Toxicity in Neuronal Cells Caused by Cerebrospinal Fluid from Pneumococcal and Gram-Negative Meningitis

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To identify neurotoxic factors in meningitis, a neuronal cell line (HN33.1) was exposed to cerebrospinal fluid (CSF) obtained from rabbits with pneumococcal meningitis or *Escherichia coli* meningitis or 2 h and 6 h after meningitis was induced by proinflammatory bacterial products (pneumococcal cell walls, endotoxin). CSF from all types of meningitis induced similar degrees of cytotoxicity. When a soluble tumor necrosis factor (TNF) receptor that completely blocked TNF-mediated toxicity at 10^{-7} M was used, all toxicity in meningitis caused by *E. coli*, endotoxin, or pneumococcal cell wall administration (2 h afterwards) was mediated by TNF. In contrast, CSF from animals with meningitis caused by live pneumococci or pneumococcal cell wall injection (6 h afterwards) retained cytotoxicity in the presence of the TNF receptor. Thus, in established pneumococcal meningitis, but not in the other forms of meningitis, TNF is not the only component toxic in this neuronal cell line.

Brain damage is the most important complication in survivors of bacterial meningitis. The spectrum of sequelae includes mental retardation, learning disabilities, and focal neurologic deficits, indicating that meningitis causes injury to neurons [1, 2]. Little is known about the processes leading to neuronal injury. Both ischemia and toxic effects of factors generated by the inflammation in the subarachnoid space have been implicated in contributing to neuronal dysfunction and injury [3–5]. However, it is difficult to identify the role of potential neurotoxic factors in vivo in a disease that results in highly complex pathophysiologic changes.

To study neuronal injury during meningitis, we developed a new approach that combines in vitro and ex vivo elements. The studies were designed to test the hypothesis that soluble factors generated by the infection and inflammation during meningitis contribute to neuronal injury. We assumed that such neurotoxic factors, in addition to acting directly on neurons, are released into the cerebrospinal fluid (CSF), thus allowing us to use CSF from animals with meningitis as the source. We used a murine neuronal cell line (HN33.1) [6], which shows important neuron-specific characteristics, and a soluble TNF receptor [7] to examine whether infected CSF contains cytotoxic factors. CSF from animals with meningitis induced by both gram-positive and gram-negative live organisms and by proinflammatory bacterial components was examined in the cell culture system.

Material and Methods

Experimental meningitis. Meningitis was induced in New Zealand White rabbits, 2–3 kg, by various stimuli as described below. Animals were anesthetized with an intramuscularly administered mixture of acepromazine, 0.5–1.0 mg/kg (Promace; Aveco, Fort Dodge, IA), ketamine, 35–50 mg/kg (Ketaset; Bristol Laboratories, Syracuse, NY), and xylazine, 2–5 mg/kg (Gemini; Rugby Laboratories, Rockville, NY). The cisterna magna was directly punctured by a 25-gauge butterfly needle, and 0.3 mL of the inoculum was injected into the CSF. After the time intervals indicated below, animals were anesthetized again, and inflamed CSF was collected by a second puncture of the cisterna magna (0.5–1.0 mL of CSF/animal). CSF was immediately centrifuged at 8000 g for 5 min, pooled for groups of 5–8 animals, and stored at −70°C. The severity of meningitis was quantitated in pooled CSF by determining CSF lactate and glucose concentrations [8] with an automatic lactate/glucose analyzer (2300 STAT; Yellow Springs Instruments, Yellow Springs, OH). Sterility of the samples before use in the cell culture system was confirmed by culturing 0.1 mL on blood agar plates.

