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Pneumococcal meningitis in the rat: evaluation of peroxynitrite scavengers for adjunctive therapy

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Abstract

We evaluated the effect of different peroxynitrite scavengers for adjunctive therapy of experimental bacterial meningitis. Twenty hours after intracisternal injection of *Streptococcus pneumoniae*, rats were treated with ceftriaxone [100 mg/kg intraperitoneal (i.p.)] and either urate (300 mg/kg i.p.), Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP, 15 mg/kg i.p.), ascorbate (100 mg/kg i.p.), or urate (300 mg/kg i.p.) + ascorbate (100 mg/kg i.p.). Six hours after initiation of treatment, the cerebrospinal fluid (CSF) pleocytosis was significantly ($p < 0.05$) reduced by urate (8697 ± 1526 cells/μl) and MnTBAP (8542 ± 4059 cells/μl) vs. ceftriaxone alone ($15{,}793 \pm 3202$ cells/μl). Brain concentrations of proinflammatory cytokines [interleukin-1β (IL-β), interleukin-6 (IL-6), and macrophage inflammatory protein-2 (MIP-2)] were also reduced by urate and MnTBAP. The intracranial hypertension was significantly reduced by MnTBAP (14.0 ± 5.4 mm Hg), but not by urate (25.5 ± 7.1 mm Hg) vs. ceftriaxone alone (22.5 ± 5.9 mm Hg). Ascorbate alone had no effect on CSF pleocytosis ($15{,}775 \pm 7058$ cells/μl), intracranial pressure (25.6 ± 8.8 mm Hg), and brain cytokine concentrations. However, the combination of urate and ascorbate was as effective as MnTBAP (CSF pleocytosis: 5392 ± 4232 cells/μl, intracranial pressure: 13.3 ± 6.9 mm Hg).

Keywords: Meningitis; Oxidative stress; Peroxynitrite; Urate; MnTBAP; Ascorbate

1. Introduction

Fifty years after the advent of antibiotics for clinical use, pneumococcal meningitis still has very high rates of morbidity (up to one-third of survivors are left with significant neurologic sequelae) and mortality (20–30%) (Schuchat et al., 1997). The unfavourable clinical outcome is often due to intracranial complications including cerebrovascular insults, raised intracranial pressure, hydrocephalus, and brain edema (Pfister et al., 1993). In order to improve the prognosis of pneumococcal meningitis, adjunctive therapeutic regimens are needed. Over the last decade, there has been a substantial body of work implying that reactive oxygen species, such as superoxide anion, hydrogen peroxide, or hydroxyl radicals play a central role in the development of intracranial complications and brain damage in experimental bacterial meningitis (reviewed in (Koedel and Pfister, 1999).

Recently, we demonstrated that reactive nitrogen species (peroxynitrite) are important mediators of meningitis-associated pathophysiological changes: nitrotyrosine residues on proteins (a marker for the formation of reactive nitrogen species, such as peroxynitrite) were detected immunohistochemically in the leptomeninges, leptomeningeal and penetrating cortical blood vessels and inflammatory cells in the brains of rats with pneumococcal meningitis and patients with bacterial meningitis (Kastenbauer et al., 1999, 2002). Pretreatment with the peroxynitrite scavenger urate attenuated meningeal inflammation, blood–brain barrier disruption, and intracranial hypertension (Kastenbauer et al., 1999, 2001). Thus, peroxynitrite is a promising target for adjunctive therapy of bacterial meningitis because it is highly cytotoxic by numerous mechanisms, including tyrosine nitration (affecting cellular signaling), lipid peroxidation (causing loss of membrane function and integrity), release of interleukin-8 (attracting neutrophils), and activation of matrix metalloproteinases (contributing to blood–brain barrier disruption) (Simon and Beckman, 2002).

