either sheep erythrocytes or casein and were incubated overnight at 37°C. As expected, no zone of clearing was observed for any strains (data not shown).
Intraocular Growth

The growth of *K. pneumoniae* wild-type NTUHK2044, the ΔmagA mutant, and the magA complement were compared in the experimental endophthalmitis model. There was no significant difference in CFUs recovered from eyes infected with any strain at 0, 3, and 9 hours after infection. At 12 hours postinfection, the wild-type strain grew to a higher density than the ΔH9004 magA mutant, reaching 7.35 log_{10} CFU/eye compared with only 5.28 log_{10} CFU/eye for mice infected with the ΔH9004 magA strain (P = 6.13 × 10^{-8}; Fig. 2). This difference was maintained until 24 hours, when the end point values for intraocular growth were 8.18 and 5.14 log_{10} CFU/eye for wild-type and ΔH9004 magA strains, respectively (P = 1.87 × 10^{-8}; Fig. 2). These results indicated that the wild-type strain grew to a greater density in the eye and maintained populations approximately 3 logs higher than the ΔH9004 magA strain. When the magA complement strain was tested, the growth curve was similar to that of the wild-type and ΔH9004 magA strains until 12 hours. At this time, the complement strain reached 6.04 log_{10} CFU/eye, which was significantly lower than wild-type (P = 1.97 × 10^{-8}; Fig. 2). The growth of the complement strain remained lower than wild-type at both 18 and 24 hours (P = 3.28 × 10^{-3} and P = 6.28 × 10^{-3}, respectively; Fig. 2) but was greater than the ΔmagA strain at 24 hours (P = 0.0075).

To ensure plasmid stability in the magA complement, bacteria were grown in LB with selection, back-diluted into LB without antibiotics, and grown overnight at 37°C. Total CFUs and the number of CFUs resistant to chloramphenicol were not different after the first or second overnight passage (passage 1, 9.38 ± 0.12 total log_{10} CFU/mL vs. 9.33 ± 0.12 ComR log_{10} CFU/mL [P = 0.26]; passage 2, 9.22 ± 0.19 total log_{10} CFU/mL vs. 9.24 ± 0.17 ComR log_{10} CFU/mL [P = 0.88]). The growth of ΔmagA containing the empty vector (pBR238Δ) in vitro was not significantly different from that of the ΔmagA strain and did not restore the HMV phenotype (data not shown); thus, we did not expect to gain further insight into the effect of MagA by comparing these two strains in vivo. In addition, eyes were infected with the ΔmagA strain, and, after 18 hours, the numbers of total and chloramphenicol-resistant colonies were not statistically different (7.05 ± 0.50 total log_{10} CFU/eye vs. 7.04 ± 0.49 log_{10} CFU/eye; P = 0.95).
7.10 ± 0.45 CFU/eye \( (P = 0.90) \). Colonies that were resistant to chloramphenicol retained their HMV phenotype after passage in vitro and in vivo, indicating that the \( magA \) complement plasmid is stable without selection both in vitro and in vivo.

**Retinal Function**

At early time points (6, 9, and 12 hours), the percentage of retained A-wave amplitude declined independently of the phenotype of the strain used because there was no difference between the groups at these time points. At 18 hours, eyes infected with wild-type \( K. \) pneumoniae retained only 37.7% A-wave amplitude, whereas eyes infected with the \( \Delta magA \) strain retained 64.4% A-wave amplitude \( (P = 2.92 \times 10^{-6}; \) Fig. 3). At 24 hours, A-wave amplitude of wild-type-infected eyes decreased to 30.3%, whereas eyes infected with the \( \Delta magA \) strain retained 74.7% \( (P = 1.97 \times 10^{-3}; \) Fig. 3). The trend of A-wave retention in eyes infected with the \( magA \) complement strain followed that of the \( \Delta magA \) strain. By 24 hours, eyes infected with the \( magA \) complement strain retained 67.9% A-wave amplitude, which was higher than in wild-type-infected eyes \( (P = 4.74 \times 10^{-5}) \) but was not different from \( \Delta magA \)-infected eyes \( (P = 0.44) \).

