Persistence of Pneumolysin in the Cerebrospinal Fluid of Patients With Pneumococcal Meningitis Is Associated With Mortality

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Poor prognosis in Pneumococcal meningitis may be associated with high pneumolysin levels in cerebrospinal fluid (CSF). In patient samples we showed that pneumolysin levels in CSF remained high after 48 hours in nonsurvivors of meningitis compared with survivors. Selective antipneumolysin treatment may present a novel therapeutic option.

Streptococcus pneumoniae accounts for 50% of bacterial meningitis in patients worldwide, carrying a 20% mortality rate in Europe and 50%–60% in sub-Saharan Africa, with up to half of the survivors left with serious neurological sequelae [1, 2]. Streptococcus pneumoniae expresses a range of protein virulence factors associated with colonization of mucosal surfaces and subsequent tissue invasion [3]. Virulence factors include pneumolysin (Ply) and neuraminidase (NanA); Ply is directly associated with neuronal damage [4]. Damage to host tissue is mediated either directly by bacterial proteins or indirectly via the host inflammatory response [5]. Pneumolysin concentrations in cerebrospinal fluid (CSF) in an animal model reach 20 ng/mL−1; a similar concentration to levels found in the CSF of pneumococcal meningitis patients, which were 1–180 ng/mL [4].

Early antibiotic treatment improves the clinical outcome in meningitis [6, 7]. Dexamethasone given as an adjuvant is beneficial in adults with acute pneumococcal meningitis in developed healthcare settings [8] but not in middle- and low-income countries [1]. We tested the hypothesis that persistent pneumococcal protein in CSF might be associated with poor outcome, despite antibiotic therapy.

METHODS

Sample Population
We previously recruited 465 patients with bacterial meningitis to a double-blinded, randomized, placebo-controlled trial of dexamethasone antibiotic adjuvant therapy [2]. Inclusion criteria in that study were a clinical suspicion of bacterial meningitis and positive cerebrospinal fluid on microscopy (defined as organisms seen on Gram stain or >100 white cells/mm3, of which >50% were neutrophils). CSF protein and glucose were semiquantitated using urine dipsticks [9]. Patients were administered ceftriaxone (2 g intravenously or intramuscularly twice daily for 10 days) and randomized to receive either dexamethasone or placebo at the time of antibiotic administration. CSF samples were taken prior to antibiotic administration on the day of admission, and a second CSF sample was taken after 48 hours of antibiotic therapy in a small subset of patients in whom this was indicated either clinically or for drug level monitoring. Paired samples of CSF were stored at −80°C. This study was approved by the Liverpool School of Tropical Medicine Research Ethics Committee and the College of Medicine Research Ethics Committee of the University of Malawi.

Quantification of Ply and NanA
Ply and NanA levels in CSF samples were assayed using standard Western blot. In brief, samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in a 1:5 dilution using phosphate-buffered saline and transferred to nitrocellulose membranes. Blots were blocked overnight with 5% skimmed milk (Sigma) and incubated with in-house anti-Ply and anti-NanA immunoglobulin G. For quantification, serial dilutions of purified Pdb (pneumolysin toxoid derivative) and NanA were included on all immunoblots. The initial concentration of Pdb was 1.2 mg/mL and NanA was 0.14 mg/mL; standard curves were constructed using band densitometry for each serial dilution and compared with band density in CSF samples (Quantity One software). Densitometry of the films was performed with Bio-Rad GS-700 imaging...
**Figure 1.**  
A and B, Concentrations of pneumococcal proteins pneumolysin (Ply) and neuraminidase A (NanA) in the cerebrospinal fluid (CSF) of patients with proven meningitis compared with controls. The lower limit of detection was 2 ng/mL for Ply and 10 ng/mL for NanA. C and D, Concentrations of pneumococcal proteins Ply and NanA in the CSF of patients with proven meningitis comparing samples taken from survivors and nonsurvivors at admission and 48 hours. The lower limit of detection was 2 ng/mL for Ply and 10 ng/mL for NanA. E, Bacterial load of *Streptococcus pneumoniae* in the CSF of patients with proven meningitis comparing survivors and nonsurvivors at admission and 48 hours.
densitometer (Bio-Rad). Ply was detected as a band at 53 kDa and NanA at 108 kDa, respectively. Densitometry results were analyzed and concentrations of antigen levels were calculated in micrograms per milliliter for NanA and milligrams per milliliter for Ply per CSF sample.

