Antioxidants Attenuate Microvascular Changes in the Early Phase of Experimental Pneumococcal Meningitis in Rats

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Background and Purpose: We tested in a rat meningitis model 1) whether pneumococcal cell wall components are capable of producing changes in regional cerebral blood flow, brain water content, and intracranial pressure similar to those we have already observed after intracisternal inoculation of live pneumococci and 2) whether antioxidants would modulate these alterations in the early phase of meningitis.

Methods: Regional cerebral blood flow as measured by laser Doppler flowmetry and intracranial pressure were monitored continuously for 4 hours after intracisternal challenge. Brain edema formation was assessed by brain water content determinations. We investigated the following groups: rats challenged intracisternally with the whole intact pneumococcal cell wall (n=7) or the pneumococcal cell wall hydrolyzed by the M1-muramidase (n=7); rats injected intracisternally with phosphate-buffered saline (n=6); rats pretreated intravenously with superoxide dismutase conjugated with polyethylene glycol (10,000 units/kg) and injected intracisternally with cell wall components (n=5) or phosphate-buffered saline (n=6); rats injected intracisternally with phosphate-buffered saline and pretreated intravenously with superoxide dismutase (10 mg/kg per hour) and injected intracisternally with cell wall components (n=6) or phosphate-buffered saline (n=7).

Results: Both pneumococcal cell wall preparations produced a significant increase in regional cerebral blood flow, intracranial pressure, and brain water content. Conjugated superoxide dismutase as well as deferoxamine prevented the increase in intracranial pressure and brain water content. In addition, the increase in regional cerebral blood flow as observed in untreated, cell wall-challenged rats (baseline, 100%; 183.1 ± 12.3% after 4 hours, mean ± SEM) was significantly attenuated by administration of both conjugated superoxide dismutase (136.6 ± 14.1%) and deferoxamine (149.8 ± 8.2%) (p < 0.05). Polyethylene glycol-conjugated superoxide dismutase alone produced an increase in regional cerebral blood flow (125.8 ± 8.7% after 4 hours). We found that polyethylene glycol per se accounts for this action.

Conclusions: These data show that pneumococcal cell wall components containing teichoic acid produce changes in regional cerebral blood flow, intracranial pressure, and brain water content and that oxygen radicals contribute to these pathophysiological alterations in the early phase of experimental pneumococcal meningitis.

KEY WORDS • cerebral blood flow • free radicals • meningitis • superoxide dismutase • rats

Despite continued improvement in antibiotic therapy and intensive care medicine the mortality rate and severe sequelae of bacterial meningitis remain high. Pneumococcal meningitis, which is the most frequent bacterial meningitis in adults, still has a fatality rate of approximately 30%. Major determinants for the prognosis are intracranial complications including cerebrovascular complications, brain edema, and increased intracranial pressure (ICP). Clinical and neuropathological studies have indicated that cerebral vessels may be involved in the inflammatory process, thus leading to the development of cerebral infarctions. Alterations in cerebral blood flow (CBF) during meningitis have been reported in humans. Cerebrovascular involvement has also been demonstrated in animal models of bacterial meningitis. An increase in regional cerebral blood flow (rCBF) was detected during the early phase of experimental bacterial meningitis, whereas in advanced stages of the disease CBF was reduced. Morphological alterations of the blood-brain barrier were found in bacterial meningitis in the rat by use of electron microscopic techniques. The complex pathophysiological mecha-
Mechanisms of the major intracranial complications, i.e., cerebrovascular complications, brain edema, and increased ICP, are not completely understood. A number of phenomena have been observed during experimental bacterial meningitis, some or all of which may contribute to the ultimate brain injury. These factors include leukocytes and their products,\textsuperscript{10} endothelial adhesion of leukocytes,\textsuperscript{11} cytokines,\textsuperscript{12-14} cyclooxygenase metabolites,\textsuperscript{5,15} and platelet-activating factor.\textsuperscript{16}

Reactive oxygen intermediates have been implicated as mediators of brain injury in a variety of conditions other than meningitis including cerebral ischemia/reperfusion injury,\textsuperscript{17-20} experimental fluid/percussion brain injury,\textsuperscript{21} cold-induced brain edema,\textsuperscript{22} cerebral arteriolar abnormalities after acute hypertension,\textsuperscript{23} and inflammation.\textsuperscript{24} Therefore, we investigated whether free radical scavengers may alter the microvascular changes, the increase in ICP, and brain water content in experimental pneumococcal meningitis. In this study we used the free radical scavengers polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and deferoxamine. PEG-SOD is a scavenger of superoxide anion and has a circulatory half-life of more than 30 hours,\textsuperscript{25} which is substantially longer than that of native SOD (8 minutes in rats).\textsuperscript{26} Deferoxamine is a powerful iron chelator and therefore inhibits iron-dependent free radical production.

