Streptococcus pneumoniae Deficient in Pneumolysin or Autolysin Has Reduced Virulence in Meningitis

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Background. The role played by pneumolysin and autolysin in pneumococcal meningitis is poorly understood.

Methods. A rat model was used to investigate the disease, in which surgical implantation of a cisternal catheter allowed bacterial instillation and cerebrospinal fluid (CSF) sampling.

Results. CSF infection of rats with wild-type pneumococci caused meningitis within 26 h, whereas isogenic mutants that do not express pneumolysin (∆Ply) or autolysin (LytA−) caused very mild or no disease. Wild-type infections resulted in pneumococci in the CSF and cortical homogenates, but a minority of the rats infected with ∆Ply or LytA− had bacteria in these locations at 26 h. Leukocyte numbers in the CSF were similar after infection with all pneumococci; however, neutrophils and monocytes predominated after wild-type infection, whereas lymphocytes and atypical lymphocytes predominated after infection with the mutants. Wild-type pneumococci caused disruption to the ependyma, but this was not observed in rats infected with ∆Ply or LytA−. Cells surrounding the ventricles in wild type–infected animals expressed caspase 3, and astrocytes had hypertrophy; both findings were absent in rats infected with the mutants.

Conclusions. This study provides strong in vivo evidence that pneumolysin and autolysin play crucial roles in the pathogenesis of pneumococcal meningitis.

Streptococcus pneumoniae is the second most frequent cause of meningitis in all age groups throughout Europe [1]. In the United States, it is the leading cause of meningitis in children [2]. Every year there are ~600,000 cases of meningitis worldwide, with ~180,000 deaths. In the developing world, mortality rates as high as 50% have been reported [3]. More than 30% of survivors of pneumococcal meningitis suffer from neurological damage, including hearing loss [4], cognitive impairment [5], and long-term defects in spatial learning [6, 7]. It is widely acknowledged that there is an urgent need to improve the treatment of pneumococcal meningitis [8].

Previously, to investigate the role played by S. pneumoniae virulence factors, we developed a ciliated ependymal cell culture model to simulate events in meningitis. The ciliated ependyma, a single-cell layer that lines the cerebral ventricles and aqueducts [9, 10], is of particular interest, because it separates infected cerebrospinal fluid (CSF) in meningitis from the brain parenchyma. Using a rat model, we have demonstrated that impairment of ciliary function and damage to ependymal cells in culture closely predict similar effects during pneumococcal meningitis in vivo [11].

We recently investigated the toxicity of the pneumococcal toxin, pneumolysin, on the ciliated ependyma. This cholesterol-dependent, cytolytic [12] toxin has been shown to be essential for bacterial survival and virulence in mouse models of pneumonia, bacteremia, and colonization [13, 14]. We found that, depending on concentration, pneumolysin inhibited ependymal ciliary beat frequency (CBF) in vitro [15] or was very toxic to ependymal cells [16, 17]. Incubation with anti-pneumolysin antibodies abolished the ependymal damaging action of pneumococci [18], suggesting that the development and use of agents to block pneumolysin may prove to be a useful adjunctive therapy for meningitis.
The aim of the present study was to determine the importance of pneumolysin in the pathogenesis of pneumococcal meningitis, because, despite our in vitro results, the effect of pneumolysin on meningitis in vivo has been controversial. In particular, the findings of our ependymal cell culture studies did not agree with those of Friedland et al. [19], who reported that a pneumolysin-deficient strain of pneumococcus induced disease in rabbits that was indistinguishable from wild-type pneumococcal infection.

To investigate the role played by pneumolysin, we used 2 mutant pneumococci: \( \Delta \text{Ply} \), which does not express pneumolysin, and LytA\(^{-}\), an autolysin-deficient mutant. The LytA autolysin degrades the pneumococcal cell wall, resulting in lysis and release of intracellular and cell wall molecules [20]. Although it is known that autolysin is crucial for pneumococcal virulence during pneumonia and septicemia [21, 22], its role in pneumococcal meningitis is unknown.

The present study provides in vivo evidence strongly supporting the hypothesis that pneumolysin plays a crucial role in the pathogenesis of pneumococcal meningitis. We have shown that after CSF infection of the adult rat, pneumococci deficient in either pneumolysin or autolysin have reduced virulence compared with wild-type bacteria. Unlike infection with wild-type pneumococci, CSF infection with mutant pneumococci did not cause widespread ependymal disruption, glial cell activation, or caspase 3 expression in the subependymal region. In addition, we have demonstrated that our previous in vitro results [17] predicted the ependymal damage seen in vivo.