Live pneumococcal meningitis. *Streptococcus pneumoniae* type 3 was used to produce meningitis [8]. The organism was grown on blood agar plates, harvested in saline, and stored at −70°C. To induce meningitis, the inoculum was thawed and diluted to 10^6 cfu/mL in normal saline. Eighteen hours after intracisternal injection of the inoculum, when animals showed signs of disease (fever, lethargy), a single dose of ceftriaxone (150 mg/kg) was administered through an ear vein to treat meningitis. Three hours later, CSF was collected and processed as described above. In control experiments designed to examine whether in vitro growth of pneumococci in CSF produced prod-

ucts that were toxic to HN33.1 cells, *S. pneumoniae* was grown for 6 h in vitro in normal rabbit CSF. Subsequently, the culture was sterilized by adding 10 μg/mL ceftriaxone for 4 h, followed by centrifugation (8000 g for 10 min) to remove residual bacteria. In a second preparation, cultures were centrifuged (8000 g for 10 min) and pneumococci were then disrupted by vigorous sonication. In both cases, sterility of the preparation was confirmed before the experiments in the cell culture system. In addition, control experiments using filter sterilization of CSF with 0.2-μm filters (Sterile Acrodisc; Gelman Sciences, Ann Arbor, MI) showed no effect of the filtration step on the cytotoxicity induced by CSF from animals with pneumococcal meningitis.

Meningitis induced by pneumococcal cell wall. Pneumococcal cell walls were prepared as described by Tuomanen et al. [9]. Briefly, a rough strain of *S. pneumoniae* (R6) was grown in Todd-Hewitt medium to stationary phase, harvested in saline, and boiled for 15 min to inactivate autolytic enzymes. Bacterial cells were then disrupted using glass beads. The resulting crude cell wall preparation was washed twice in boiling 2% SDS, incubated with DNase, RNase, and trypsin, extensively washed in distilled water, suspended in saline, and stored at −70°C. Cell wall preparation (10–30 μg, equivalent to ∼10⁸ cfu) was injected intracisternally in 0.3 mL; 2 and 6 h later, CSF was collected and processed as described above. In control experiments, the same cell wall preparation was added to the cell culture medium.

Live Escherichia coli meningitis. A K1-positive strain of *E. coli* was used for these experiments. The organism was grown in trypticase soy broth to logarithmic phase, washed, and resuspended in saline, and rabbits were injected intracisternally with 2 × 10⁶ cfu; 14 h later, when animals were clinically ill, antibiotic treatment was initiated with one intravenous dose of ceftriaxone or cefotaxime (150 mg/kg). Three hours later, CSF was collected and processed as described above. In control experiments, supernatants from *E. coli* cultures grown in vitro in CSF were used after sterilization of the culture with 10 μg/mL ceftriaxone and centrifugation (8000 g for 5 min).

Meningitis induced by endotoxin. Endotoxin from rough *Salmonella minnesota* Re 595 was purchased from Sigma (St. Louis). Stock solutions were prepared by suspending 10 mg of endotoxin in 2 mL of 20 mM EDTA, followed by sonification. Aliquots were kept at −70°C. To induce meningitis, stock solution was diluted 1:100 in saline and filter sterilized (0.2-μm filters), and 10 μg of endotoxin was injected intracisternally; 2 and 6 h later CSF pools were generated. The identical endotoxin preparation was also used directly in the cell culture system at a concentration of 30% (vol/vol).

Other reagents. Recombinant human (rh) TNFα was a gift from Genentech (South San Francisco). Stock solutions were made in saline containing 0.2% bovine serum albumin and kept at 4°C. Appropriate final concentrations were prepared in culture medium. Recombinant human soluble TNF receptor consisted of the extracellular portion of the 55-kDa human TNF receptor, as previously described [7, 10]. Recombinant human interleukin-1α (rhIL-1α) was a gift from Hoffmann-La Roche (Nutley, NJ). Stock solutions in distilled water were kept at 4°C, and final concentrations were prepared in culture medium. Glutamate was purchased from Sigma, and solutions were prepared

freshly for each experiment in culture medium. Arachidonic acid was purchased from Sigma and was dissolved in Krebs-Ringer buffer to yield a 1 mM concentration.

