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RESEARCH ARTICLE

Death Is Associated with Complement C3 Depletion in Cerebrospinal Fluid of Patients with Pneumococcal Meningitis

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ABSTRACT **Pneumococcal meningitis can lead to death or serious neurological sequelae as a result of the host inflammatory response. We investigated the association between host response protein expression and outcome in patients with pneumococcal meningitis. Cerebrospinal fluid (CSF) was obtained from 80 patients with pneumococcal meningitis (40 nonsurvivors and 40 survivors) and 10 normal controls. Candidate proteins were analyzed for an association with survival. Complement C3 levels were 5-fold lower in nonsurvivors than in survivors ($P < 0.05$). This C3 reduction was not associated with lower levels in serum, indicating a compartmentalized CSF response. Transferrin levels were significantly higher in CSF (but not serum) from nonsurvivors than in CSF from survivors, suggestive of blood-brain barrier damage. Classical apoptosis proteins caspase 3 and apoptosis-inducing factor were not present in CSF. Expression of creatine kinase BB in clinically infected CSF suggested neuronal necrosis, but there was no clear association between level of expression and clinical outcome. Increased blood-brain barrier permeability and complement C3 depletion may have a role in determining outcome from bacterial meningitis. Therapeutic use of citicoline or caspase inhibitors is unlikely to have beneficial effects in patients with meningitis.**

IMPORTANCE **We previously identified proteins associated with clinical outcome in patients diagnosed with pneumococcal meningitis in a pilot proteomics study of cerebrospinal fluid (CSF). In this article, we have quantitatively assayed specific proteins identified from this previous proteomics analysis along with proteins associated with cell death by using Western blotting.**

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Despite many major advances in our understanding of the pathogenesis of pneumococcal meningitis, there has been little therapeutic progress in the last 60 years. This is predominantly due to the fact that bacterial cell wall products released in the cerebrospinal fluid (CSF) initiate a cascade of destructive host immune responses that are not ameliorated by currently available treatments.

We have shown that complement C3 depletion and increased blood-brain barrier permeability may have a role in determining the outcome of bacterial meningitis. In addition, therapeutic use of citicoline or caspase inhibitors is unlikely to have a beneficial effect in patients with meningitis, as these pathways were not demonstrably disrupted.

Streptococcus pneumoniae is the most common pathogen associated with bacterial meningitis beyond the neonatal period (1). In Malawi, pneumococcal meningitis has a high fatality rate of 67% (2). Survivors often develop long-term neurological sequelae, including hearing loss and focal neurological deficits (3, 4). Our previous pilot proteomic study of CSF in patients with pneumococcal meningitis revealed that clinically infected CSF contains several thousand proteins of central nervous system (CNS) and serum origin. A proteomic comparison of nonsurvivors and survivors allowed detection of proteins associated with survival (5). It was therefore necessary to determine if this correlation, and subsequent hypotheses generated, could be confirmed in a larger sample size.

CSF proteins originate from serum and from local intracranial production. A breakdown in the integrity of the blood-brain barrier allows increased levels of serum proteins to access the CSF (6–8). This contributes to the development of increased intracranial pressure, hydrocephalus, brain edema, and cerebral ischemia (9, 10), all of which can cause neuronal cell death (11, 12). In animal models, cell death was found to occur via three distinct pathways: classic caspase 3-dependent cell death (i.e., apoptosis), caspase 3-independent cell death (i.e., pyknosis), and necrosis (13, 14). Caspase inhibitors and citicoline, an intermediate in the synthesis of phosphorylcholine in mitochondrial and cell membranes, prevented neuronal damage when given before and after bacterial infection in animal models of meningitis. These products might be used therapeutically for protection against neuronal cell death by apoptosis if this mechanism was shown to be important in humans (15–17). Accurate identification of proteins associated with specific mechanisms of neuronal cell death in human CSF could therefore provide evidence to support clinical trials of anti-apoptotic drugs for improved patient outcome.