**METHODS**

**Bacteria.** The wild-type pneumococcus was the serotype 2 strain, D39. The isogenic mutants, with inactivated genes for pneumolysin (\( \Delta \text{Ply} \)) and autolysin (LytA\(^{-}\)), have been described elsewhere [23, 24]. To standardize the virulence, all bacteria were passed through intravenously infected mice before use [11]. Pneumococcal numbers were determined by colony counting, as described elsewhere [11]. All mutant bacterial growth rates have been measured in vitro and were not different from those for the wild type (data not shown).

**Model of meningitis.** All of the experiments on animals were approved by a UK Home Office project license guidelines and by the University of Leicester ethics committee. Adult male Wistar rats (350 – 450 g; Harlan UK) were injected with 104 cfu of \( S. \text{pneumoniae} \) in a total volume of 50 \( \mu \)L into the cisternal CSF via a previously implanted guide tube [11]. The guide tube was fixed between the occipital bone and the atlas arc onto the atlanto-occipital membrane, so that injection of the infectious dose could be directed to the cisternal CSF and not the brain tissue. Control rats were injected into the CSF with 50 \( \mu \)L of sterile PBS. Rats were assessed for clinical signs (weight loss, piloerection, hunching, and lethargy), and when they reached the 2+ lethargic stage of the disease [25] (or at a time appropriate for the experiment) they were euthanized by sodium pentobarbital overdose. After the animals were euthanized, CSF was removed from the cisterna magna via the guide tube, using a gas-tight sterile syringe. Brains were carefully removed and dissected. The prefrontal cerebral cortex was removed and homogenized for colony counting [11], and the cerebellum was removed for vibrotome sectioning (250-\( \mu \)m sections) and

**Figure 1.** Time course of clinical scores (0 indicates normal healthy animal; 1+, mild lethargy; 2+, severe lethargy). Each line represents an individual rat infected with wild-type (A), pneumolysin-deficient (\( \Delta \text{Ply} \)) (B), or autolysin-deficient (LytA\(^{-}\)) (C) pneumococci. *Significantly different \((P < .05)\) clinical scores in wild type–infected rats at 26 h, compared with \( \Delta \text{Ply} \)- and LytA\(^{-}\)-infected rats.
ependymal assessment within the fourth ventricle, as described elsewhere [11]. Blood was obtained by cardiac puncture, and numbers of pneumococci were determined by colony counting [11].

**CSF parameters.** Cells in the CSF were counted in either a Fuchs-Rosenthal counting chamber (samples $>10^3$ cells/mL) or a hemocytometer (samples $>10^3$ cells/mL). The cells in the CSF (20 μL) were fixed with methanol (20 μL) for 30 min before addition of Giemsa stain (Fisher Chemicals; 20 μL; 1:4 dilution in filter-sterilized PBS). The counting was done after a 10-min incubation at room temperature, and individual cells were identified, according to their morphological characteristics, using light microscopy [26]. The cell types that could be readily identified and counted were neutrophils, identified by their multilobed nucleus; monocytes, identified by a cleft nucleus; and lymphocytes, identified by a circular nucleus. Atypical lymphocytes were morphologically unusual nucleated immune cells, as described elsewhere [27, 28].

**CBF measurement.** The ependymal CBF was measured as described elsewhere [16]. Briefly, beating cilia on ependymal edges were recorded by a high-speed video camera (Kodak Ektapro motion analyzer) at a frame rate of 400/s. The camera allowed video sequences to be downloaded at reduced frame rates, from which the CBF was determined directly.

**Electron microscopy.** Ependymal slices were fixed and processed for transmission electron microscopy, as described elsewhere [15].

**Immunocytochemistry and confocal microscopy.** Brains were fixed by cardiac perfusion of paraformaldehyde (4% wt/vol) and stored at 4°C in the same fixative. Sections (80 μm thick) were cut on a Leica VT1000 microtome, blocked for 1 h in normal goat serum (1:500), incubated overnight at 4°C in primary antibody (rabbit anti–active caspase 3 [1:500] or glial fibrillary acidic protein [1:500; Becton Dickinson]), washed again, incubated overnight at 4°C in secondary antibody (goat anti–rabbit IgG conjugated to Alexa Fluor 488; 1:500) washed, and
then mounted in n-propyl-gallate (5% wt/vol) in absolute glycerol. Negative control sections had the primary antibody omitted. Sections were inspected using an LSM 510 META confocal microscope (Zeiss) with a 488 laser line, a 505–550-nm bandpass emission filter, and a /H11003 40 oil-immersion objective lens (numerical aperture, 1.3).