Neuronal cell line HN33.1. This cell line was generated by the fusion of hippocampal neurons from young adult mice with a neuroblastoma cell line (gift of B. Wainer, University of Chicago) [6]. HN33.1 cells show important features characteristic of neurons, such as the presence of neuritic processes, neurofilament protein, and membrane excitability typical for hippocampal neurons. In these regards the cell line is distinct from the neuroblastoma parent cell line [6]. HN33.1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 100 units/mL penicillin G and 100 μg/mL streptomycin in tissue culture 96-well microtiter plates (Falcon Primaria; Becton Dickinson, Lincoln Park, NJ) at 37°C in room air containing 5% CO₂. When the cells reached 75%–90% confluence, the culture medium was changed to fresh medium and the cells were exposed to the various stimuli for 48 h. The highest concentration of CSF and bacterial supernatants examined in these experiments was 30% (vol/vol). All experiments were done in triplicate wells, and culture medium alone and native CSF were included as controls.

Cytotoxicity assay. Cytotoxicity was assessed by measuring leakage of lactate dehydrogenase (LDH) into the culture supernatant by a commercial assay (Sigma). Centrifuged medium (100 μL) from each cell culture well was added to 1 mL of reagent solution according to the manufacturer's instructions, and the reduction of NAD resulting from oxidation of lactate to pyruvate was measured at 340 nm on a spectrophotometer (DU-70; Beckmann, Duarte, CA). LDH contained in the culture medium (mean, 59 units/mL) and in CSF-containing medium (mean, 115 units/mL for live pneumococcal meningitis, the pool with the highest LDH content) was subtracted and the net LDH release was then expressed as percentage of control wells (LDH releases in control wells = 100%). Cytotoxicity was routinely confirmed in all experiments by simultaneous microscopic quantitation of dead cells stained with 0.4% trypan blue for 5 min.

Statistics. Results of an individual experiment were calculated as mean ± SD for triplicate wells. Results presented here are mean ± SD of four to eight independent experiments. Comparison between groups was done by Student's *t* test, adjusted for multiple comparisons by the Bonferroni correction. Data that did not show a normal distribution were analyzed by the Mann-Whitney rank sum test.

Results

Characteristics of CSF pools. Infection with either *S. pneumoniae* or *E. coli* produced profound inflammatory changes in the CSF, with polymorphonuclear pleocytosis and markedly increased lactate concentrations and reduced glucose concentrations (table 1). Lactate concentrations were higher in animals infected with *E. coli* than in animals infected with *S. pneumoniae* ($P < .01$), while glucose concentrations were similar. Lactate and glucose concentrations in CSF 2 h after intracisternal injection of pneumococcal cell

Table 1. Lactate and glucose concentrations in CSF pools from rabbits with different forms of bacterial meningitis.

Pool (n)	Lactate (mmol/L)	Glucose (mg/100 mL)
Normal controls (6)	1.7 ± 0.2*	116 ± 4
Pneumococcal meningitis		
Live *Streptococcus pneumoniae* (5)	11.2 ± 1.9†	71 ± 36‡
Cell wall 2 h (4)	2.9 ± 0.8	146 ± 15‡
Cell wall 6 h (4)	8.3 ± 1.5	114 ± 24
Gram-negative meningitis		
Live *Escherichia coli* (3)	14.5 ± 1.6†	70 ± 24‡
LPS 2 h (4)	3.2 ± 0.4	105 ± 20
LPS 6 h (4)	9.7 ± 1.5	80 ± 45‡

NOTE. LPS = lipopolysaccharide, n = no. of CSF pools used.
* $P < .01$ vs. all meningitic CSF.
† $P < .01$.
‡ $P < .01$ vs. controls.

wall or endotoxin showed only mild abnormalities compared with normal control levels (table 1). These CSF pools, however, contain high concentrations of various cytokines, including TNF at $\sim 2 \times 10^{-9}$ M concentration, IL-1, and IL-6 [11–13]. Four hours later, both bacterial stimuli had induced a typical inflammatory response, with CSF pleocytosis and markedly increased lactate concentrations that were similar to those found in animals infected with live organisms (table 1). Pneumococcal cell wall did not cause a reduction in CSF glucose concentrations, while endotoxin did (table 1).