In our previous study, we found reduced complement C3 levels

TABLE 1 Clinical details of survivors and deceased patients[a]

Parameter	Subject group (*n*)		
	Normal (10)	Nonsurvivors (40)	Survivors (40)
Age, yr [mean (SD)]	27.8 (9.5)	30.1 (8.4)	33.3 (11.2)
Male sex, *n*	3	23	16
GCS [median (SD)]	9.0 (4.7)	8.0 (3.2)	11.0 (3.2)
Median time to presentation [h (range)]	60 (12–96)	72 (15–336)	48 (10–192)
Previous antimicrobials, no.	2	7	6
Steroid treatment [no. receiving (no. receiving placebo)]		17 (23)	21 (19)
HIV positive [no. (% of those tested)]		35 (100)	37 (100)
HIV status not known, no.	10	5	3
Survival at day 10, %	100	0	100
Hemoglobin [g/dl (range)]		10.82 (2.8–15)	11.01 (5.5–17.7)
CSF leukocytes [count/μl (range)]		1,376.3 (20–9,980)	2,088.4 (15–10,500)
Neutrophils [count (% of those tested)]		92.17 (67–100)	91.97 (76–99)

[a] Clinical data collected from patients providing CSF samples that were used in this analysis. All meningitis subjects were diagnosed as having pneumococcal meningitis caused by *Streptococcus pneumoniae*, all subjects were HIV positive, and all samples were collected before any treatment commenced. Normal CSF was obtained from patients who tested negative for meningitis or any other pathogen. The HIV status of the normal patients was not known. Hemoglobin levels have been included as low hemoglobin levels are associated with both advanced AIDS and poor outcome. GCS, Glasgow coma scale.

in patients who died of pneumococcal meningitis (5). C3 plays a central role in the activation of the complement system. Processing by C3 convertase is the central reaction in both classical and alternative complement pathways. C3 aids innate immunity either by the coating of pathogens with C3b and iC3b, which stimulates phagocytosis, or by the release of proinflammatory mediators C3a and C5a (18). Pathological sequelae are mediated in part by the intrathecal activation of the complement cascade in response to a bacterial challenge (19). The relationship between complement C3 activity and pneumococcal meningitis is not fully understood. Classical and lectin pathways are most commonly associated with pneumococcal disease (20). Many pathogenic serotypes of pneumococci are poor activators of the alternative complement pathway (21) or degrade C3b into less opsonically active components (22) and are therefore able to resist phagocytosis. The importance of opsonization, however, is strongly supported by the finding that the impairment of either the phagocytic system or opsonin production predisposes the host to pneumococcal disease (23, 24).

This study tested three hypotheses: (i) candidate proteins previously identified by pilot proteomics can be confirmed in a larger comparison of survivors and nonsurvivors, (ii) apoptotic proteins in CSF are associated with death, and (iii) necrosis proteins found in pneumococcal meningitis-affected CSF are associated with death.

RESULTS

Clinical details of patients. CSF from 80 patients with microbiologically proven pneumococcal meningitis was analyzed by Western blotting. Forty of these CSF samples were from patients who survived with no neurological impairment. The remaining 40 subjects all died within 10 days. Clinical details of the patients are provided in Table 1.

CSF total protein concentration. CSF from patients with meningitis had a mean total protein concentration of 5.92 mg liter^{-1} compared to control (mean, 0.23 mg liter^{-1}; $P < 0.0001$). Nonsurvivors had a total protein concentration between 2.20 and 11.30 mg liter^{-1} (mean, 6.38 mg liter^{-1}). Survivors had a total protein concentration between 1.12 and 10.89 mg liter^{-1} (mean, 5.46 mg liter^{-1}). The mean total protein concentration was higher in nonsurvivors than in survivors, but the two concentrations were not significantly different ($P = 0.09$; see Fig. S1 in the supplemental material).

Expression of proteins of plasma origin in cerebrospinal fluid. (i) Expression of transferrin in cerebrospinal fluid and blood serum. Transferrin was measured in normal CSF (mean, 0.08 mg liter^{-1}), nonsurvivor CSF samples (mean, 1.25 mg liter^{-1}), and survivor CSF samples (mean, 0.67 mg liter^{-1}) (Fig. 1). The mean concentration of transferrin in normal CSF was significantly lower than that in CSF from patients diagnosed with meningitis ($P < 0.0001$). The mean concentration of transferrin in CSF from nonsurvivors was 2-fold higher than that in CSF from survivors ($P < 0.0004$).