Statistics. All data were nonparametric, and groups were compared using the Kruskal-Wallis test. For comparisons between 2 groups, a 2-proportion method was used in which “trial” indicated the number of rats tested and “success” indicated the number with positive bacterial counts.

RESULTS

Development of disease. All rats infected with wild-type pneumococci developed signs of meningitis that followed a time course notably different from the course after infection with pneumolysin-negative ΔPly or autolysin-negative LytA−. After 6 h, the wild type–infected rats showed slight piloerection and/or slight hunching and lethargy. By 26 h, all 7 of these rats were severely lethargic (2+) and were euthanized. In contrast, 6 h after infection with ΔPly or LytA−, the rats showed no signs of disease, and at 26 h the majority of them (10/11) still showed no disease signs (figure 1B and 1C). All of these animals were assessed for 72 h after infection, before they were killed. Two rats infected with ΔPly (1 after 48 and 1 after 72 h) (figure 1B) and 2 infected with LytA− (1 after 26 and 1 after 48 h) (figure 1C) progressed to a severely (2+) lethargic stage and were euthanized.

The decreased extent of disease seen at 26 h after infection with ΔPly or LytA− was accompanied by significantly fewer pneumococci in the CSF and cerebrocortical homogenates compared with wild-type infection (P < .05) (figure 2A and 2B). However, at 72 h after infection, the numbers of ΔPly pneumococci in the CSF were not significantly different from the 26-h data for the wild-type infections (P > .05) (figure 2A), but the numbers of ΔPly pneumococci in the cerebrocortical homogenates at 72 h were still significantly lower than the 26-h data for the wild-type infections (P < .05) (figure 2B). In rats infected with LytA−, the numbers of bacteria in the CSF and cerebrocortical homogenates had increased at 72 h after infection but remained significantly lower than the numbers in rats infected

Figure 3. Leukocytes (A), neutrophils (B), monocytes (C), lymphocytes (D), and atypical lymphocytes (E) in the cerebrospinal fluid (CSF) after cisternal infection with wild-type (WT), pneumolysin-deficient (ΔPly), or autolysin-deficient (LytA−) pneumococci. Control rats given cisternal PBS had no leukocytes in their CSF at 26 or 72 h. Individual points represent the nos. of cells in the CSF from individual rats, and horizontal lines represent medians. *Significantly fewer (P < .05) rats with cells in the CSF, compared with WT-infected rats.
with wild-type pneumococci after just 26 h ($P < .05$) (figure 2A and 2B). Significantly more rats had bacteremia after infection of the CSF with wild-type pneumococci than after infection with either $\Delta$Ply (at 26 and 72 h) or LytA$^-$ (at 26 h) ($P < .05$) (figure 2C).

**Inflammatory cells in the CSF.** No leukocytes were detected in the CSF of 4 sham-infected control rats. In all infections with pneumococci, the number of cells present in the CSF was significantly higher than that in sham-infected control rats ($P < .05$). There was no significant difference in the total leukocytes in the CSF of each infection group, at either time point ($P > .05$) (figure 3A). However, a significantly higher proportion of rats in the wild type–infected group had neutrophils in the CSF, compared with the mutant pneumococci–infected group, at both 26 and 72 h ($P < .05$) (figure 3B).

Interestingly, at 26 h, there were more monocytes in the CSF of the wild type–infected rats than in that of rats infected with $\Delta$Ply (at 26 h) or LytA$^-$ (at 26 and 72 h) (figure 3C). The CSF of rats infected with the mutant bacteria contained lymphocytes and atypical lymphocytes. However, the proportions of rats in the mutant-infected groups were not significantly different from that in the wild type–infected group ($P > .05$) (figure 3D and 3E).

**Ependymal damage.** By 26 h after infection with wild-type pneumococci, there was loss of cilia and ependymal cell detachment, opening of gap junctions, and disruption of ependymal organization in the fourth ventricle of the brain (figure 4A). In contrast, the ependymas of rats infected with $\Delta$Ply or LytA$^-$ were undamaged at 26 h (data not shown) and at 72 h (figure 4B and 4C) and did not differ from those in the sham-infected control rats (figure 4D).

