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**CONCISE COMMUNICATION**

Experimental Pneumococcal Meningitis in Mice: A Model of Intranasal Infection

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Effective laboratory animal models of bacterial meningitis are needed to unravel the pathophysiology of this disease. Previous models have failed to simulate human meningitis by using a directly intracerebral route of infection. Hyaluronidase is a virulence factor of *Streptococcus pneumoniae*. In this study, a novel model of murine meningitis is described. Intranasal administration of *S. pneumoniae* with hyaluronidase induced meningitis in 50% of inoculated mice, as defined by a positive cerebrospinal fluid (CSF) culture and an inflammatory infiltrate in the meninges. None of the mice inoculated without hyaluronidase developed meningitis. Hyaluronidase was found to facilitate pneumococcal invasion of the bloodstream after colonization of the upper respiratory tract. Meningitis was characterized by pleocytosis of CSF and the induction of proinflammatory cytokines and CXC chemokines in brain tissue. These results indicate that this murine model mimics important features of human disease and allow for the use of this model for studying issues related to the pathophysiology and the treatment of pneumococcal meningitis.

Bacterial meningitis due to *Streptococcus pneumoniae* is associated with significant mortality and morbidity, despite antibiotic treatment [1]. Knowledge of the pathogenesis of pneumococcal meningitis is important not only because of the severity of the disease but also because resistance to penicillin and other antimicrobial agents has increased dramatically over the past decade [2]. An animal model simulating human disease is essential to study the pathophysiology of meningitis and to investigate the effectiveness of vaccines and potential new adjunctive therapeutic strategies. Various animal models have been established [3, 4]. Mice models generally involve direct intracerebral injection of bacteria and therefore fail to resemble many aspects of bacterial invasion in human disease.

Hyaluronidase is a virulence factor of many bacteria, including *S. pneumoniae*. Hyaluronidase is an endoglycosidase that cleaves hexosaminidic linkages, thereby degrading hyaluronic acid, a component of the extracellular matrix. *S. pneumoniae* strains demonstrate a strong correlation between hyaluronidase activity and the capacity to induce meningitis [5–7].

In this study, we sought to establish a mouse model of pneumococcal meningitis that mimics the common route of infection in human disease (i.e., colonization of the upper respiratory tract mucosa, which is followed by invasion of bacteria into the bloodstream and subsequent invasion of the central nervous system [CNS] via the blood-brain barrier [BBB]) [8, 9]. For that reason, we added increasing doses of purified hyaluronidase to intranasally inoculated *S. pneumoniae*. In addition, to assess the similarity of this murine model of pneumococcal meningitis to human disease, we describe the local inflammatory response by analyzing leukocyte influx and by measuring concentrations of inflammatory mediators.

**Materials and Methods**

**Bacteria.** *S. pneumoniae* (serotype 6A), isolated from a meningitis patient, was cultured overnight at 37°C in 80 mL of brain heart infusion broth (BHI). At midlogarithmic growth phase, 5 mL of this suspension was transferred to 25 mL of BHI. The bacterial suspensions were grown at 37°C until an optical density of 1.0 at a wavelength of 620 nm was achieved. Subsequently, the suspension was washed twice in sterile isotonic saline and was resuspended in sterile isotonic saline, which corresponded to $3 \times 10^8$ cfu/mL. The exact number of colony-forming units was determined retrospectively by the growth of serial dilutions on blood agar plates. Synthetic hyaluronidase (0, 180, 360, or 560 U; Sigma) was added to the inoculum.

**Mice.** Female Swiss mice, weighing 12–14 g, were used.

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All animal experimentation guidelines were followed and were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam.

