The Free Radical Scavenger α-Phenyl-*Tert*-Butyl Nitrone Aggravates Hippocampal Apoptosis and Learning Deficits in Experimental Pneumococcal Meningitis

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The effect of adjuvant therapy with the radical scavenger α-phenyl-*tert*-butyl nitrone (PBN; 100 mg/kg given intraperitoneally every 8 h for 5 days) on brain injury and learning function was evaluated in an infant rat model of pneumococcal meningitis. Meningitis led to cortical necrotic injury (median, 3.97% [range, 0%–38.9%] of the cortex), which was reduced to a median of 0% (range, 0%–30.9%) of the cortex ($P < .001$) by PBN. However, neuronal apoptosis in the hippocampal dentate gyrus was increased by PBN, compared with that by saline (median score, 1.15 [range, 0.04–1.73] vs. 0.31 [range, 0–0.92]; $P < .001$). Learning function 3 weeks after cured infection, as assessed by the Morris water maze, was decreased, compared with that in uninfected control animals ($P < .001$). Parallel to the increase in hippocampal apoptosis, PBN further impaired learning in infected animals, compared with that in saline-treated animals ($P < .02$). These results contrast with those of an earlier study, in which PBN reduced cortical and hippocampal neuronal injury in group B streptococcal meningitis. Thus, in pneumococcal meningitis, antioxidant therapy with PBN aggravates hippocampal injury and learning deficits.

Bacterial meningitis continues to be a devastating disease with high mortality (5%–40%) and frequent neurologic sequelae. In a study of 130 children, assessed several years after bacterial meningitis, 8.5% had major deficits (IQ < 70, seizures, spasticity, or profound hearing loss) and a further 18.5% showed minor deficits (IQ, 70–80) [1]. The most common causal pathogen of bacterial meningitis is *Streptococcus pneumoniae,* which accounts for about half the cases and has the highest mortality and incidence of sequelae [2–4].

Impaired learning, in both humans and animals, can result from damage to the hippocampus [5–7]. In a recent histopathologic study of humans who died of bacterial meningitis, apoptosis of granular cells in the dentate gyrus was shown [8]. Volumetric measurements of the hippocampus by magnetic resonance revealed unilateral and bilateral hippocampal atrophy in patients who survived bacterial meningitis [9]. Apoptosis of granular cells is a characteristic feature of bacterial meningitis in human disease and in several animal models of meningitis due to the most common pathogens, including *S. pneumoniae,* group B streptococci (GBS), and *Listeria monocytogenes* [8, 10–12].

Neuronal damage during bacterial meningitis is caused by a complex cascade of inflammatory mechanisms, in which the production of reactive oxygen species (ROS) plays an important part. Vascular reactivity, permeability of the blood-brain barrier, and neurotoxicity of excitatory amino acids have been linked to the production of ROS [10, 13–15]. ROS are released by the host from stimulated granulocytes, macrophages, and microglia, and *S. pneumoniae* has been shown to use pyruvate oxidase to produce ROS [16]. Inflammatory cytokines are under the control of transcription factors modulated by ROS [17–19]. In experimental bacterial meningitis, the presence of ROS has been localized to the ventricular and subarachnoid space and along penetrating cortical vessels [10].

Thus far, experimental evidence has identified antioxidants as a promising approach for adjunctive therapy of bacterial meningitis [13, 20]. α-phenyl-*tert*-butyl nitrone (PBN) reacts with ROS to form stable adducts. It is evenly distributed among a wide range of tissues and shows high concentrations in the cerebrospinal fluid (CSF) because of its lipophilicity [21]. PBN is nontoxic at the levels required for efficient scavenging of ROS in the brain [10, 22]. In an infant rat model of GBS meningitis, PBN effectively decreased oxidative injury and attenuated neuronal injury in the cortex and hippocampus [10].

The aim of the present study was 2-fold. First, we evaluated the neuroprotective efficacy of PBN in an infant rat model of meningitis due to *S. pneumoniae,* because PBN proved to be beneficial in GBS meningitis. Second, we determined whether

Received 12 May 2000; revised 5 September 2000; electronically published 8 December 2000.

Presented in part: European Congress on Clinical Microbiology and Infectious Diseases, Stockholm, 28–31 May 2000 (abstract 3/282).

The animal studies were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland, and followed National Institutes of Health guidelines for the performance of animal experiments.

Financial support: Swiss National Science Foundation (32-61654 and NRP-4038-52841); National Institutes of Health (NS-32553 and NS-34028).