Results were similar when B-waves were examined. There was a gradual decrease over the first 12 hours in wild-type-infected eyes that was mirrored in the \( \Delta magA \)-infected eyes. By 12 hours, the percentage of retained B-wave was 62.3% and 58.8% for wild-type and \( \Delta magA \)-infected eyes, respectively \( (P = 0.66) \). Over the next 12 hours, the B-wave amplitude of wild-type-infected eyes declined to 15.3%. However, at 18 and 24 hours, eyes infected with the \( \Delta magA \) strain had significantly higher B-wave amplitudes \( (37.2\% \text{ vs. } 20\% \text{ and } 44.6\% \text{ vs. } 15.3\% \) \( (P = 8.86 \times 10^{-5} \text{ and } P = 0.001) \) than wild-type-infected eyes. The trend of B-wave amplitude loss in \( magA \)-complement-infected eyes closely followed that of the eyes infected with \( \Delta magA \) \( K. \) pneumoniae. At 24 hours there was no difference in retained B-wave amplitude compared with \( \Delta magA \)-infected eyes \( (P = 0.24) \).

**FIGURE 3.** Retinal function during experimental \( K. \) pneumoniae endophthalmitis. Eyes were injected with 100 CFU of wild-type, \( \Delta magA \), or \( magA \) complement \( K. \) pneumoniae and were dark adapted at least 6 hours before electroretinography. Infected eyes were compared with the contralateral mock-injected or absolute control eye and were reported at percentage of amplitude of A-wave or B-wave retained. *\( P < 0.05 \) for wild-type versus \( \Delta magA \). †\( P < 0.05 \) for wild-type versus \( magA \) complement. Two-tailed, two-sample \( t \) tests assuming equal variance were used to statistically compare groups.

**Inflammation**

We previously reported that in experimental \( Bacillus cereus \) endophthalmitis, polymorphonuclear leukocytes (PMNs) represent the predominant immune cell infiltrating the eye after infection.\(^{16}\) Infiltration of PMNs was measured by an abundance of MPO. There was no significant difference in the amount of MPO detected in eyes infected with wild-type or \( \Delta magA \) at 0, 3, 6, 12, or 18 hours (Fig. 4). At 9 hours, eyes infected with wild-type \( K. \) pneumoniae contained significantly more MPO \( (266 \text{ ng/eye}) \) than either the \( \Delta magA \) \( (62.5 \text{ ng/eye}) \) or the complement \( (85.8 \text{ ng/eye}) \) strains \( (P = 0.003 \text{ and } P = 0.01) \). By 12 hours, there was at least a 20-fold increase in MPO over baseline levels that was independent of the infecting strain. However, at 24 hours, eyes infected with the wild-type strain contained 709.2 ng/eye MPO compared with only 343.2 ng/eye elicited by the \( \Delta magA \) strain \( (P = 0.031) \). This difference is consistent with the histologic data at 24 hours, which showed more ocular architectural damage. \( K. \) pneumoniae does not produce hemolytic or proteolytic toxins; therefore, much of the histologic pathology may be due to the immune influx in response to bacteria in the vitreous. The abundance of MPO elicited by the \( magA \) complement strain generally followed the \( \Delta magA \) strain and was not significantly different at any time point analyzed.

Representative histologic sections of experimental \( K. \) pneumoniae endophthalmitis are shown in Figure 5. Eyes infected with any strain were indistinguishable from control eyes at 6 hours because all retinal layers were intact and the vitreous was clear. At 12 hours, eyes infected with the \( \Delta magA \) strain exhibited mild optic nerve inflammation and minimal fibrin deposition, whereas eyes infected with the wild-type strain exhibited marked fibrin deposition and cellular infiltration in both the anterior and the posterior chambers. At 18 hours, there was significant inflammation in eyes infected with the wild-type strain, with anterior chamber fibrin deposition, separation of the corneal stroma from the epithelium, and separation of ganglion cell and inner plexiform layers in the retina. There was only mild disruption of the retinal ganglion cell layer in \( \Delta magA \)-infected eyes at 18 hours. At 18 hours, there was also cellular infiltration into the vitreous of infected eyes regardless of the infecting strain. The amount of inflammation in wild-type-infected eyes was significant at 24 hours and resulted in phthisis.
fected with the ΔmagA- or magA-complemented strains remained intact.

DISCUSSION

Endogenous bacterial endophthalmitis is a potentially blinding disease, especially when caused by Gram-negative pathogens. In the human population, endogenous *K. pneumoniae* endophthalmitis is a frequent sequela of an underlying infection, and endophthalmitis caused by *K. pneumoniae* is typically of endogenous origin. However, there are no well-characterized models of endogenous bacterial endophthalmitis. We therefore chose to directly inoculate eyes with *K. pneumoniae* to minimize complicating factors associated with systemic disease and to evaluate the ability of various strains to cause disease once they gain access to the intraocular environment. The limitation to this model includes the absence of predisposing factors described in the clinical literature that are associated with systemic disease, including diabetes and hepatobiliary disease.20–22 The direct inoculation model facilitated a clear with systemic disease, including diabetes and hepatobiliary results suggest that other factors in the NTUHK2044 background may cause this particular *K. pneumoniae* clone to be more inflammatory, more virulent, or both.