Transferrin was measured in serum samples from nonsurvivors (mean, 1.72 mg liter^{-1}) and survivors (mean, 1.89 mg liter^{-1};

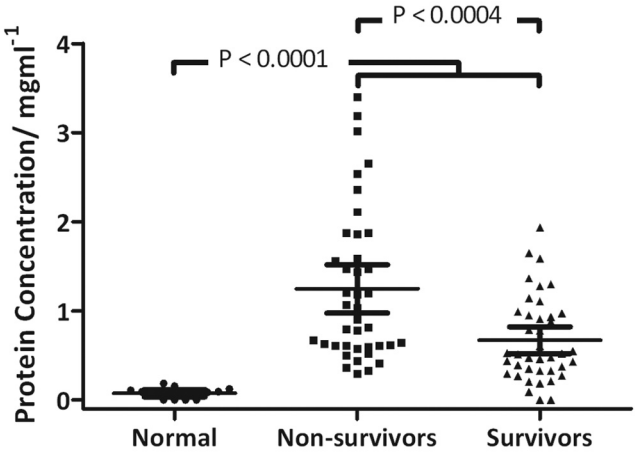

FIG 1 Transferrin levels in CSF. Transferrin was measured in normal CSF (mean, 0.08 mg liter^{-1}). Transferrin was found in nonsurvivor CSF samples (mean, 1.25 mg liter^{-1}) and survivor CSF samples (mean, 0.67 mg liter^{-1}). There was a statistically significant difference between the mean level of transferrin in nonsurvivors and that in survivors ($P < 0.0004$). The mean level of transferrin in nonsurvivor CSF was 2-fold higher than that in survivor CSF. There was a statistically significant difference between the mean level of transferrin in meningitis-affected CSF and that in normal CSF ($P < 0.0001$).

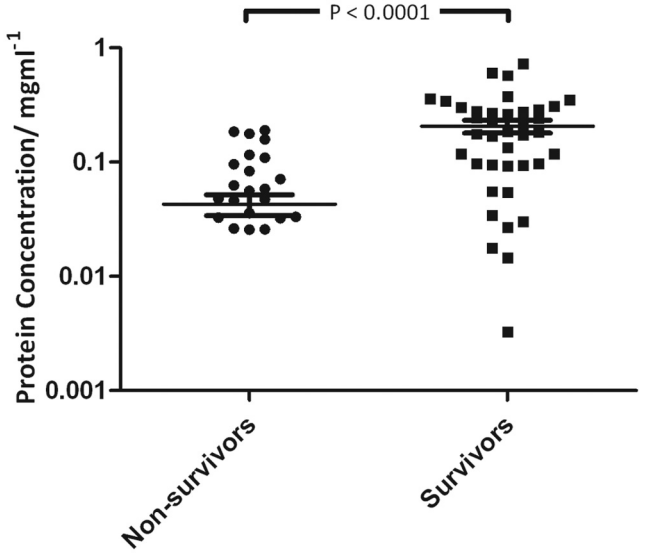

FIG 2 Complement C3 levels in CSF. C3 was not found in normal CSF ($n = 10$). C3 was found in nonsurvivor CSF samples ($n = 40$; mean, 0.04 mg liter^{-1}) and survivor CSF samples ($n = 40$; mean, 0.21 mg liter^{-1}). There was a statistically significant difference between the mean concentration of C3 in nonsurvivors and that in survivors ($P < 0.0001$). The mean level of C3 in nonsurvivor CSF was 5-fold lower than that in survivor CSF.