**Quantification of Bacterial Load**

To extract pneumococcal DNA, 100 μL of CSF was removed and subjected to a lysozyme-buffer digestion protocol (20 mmol/L Tris.Cl pH 8.0, 2 mmol/L EDTA, 1.2% Triton-X-100, 100 μL of 10× lysozyme, and 10 μL of 10× lysostaphin), incubated for 1 hour at 56°C. DNA was extracted using Qiagen mini blood and tissue kits (Qiagen, Germany). Pneumococcal standards for real-time polymerase chain reaction (PCR) were created using a known number of copies of synthetic autolysin gene (LytA) (Eurofins, Germany). Standard DNA was diluted logarithmically to create serial standards at 1×109 to 1×101.

Both standard and experimental DNA were amplified using the ABI7300 protocol (Applied Biosystems), and the bacterial loads, expressed in copies per milliliter were extrapolated from the cycle threshold values, calculated by using ABI software. Statistical analysis was performed using the Mann–Whitney U test. Data are expressed as median with interquartile range (IQR).

**RESULTS**

One hundred fourteen study participants in our clinical study had culture-confirmed pneumococcal meningitis. Of these, 43 had both t = 0 and t = 48 samples available (Table 1). Eight control patients with headache provided CSF with no evidence of meningitis. In sum, 28 of 43 study patients survived to day 10, and 15 died (mortality rate 34.8%); no patients died in the control group. All patients had therapeutic levels of ceftriaxone in the CSF, and all isolates were fully sensitive (mean minimum inhibitory concentration, 0.038 μg/mL; range, 0.0003–0.19 μg/mL).

Ply and NanA concentrations were measured in all samples. Ply and NanA were detected only in the patient samples (median Ply, 1.115 mg/mL [IQR, 0.05–0.27]; median NanA, 0.57 μg/mL [IQR, 0.06–1.04]; control samples: Ply, 0 ± 0 mg/mL and NanA, 0.0 ± 0 μg/mL) (Figure 1A and 1B).

**CSF Ply**

Survivors had median Ply concentrations at t = 0 of 0.03 mg/mL (IQR, 0.01–0.12) and t = 48 hours of 0.01 mg/mL (IQR, 0.00–0.05) (P = .041) (Figure 1C). Nonsurvivors at the same time points had median Ply concentrations of 0.075 mg/mL (IQR, 0.01–0.13) and 0.07 mg/mL (IQR, 0.01–0.10) at P = .98 (Figure 1C). The difference between Ply levels at t = 48 between the survivors and nonsurvivors was significant at P = .006.

**CSF NanA**

Survivors’ CSF samples at t = 0 and t = 48 after antibiotic treatment had no significant difference in their median NanA concentrations of 0.4 μg/mL (IQR, 0.0–0.7) and 0.2 μg/mL (IQR, 0.0–0.5), respectively, at P = .39 (Figure 1D). The nonsurvivors at the same time points had values of 0.3 μg/mL

Table 1. **Demographic and Clinical Details of Study Participants**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>Survivors DEX</th>
<th>Survivors Placebo</th>
<th>Nonsurvivors DEX</th>
<th>Nonsurvivors Placebo</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>43</td>
<td>20 (46.5%)</td>
<td>8 (18.6%)</td>
<td>8 (18.6%)</td>
<td>7 (16.3%)</td>
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<tr>
<td>HIV-positive</td>
<td>34 (79%)</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Median age</td>
<td>29 (IQR, 23–36)</td>
<td>27.5</td>
<td>34</td>
<td>31</td>
<td>28</td>
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<tr>
<td>Female</td>
<td>24 (55.8%)</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Median Glasgow coma score at presentation</td>
<td>10/15 (IQR, 8–14)</td>
<td>13/15</td>
<td>10.5/15</td>
<td>8.5/15</td>
<td>11/15</td>
</tr>
<tr>
<td>CSF WCC, cells/mm³</td>
<td>640 (IQR, 110–2720)</td>
<td>920</td>
<td>724.5</td>
<td>560</td>
<td>365</td>
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<tr>
<td>CSF protein, trace = 30 g/L, 1+ = 100 g/L, 2+ = 500 g/L</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td></td>
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<tr>
<td>CSF glucose, Neg = &lt;2.8 mmol/L, trace/1+ = 2.8 mmol/L, 2 = 5.5 mmol/L</td>
<td>Neg</td>
<td>Neg</td>
<td>Trace ×2</td>
<td>Trace ×1</td>
<td></td>
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<td>Serotype of Streptococcus pneumoniae known (No. of patients with that serotype)</td>
<td>4 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>6 (1)</td>
<td></td>
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<td></td>
<td>9 (2)</td>
<td>3 (1)</td>
<td>7 (1)</td>
<td>1 (14)</td>
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<td></td>
<td>15 (1)</td>
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Abbreviations: CSF, cerebrospinal fluid; DEX, dexamethasone; HIV, human immunodeficiency virus; IQR, interquartile range; WCC, white cell count.
(IQR, 0.125–0.6) and 0.3 µg/mL (IQR, 0.1–0.5), respectively, at $P = .56$ (Figure 1D).