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The Journal of Infectious Diseases 2001; 183:247–52

0022-1899/2001/18302-0011$02.00

the extent of hippocampal apoptosis observed in the acute disease correlates with learning function in meningitis survivors treated with PBN or saline.

Materials and Methods

Model of meningitis. Nursing Sprague-Dawley rat pups ($n = 117$) were infected on postnatal day 11 by intracisternal injection with 10 μL of saline containing 6.40 ± 0.14 $\log_{10}$ cfu/mL *S. pneumoniae* (mean ± SD) by means of a 32-gauge needle, as described elsewhere [23, 24]. Infected animals were randomized for treatment with PBN (Calbiochem), 100 mg/kg intraperitoneally every 8 h ($n = 57$), or an equal volume (0.3 mL) of saline intraperitoneally every 8 h ($n = 60$), starting at the time of infection, for a duration of 5 days. Noninfected animals ($n = 38$) were injected intracisternally with 10 μL of sterile pyrogen-free saline and also were randomized to receive PBN ($n = 8$) or saline ($n = 30$), as described above. Eighteen hours after intracisternal inoculation, animals were weighed and assessed clinically. Clinical status was scored as follows: 5, normal motor activity and animal turned upright in <5 s when put on its back; 4, decreased spontaneous activity but still turned upright in <5 s; 3, turned upright in >5 s; 2, did not turn upright; and 1, did not move. To document meningitis, 10 μL of CSF was obtained by puncture of the cisterna magna and was cultured quantitatively. Infected animals then were treated with ceftriaxone (Roche Pharma), 100 mg/kg subcutaneously every 12 h for 4 days.

Histopathology. For histopathologic examination, 52 animals (infected and PBN-treated, $n = 18$; infected and saline-treated, $n = 18$; uninfected and PBN-treated, $n = 8$; uninfected and saline-treated, $n = 8$) were evaluated at 22.5 ± 2.0 h (mean ± SD) after infection, as described elsewhere [23, 25]. The area of cortical brain damage was calculated as a percentage of the total cortex in each section and was expressed as the mean value per animal. Cells with morphologic changes compatible with apoptosis were counted in 3 visual fields (×400) in each of the 4 blades of the dentate gyrus, and the following scoring system was applied: 0–5 cells = 0; 6–20 cells = 1; >20 cells = 2. An average score per animal was calculated from all sections evaluated. All histopathologic evaluations were done by an investigator who was unaware of the clinical, microbiologic, and treatment data for the respective animal.

Assessment of learning function. For the water maze procedure, a round gray tank 1.8 m in diameter (surface, 2.54 m^2) and 0.7 m in height, filled with water at a temperature of 24–26°C to a depth of 48 cm, was used. The water was darkened by the addition of nontoxic food coloring. A video camera was fastened to the ceiling above the center of the pool. Before the test, gross vestibulomotor dysfunction of animals to be assessed in the water maze was excluded by use of a rotating rod. Rats were placed on a foam cylinder (circumference, 22 cm), which was fastened to a motor with adjustable speed and was placed 20 cm above the table surface. Animals had to stay on the cylinder at different speeds (4, 8, 12, and 16 rpm) for ≥10 s, to qualify for the water maze test. For assessment of learning function, swimming patterns of the rats were registered with the video tracking system (Ethovision; Noldus Information Technology). The water surface was virtually divided into 4 inner quadrants and a periphery with a width of 18 cm. An adjustable platform, measuring 16×13 cm and covered with a black, rough mat, was placed in the center of the first quadrant 0.5 cm below the water surface. Four entry zones, situated each between 2 quadrants, were marked outside the pool. Three posters of 0.6×0.3 m with different black-and-white patterns (horizontal stripes, diagonal stripes, and circles) were placed on 3 different walls 0.5 m from the edge of the pool, to serve as visual cues. The room was illuminated by indirect light from the ground.

Thirty-two-day-old survivors of meningitis (PBN-treated, $n = 27$; saline-treated, $n = 31$) and uninfected littermate controls ($n = 22$) were transferred to the experiment room, where they were given 24 h to acclimatize in 12-h light/dark cycles; the light was switched on at 8 a.m. Animals were provided with water and food ad libitum.