In the present study, we evaluated three different peroxynitrite scavengers [urate, Mn(III)tetrakis(4-benzoic acid)-

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porphyrin (MnTBAP), and ascorbate], each in combination with the antibiotic ceftriaxone, for adjunctive therapy of severe pneumococcal meningitis in the rat. Urate, which comprises 30–65% of the total peroxyl radical-scavenging ability of human plasma (Wayner et al., 1987), has been shown to be very effective in detoxifying both hydroxyl radicals and peroxynitrite, while not reacting with the weaker oxidants hydrogen peroxide or superoxide anion (Hooper et al., 1998). Furthermore, the increased oxidative degradation of urate to allantoin during bacterial meningitis demonstrates its antioxidative action in this disease (Kastenbauer et al., 2002). In vitro studies have shown synergistic effects of urate with other antioxidants, such as ascorbate (Aruoma and Halliwell, 1989; Becker, 1993), which is the dominant antioxidant in cerebrospinal fluid (CSF) (Rice, 2000) and a peroxynitrite scavenger itself (Kirsch and de Groot, 2000). Mn(III)tetrakis MnTBAP belongs to a novel class of catalytic antioxidants, the metalloporphyrins; it is known to act as a peroxynitrite decomposition catalyst and superoxide dismutase mimetic (Patel and Day, 1999).

2. Material and methods

2.1. Rat model of pneumococcal meningitis

We used a well-characterized rat model of pneumococcal meningitis, which was previously described in detail (Lorenzl et al., 1996). Adult male Wistar rats (270–350 g) were anesthetized with halothane (Hoechst, Frankfurt, Germany), and meningitis was induced by transcutaneous intracisternal injection of 150 μl phosphate-buffered saline (PBS, pH 7.4) containing 10^6 colony forming units ml^{-1} of *Streptococcus pneumoniae* type 3. Controls were injected with 150 μl sterile PBS. Each rat was put into an individual cage, allowed to wake up, and fed with a standard diet and water ad libitum. Twenty hours after infection, clinical signs of meningitis (weight loss, obtundation, piloerection, seizures, reduced motor skills, reduced motor activity) were evident in infected animals, but not in uninfected controls. Then, rats were treated with 100 mg/kg intraperitoneal (i.p.) ceftriaxone (Rocephin, Hoffmann–La Roche, Germany) and with or without the respective adjunctive agent which was applied simultaneously (experimental groups, see Table 1). Six hours after initiation of antibiotic therapy, rats were anesthetized with 100 mg/kg intraperitoneal (i.p.) thiopental (Trapanal, Byk Gulden, Germany), tracheotomized and artificially ventilated with a small animal ventilator (Model Ap-10, Effenberger, Pfaffing, Germany). Body temperature was monitored with a rectal thermometer and kept at