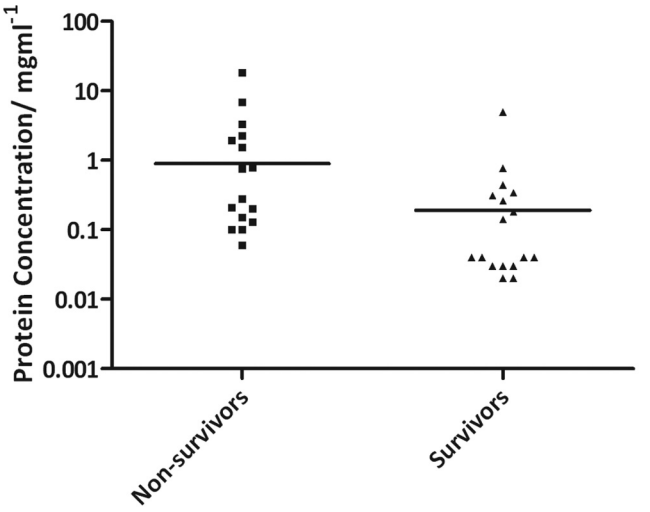

FIG 3 Creatine kinase BB levels in CSF. CKBB was discovered in over 40% of the CSF samples. CKBB levels were not significantly higher in nonsurvivors (mean, 0.89 mg liter^{-1}, inclusive of all samples) than in survivors (mean, 0.19 mg liter^{-1}, inclusive of all samples) ($P = 0.16$). CKBB was not found in normal CSF.

$P = 0.183$) (see Fig. S2 in the supplemental material). We tested for correlation between the concentration of transferrin in serum from patients and the corresponding CSF concentration in both nonsurvivors and survivors and found none (see Fig. S3 and S4). The mean concentration of transferrin in serum from nonsurvivors was 1.5-fold higher than the corresponding level in CSF, but the mean concentration of transferrin in survivors was 3-fold higher in serum than in the corresponding CSF.

(ii) Expression of complement C3 in cerebrospinal fluid and serum. Complement C3 was not found in normal CSF. C3 levels in nonsurvivor CSF samples (mean, 0.04 mg liter^{-1}) were significantly lower than those in survivor CSF samples (mean, 0.21 mg liter^{-1}; $P < 0.0001$) (Fig. 2).

There was no statistically significant difference between the mean concentration of C3 in serum of nonsurvivors (mean, 1.47 mg liter^{-1}) and that in the serum of survivors (mean, 1.50 mg liter^{-1}; $P = 0.81$) (see Fig. S5 in the supplemental material). There was a statistically significant difference between the mean concentrations of C3 in CSF and in the corresponding serum samples ($P < 0.0001$) see Fig. S6 and S7).

(iii) Expression of beta-2-glycoprotein in cerebrospinal fluid. Beta-2-glycoprotein was measured in normal CSF (mean, 0.13 mg liter^{-1}), nonsurvivor CSF samples (mean, 0.83 mg liter^{-1}), and survivor CSF samples (mean, 0.69 mg liter^{-1}). There was a significant difference between the mean beta-2-glycoprotein levels in CSF from patients diagnosed with pneumococcal meningitis and those in normal CSF ($P < 0.0001$) but not between nonsurvivors and survivors ($P = 0.10$).

Cerebrospinal fluid levels of apoptosis proteins. Neither caspase 3 nor apoptosis-inducing factor (AIF) was detected in the 80 meningitis-associated CSF samples (nonsurvivors or survivors) or the 10 normal CSF samples (see Fig. S8 in the supplemental material). Western blotting for both caspase 3 and AIF was repeated with two primary antibodies directed against different regions of the respective protein structures with the same outcome. The specificity of the antibodies was confirmed using an antigenic blocking peptide. The blocking peptide binds to the active site of the antibody, which inhibits the antibody activity, leading to quenching of protein expression. The sensitivity of detection was increased by the use of polyclonal antibodies directed against caspase 3 and AIF at optimum levels of concentration and exposure to the CSF proteins.

The limit of detection was determined by serial dilution of the positive control. Both AIF and caspase 3 were detectable in the positive cell lysate at protein concentrations as low as 100 ng μl^{-1}. The visualization of the proteins in CSF upon spiking normal CSF with the control lysate would suggest that the proteins are not being confounded by larger proteins in CSF (see Fig. S9 in the supplemental material).