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Induction of meningitis. Mice were lightly anesthetized by inhalation of isoflurane (Abbott), and 50 mL of bacterial suspension was inoculated intranasally or intravenously. Control mice were inoculated with isotonic saline (n = 2), sterile hyaluronidase (n = 8), or S. pneumoniae only (i.e., without hyaluronidase; n = 8). At 48, 72, or 96 h after inoculation, mice were anesthetized with Hypnorm (Janssen Pharmaceutica) and midazolam (Roche), after which blood samples were obtained through cardiac puncture, and cerebrospinal fluid (CSF) was collected by puncture from the cisterna magna. White blood cells (WBCs) in CSF were counted immediately in a hematometer (Emergo). Cytospins were made of CSF and were stained using DiffQuick (Dade), according to the manufacturer’s instructions.

Brains were removed, half of which were fixed in 10% buffered formalin for histopathologic study. The other half were homogenized in sterile saline. Homogenized brain tissue was incubated with lysis buffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl\textsubscript{2}, 2 mM Triton [X-100], Pepstatin A [20 ng/mL; pH 7.4], Leupeptin [20 ng/mL; pH 7.4], and Aprotinin [20 ng/mL; pH 7.4]) for 30 min and were centrifuged for 15 min at 1500 g. After plating on blood agar, blood samples were transferred to EDTA tubes (Becton Dickinson) and were centrifuged at 1500 g for 10 min. Supernatants of brain homogenates were stored at −20°C for cytokine measurements. In 2 experiments, blood samples were obtained twice daily through the experimentation period (at 7, 24, 31, 48, 55, and 72 h after inoculation) from the tail vein and were plated on blood agar, to provide insight into the development of bacteremia.

Quantiﬁcation of bacterial outgrowth. Serial 10-fold dilutions in sterile isotonic saline were made of CSF and blood and were plated onto blood agar plates. Plates were incubated for 18 h at 37°C, after which colonies were counted.

Histopathologic study. Brains were ﬁxed in 10% buffered formalin, and, after parafﬁn embedding, 4-mm sections were stained with hematoxylin-eosin. All slides were coded and were scored semiquantitatively by a pathologist who had no knowledge of the inocula or CSF culture.

ELISA. Levels of cytokines were measured by using commercially available ELISAs, according to the manufacturer’s recommendations (KC, macrophage inﬂammatory protein [MIP]-2, interleukin [IL]-1α, IL-1β, IL-6, interferon-γ, and tumor necrosis factor (TNF)-α; all obtained from R&D Systems).

Statistical methods. Data are expressed as mean ± SE. Statistical analysis was performed by using Mann-Whitney U test. P < .05 was considered to be signiﬁcant.

Results

Induction of meningitis. Mice were considered to have meningitis when the CSF culture was positive, and histopathologic evidence for meningitis was present. To identify the optimal inoculum, mice were inoculated intranasally with $1 \times 10^4$, $5 \times 10^4$, $1 \times 10^5$, or $5 \times 10^5$ cfu of S. pneumoniae and 180 U of hyaluronidase, and were killed after 24, 48, 72, or 96 h. At 24 h after inoculation, none of the mice showed positive CSF cultures or histopathologic signs of meningitis. At all other timepoints (48, 72, and 96 h), 50% of all mice inoculated with $5 \times 10^4$ or $1 \times 10^5$ cfu of S. pneumoniae and 180 U of hyaluronidase had developed meningitis (table 1). No further increase in disease rate was found at 72 h after inoculation with the addition of 360 or 540 U of hyaluronidase to the inoculum. None of the mice inoculated intranasally with S. pneumoniae only (i.e., without hyaluronidase) developed meningitis.

At 72 h after inoculation with $5 \times 10^5$ cfu of S. pneumoniae and 180 U of hyaluronidase, the mean WBC count of CSF of mice with positive CSF cultures was 1900 ± 600 × 10\textsuperscript{4} cells/mL of CSF, whereas the mean WBC count of mice with negative cultures was 1.5 ± 0.5 × 10\textsuperscript{4} cells/mL of CSF (P < .05). Differentiation of this pleocytosis showed 70%–75% neutrophils, 20%–25% monocytes, and 2%–5% lymphocytes.