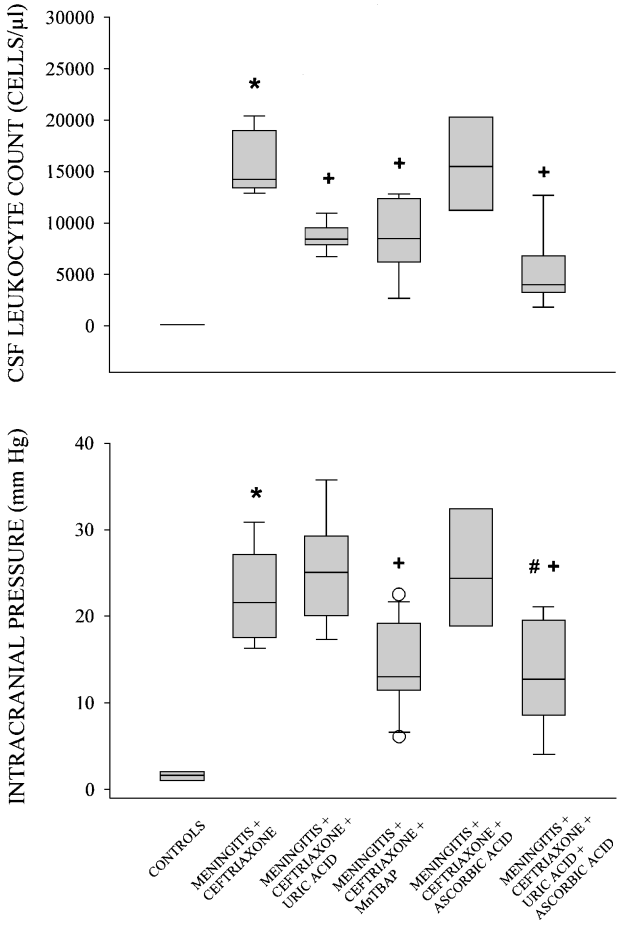

Fig. 1. Effect of adjunctive treatment with peroxynitrite scavengers on CSF leukocyte counts (upper panel) and intracranial pressure (lower panel). Leukocyte counts were reduced by adjunctive therapy with urate and MnTBAP, but not ascorbate, compared with animals treated only with ceftriaxone. The intracranial hypertension was reduced by MnTBAP, but not by urate. Urate in combination with ascorbate, however, reduced the intracranial hypertension compared with urate alone. The graph shows the median and the 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars. Open circles represent the 5th and the 95th percentile. $*p<0.05$ compared with uninfected controls; $+p<0.05$ compared with meningitis + ceftriaxone; $\#p<0.05$ compared with meningitis + ceftriaxone + urate.

Table 1
Experimental groups

Experimental group (*n*)	Intracisternal inoculum	Systemic (i.p.) treatment, 20 h after infection
Controls (4)	PBS	none
Meningitis + ceftriaxone (10)	Pneumococci	100 mg/kg ceftriaxone
Meningitis + ceftriaxone + urate (8)	Pneumococci	100 mg/kg ceftriaxone + 300 mg/kg urate
Meningitis + ceftriaxone + MnTBAP (11)	Pneumococci	100 mg/kg ceftriaxone + 15 mg/kg MnTBAP
Meningitis + ceftriaxone + ascorbate (4)	Pneumococci	100 mg/kg ceftriaxone + 100 mg/kg ascorbate
Meningitis + ceftriaxone + urate + ascorbate (6)	Pneumococci	100 mg/kg ceftriaxone + 300 mg/kg urate + 100 mg/kg ascorbate

37.5 ± 0.5 °C with a heating pad. A catheter was inserted into the left femoral artery for continuous monitoring of mean arterial blood pressure, for blood sampling, and for blood gas analyses. A burr hole was made in the occipital bone and a catheter was introduced into the cisterna magna for continuous intracranial pressure monitoring and collection of CSF samples. CSF was cultured quantitatively to determine bacterial titers. The CSF leukocytes were counted in the Neubauer chamber. A differentiation of the CSF leukocytes was not performed. However, we know from previous studies in our rat model that during this acute stage of pneumococcal meningitis, the CSF pleocytosis is almost exclusively due to an increase of neutrophil granulocytes.

At the end of the experiment, rats were deeply anaesthetized with thiopental and were perfused transcardially with 100 ml of ice-cold PBS. The brains were extracted and rapidly frozen in tissue-freezing medium (−80 °C; Leica Instruments, Nussloch, Germany).

2.2. Determination of cytokines and chemokines in rat brain homogenate

Nine 100-μm cryostat-cut brain sections were sonicated in 10 mM HEPES buffer (pH 7.9, 10 mM KCl, 1.5 mM MgCl) containing a proteinase inhibitor cocktail (aprotinin, PMSF, leupeptin, pepstatin), centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatants were collected. Total protein concentration of the supernatant was determined according to Bradford. The concentrations of immunoreactive interleukin-1β (IL-β) and interleukin-6 (IL-6) (R&D Systems, Wiesbaden, Germany, detection limit 10 and 50 pg/ml, respectively) and the chemokine macrophage inflammatory protein-2 (MIP-2, Biosource International, Solingen, Germany, detection limit 5 pg/ml) were determined by ELISA and expressed as pg/mg protein.

2.3. Statistical analysis

Statistical analysis was performed by nonparametric procedures including the Kruskal–Wallis ranking test as well as the Mann–Whitney *U*-test (combined with α-adjustment for multiple comparisons) to detect differences between the experimental groups. Experimental groups were compared as follows: (a) uninfected controls vs. meningitis + ceftriaxone; (b) meningitis + ceftriaxone vs. meningitis + ceftriaxone + urate and vs. meningitis + ceftriaxone + MnTBAP; (c) meningitis + ceftriaxone vs. meningitis + ceftriaxone + ascorbate; (d) meningitis + ceftriaxone + urate vs. meningitis + ceftriaxone + urate + ascorbate; (e) meningitis + ceftriaxone vs. meningitis + ceftriaxone + urate + ascorbate.