Cerebrospinal fluid levels of the necrosis protein CKBB. Creatine kinase BB (CKBB) was not detected in normal CSF samples but was present in over 40% of CSF samples from patients diagnosed with meningitis. The mean concentrations of CKBB in nonsurvivors and survivors were 0.89 mg liter^{-1} and 0.19 mg liter^{-1}, respectively, but there was no statistically significant difference between the two groups ($P = 0.16$), owing to the small number of samples with detectable CKBB as shown in Fig. 3.

Proteomic candidate cerebrospinal fluid markers of clinical outcome. Retinoid X receptor (RXR) gamma was not found in normal CSF but was found in 73% of nonsurvivor CSF samples (mean, 0.50 mg liter^{-1}, inclusive of all samples) and 83% of survivor CSF samples (mean, 0.36 mg liter^{-1}, inclusive of all samples; $P = 0.48$).

Zinc finger protein 179 was not found in normal CSF but was found in 98% of nonsurvivor CSF samples (mean, 0.52 mg liter^{-1}, inclusive of all samples) and 70% of survivor CSF samples (mean, 0.41 mg liter^{-1}, inclusive of all samples; $P = 0.59$).

Chitotriosidase was not found in normal CSF. Chitotriosidase was found in 50% of nonsurvivor CSF samples (mean, 0.88 mg li-

ter^{-1}, inclusive of all samples) and 75% of survivor CSF samples (mean, 0.47 mg liter^{-1}, inclusive of all samples; $P = 0.09$).

DISCUSSION

In our previous pilot proteomic analysis of CSF in patients diagnosed with pneumococcal meningitis, we observed several proteins having an association with survival (5). In this study, we have shown that a subset of these proteins were detectable in a larger sample size; however, only the proteins transferrin and complement C3 showed a significant association with survival.

Blood-brain barrier integrity and transferrin. The blood-brain barrier is responsible for maintaining biochemical homeostasis within the CNS (25). Penetration of the blood-brain barrier by a bacterial pathogen reflects a complex interplay between the host endothelium and microbial surface components (26).

Transferrin is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization (27, 28). The increased level of transferrin in nonsurvivors was an indicator of blood-brain barrier damage, and this damage may have been a contributing factor to death from pneumococcal meningitis. The observation, however, that CSF levels did not correlate with serum levels implies that, although transferrin levels in CSF relate to blood-brain barrier integrity and simple diffusion, there has not been a complete breakdown of the blood-brain barrier regulation of proteins in most cases.

Another possible explanation for the lack of association between CSF and plasma transferrin levels may be the negative acute-phase property of transferrin, i.e., transferrin has a tendency to decrease during inflammation and CSF/serum time courses of transferrin regulation are likely not synchronous (27).

This partial breakdown of the blood-brain barrier correlated with the observation that the levels of the serum protein beta-2-glycoprotein (important in processes such as coagulation and atherosclerosis) did not vary between nonsurvivors and survivors, indicating that the blood-brain barrier retains some ability to filter serum (29).

Complement C3 depletion and outcome. Complement C3 was observed to be lower in nonsurvivors than in survivors. To confirm that this change in C3 concentration was localized to the CNS, analysis was carried out on corresponding blood samples. The lack of any association between C3 in serum and that in CSF indicated that changes in the level of C3 were localized to CSF.

The first evidence for a functional role of the complement system in limiting pneumococcal outgrowth within the CNS was described by Tuomanen et al. (23). In rabbits depleted of C3 by cobra venom factor, intracisternal inoculation of *S. pneumoniae* resulted in higher bacterial titers than those in complement-sufficient control animals (30). Thus, lower levels of C3 in nonsurvivors than in survivors may have resulted in higher bacterial titers or may have been due to greater consumption of C3 in more severe illness. A positive-feedback mechanism can be proposed where nonsurvivors had very high numbers of bacterial CFU in their CSF, which led to activation and breakdown of C3. Some pneumococcal species can degrade C3 directly; for example, Angel et al. demonstrated that type 3 pneumococci express C3-degrading activity associated with the cell wall (22, 31). Few of the infections in this study were due to type 3, however, as this type is not common in Malawi (32).