Cytotoxicity induced by pools of infected CSF. CSF from all six types of meningitis produced substantial cytotoxicity in HN33.1 cells when the cells were exposed to culture medium containing 30% CSF (vol/vol) for 48 h. Net LDH release averaged 40.5 units/mL of supernatant in control wells and increased to 311.3 units/mL in wells exposed to CSF from animals with live pneumococcal meningitis. Values for other CSF pools were similar, and LDH release, when normalized for controls, increased by ~600%–900% (table 2). There was no significant difference in the degree of cytotoxicity between the six CSF preparations examined (table 2). Results of LDH measurements were confirmed by examination of the cultures after trypan blue staining, with at least 30%–50% of the exposed cell showing evidence of injury, while <1% of control cells were trypan blue-positive. Native CSF at 30% (vol/vol) and CSF from infected animals at concentrations of <10% (vol/vol) did not lead to increased LDH release or evidence of injury by trypan blue staining, while there was increasing toxicity at concentrations of 15%–30% (vol/vol) of infected CSF. Filter sterilization of the CSF pools did not affect the degree of cytotoxicity.

Effect of soluble TNF receptor on cytotoxicity of meningitic CSF. We found that rhTNFα at concentrations as low as 5 × 10^{-11} M induced significant release of LDH from HN33.1 cells, while maximal LDH release was induced by 5 × 10^{-8}

M. Since CSF from animals with experimental meningitis due to various bacterial stimuli has been shown to contain $\sim 10^{-9}$ M TNFα [11–13], we used a soluble TNF receptor to determine to what extent the cytotoxicity of CSF could be accounted for by the presence of TNF [7]. Experiments with rhTNF showed that between 10- and 100-fold molar excess of the receptor completely blocked the cytotoxicity of rhTNFα (table 2). We therefore used the receptor at 10^{-8} and 10^{-7} M concentrations in the experiments with CSF from rabbits with meningitis. The receptor alone was not toxic when added to the culture. At the higher concentration examined, the receptor completely blocked the cytotoxicity of CSF from animals with meningitis caused by *E. coli*, endotoxin, or injection of pneumococcal cell wall (after 2 h) (table 2). In contrast, cytotoxicity of CSF from animals with *S. pneumoniae* meningitis was only partially blocked by the receptor ($P < .001$ compared with rhTNF) (table 2). Cytotoxicity from CSF obtained 6 h after injection of pneumococcal cell wall was also not completely blocked by the TNF receptor ($P = .01$ vs. rhTNF) (table 2).

Cytotoxicity of bacterial products. Since CSF from animals with pneumococcal but not gram-negative meningitis seemed to contain a factor other than TNF that is cytotoxic for HN33.1 cells, we examined whether various sterile preparations obtained from pneumococcal cultures were toxic to the cell line in vitro. Adding the cell wall preparation used to produce meningitis at 30% (vol/vol) to the cell culture led to an increased release of LDH to 250.1% ± 26.8% of controls ($P < .01$), while concentrations of ≤20% were not toxic. In contrast, CSF in which pneumococci had been grown in vitro and then killed by antibiotics, pneumococci disrupted by sonication, or rough, heat-killed pneumococci (strain R6)

Table 2. Lactate dehydrogenase (LDH) release from HN33.1 cells after exposure to recombinant human tumor necrosis factor (rhTNFα) or CSF from various forms of meningitis in the absence or presence of soluble TNF receptor.