Unless otherwise stated, data are expressed as mean ± standard deviation. $p < 0.05$ was considered significant.

2.4. Reagents

Unless otherwise stated, all reagents and chemicals were obtained from Sigma (Deisenhofen, Germany).

3. Results

Six hours after antibiotic treatment (i.e., 26 h after intracisternal injection of live pneumococci), CSF leukocyte counts and intracranial pressure were significantly increased in infected animals compared with uninfected controls (Fig. 1). In brain homogenates, significantly elevated levels of the proinflammatory cytokines interleukin-1β and interleukin-6, and of the neutrophil-attractant chemokine MIP-2 were found in infected animals (Table 2).

Adjunctive therapy with the natural antioxidant and peroxynitrite scavenger urate reduced the CSF leukocyte counts and brain concentrations of interleukin-1β, interleukin-6, and MIP-2, compared with infected animals treated with ceftriaxone only (Fig. 1, Table 2). However, urate failed to reduce the intracranial hypertension (Fig. 1).

The synthetic peroxynitrite decomposition catalyst MnTBAP also reduced the CSF leukocyte counts and brain concentrations of interleukin-1β,interleukin-6, and MIP-2 compared with animals treated with ceftriaxone alone (Fig. 1, Table 2). In addition, MnTBAP also reduced the intracranial hypertension (Fig. 1).

Adjunctive treatment with the natural antioxidant ascorbate had no effect on CSF leukocyte counts, intracranial hypertension, or brain cytokine concentrations compared

Table 2
Effect of adjunctive treatment with peroxynitrite scavengers on mean arterial blood pressure (MABP), brain concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6), and macrophage inflammatory protein-2 (MIP-2) and CSF bacterial titers (colony-forming units, cfu)

Group	MABP (mmHg)	Brain IL-1β (pg/mg protein)	Brain IL-6 (pg/mg protein)	Brain MIP-2 (pg/mg protein)	log cfu/ml CSF
Controls	119 ± 19	10.4 ± 8.5	10.0 ± 6.9	23.7 ± 20.4	negative
Meningitis + ceftriaxone	77 ± 22 *	298.6 ± 157.8 *	141.1 ± 89.8 *	191.5 ± 136.1 *	5.04 ± 1.03
Meningitis + ceftriaxone + urate	77 ± 12	130.4 ± 57.7 * *	75.8 ± 34.4 * *	60.4 ± 17.5 * *	5.34 ± 1.05
Meningitis + ceftriaxone + MnTBAP	73 ± 13	133.8 ± 103.5 * *	57.2 ± 34.1 * *	53.3 ± 21.6 * *	4.85 ± 1.36
Meningitis + ceftriaxone + ascorbate	57 ± 8	173.5 ± 42.4	132.9 ± 142.3	115.5 ± 64.6	4.97 ± 0.77
Meningitis + ceftriaxone + urate + ascorbate	78 ± 15	88.1 ± 81.8 * *	39.9 ± 24.6 * *	70.6 ± 45.4 * *	5.38 ± 0.85

The groups which were compared statistically are given in the text (Section 2.3).
* $p < 0.05$ compared with uninfected controls.
** $p < 0.05$ compared with meningitis + ceftriaxone.

with infected animals treated with ceftriaxone alone (Fig. 1, Table 2).

Since ascorbate was reported to enhance the beneficial effect of urate in vitro, we then evaluated a combination therapy of urate and ascorbate. This combination significantly reduced the intracranial hypertension compared with animals treated with urate alone (Fig. 1), suggesting that the failure of urate alone to reduce the intracranial pressure may have been due to a lack of the co-antioxidant ascorbate.