Patients were recruited at the point of admission to the hospital. Clinical information indicative of AIDS preceding the acute episode was sought in all patients. Twenty-four patients had evidence of chronic illness or one or more HIV-associated infections (11 had a history of tuberculosis [TB] treatment, 10 had shingles, 3 had pneumonia, and 3 had meningitis). We can only speculate as to the nature of the effect of HIV and of encephalopathy caused by HIV on the final results, as CNS HIV infection and immune activation have been demonstrated to also lead to brain injury (33).

Serum albumin levels are used to distinguish viral meningitis from bacterial meningitis in CSF (34). Unfortunately, the albumin data from patients were not available owing to the limited hospital laboratory support available at the study site. These albumin data could have been used to compare the blood plasma levels with the final CSF levels. This would allow a measure of the compartmentalization of the CSF protein changes and also a measure of the effect of viral infection prior to meningitis.

Cell death markers in cerebrospinal fluid. Despite animal models indicating that caspase 3 and AIF would be detectable in CSF during pneumococcal meningitis in humans (17, 35, 36), there was no evidence observed of these classical apoptosis markers. The data obtained from the analysis of apoptosis proteins indicate that since caspase 3 and AIF are not present, there was no leakage of cell death material into CSF. This was surprising considering the level of data in animal models which suggest that these proteins would be identified in CSF. It was possible, however, that these were longer-term animal models where the levels of bacteria and pneumolysin were lower than those in the nonsurvivor groups analyzed in this study (35, 37).

It was also possible that small foci of apoptosis did not result in a large increase of caspase 3 or AIF, and this could be confirmed with suitable postmortem material. Alternatively, an increase in metabolic proteins, previously identified in the pilot proteomics analysis (5), may have led to an increase in proteosomal degradation of AIF and caspase 3. These degradation pathways may occur via a ubiquitination process such as that mediated by X-linked inhibitor of apoptosis (XIAP) utilizing E3 ubiquitin ligase activity. This enables apoptosis inhibitors to catalyze ubiquitination of themselves, caspase 3, caspase 7, and AIF (38).

Caspase inhibitors and citicoline may have neuroprotective effects and may potentiate neurorecovery. This has led to the evaluation of caspase inhibitors and citicoline as potential treatment for neuronal injury during meningitis. On the basis of our data, however, there is no theoretical rationale for the use of caspase inhibitors or citicoline in patients with pneumococcal meningitis.

Further exploration may require development of an *in vitro* model of the CSF inflammatory process to determine if CSF from nonsurvivors causes injury to neurons and endothelial cells more than does that from survivors. The addition of caspase inhibitors to this model can be used to see if a difference is observed. Anti-apoptosis treatment could be used to determine if the lack of caspase or AIF in CSF is related to tissue damage. This will allow the opposite conclusion to be validated.

In rodent models, neuronal necrosis has been shown to involve caspase 3 and AIF (39). Therefore, the absence of these proteins provides evidence that necrosis also does not occur at significant levels. To distinguish necrosis from apoptosis, we used creatine kinase as a specific marker of neuronal necrosis. CKBB is the brain-specific isotype of the protein (40). This protein is a cytoplasmic protein released during tissue necrosis. CKBB can leak out of cells under ischemic episodes or injury to the brain (41). Our

data suggest that CKBB is not a useful prognostic indicator in pneumococcal meningitis.

Proteins of cerebrospinal fluid origin showed no association with outcome. The proteins analyzed as indicators of proteins in CSF included RXR gamma, zinc finger protein 179, and chitotriosidase. RXR gamma receptors are involved in the expression of anti-inflammatory activity by immune cells and mediate the cellular effects of retinoid compounds (42). Zinc finger protein 179 is predominantly expressed in the brain. The gene for this protein encodes a member of the RING finger protein family of transcription factors and is associated with neuronal differentiation. The protein has been implicated in a variety of functions such as transcriptional regulation, DNA repair, site-specific recombination, and signal transduction (43). RXR gamma and zinc finger protein 179 are located primarily in the nucleus. Their low-level detection may be due to an incomplete leakage of cell material into the CSF, although the similar levels of these proteins suggest that their expression has no clear association with outcome (42, 43).