Stimulus	Receptor concentration		
	None (n)	10^{-8} M (n)	10^{-7} M (n)
rhTNFα (10^{-9} M)	534 ± 237 (8)	136 ± 71 (8)	74 ± 20 (4)*
Meningitic CSF (30%)			
Live *Streptococcus pneumoniae*	750 ± 378 (9)	334 ± 152 (9)†	323 ± 122 (6)†
Cell wall 2 h	995 ± 257 (6)	113 ± 38 (6)	80 ± 43 (3)
Cell wall 6 h	779 ± 307 (9)	279 ± 205 (9)*	248 ± 205 (6)*
Live *Escherichia coli*	713 ± 271 (7)	113 ± 41 (7)	108 ± 9 (4)
LPS 2 h	705 ± 445 (4)	57 ± 43 (4)	ND
LPS 6 h	896 ± 396 (7)	106 ± 62 (7)	90 ± 43 (4)

NOTE. Data are % of control. LPS = lipopolysaccharide; n = no. of independent experiments. ND = not done.
† $P < .009$ vs. other groups except cell wall 6 h.
* $P = .01$ (by Mann-Whitney rank sum).

were not toxic at the highest concentrations examined ($\sim 10^7$ cfu/mL, corresponding to the CSF titers in vivo). Endotoxin at 10 μg/mL and CSF in which *E. coli* had been grown in vitro at 30% (vol/vol) concentrations also were not toxic.

Cytotoxicity of other components of infected CSF. Components of infected CSF that can potentially be cytotoxic include cytokines other than TNF (e.g., interleukins), excitatory amino acids (glutamate and aspartate), and arachidonic acid released from neutrophil cell membranes. IL-1α at concentrations up to 10^{-8} M and the excitatory amino acid glutamate at concentrations up to 1 mM were not toxic to HN33.1 cells. Arachidonic acid at 1×10^{-3} M and 5 $\times$ 10^{-4} M led to significant increases in LHD (359.3% ± 11.9% and 230.1% ± 35.3% of controls, respectively; $P <$ .01), while lower concentrations were not toxic.

Discussion

The spectrum of neurologic sequelae caused by bacterial meningitis provides good evidence for the occurrence of neuronal injury during meningitis. Work in animal models has provided insights into the molecular mechanisms of inflammation and into the pathophysiology of the disease [4, 5], but in general animal models have not conclusively demonstrated a causal relationship between these changes and neuronal injury. The main purpose of the present studies was therefore to develop an in vitro approach to examine the process of neuronal injury during bacterial meningitis.

Clinical studies showing a beneficial effect of dexamethasone in children with bacterial meningitis [14, 15] and animal studies indicate that components of the inflammation generated during meningitis contribute to brain injury [16–18]. These observations prompted us to explore in vitro the presence of cytotoxic factors generated during meningitis. Since the primary site of infection and inflammation in meningitis is the subarachnoid space, we examined CSF from animals with meningitis. Ultimately, it will be necessary to show in vivo that cytotoxic factors identified in the CSF during meningitis produce their toxic effects on neurons within brain tissue. However, there are clearly similarities between the disease process in the subarachnoid space and events in the brain. In both animal models and humans with meningitis the inflammatory process is not confined to the subarachnoid space but involves focal areas of the brain, thereby generating inflammatory products directly within brain tissue [19–21]. Furthermore, proteins can diffuse from the CSF into the brain [22]. This diffusion likely explains why the injection of cytokines into the CSF of experimental animals has profound effects on the brain and induces diffuse brain edema [17].

We induced meningitis with both live organisms and products of these meningeal pathogens that are responsible for initiating the inflammatory response during meningitis [9,

23] to examine whether our system detected biologic differences between, for example, pneumococcal and *E. coli* meningitis or between meningitis induced by live organisms and by sterile bacterial products. We were initially surprised that CSF from animals with all forms of meningitis was neurotoxic in vitro to very similar degrees, despite the fact that changes in the CSF formula were much less pronounced 2 h after intracisternal injection of a sterile bacterial stimulus than they were 20 h after induction of meningitis by live organisms. However, the results can be explained by the importance of TNF in this system that used a TNF-sensitive cell line. Cytokines in the CSF peak $\sim$2 h after intracisternal injection of proinflammatory bacterial products, and after 6 h the concentrations are still in a range cytotoxic for HN33.1 cells [11, 12]. In animals with meningitis induced by live organisms, TNF concentrations are especially high shortly after beginning antibiotic therapy, which leads to increased release of TNF through the liberation of bacterial products [11, 24, 25]. Thus, all CSF samples tested in our study contained high TNF concentrations sufficient to induce close to maximal cytotoxicity in the HN33.1 cells.