During days 1–4, animals performed 5 training trials per day, with the invisible platform in a fixed position throughout the test. Each rat was put into the water with its head directed toward the wall of the tank. If an animal found the platform within 90 s, it was allowed to stay on it for 15 s before it was returned to the cage. If the rat did not find the platform within 90 s, it was guided there by hand and was allowed to stay on it for 15 s. Between trials, animals rested for 45 min. Entry zones were randomized with a dice for each trial. Tracks were recorded by the video tracking system, with 5 samples per second. The total distance moved and the time to reach the platform were documented for each trial.

Statistical methods. Bacterial titers in the CSF were compared with the unpaired Student's t test. Survival curves were analyzed by use of the Kaplan-Meier method. Incidence of seizures, spontaneous death, and cortical injury were compared with Fisher's exact test. The score of hippocampal apoptosis was analyzed with the Mann-Whitney U test. The clinical activity score and weight at 18 h and before the water maze task were compared with 1-way analysis of variance and the Newman-Keuls post hoc test for multiple comparison. Distance and time in the water maze task were calculated with repeated-measures analysis of variance, and pairwise comparison was done with the Tukey-Kramer adjustment. SAS version 8.0 (SAS Institute) software was used. $P \leq .05$ was considered to be significant.

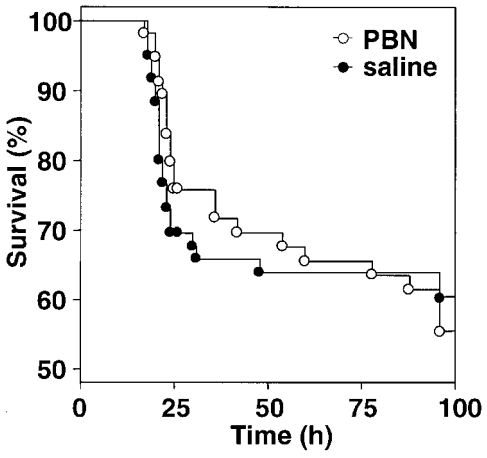

Figure 1. Survival curves for rats with *Streptococcus pneumoniae* meningitis, treated with α-phenyl-*tert*-butyl nitrone (PBN) or saline.