All mice that demonstrated growth of S. pneumoniae in CSF also showed clear histopathologic evidence of meningitis, namely, a significant inflammatory infiltrate predominantly composed of polymorphonuclear leukocytes in the meninges with a preferential concentration around leptomeningeal blood vessels, at 48 h after inoculation (figure 1). The inflammation was more pronounced at the base of the brain than at the hemispheric convexities. After 72 and 96 h, the inflammatory infiltrates in the meninges were more pronounced and diffuse, with the development of foci of necrotizing cerebritis and formation of small brain abscesses in 70% of mice with meningitis. Control mice, as well as mice with negative CSF culture, did not show any signs of meningitis or other histopathologic lesions.

Mice did not show any signs of illness for the first 40–48 h after inoculation with S. pneumoniae. Thereafter, 30% of mice developed signs of systemic toxicity. No neurological signs were observed.

Route of infection. Blood samples obtained from all mice that had been killed were cultured, to evaluate the route of infection. Of mice inoculated with $5 \times 10^4$ to $10 \times 10^4$ cfu of S. pneumoniae and 180 U of hyaluronidase, all mice (n = 24) that developed meningitis were bacteremic at time of death, whereas 6 mice with positive blood cultures did not show any signs of meningitis at time of death. Therefore, 80% of bacteremic mice developed meningitis. Of 32 mice from which blood samples
Figure 1. Representative histopathology of hematoxylin-eosin-stained brain tissue of a mouse that developed meningitis after inoculation with *Streptococcus pneumoniae* and hyaluronidase (A) and of a mouse that was inoculated with hyaluronidase alone (B); original magnification, 10 × 3.3. Cerebral cortex is depicted by a broad arrow; meninges by a thin arrow. Slides are from brain tissue obtained 96 h after inoculation.

Induction of cytokines and chemokines. The development of meningitis was associated with an increase in concentrations of inflammatory cytokines such as TNF-α, IL-1α, and IL-1β and IL-6, KC, and MIP-2 in brain tissue (table 2). TNF-α, IL-1α, IL-6, and MIP-2 concentrations correlate with bacterial counts in CSF (all $P < .01$), and IL-1β levels correlate with severity of histological changes ($P < .05$).

Discussion

In this study, we describe a new murine model of meningitis, in which pneumococci with hyaluronidase are introduced intranasally.

The pleocytosis of the CSF and the differentiation of the cells that we found are comparable with human meningitis. Furthermore, the histopathologic findings during the course of the disease in our model closely mimic the progression of meningitis in untreated patients.

We were able to induce meningitis in 50% of mice inoculated intranasally with *S. pneumoniae* and hyaluronidase, whereas none of the mice inoculated with *S. pneumoniae* alone developed meningitis. After intranasal administration, substances can be delivered to the CNS either directly, via the olfactory nerve or olfactory epithelium, or indirectly, via the systemic circulation [10]. In our experiments, all mice with meningitis were bacte-remic at time of death, and sequential blood cultures showed