Pneumococcal cell wall components have been shown to induce meningeal inflammation in a rabbit model.\textsuperscript{27} In this study, we investigated the capacity of pneumococcal cell wall components to produce meningeal inflammation and similar changes in rCBF, ICP, and brain water content as observed after intracisternal injection of live pneumococci in the rat.\textsuperscript{6}

Materials and Methods

The meningitis model used in the present study was previously described in detail.\textsuperscript{8} Briefly, the experiments were carried out in 64 male Wistar rats weighing 250–350 g. Rats were anesthetized with 100 mg/kg thiobutabarbital (Inactin) intraperitoneally. Anesthesia was maintained by supplementary administration of thiobutabarbital as required. The rats were tracheotomized and artificially ventilated (small animal ventilator model 683; Harvard Instrument Co., South Natick, Mass.). Cannulas were inserted into the left femoral artery for blood pressure monitoring (via a Statham P23 pressure transducer) and blood sampling and into the left femoral vein for fluid and drug administration. Serial blood samples for determination of Po\textsubscript{2}, Pco\textsubscript{2}, pH (AVL gas check, model 940), and hematocrit (hematocrit centrifuge, Hettich, Freiburg, FRG) were collected. Body temperature was maintained at 38°C with an adjustable heating pad. The rats were placed in a stereotaxic head frame, the cranium was exposed via a midline incision, and a burr hole was drilled in the occipital bone. A catheter connected to a Statham P23 pressure transducer was inserted into the cisterna magna. In the right parietal bone, a craniotomy with a diameter of 5 mm was made for the placement of the laser Doppler probe (model BPM 403; TSI Inc., St. Paul, Minn.), which was fixed with a stainless steel cap. The dura was left intact in all preparations. Laser Doppler flowmetry permits on-line measurements of blood flow changes compared with a baseline value of 100%.\textsuperscript{28} Mean arterial blood pressure (MABP), ICP, end-expiratory CO\textsubscript{2} (measured by an infrared CO\textsubscript{2} analyzer, model 2200; Heyer, Bad Ems, FRG), and rCBF were continuously recorded on a multichannel paper strip recorder (model BD 101; Kipp & Zonen, Delft, Holland) for 4 hours after intracisternal challenge. In addition, these parameters were transferred to a personal computer system and analog digitally converted for signal processing. Because of the fluctuation of the laser Doppler flowmetry signal, the data were averaged every 30 seconds during the experiment. In our previous experiments using live pneumococci, the increase in rCBF reached a plateau 4–6 hours after infection with only slight fluctuations in the mean values.\textsuperscript{6} Therefore, the current investigations were limited to the observation period 4 hours after intracisternal injection of pneumococcal cell walls. When a stable baseline of rCBF and ICP was achieved for 30 minutes, 75 μl of cerebrospinal fluid was removed through the intracisternal catheter.

Methods for the two different preparations of pneumococcal cell walls used in this study were previously described in detail: 1) highly purified intact pneumococcal cell wall containing the choline teichoic acid (PCW) and 2) pneumococcal cell wall solubilized by the M1-muramidase (PCW-M). This preparation contains the equivalent of wall material in the form of disaccharide peptides and their oligomers with and without attached choline teichoic acid. Both preparations had a pH of 7.4.