Chitotriosidase is an enzyme which degrades chitin and chitotriose and may participate in the defense against nematodes and other pathogens (44). Chitotriosidase can be considered an inflammatory protein since it is secreted only by activated macrophages and may be used as an important predictor of neuronal disease severity (45). It was expected that chitotriosidase would have greater expression in CSF associated with clinically proven pneumococcal meningitis, but the expression was very low or absent.

Conclusions. This study has shown that blood-brain barrier damage appears to alter the level of protein in CSF and acts as a confounding factor in protein analysis of the CNS during meningitis. The increased level of transferrin in nonsurvivors suggested blood-brain barrier damage but did not provide a direct marker for the degree of damage.

In addition, this study has shown that CSF levels of complement C3 are associated with outcome in pneumococcal meningitis. The data suggest that complement C3 plays an important role in the pathogenesis of bacterial meningitis and may serve as a prognostic marker.

The absence of standard apoptosis markers in CSF suggested that treatment involving citicoline and caspase inhibitors is unlikely to provide benefit to patients with pneumococcal meningitis. The presence of CKBB indicated that cortical necrosis occurs to a significant degree in many patients with pneumococcal meningitis, but this was not clearly associated with outcome.

MATERIALS AND METHODS

Patient information. (i) Patients and sample collection. Patients were recruited to a double-blind, randomized, placebo-controlled trial of dexamethasone adjuvant therapy. CSF and blood serum samples collected from patients as part of clinical diagnosis were saved for later study. The study was conducted in adults with bacterial meningitis presenting at the Queen Elizabeth Central Hospital in Blantyre, Malawi, between May 2002 and January 2005 (46). The study was approved by the research ethics committees of the University of Malawi College of Medicine and the Liverpool School of Tropical Medicine. Patients provided written informed consent, or if they were unable to read or write, they provided independently witnessed verbal consent before recruitment. Patients were treated in the hospital for a minimum of 10 days and were evaluated at 40 days and at 6 months. Clinically evident adverse events were recorded systematically throughout the trial period. At follow-up, patients had a standardized neurologic examination and a hearing assessment. Patients who did not return for follow-up appointments were visited at home. No other underlying diseases were specifically sought other than HIV and malaria (46).

(ii) Patient categories. Clinical data allowed the patients in the meningitis trial to be classified into 3 clear phenotypes: survival to 40 days with no neurological impairment or recorded disability ($n = 103$), deafness or disability at 40 days ($n = 113$), and death within 40 days ($n = 157$). We chose to study only patients who had confirmed pneumococcal meningitis (Gram-positive diplococci seen in CSF or pneumococci cultured from either CSF or blood) and to compare those who survived free of neurological deficit with those who died, as we anticipated that these phenotypes would show the clearest differences. Control CSF was obtained with consent from patients who presented to the hospital with a headache but subsequently tested negative for meningitis or any other pathogen. CSF samples were stored at −20°C within an hour of sampling and at −80°C from 24 h until analysis.

Protein target selection. (i) Proteomically derived targets. Proteins were previously identified as having different levels of expression between nonsurvivors and survivors in a pilot proteomic comparison of CSF (5). These proteins were formally identified after manual excision from stained two-dimensional (2D) gels. The proteins were subject to in-gel tryptic digestion and sequenced by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) or liquid chromatography (LC)-MS. Peptide data were analyzed with Mascot software (Matrix Science, London, United Kingdom). In this comparison of CSF from patients who survived with CSF from those who died, we chose to compare specific proteins that showed important differences (>3-fold expression) in exploratory 2D gel analysis. The targets selected from these proteins for further analysis included three proteins of plasma origin and three proteins of CSF origin. The three plasma proteins analyzed were (i) transferrin, (ii) complement C3, and (iii) beta-2-glycoprotein. The three proteins of cerebrospinal fluid origin analyzed were (i) retinoic acid X receptor gamma, (ii) zinc finger protein 179, and (iii) chitotriosidase.