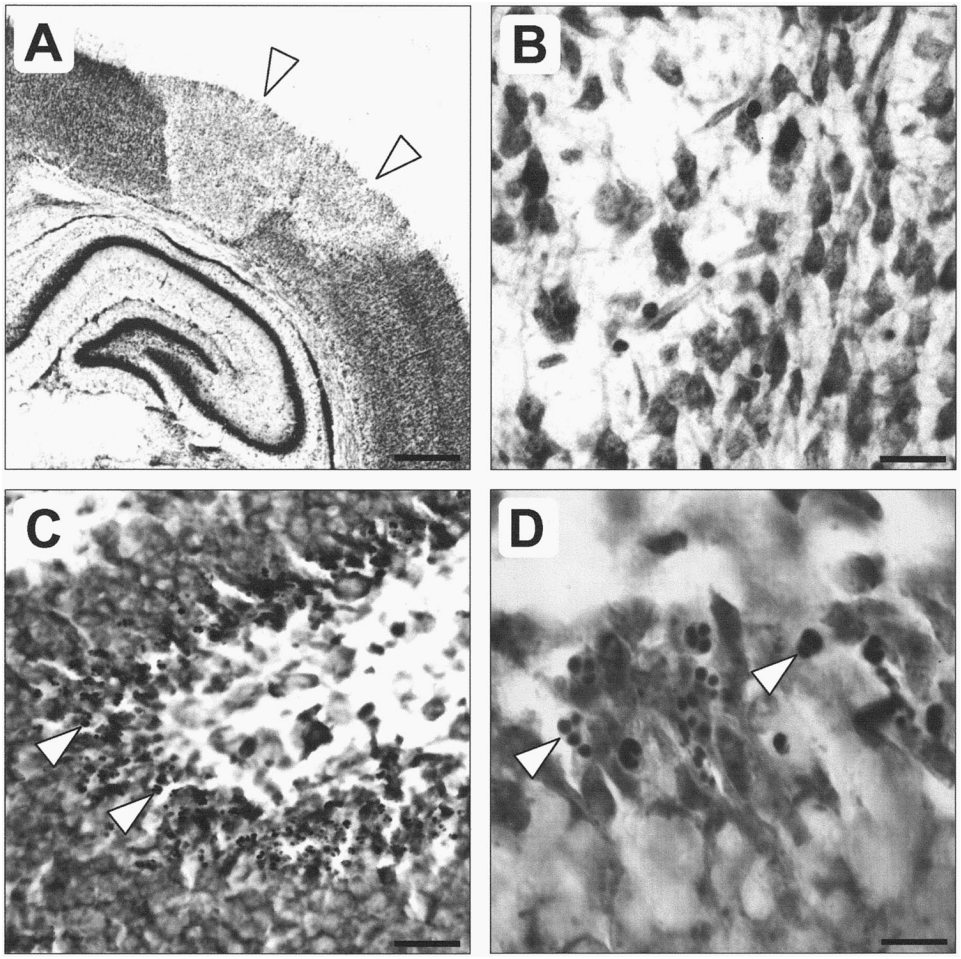

Figure 2. Brain histopathology of infant rats with pneumococcal meningitis 22.5 h after infection (Nissl stain). *A,* Section of cortex and underlying hippocampus, at ×20 magnification. Cortex shows area of reduced neuronal density with wedge-shaped distribution *(arrowheads)*. *Bar,* 0.5 μm. *B,* Border between areas of reduced *(left)* and normal *(right)* neuronal density, at ×400 magnification. *Bar,* 25 μm. *C,* Section of dentate gyrus, at ×200 magnification. *Arrowheads,* Apoptotic neurons in granule cell layer; *bar,* 100 μm. *D,* Apoptotic cells showing typical condensed, fragmented nuclei *(arrowheads),* at ×400 magnification. *Bar,* 25 μm.

Results

Characteristics of disease. By 18 h after infection, all animals had meningitis, as evidenced by lethargy and growth of *S. pneumoniae* from CSF. Treatment with PBN, compared with treatment with sterile saline, had no effect on bacterial titers in CSF (mean ± SD, 7.42 ± 1.06 vs. 7.56 ± 0.87 $\log_{10}$ cfu/mL; not significant). The clinical score was a mean ± SD of 4.1 ± 0.6 for PBN-treated animals versus 3.9 ± 0.6 for saline-treated animals (not significant). Uninfected animals weighed a mean ± SD of 30.3 ± 3.7 g; infected and PBN-treated animals weighed 25.7 ± 3.6 g; and infected and saline-treated animals weighed 26.7 ± 3.2 g ($P < .001$, each infected group vs. uninfected; not significant, infected and PBN-treated vs. infected and saline-treated). Survival curves were similar in the 2 infected groups (figure 1), and spontaneous death occurred in 23 of 57 PBN-treated animals and in 24 of 60 saline-treated animals (not significant). Seizures were observed more frequently in saline- than in PBN-treated animals (15 of 60 vs. 3 of 57; $P < .005$). At the age of 32 days, uninfected control animals weighed a mean ± SD of 138.0 ± 12.7 g; infected and PBN-treated animals weighed 116.6 ± 22.0 g; and infected and saline-treated animals weighed 115.6 ± 24.4 g ($P < .001$, each infected group vs. uninfected; not significant, infected and PBN-treated vs. infected and saline-treated).

Histopathology. Histopathologic aspects of cortical and hippocampal injury are shown in figure 2. Cortical injury, defined as reduced neuronal density with morphologic features of necrosis, was seen in 83.3% of infected and saline-treated animals (median, 3.97% [range, 0%–38.9%] of the cortex). This proportion was reduced to 22.2% of infected and PBN-treated animals (median, 0% [range, 0%–30.9%] of the cortex; $P < .001$; figure 3*A*). In contrast, apoptosis in the dentate gyrus was