We investigated the following groups: group 1: rats challenged intracisternally with 75 μl of 200 μg PCW (n=7); group 2: rats injected intracisternally with 75 μl of 200 μg PCW-M (n=7); group 3: rats injected intracisternally with 75 μl phosphate-buffered saline (PBS; controls, n=6); group 4: rats pretreated with an intravenous bolus injection of PEG-SOD (10,000 units/kg i.v.; Sigma Chemie, Deisenhofen, FRG) and injected immediately thereafter intracisternally with 200 μg PCW-M (n=5); group 5: rats pretreated with an intravenous bolus injection of PEG-SOD (10,000 units/kg i.v.) just before intracisternal injection with PBS (n=6); group 6: rats continuously treated with an intravenous infusion of deferoxamine mesylate (10 mg/kg per hour i.v.; Sigma) and injected intracisternally with 200 μg PCW-M (n=6); group 7: rats continuously treated with an intravenous infusion of deferoxamine mesylate (10 mg/kg per hour i.v.) and injected intracisternally with PBS (n=7). Because we found an effect of PEG-SOD alone on rCBF, we investigated additional groups including rats pretreated intravenously with PEG (10% solution, 1.2 ml/kg, Sigma) and injected intracisternally with PBS (group 8; n=5), and rats continuously treated intravenously with free SOD (22,000 units/kg per hour from bovine erythrocytes, Sigma) and injected intracisternally with PBS (Group 9; n=6).

At 4 hours after intracisternal inoculation the rats were killed by exsanguination. Their brains were removed, weighed in a glass dish, and dried in a stove for 16 hours at 130°C to a stable weight. Brain water content was calculated by (wet weight–dry weight)/wet weight×100.

We examined separate groups of animals to detect the capacity of pneumococcal cell wall components to induce meningeal inflammation in the rat. The brains of
rats injected with PCW (n=3), PCW-M (n=3), and PBS (n=3) were examined histologically. Coronal slices 5-mm thick were obtained subsequent to immersion in 4% neutral buffered formaldehyde solution. Paraffin sections, 5-μm thick, of each tissue block were stained by hematoxylin and eosin.

The following groups were compared for rCBF, ICP, and brain water content using one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparisons: groups 1-3; 3-5; 3, 6, and 7; 3, 5, 8, and 9. rCBF and ICP data were compared across time from the point of intracisternal injection every half hour for 4 hours. One-way ANOVA with repeated measures was used to identify changes of rCBF and ICP within each group. A value of p<0.05 was regarded as significant. Data are presented as mean±SEM.

Results

The physiological parameters were within normal ranges in all groups (data not shown). PEG-SOD and deferoxamine caused a significant decrease in MABP (p<0.05) within 4 hours after administration: group 4, 105.4±4.3 mm Hg at baseline, 95.8±5.0 mm Hg at 4 hours; group 5 103.5±2.4 mm Hg at baseline, 88.2±4.2 mm Hg at 4 hours; group 6 115.0±3.3 mm Hg at baseline, 92.2±2.9 mm Hg at 4 hours; group 7, 118.3±4.3 mm Hg at baseline, 106.1±4.3 mm Hg at 4 hours.

Histological examination showed meningeal polymorphonuclear inflammation in both groups of rats inoculated with either PCW or PCW-M. The inflammatory infiltrates were slight and mainly located in the leptomeningeal space of the posterior fossa and the base of the cerebrum. In one PCW-injected rat some inflammatory cells were also found in the cerebellar interhemispheric fissure. This is in accordance with previous findings using live pneumococci illustrating slight meningeal inflammation at 4 hours after intracisternal challenge and marked infiltrates 6-14 hours after injection. In PBS-injected rats no meningeal inflammation was detected.

rCBF and ICP did not change in PBS-injected rats (group 3) throughout the experiment. There was a significant increase in rCBF and ICP in rats injected with either PCW or PCW-M (Figure 1). rCBF increased significantly to 118.6±6.5% as early as 0.5 hours after inoculation of PCW (p<0.05 compared with controls, 97.7±3.5%; Figure 1). rCBF also began to increase 0.5 hours after inoculation in the rats challenged with PCW-M and differed significantly from the controls at 1 hour after inoculation (p<0.05, 123.6±6.9% compared with controls, 100.2±4.3%). At 4 hours after inoculation, rCBF increased to 150.1±12.0% in the rats challenged with PCW and to 183.1±12.3% in the rats challenged with PCW-M (p<0.05 compared with controls, 97.8±4.6%). The rCBF at 4 hours after inoculation was significantly higher in the rats inoculated with PCW-M than in the rats challenged with PCW (p<0.05). There was a significant increase in ICP from 2.2±0.6 mm Hg (baseline) to 8.2±1.1 mm Hg at 4 hours after injection of PCW and from 2.8±0.4 mm Hg to 9.8±1.1 mm Hg at 4 hours after intracisternal injection in rats challenged with PCW-M (p<0.05 compared with controls). Brain water content was significantly elevated in rats challenged with PCW (79.24±0.06%) and in rats inoculated with PCW-M (79.33±0.07%) compared with PBS-injected rats (78.89±0.03%; p<0.05).