<table>
<thead>
<tr>
<th>Cytokine, time in h</th>
<th>Meningitis</th>
<th>No meningitis</th>
<th>$P^*$</th>
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<tbody>
<tr>
<td>IL-1α 48</td>
<td>5.65 ± 0.59</td>
<td>4.93 ± 0.33</td>
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</tr>
<tr>
<td>72</td>
<td>5.92 ± 2.21</td>
<td>2.86 ± 0.40</td>
<td>.186</td>
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<tr>
<td>96</td>
<td>5.28 ± 0.30</td>
<td>4.45 ± 0.16</td>
<td>.033</td>
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<tr>
<td>IL-1β 48</td>
<td>1.68 ± 0.20</td>
<td>1.33 ± 0.13</td>
<td>.155</td>
</tr>
<tr>
<td>72</td>
<td>2.43 ± 0.27</td>
<td>0.83 ± 0.09</td>
<td>.050</td>
</tr>
<tr>
<td>96</td>
<td>4.37 ± 0.67</td>
<td>2.21 ± 0.17</td>
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<tr>
<td>IL-6 48</td>
<td>7.00 ± 3.66</td>
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<td>72</td>
<td>25.56 ± 13.3</td>
<td>3.84 ± 0.83</td>
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<td>96</td>
<td>9.40 ± 1.29</td>
<td>0.86 ± 0.11</td>
<td>.006</td>
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<tr>
<td>TNF-α</td>
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<tr>
<td>48</td>
<td>0.77 ± 0.13</td>
<td>0.45 ± 0.06</td>
<td>.061</td>
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<tr>
<td>72</td>
<td>1.15 ± 0.36</td>
<td>0.32 ± 0.07</td>
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<tr>
<td>96</td>
<td>1.08 ± 0.20</td>
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<td>4.2 ± 0.3</td>
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<td>72</td>
<td>21.2 ± 7.5</td>
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<tr>
<td>96</td>
<td>11.1 ± 1.0</td>
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<tr>
<td>MIP-2</td>
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<td></td>
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<tr>
<td>48</td>
<td>8.6 ± 5.2</td>
<td>2.3 ± 0.2</td>
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<td>96</td>
<td>9.2 ± 2.8</td>
<td>2.0 ± 0.1</td>
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</table>

**Table 2.** Brain cytokine concentrations in mice that developed and did not develop meningitis.

NOTE. Data are mean levels (ng/g) ± SE, unless otherwise indicated. Results are from 4–10 mice/group. Time point after intranasal inoculation with $5 \times 10^3$–$10^4$ cfu of *Streptococcus pneumoniae* with or without hyaluronidase. IL, interleukin; MIP, macrophage inflammatory protein; TNF-α, tumor necrosis factor.

*$P$ values indicate differences between groups, using Mann-Whitney U test.
bacterial growth at time points when CSF cultures were still negative. Therefore, in our model, *S. pneumoniae* seems to gain access to the subarachnoid space via the circulation.

Invasion of the CNS by pneumococci is initiated by colonization of the nasopharyngeal mucosa. Pneumococcal surface adhesin A, pneumococcal surface protein A, and pneumolysin are surface proteins of pneumococci, which are believed to play an important role in adherence to the mucosa [9]. By a proteolytic step, pneumococci are capable of penetrating the basement membrane and extracellular matrix, after which they may enter the circulation. Plasminogen binding by bacteria has been suggested to contribute to this phenomenon [11]. In our model, the addition of hyaluronidase probably facilitates invasion by loosening the barrier function of the nasopharyngeal mucosa. Hyaluronidase does not seem to influence the passage of the bacteria across the BBB, in light of the fact that we could not demonstrate any difference in bacterial migration across the BBB in the presence of hyaluronidase after intravenous administration of pneumococci. Therefore, we assume that the function of hyaluronidase in this model is predominantly facilitation of systemic invasion after nasopharyngeal colonization.

The elevation of proinflammatory cytokines in brain tissue during meningitis highlights the similarity of the model with human disease. TNF-α, IL-1α, IL-1β, and IL-6 were demonstrated elsewhere to be markedly elevated in human CSF during bacterial meningitis [12, 13].

An important hallmark of bacterial meningitis is the development of pleocytosis of CSF. Chemokines are considered to be highly relevant in activating and attracting specific leukocyte subsets and play a critical role in meningeal inflammation [14]. CXC chemokines have been implicated in the development of neutrophilic pleocytosis in CSF during bacterial meningitis [14, 15]. In mice, the prototypic CXC chemokines are KC and MIP-2. We found elevated MIP-2 and KC concentrations in homogenates of brain tissue of mice with meningitis. No reports are available on the production of KC and MIP-2 in mice during pneumococcal meningitis.

Pneumococcal meningitis is a life-threatening community-acquired infection. The model for this disease described in this study is unique, because it mimics the route of infection in humans. The model is efficient and reproducible and deals with an experimentally convenient latency, because mice develop meningitis 2 days after inoculation. As such, it should be an excellent model for addressing issues related to this clinically important disease.

References