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

Emma C. Wall,1,a Stephen B. Gordon,1,a Samia Hussain,2 Upali R. S. Goonetilleke,1 Jenna Gritzfeld,1 Matthew Scarborough,3 and Aras Kadioglu2

1Clinical Research Group, Liverpool School of Tropical Medicine, 2Institute of Infection and Global Health, University of Liverpool; and 3Nuffield Department of Medicine, John Radcliffe Infirmary, Oxford, United Kingdom

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/mm², 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

Received 31 March 2011; accepted 17 November 2011; electronically published 11 January 2012.

© The Author 2012. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please email: journals.permissions@oup.com. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1093/cid/cir926
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, 0.0–0.05) (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

| Characteristics | Total | Survivors DEX | Survivors Placebo | Nonsurvivors DEX | Nonsurvivors Placebo |
|-----------------|-------|----------------|-------------------|------------------|----------------------|
| No. of patients | 43    | 20 (46.5%)     | 8 (18.6%)         | 8 (18.6%)        | 7 (16.3%)            |
| HIV-positive    | 34 (79%) | 15              | 7                 | 5                | 7                    |
| Median age      | 29 (IQR, 23–36) | 27.5            | 34                | 31               | 28                   |
| Female          | 24 (55.8%) | 12              | 4                 | 3                | 5                    |
| Median Glasgow coma score at presentation | 10/15 (IQR, 8–14) | 13/15            | 10.5/15           | 8.5/15             | 11/15                |
| CSF WCC, cells/mm² | 640 (IQR, 110–2720) | 920             | 724.5             | 560              | 365                  |
| CSF protein, trace = 30 g/L, 1+ = 100 g/L, 2+ = 500 g/L | 2+ | 2+             | 2+                | 2+               | 2+                   |
| CSF glucose, Neg = <2.8 mmol/L, trace/1+ = 2.8 mmol/L, 2 = 5.5 mmol/L | Neg | Neg           | Trace ×2          | Trace ×1          |                      |
| Serotype of *Streptococcus pneumoniae* where known (No. of patients with that serotype) | 4 (1) | 1 (1)         | 1 (1)             | 6 (1)            |                      |
| | 9 (2) | 3 (1)         | 7 (1)            |                  |                      |
| | 7 (1) |              |                  | 1 (14)           |                      |
| | 33 (1) |              |                  |                  |                      |
| | 1 (2) |              |                  |                  |                      |
| | 15 (1) |              |                  |                  |                      |

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 10^5) 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 10^3 to 3.7 \times 10^6) and at \( t = 48 \) hours it was \( 1.3 \times 10^5 \) (IQR, 1.4 \times 10^5 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 of CSF [4]. Ply levels were also significantly higher than NanA levels in the same patients. This could be due to the release of Ply upon bacterial lysis. Ply is necessary for microvascular invasion of pneumococci across the blood-brain barrier and is a powerful stimulator of the CSF inflammatory response in pneumococcal meningitis [11]. Animals infected with wild-type pneumococci intracisternally became unwell within 26 hours, whereas animals infected with pneumolysin-deficient 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 greater 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**

1. van de Beek D, de Gans J, Spanjaard L, Weisfelt M, Reitsma JB, Vermeulen M. Clinical features and prognostic factors in adults with bacterial meningitis. N Engl J Med 2004; 351:1849–59.
2. Scarborough M, Gordon SB, Whitty CJ, et al. Corticosteroids for bacterial meningitis in adults in sub-Saharan Africa. N Engl J Med 2007; 357:2441–50.
3. Kadioglu A, Weiser J, Paton JC, Andrew PW. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 2008; 6:288–301.
4. Spreer A, Kerstan H, Bottcher T, et al. Reduced release of pneumolysin by Streptococcus pneumoniae in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. Antimicrob Agents Chemother 2003; 47:2649–54.
5. Goonetilleke UR, Scarborough M, Ward SA, Gordon SB. Proteomic analysis of cerebrospinal fluid in pneumococcal meningitis reveals potential biomarkers associated with survival. J Infect Dis 2010; 202:542–50.
6. Weisfelt M, de Gans J, van der Poll T, van de Beek D. Pneumococcal meningitis in adults: new approaches to management and prevention. Lancet Neurol 2006; 5:332–42.
7. Booy R, Habibi P, Nadel S, et al. Reduction in case fatality rate from meningococcal disease associated with improved healthcare delivery. Arch Dis Child 2001; 85:386–90.
8. van de Beek D, Farrar J, de Gans J, et al. Adjunctive dexamethasone in bacterial meningitis: a meta-analysis of individual patient data. Lancet Neurol 2010; 9:254–63.
9. Moosa AA, Quortum HA, Ibrahim MD. Rapid diagnosis of bacterial meningitis with reagent strips. Lancet 1995; 345:1290–1.

10. Carrol ED, Guiver M, Nkhoma S, et al. High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease. Pediatr Infect Dis J 2007; 26:416–22.

11. Zysk G, Schneider-Wald BK, Hwang JH, et al. Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by \textit{Streptococcus pneumoniae}. Infect Immun 2001; 69: 845–52.

12. Hirst RA, Gosai B, Rutman A, et al. \textit{Streptococcus pneumoniae} deficient in pneumolysin or autolysin has reduced virulence in meningitis. J Infect Dis 2008; 197:744–51.

13. Wellmer A, Zysk G, Gerber J, et al. Decreased virulence of a pneumolysin-deficient strain of \textit{Streptococcus pneumoniae} in murine meningitis. Infect Immun 2002; 70:6504–8.

14. Uchiyama S, Carlin AF, Khoorsavi A, et al. The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. J Exp Med 2009; 206:1845–52.