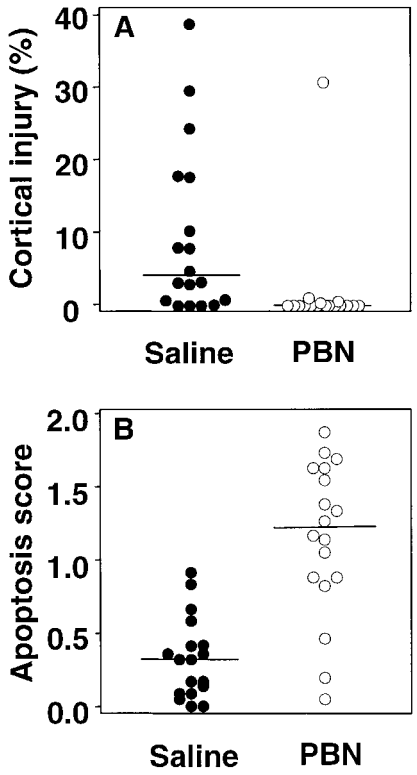

Figure 3. Effect of treatment with radical scavenger α-phenyl-*tert*-butyl nitrone (PBN) on neuronal injury in infant rats with pneumococcal meningitis. *A,* Histopathology at 22.5 ± 2 h after infection showed extensive cortical injury in 83.3% of infected and saline-treated animals (median, 3.97% [range, 0%–38.9%] of cortex) but in only 22.2% of animals treated with PBN (median, 0% [range, 0%–30.9%] of cortex; $P < .001$). *B,* In contrast, treatment with PBN increased apoptotic neuronal injury in hippocampal dentate gyrus, compared with saline (median score, 1.15 [range, 0.04–1.73] vs. 0.31 [0–0.92]; $P < .001$). Histopathology of uninfected animals (not shown) revealed neither cortical nor hippocampal neuronal injury.

more frequent in the infected and PBN-treated animals than in the infected, saline-treated group (median score, 1.15 [range, 0.04–1.73] vs. median score, 0.31 [range, 0–0.92]; $P < .001$; figure 3*B*). Uninfected animals, treated with PBN or saline, showed neither cortical nor hippocampal injury.

Water maze experiment. All animals had normal vestibulomotor function, as evidenced by their ability to stay on the rotating rod. The distance to reach the platform decreased in all 3 groups over time (figure 4), which indicated that all animals had the capacity to learn the location of the platform. Uninfected control animals, however, learned significantly ($P < .001$) more quickly than did the survivors of meningitis. Among those, treatment with PBN, compared with treatment with saline, led to a significantly inferior performance ($P < .02$). The level of performance of uninfected animals on day 2 was approximated by infected and saline-treated animals on day 3 and by infected and PBN-treated animals on day 4 only. Similar results were obtained when we analyzed the time the animals needed to reach the platform (figure 4), with a significant difference among all 3 groups ($P < .001$) and a trend for inferior performance of the infected and PBN-treated group, compared with that of the infected and saline-treated group ($P = .061$).

Discussion

The present study shows a detrimental effect of the radical scavenger PBN in experimental *S. pneumoniae* meningitis, leading to an increase in hippocampal apoptosis and, in consequence, to impaired learning function. This is the first report to relate the severity of hippocampal apoptosis as a conse-

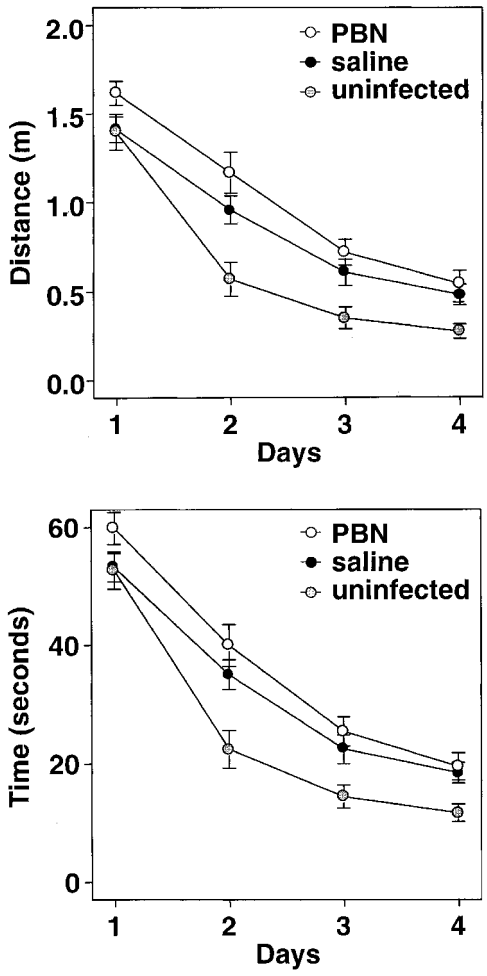

Figure 4. Assessment of learning function, by Morris water maze. Plots of distance and time to reach platform. Data are mean ± SE. Analysis of distance to reach platform showed decrease over time, which indicates that all animals had function to learn location of the platform. Uninfected control animals learned significantly more quickly than did survivors of meningitis ($P < .001$). Among these, treatment with α-phenyl-*tert*-butyl nitrone (PBN) led to inferior performance, compared with treatment with saline ($P < .02$).

quence of bacterial meningitis to reduced learning function in the Morris water maze.