(ii) Cell death pathway targets. Proteins associated with apoptosis and necrosis were analyzed in CSF. We analyzed CSF for the apoptosis-associated proteins apoptosis-inducing factor (AIF) and caspase 3 (35). We analyzed CSF for the protein creatine kinase BB (CKBB) as evidence of neuronal necrosis (45).

Western blotting of cerebrospinal fluid. Archived CSF was thawed at 4°C. The concentration of protein in each CSF sample was determined using the Bradford assay. SDS-PAGE gels of whole CSF protein were separated using optimized gel concentrations for each Western blot. The transfer membranes used in all the experiments of this analysis were nitrocellulose paper (NCP; GE Life Sciences, Piscataway, NJ). AIF and caspase 3 protein blots were retested using polyvinylidene difluoride (PVDF) membranes (GE Life Sciences).

(i) Antibodies. Primary antibodies used in this analysis were directed against caspase 3 (9665 and sc-22140) and apoptosis-inducing factor (AIF; 4642 and sc-9416) and came from Cell Signaling (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Creatine kinase BB (CKBB; ab38212), zinc finger protein 179 (ab42499), retinoic acid X receptor gamma (ab15518), chitotriosidase (CHIT1; ab72574), and transferrin (ab1223) antibodies came from Abcam (Cambridge, United Kingdom). Beta-2-glycoprotein (MCA2114; AbD Serotec, Oxford, United Kingdom) and complement C3 (C7761-1VL; Sigma-Aldrich, Poole, United Kingdom) antibodies were also used. Secondary antibodies were purchased from Dako (Cambridge, United Kingdom) and Nordic Immunology (Tilburg, Netherlands).

(ii) Positive-control protein markers. The positive-control lysate used for caspase 3 was Jurkat apoptosis cells treated with etoposide (9663; Cell Signaling). For AIF, CKBB, and RXR gamma, the positive-control cell lysate was HeLa whole-cell lysate (sc-2200; Santa Cruz Biotechnology). For zinc finger protein 179, the positive-control lysate used was HepG2 cell lysate (sc-2227; Santa Cruz Biotechnology). For chitotriosidase, a re-

combinant chitotriosidase protein was used (3559-GH; R&D Systems, Minneapolis, MN).

Blood plasma (obtained from a healthy volunteer) was used as a control for complement C3, transferrin, and beta-2-glycoprotein. For transferrin, a recombinant transferrin protein was used as an additional positive control (Sigma-Aldrich; 90190).

Quantitative measure of protein expression in cerebrospinal fluid. The blot images were captured using a gel documentation camera (Syngene, Cambridge, United Kingdom) and analyzed using the open source software ImageJ (v.1.43; NIH, Bethesda, MD; free download available at http://rsb.info.nih.gov/ij/) (47). In these Western blots, the reference band used was the positive control for the protein under analysis. The expression value obtained for CSF was converted to an approximate quantity using a calibration curve generated from the positive control (http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). This value was then converted to a native concentration in CSF by correction of the dilution of CSF used in the Western blot assay. The quantity of protein measured was estimated for each CSF sample as a relative intensity. Data regarding expression are given in Table S1 in the supplemental material.

Quantitative analysis of transferrin and complement C3 in blood serum. Analysis was performed on corresponding serum samples collected as part of routine diagnosis. Analysis was performed using an immunoturbidimetric assay. Measurements were collected using a Cobas c501 modular analyzer (Roche, West Sussex, United Kingdom). Precinorm protein kits (Roche) were used for both complement C3 and transferrin analysis. The measurement range for C3 was 0.9 to 1.8 g liter^{-1}, and that for transferrin was 2.0 to 3.6 g liter^{-1}.

Statistical analysis. Pairwise comparisons were performed using an unpaired *t* test with Welch's correction. This was calculated using Prism software (v.5.0; GraphPad, La Jolla, CA).

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The authors do not have any commercial or other associations that might pose a conflict of interest.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00272-11/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.1 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 0.1 MB.
Figure S6, DOCX file, 0.1 MB.
Figure S7, DOCX file, 0.1 MB.
Figure S8, DOCX file, 0.1 MB.
Figure S9, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

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