The virulence of many ocular pathogens can be attributed to the production of toxins. *Staphylococcus aureus* strains deficient in α-toxin are significantly attenuated in their ability to cause endophthalmitis.23 *S. aureus* and *B. cereus*, which do not produce toxins in a density-dependent (i.e., deficient in agr and plcR, respectively) manner, eventually caused damage similar to wild-type bacteria, but intraocular infections were highly attenuated.24,25 In contrast, *K. pneumoniae* are not known to produce membrane-damaging toxins such as hemolysins, cytolysins, or phospholipases. To confirm this, we cultured *K. pneumoniae* on casein and blood agar, and no zones of clearing were observed with any of the strains used in this study. In experimental *B. cereus* endophthalmitis, it has been previously shown that PMNs are the major cell population infiltrating the posterior segment and that the increase of CD18+/Gr-1+ cells correlated with an increase in MPO detected over time.16 Much of the intraocular virulence of *B. cereus* is attributed to its potent toxins and to its inflammatory cell wall. Despite the lack of these classical virulence factors, NTUHK2044 elicits an amount of inflammation during experimental endophthalmitis similar to or greater than that elicited by *B. cereus*. It is likely that other bacterial products,
such as endotoxin (LPS) on the surface of *K. pneumoniae*, may account for the robust inflammatory response in the absence of classical toxin production.

It has been reported that *magA* is required to mask LPS recognition by TLR-4 in vitro.\(^{26}\) In that report, cells deficient in TLR-4 were unable to secrete TNF-α when stimulated with UV-inactivated *magA*\(^*\) bacteria. However, a cell line competent for TLR-4 secreted more TNF-α when treated with heat-killed bacteria than when treated with UV-inactivated bacteria, suggesting that the integrity of the capsule is essential for evading TLR-4 recognition.\(^{26}\) Here we report that an equivalent amount of MPO was elicited by all strains at both 12 and 18 hours. Interestingly, at these time points, there were 1.5- to 3-fold less bacteria in the Δ*magA* and *magA* complement groups. In the case of the Δ*magA* strain, this suggests that the lack of *magA* renders the bacterium more immunogenic, in accordance with previous findings. Although the *magA* complement strain did produce capsule that could be visualized and quantified, it is possible that the trans-complementation of *magA* did not restore the proper localization of the polysaccharide capsule. We speculate that the capsule, while present, may not be completely intact so as to function as a barrier to the innate immune response. This may account for the fact that the Δ*magA* and *magA* complement groups elicited amounts of inflammation similar to those of the wild-type strain but at much lower bacterial loads. Toll-like receptor 4 is the pathogen-associated molecular pattern receptor for bacterial LPS and is expressed in the human eye and is localized to resident perivascular antigen-presenting cells and the retinal pigment epithelium. Expression and localization of TLR-4 is not well understood in the mouse eye. It is unclear whether TLR-4 is important in the clearance of Gram-negative bacteria during endophthalmitis. It is possible that TLR-4 dependent recruitment of immune cells into the eye after infection with Gram-negative bacteria could exacerbate visual outcomes by causing bystander damage to nearby retinal neurons. We have undertaken studies to determine the role of TLR-4 in experimental endophthalmitis, and preliminary results suggest that in TLR-4\(^*\) mice, the infiltration of immune cells is delayed several hours compared with wild-type mice when infected with *K. pneumoniae*.

Prompt diagnosis and treatment of endophthalmitis are important to preserve visual function. Visual acuity in patients with endogenous *K. pneumoniae* endophthalmitis is generally poor, and practitioners should be aware of this complication in patients treated for pyogenic abscesses caused by *K. pneumoniae*. Although aggressive treatment, including vitrectomy and lensectomy for treatment of *K. pneumoniae* endophthalmitis, was shown to be effective when performed 8 hours from onset of ocular symptoms,\(^{27}\) a retrospective study on endogenous endophthalmitis found that 74% of eyes infected with *Klebsiella retai*na, retain, at best, hand motion perception.\(^{14}\) In recent years, multiply drug-resistant *K. pneumoniae* have gained significant attention. Strains have been described that are resistant to most clinically relevant antibiotics. Considering the increasing frequency of community-acquired *K. pneumoniae* infections and their secondary consequences, it will be important to find new therapeutic targets to aid in the control of this organism.

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**References**