The increase in ICP, as observed in untreated, PCW-M-challenged rats, was inhibited by PEG-SOD (Figure 2). In addition, the increase in brain water content was prevented by PEG-SOD in PCW-M-challenged rats (79.02±0.07%; p<0.05 compared with 79.33±0.07% in untreated rats challenged with PCW-M and compared with 78.92±0.09% in rats pretreated with PEG-SOD and injected intracisternally with PBS). The increase in rCBF as seen in untreated, PCW-M-challenged rats was greatly attenuated by PEG-SOD (Figure 2). At 4 hours after intracisternal injection, rCBF increased to 136.6±14.1% (p<0.05 compared with 183.1±12.3% in untreated, PCW-M-challenged rats).

We found an increase in rCBF from 100% (baseline) to 125.6±8.7% at 4 hours in rats pretreated with intravenous PEG-SOD and challenged intracisternally with PBS (group 5; Figure 3). Likewise, PEG alone caused a significant increase in rCBF (136.4±13.3% at 4 hours after intracisternal injection, Figure 3). There was no change in rCBF in rats injected intracisternally with PBS (97.8±4.6% at 4 hours after intracisternal injection) and in rats treated with free SOD and injected...
intracisternally with PBS (99.9±4.3% at 4 hours after intracisternal injection). There was no effect of PEG-SOD, PEG, or free SOD on ICP (Figure 3). In addition, brain water content did not significantly differ in rats pretreated with PEG-SOD and challenged intracisternally with PBS (78.92±0.09%), rats treated continuously with free SOD and injected intracisternally with PBS (79.01±0.03%), and rats injected intracisternally with PBS (78.89±0.03%; p>0.05).

The increase in ICP, as observed in untreated, PCW-M-challenged rats, was prevented by deferoxamine (Figure 4). Brain water content tended to be lower in rats treated with deferoxamine and injected with PCW-M (78.92±0.27%) than in untreated, PCW-M-challenged rats (79.33±0.07%; p>0.05). At 4 hours after intracisternal injection, rCBF data in rats treated with deferoxamine and injected intracisternally with PCW-M (149.8±8.2%) differed significantly from those in rats treated with deferoxamine and injected intracisternally with PBS (109.3±7.6%) and those in rats untreated and challenged intracisternally with PCW-M (183.1±12.3%; Figure 5). Deferoxamine alone did not affect rCBF, ICP, or brain water content (group 7).

Discussion

There are two major findings in this study. First, similar to live pneumococci, intracisternal injection of pneumococcal cell wall components in a rat meningitis model also caused alterations in rCBF, brain water content, and ICP. Second, both antioxidants used in this study greatly attenuated these changes. These findings indicate that microvascular changes and increased ICP.
develop without the necessity of live pneumococci, and they may have clinical relevance. Despite progress in antibiotic therapy, mortality and morbidity due to pneumococcal meningitis have not decreased during the last decades. This is perhaps not surprising because the antibiotics most frequently used for the chemotherapy of meningitis are known to induce autolysis, resulting in the release of inflammatory cell components. Improvements of antibiotics so that they retain their effect without lysis may substantially improve the prognosis of the disease. It may also be rewarding to identify agents capable of intervening with mediators that are believed to play a key role in secondary brain damage caused by brain edema, vascular complications, and increase in ICP.