The use of a soluble 55-kDa TNF receptor proved to be a valuable tool to further examine the cytotoxicity of infected CSF in our in vitro model [7, 10]. The complete blockage of the cytotoxicity of rabbit TNF in inflamed CSF by the human receptor molecule documented that the neutralizing capacity of the receptor was less susceptible to species variations of the TNF molecule than was a mouse monoclonal antibody (provided by Cutter Laboratories, Berkeley, CA), which effectively neutralized rhTNFα in our system but was ineffective in blocking the cytotoxicity of CSF from rabbits with meningitis (data not shown). The results obtained with the receptor indicate that TNF was the only component toxic to the HN33.1 cells in the CSF from animals with meningitis caused by gram-negative stimuli (live bacteria and endotoxin) and early after the induction of meningitis by pneumococcal cell wall. In contrast, significant cytotoxicity remained despite high doses of TNF receptor in CSF from animals with established pneumococcal meningitis. This suggests a fundamentally different neurotoxic potential in pneumococcal than in gram-negative meningitis. It remains to be determined whether this non–TNF-mediated cytotoxicity of pneumococcal meningitis contributes to the high rate of central nervous system injury and mortality observed in pneumococcal meningitis [26–28].

The reasons for the different cytotoxic profiles of pneumococcal and *E. coli* meningitis are unclear. CSF inflammation was not more pronounced in pneumococcal meningitis, and TNF concentrations in CSF during pneumococcal meningitis are not higher than in *E. coli* meningitis (Tureen JH, Lau A, personal communication). Also, the difference apparently is not explained by direct toxicity of components derived from pneumococci. The fact that non–TNF-mediated toxicity could be induced by the cell wall preparation indi-

cates that bacterial products such as pneumolysin or lipo-teichoic acid were not responsible for this cytotoxicity, since they are inactivated during preparation of the cell walls. This is also supported by the lack of toxicity of supernatants of mechanically disrupted or lysed pneumococci. Pneumococcal cell walls showed some toxicity in vitro, but the cytotoxic concentration of these fragments was at least 10 times higher than that in CSF, and CSF obtained 6 h after intracisternal injection of the pneumococcal cell wall but not the 2-h sample with the higher cell wall concentration contained the non–TNF-mediated cytotoxic component. While these results cannot conclusively rule out a role of a pneumococcal factor in the non–TNF-mediated toxicity, they are compatible with the hypothesis that pneumococci, but not gram-negative bacterial stimuli, induce a host factor other than TNF that is cytotoxic in the HN33.1 cells. The nature of this factor is unclear, but several candidates seem unlikely on the basis of our results. IL-1 is one of the cytokines found in the CSF during meningitis [12, 29], and in human macrophages pneumococcal cell walls are more potent stimuli of IL-1 than is endotoxin [30]. However, IL-1 failed to show any cytotoxicity in HN33.1 cells. Excitatory amino acids such as glutamate can cause neuronal injury via N-methyl-D-aspartate receptors [31] and are increased in the CSF during meningitis (unpublished data), but HN33.1 cells are resistant to glutamate. Finally, we examined arachidonic acid because of its known cytotoxic effect on neurons [32, 33], but the concentrations that were cytotoxic to HN33.1 cells far exceeded those that can be expected in infected CSF.

The identification of a cytotoxic factor associated with pneumococcal meningitis poses several interesting questions to be addressed in future studies. The chemical identity of this factor and its specificity for cells of neuronal origin will have to be worked out. Furthermore, it will be important to examine this factor in primary neuronal cultures that may be more indicative of the situation in vivo than the cell line used here. Finally, once the neurotoxic factor is identified, we hope to be able to determine its role in causing neuronal injury in vivo.

Acknowledgment

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