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**Figure 4.** Transmission electron micrographs of the ependyma within the fourth ventricle of rats infected with wild-type pneumococci (A), pneumolysin-deficient pneumococci ($\Delta$Ply) (B), autolysin-deficient pneumococci (LytA$^-$) (C), or PBS (D). In panel A, open gap junctions, detachment of the ependymal cells, and loss of cilia can be observed. Cilia are healthy in panels B (cross-section of ciliary axoneme), C, and D (arrows). Scale bars indicate 10 $\mu$m, and each micrograph is representative of a total of 145 micrographs.
At 26 h after infection with wild-type pneumococci, where the ciliated ependyma in the fourth ventricle remained intact CBF was significantly reduced ($P < .05$) (table 1). In contrast, in rats infected with $\Delta$Ply or LytA$^{-}\text{Ply}$ CBFs were the same as those in the sham-infected control rats at 26 or 72 h after infection ($P > .05$) (table 1).

**Immunohistochemistry.** Animals that were sham infected with PBS appeared clinically normal and were immunonegative when stained with antibody to active caspase 3 (data not shown). Animals infected with wild-type pneumococci had hypertrophied astroglial cells (figure 5A), relative to similar areas of the lateral ventricle in nondiseased animals infected with LytA$^{-}\text{Ply}$ (figure 5B). Wild type–infected rats also had marked caspase 3 reactivity in ependymal and subependymal cells (figure 5C and 5D). An antibody-negative control is shown, in which the primary antibody was omitted, without (figure 5E) and with (figure 5F) nuclear counterstain. Animals infected with LytA$^{-}$ or $\Delta$Ply pneumococci had no detectable caspase 3 immunostaining and no hypertrophy of their astroglial cells.

**DISCUSSION**

In the present study, we investigated the role of pneumolysin and autolysin in the development of meningitis and ependymal damage in vivo. All of the rats infected with wild-type pneumococcus developed severe signs of disease (2+ lethargic) by 26 h after infection of the CSF, and the disease was accompanied by loss of ependymal cilia and slowing of CBF, as predicted by our previous work [11]. In contrast, the majority of animals infected with mutant pneumococci showed no signs of the disease. All of the wild type–infected rats had increased numbers of pneumococci in the CSF and the cerebrocortical homogenates, but after infection with mutant pneumococci only a minority of animals had bacteria in the CSF and cerebrocortical homogenates.

The autolysin-deficient pneumococci showed reduced virulence even though they synthesize pneumolysin. The reason for the reduced virulence may be an inability to release intracellular pneumolysin and cell wall components, such as peptidoglycan and teichoic acid. Similar attenuation of virulence was found with the $\Delta$Ply mutant, which does not produce pneumolysin. These results strongly support the hypothesis that pneumolysin

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**Table 1. Ciliary beat frequency (CBF) in the fourth ventricles of rats infected with pneumococci.**

<table>
<thead>
<tr>
<th>Infection, time after infection</th>
<th>CBF, Hz</th>
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<tbody>
<tr>
<td>Sham, 26 h ($n = 6$)</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>Wild type, 26 h ($n = 21$)</td>
<td>26 ± 12$^a$</td>
</tr>
<tr>
<td>$\Delta$Ply, 26 h ($n = 42$)</td>
<td>38 ± 0.6</td>
</tr>
<tr>
<td>$\Delta$Ply, 72 h ($n = 15$)</td>
<td>35 ± 10</td>
</tr>
<tr>
<td>LytA$^{-}$, 26 h ($n = 18$)</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>LytA$^{-}$, 72 h ($n = 18$)</td>
<td>38 ± 0.8</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SD values. $\Delta$Ply, pneumolysin-deficient pneumococci; LytA$^{-}$, autolysin-deficient pneumococci.

$^a$ Significantly different ($P < .05$) from CBFs in animals given mutant pneumococci or sham infected. There was no significant difference between CBFs of $\Delta$Ply- and LytA$^{-}\text{Ply}$-infected animals ($P > .05$).
has a crucial impact on meningitis, and they are in contrast to the findings of Friedland et al. [19].