In these experiments we used the antioxidants PEG-SOD and deferoxamine. Superoxide anion radical and hydrogen peroxide may interact via the iron-catalyzed Haber-Weiss reaction to produce the highly active hydroxyl radical, which, in turn, may initiate lipid peroxidation and cellular injury. SOD inactivates superoxide anion radical to produce hydrogen peroxide, thus preventing the generation of hydroxyl radical by removing one reagent of the Haber-Weiss reaction. In addition, SOD may prevent the formation of toxic peroxynitrite from superoxide and nitric oxide. Deferoxamine is a powerful chelator of iron (III) and therefore inhibits iron-dependent free radical reactions, in particular the formation of hydroxyl radical via the Haber-Weiss reaction. In addition, deferoxamine was shown to inhibit peroxynitrite-mediated oxidation via a direct reaction between deferoxamine and peroxynitrite rather than iron chelation. Other mechanisms of deferoxamine as shown in vitro include binding of other metal ions, reaction with superoxide, and influence on eicosanoid synthesis. However, these effects seem to be of no relevance in vivo in the dosages commonly used in animal models. The action of deferoxamine was investigated in several animal models of tissue injury. It has been shown to be beneficial in several conditions including allergic encephalomyelitis in the rat, cold-induced brain edema, and cardiac arrest in the rat.

We found that both PEG-SOD and deferoxamine prevented the increase in brain water content and ICP that was observed in the untreated, PCW-M-challenged rats. Whereas the increases in ICP and brain...
water content were completely blocked, the increase in rCBF was significantly attenuated by these antioxidants. In infected rats pretreated with PEG-SOD, there was an increase in rCBF to 136.6±14.1% at 4 hours after intracisternal challenge. Possible explanations for this finding might be the fact that PEG-SOD was not able to completely scavenge the oxygen radicals or an effect of PEG-SOD alone on rCBF. Both the native copper-zinc SOD and PEG-SOD may be incapable of crossing the blood–brain barrier sufficiently because of their large size and therefore may be unable to reach the site of radical production.36,37 In vitro 4 hours were required for a significant uptake of PEG-SOD into cultured endothelium.38 We believe that the most likely explanation for the increase in rCBF in PEG-SOD pretreated, infected rats is the effect of PEG-SOD alone on rCBF, which was demonstrated in our experiments (Figure 3). Our results suggest that PEG per se accounts for the effect of PEG-SOD on rCBF. In a recent study in piglets, Helfaer et al39 demonstrated a higher CBF after intravenous PEG administration compared with saline, as measured by the microsphere technique.

Deferoxamine significantly modulated the increase in rCBF in infected rats. The fact that deferoxamine did not completely prevent changes in rCBF may be explained by the production of radicals such as superoxide anion, which are not scavenged in vivo by deferoxamine or by too low a dosage, as used in this study. A higher dosage of deferoxamine (30 mg/kg i.v. bolus injection and 15 mg/kg per hour i.v.) was initially used in a group of four animals (data not shown). However, we found a serious depressive effect of deferoxamine on the MABP of four animals (data not shown). However, we found a serious depressive effect of deferoxamine on the MABP of four animals (data not shown). However, we found a serious depressive effect of deferoxamine on the MABP of four animals (data not shown). However, we found a serious depressive effect of deferoxamine on the MABP of four animals (data not shown).

The definite site of radical generation during bacterial meningitis is still unknown. Our results indicate that both PEG-SOD and deferoxamine gain access to the site of oxygen radical production. Oxygen-derived free radicals may be generated by a variety of biochemical pathways, including the NADPH oxidase–dependent reaction within phagocytic cells, the xanthine oxidase pathway, and the cytochrome P-450 pathway.28

The suppressing effect of PEG-SOD on rCBF, ICP, and brain water content in pneumococcal cell-wall-induced meningitis is in accordance with our findings in previous experiments using free SOD in meningitis induced by live pneumococci.6 Taken together, these studies support the hypothesis that oxygen-derived free radicals are major mediators of changes in rCBF, ICP, and brain water content during the early phase of pneumococcal meningitis. Further studies are warranted to investigate the effect of antioxidants on meningeval inflammation and to detect their possible influence in later stages of the disease.

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**Editorial Comment**

Pneumococcal meningitis remains a serious and often fatal disease despite effective antimicrobial therapy. The precise reasons for this high mortality are not well understood. Increased vascular permeability, brain edema, and increased intracranial pressure may contribute to the adverse outcome from this disease.

Activated leukocytes are a well-known source of oxygen radicals. These radicals induce vascular injury in cerebral vessels with consequent vasodilation, increased vascular permeability, and edema. In the accompanying article, Pfister and colleagues show that antioxidant therapy is effective in ameliorating the changes in regional cerebral blood flow, intracranial pressure, and brain water content induced by pneumococcal cell wall components. These findings provide an experimental basis for possibly testing the effectiveness of antioxidant therapy in human pneumococcal meningitis.

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