**CSF Bacterial Load by PCR**

All CSF samples were sterile by culture at 48 hours. The median CSF bacterial load measured by PCR copy number was significantly reduced at $t = 48$ compared with $t = 0$ in survivors; $t = 0$ copy number was $3.9 \times 10^3$ copies/mL (IQR, $5.3 \times 10^4$ to $1.15 \times 10^6$) and $t = 48$ copy number was $3.4 \times 10^4$ (IQR, $1.56 \times 10^4$ to $8.9 \times 10^4$) copies/mL at $P < .005$ (Figure 1E).

In nonsurvivors the median CSF bacterial copy number at $t = 0$ was $3.6 \times 10^5$ (IQR, $8.4 \times 10^4$ to $3.7 \times 10^6$) and at $t = 48$ hours it was $1.3 \times 10^5$ (IQR, $1.4 \times 10$ to $5.4 \times 10^5$) at $P = .19$ (Figure 1E). The differences in bacterial loads between survivors and nonsurvivors at admission ($P = .89$) and 48 hours after admission ($P = .07$) were both nonsignificant.

**DISCUSSION**

In this study, Ply levels were significantly reduced between admission and 48 hours in survivors but not in nonsurvivors. Nonsurvivors had higher CSF Ply levels at $t = 0$. Levels of NanA did not change significantly between the 2 groups over the 2 time periods. The bacterial load measured by culture was reduced to zero in both groups. The bacterial load measured by copy number fell in both survivors and nonsurvivors, with a significant fall only in the survivor group. In the nonsurvivor group, high levels of Ply persisted and were associated with a nonsignificant fall in the number of bacteria present. In the survivor group Ply fell with bacterial load. NanA levels did not correlate with bacterial copy number or outcome. The sterilization of CSF in both groups suggests that ceftriaxone caused effective bacterial killing in both groups as expected. This study does not explain the persistence of Ply in the nonsurvivor group but suggests an association with mortality. Absolute values of Ply do not predict outcome. The lack of statistically significant differences between the admission bacterial load in survivors and nonsurvivors is in contrast with a larger data set (82 CSF samples) from Malawi in children [10] and is likely due to the smaller numbers in this study.

Ply levels seen in our study were very high and consistent with neurotoxicity (median, 1 mg/mL). For comparison, CSF Ply levels in animal models of pneumococcal meningitis were 20 ng/mL and 3.92 ng/mL in survivors and nonsurvivors, respectively, at $P = .56$ (Figure 1D).

Isogenic mutants remained asymptomatic or survived for significantly longer. Attenuation of virulence was observed with Ply and autolysin mutants in other animal studies [12, 13]. The association of high levels of CSF Ply and patient nonsurvival is a novel finding and highlights the crucial role of Ply in pathogenesis of meningitis.

NanA in animal models has been shown to have conflicting roles in inflammation; one study demonstrated that NanA mediated pneumococcal invasion of blood-brain barrier endothelial cells [14], and in another NanA deficiency was found to have no influence on the clinical course of the disease nor had any effect on systemic bacterial dissemination [15]. Our findings suggest NanA does not have a clear pathogenic role in human pneumococcal meningitis.

In conclusion, we found that all pneumococcal meningitis patients had higher levels of Ply and NanA than those expected from extrapolated animal models. The persistence of high levels of Ply in nonsurvivors despite falling numbers of bacteria suggests that this protein is involved in severe pathogenesis. Blocking or inhibiting pneumolysin during acute meningitis may represent a future therapeutic option to improve mortality.

**Notes**

**Financial support.** This work was supported by grants from the Meningitis Research Foundation (to M. S.), the Dunhill Medical Trust (to A. K.), and the Wellcome Trust (Clinical PhD Fellowship to E. C. W. and Career Development Fellowship, grant number 061231 to S. B. G.).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**


704 • CID 2012:54 (1 March) • BRIEF REPORT