The finding that pneumolysin-deficient pneumococci were less virulent in meningitis is consistent with the findings of Wellmer et al. [29], who showed reduced clinical signs in mice when pneumococci were injected directly into the brain parenchyma as opposed to the CSF. We chose inoculation of bacteria into the CSF to mimic the meningitic process more closely. Although this route may not precisely mimic hematogenous spread, it is likely to closely mimic meningitis caused by direct extension from infected sinuses, otitis media, or mastoiditis after skull fracture and in children with neurocutaneous connections. Braun et al. [30], who also used cisternal inoculation of pneumococci deficient in pneumolysin, found reduced virulence in a rabbit model of meningitis. Further evidence for an important role of pneumolysin in meningitis comes from an in vitro study showing that pneumococci deficient in pneumolysin were unable to penetrate the microvascular endothelium, which indicates that the toxin was essential for breaching the blood-brain barrier [31].

The pattern of leukocytes present in the CSF after infection was dependent on the type of pneumococci used to infect the CSF, but the precise mechanisms that regulate leukocyte invasion are poorly understood. The recruitment of neutrophils and monocytes to the CSF after infection with wild-type pneumococci was expected [32, 33]. It can be predicted that the different patterns of leukocytes in the CSF after the various infections reflect different cytokines released. A recent study has shown that during pneumococcal meningitis monocytes enter the brain parenchyma from the circulation, where they differentiate into microglial cells and contribute to the neuroimmune response [32]. The different patterns of cell influx have been shown to reflect differential patterns of cytokine/chemokine released [33]. The exact patterns of mediators released in response to wild-type and mutant pneumococci warrant further study. It is noteworthy that rats infected with mutant pneumococci have increased numbers of inflammatory cells in the CSF without clinical signs.

Although infection of the CSF with wild-type pneumococci caused slowing of ependymal CBF and ependymal damage, this was not seen in rats infected with mutant pneumococci. Ciliary slowing and ependymal damage were associated with expression of a marker for apoptosis, caspase 3, and glial cell activation in the subependymal region. In contrast, the ependyma was intact after infection with the ΔPly and LytA− mutants, and caspase 3 expression was not seen. This finding lends support to our hypothesis that damage to ciliary function and to the integrity of the ependymal layer after pneumococcal infection in an in vitro ependymal cell culture model predicts ependymal damage in vivo [11].

The active caspase 3 immunostaining in animals infected with wild-type bacteria shows that in the acute phase of infection some ependymal cells initiate an apoptotic cell death program. Cells surrounding the damaged ependyma were also positively stained, which shows that adjacent brain tissue was also affected and would experience subsequent cell loss. In the acute stage of meningitis, we did not observe any necrosis surrounding the ventricles of the rat brains (data not shown). Rats infected with wild-type pneumococci also had hypertrophied astrocytes in the subependymal region, which, in time, may form a permanent glial scar. There is evidence in the literature that, once damaged, the ependyma undergoes limited repair [34, 35]. Therefore, further studies using a model of meningitis after antibiotic recovery are indicated to determine whether ependymal regeneration occurs after pneumococcal meningitis.

Although a small minority of rats developed signs of meningitis after infection with mutant bacteria, these animals displayed no evidence of ependymal damage, caspase 3 expression, or glial cell activation in the subependymal region. These findings suggest that other pneumococcal mechanisms of damage or other host responses may cause the clinical signs. In a natural setting, pneumococci are unlikely to fail to express pneumolysin and autolysin; therefore, ependymal damage remains a good predictor of clinical signs of meningitis in wild-type infections.

The development of disease signs in rats infected with mutant bacteria may be due to other pneumococcal factors, such as meningeal inflammation triggered by the bacterial cell wall components [19]. The similar degree of attenuation of the ΔPly and LytA− mutants and the similar inflammatory responses induced by these mutants support the argument that pneumolysin is more potent than cell wall fragments in inducing pathogenesis. If autolysin-derived cell wall fragments played a significant role, then less attenuation might be expected in the autolysin-sufficient ΔPly.

In summary, the present study showed that, to cause reproducible severe meningitis in the adult rat, pneumococci need to produce pneumolysin. The data support the hypothesis that pneumolysin plays a major role in the pathogenesis of pneumococcal meningitis and could be a valuable target for adjunctive therapy. Autolysin-deficient bacteria are likely to be less efficient in releasing pneumolysin and are also unable to degrade and release cell wall components, which probably explains the reduction in virulence observed with these bacteria. Pneumolysin could exert a direct effect on the disease as a result of its known cytotoxic activity, but the toxin also is likely to trigger a cascade of host inflammatory mediators. Further investigation is under way to determine whether pneumolysin and LytA neutralization will provide a useful adjunct to antibiotic treatment.

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References