The histopathologic aspect of the cortical damage in meningitis models with *S. pneumoniae* or GBS is usually a well-demarcated wedge-shaped area of injury, which suggests an ischemic mechanism caused by vasculitis and intravascular microthrombi. Production of ROS in the cortex during meningitis seems to be caused by ischemia and reperfusion, which is similar to the mechanisms in other brain diseases, such as brain concussion or stroke, in which ROS are thought to be mediators of injury [26–29]. Treatment with PBN, as well as other types of antioxidants, proved to be beneficial in preventing microvascular changes and cortical necrotic injury in various animal models of meningitis, independent of the infecting pathogen [20, 23, 30, 31].

The pathways that lead from bacterial invasion of the central nervous system to hippocampal neuronal apoptosis in bacterial meningitis are not well understood. The fact that PBN is neuroprotective for the hippocampus in GBS meningitis, whereas it is neurotoxic in the present study of *S. pneumoniae* meningitis, suggests that pathogens may interact via different pathways at the level of cellular signaling and that, depending on the precise nature of that interaction, scavenging of ROS by PBN favors or attenuates apoptosis.

Two hypotheses may explain our observations. First, bacterial pathogens seem to interact differently with ROS. In our present study, CSF titers of *S. pneumoniae* were similar in the PBN and saline treatment groups, which indicates that their growth remains unaffected by the presence of a potent antioxidant in vivo. In contrast, the CSF titer of GBS in saline-treated rats was 100-fold lower than that in PBN-treated animals at 18 h after infection, which suggests that ROS play a role as a host defense mechanism against GBS [10]. Recently it was shown that *S. pneumoniae* possess a distinct mechanism that decreases measurable superoxide production during incubation with stimulated neutrophils [32, 33]. The preservation of a nonoxidative environment, for example, through production of superoxide dismutase, might prevent the deactivation of the *S. pneumoniae*–specific virulence factor pneumolysin [34, 35]. Moreover, apoptotic neuronal death involves several types of cellular signaling mechanisms—such as glutamate receptors, protein kinases, and transcription factors—which are all sensitive to the redox status of the cell [36, 37]. Thus, combination of antioxidant treatment and the presence of *S. pneumoniae* in our model may create a reductive environment that favors virulent properties of *S. pneumoniae* and can directly lead to neuronal death itself [37].

The second hypothesis relies on a direct pathogen-specific signaling pathway via the recently discovered human toll-like receptor (TLR). This is suggested by the finding that the human TLR-2 is a signaling receptor for *S. pneumoniae* but not for GBS [38, 39]. Human TLRs are transmembrane proteins that, among other functions, are known to mediate translocation and activation of NF-κB by gram-positive bacteria. PBN blocks the predominantly antiapoptotic effect of NF-κB activation [40, 41] and may hence shift the balance from antiapoptotic to proapoptotic signaling. Apart from NF-κB activation, TLR-2–gated discrimination among different pathogens may account for alternate signaling pathways with different susceptibility to ROS. Discrimination among specific pathogens has been shown elsewhere by the documentation of pathogen-dependent patterns of cytokine activation in bacterial meningitis [42]. Thus, the TLR pathway may ultimately determine the pro- or antiapoptotic effect of therapeutic intervention with antioxidants. These opposite effects of the same agent depending on the pathogen strongly suggest a critical interplay between microbial and host factors, which are currently under investigation. We suspect that the elucidation of these processes will lead to the identification of novel factors important in the pathogenesis of bacterial infections.

Neither increased nor decreased apoptosis has been observed with other antioxidants given during *S. pneumoniae* meningitis [23], which can be explained by the shorter circulatory half-life and lower blood-brain barrier passage of these drugs, compared with that of PBN.

In conclusion, we found that treatment with the radical scavenger PBN during *S. pneumoniae* meningitis in rat pups attenuated cortical necrosis but increased apoptotic neuronal death in the hippocampus. This is in contrast to what was seen in GBS meningitis, in which PBN protected neurons from both forms of injury. Increased hippocampal damage by PBN led to further decrease in learning function of survivors in the Morris water maze. Antioxidant treatment is therefore not uniformly beneficial in bacterial meningitis. Pathogen- and host-related mechanisms that influence the redox status and transcription factors of neuronal cells may be involved.

Acknowledgments

We thank Philipp Joss and Oliver Schütz (Institute for Infectious Diseases, University of Bern, Bern, Switzerland) for technical support.

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