4. Discussion

The first important finding of this study was that treatment with antioxidants in addition to ceftriaxone (adjunctive therapy) had a marked anti-inflammatory effect. Treatment of bacterial meningitis with antibiotics alone can cause an acute exacerbation of the inflammatory response in the subarachnoid space, most likely because antibiotic-induced bacterial lysis liberates large amounts of proinflammatory bacterial cell wall components in the CSF (Tuomanen et al., 1985). This inflammatory burst is characterized by an increase of CSF leukocyte counts and cytokine concentrations (Tuomanen et al., 1989) and can cause a rapid clinical deterioration of bacterial meningitis patients following antibiotic therapy. The reduction of the proinflammatory cytokines interleukin-1β, interleukin-6, and MIP-2 by adjunctive antioxidant therapy is a relevant finding, because these mediators have been shown to contribute to the development of intracranial complications during bacterial meningitis (Tauber and Moser, 1999). Anti-inflammatory effects have also been reported for other antioxidants and, on occasion, were attributed to an inhibition of the redox-sensitive transcription factor NFκB (Flohe et al., 1997), which has also been shown to be a potent regulator of proinflammatory cytokines during experimental pneumococcal meningitis (Koedel et al., 2000).

Second, we observed a striking discrepancy between urate and MnTBAP with respect to their effect on the elevated intracranial pressure. Furthermore, the beneficial effect of urate on the intracranial hypertension was fully restored by supplementation of ascorbate, which was not protective by itself. In one of our previous studies, a single intraperitoneal injection of 300 mg/kg urate before infection (pretreatment) clearly reduced the intracranial hypertension (and CSF pleocytosis and blood–brain barrier disruption) in the same rat model of pneumococcal meningitis (Kastenbauer et al., 1999). Also, ascorbate in the dosage of 100 mg/kg has been repeatedly demonstrated to be protective in rat models of, e.g., ischemia or hemorrhagic shock (Ekman et al., 1994; Seo and Lee, 2002). Because in vitro studies had reported that the antioxidative capacity of urate may depend on the presence of co-antioxidants such as ascorbate (Aruoma and Halliwell, 1989), and since we had observed a severe depletion of CSF ascorbate during bacterial meningitis (Kastenbauer et al., 2002), we studied the combined therapy of urate and ascorbate. The addition of ascorbate restored the beneficial effect of urate on the intracranial hypertension, demonstrating for the first time in vivo that supplementation of ascorbate may enhance the beneficial effects of urate. Thus, the failure of urate to reduce the intracranial hypertension during the advanced stage of pneumococcal meningitis (posttreatment) may be due to a depletion of co-antioxidants, such as ascorbate.

Third, adjunctive therapy with peroxynitrite scavengers was safe with respect to CSF bacterial titers. Similar to other oxidants, peroxynitrite was demonstrated to have a bactericidal effect in vitro (reviewed in Koedel and Pfister, 1999). However, the similar CSF bacterial titers in our study demonstrate that scavenging peroxynitrite does not accelerate bacterial growth or delay CSF sterilization by ceftriaxone.

In conclusion, treatment with scavengers of peroxynitrite, such as those used in this study (MnTABP or urate, the latter possibly in combination with ascorbate), has marked anti-inflammatory effects, can reduce the intracranial hypertension and is safe with respect to CSF bacterial titers. Therefore, these substances hold much promise for the adjunctive therapy of bacterial meningitis.

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References

Aruoma, O.I., Halliwell, B., 1989. Inactivation of alpha 1-antiproteinase by hydroxyl radicals. The effect of uric acid. FEBS Lett. 244, 76–80.

Becker, B.F., 1993. Towards the physiological function of uric acid. Free Radic. Biol. Med. 14, 615–631.

Ekman, T., Risberg, B., Bagge, U., 1994. Ascorbate reduces gastric bleeding after hemorrhagic shock and retransfusion in rats. Eur. Surg. Res. 26, 187–193.

Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M.G., Packer, L., 1997. Redox regulation of NF-kappa B activation. Free Radic. Biol. Med. 22, 1115–1126.

Hooper, D.C., Spitsin, S., Kean, R.B., Champion, J.M., Dickson, G.M., Chaudhry, I., Koprowski, H., 1998. Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. Proc. Natl. Acad. Sci. U. S. A. 95, 675–680.

Kastenbauer, S., Koedel, U., Pfister, H.W., 1999. Role of peroxynitrite as a mediator of pathophysiological alterations in experimental pneumococcal meningitis. J. Infect. Dis. 180, 1164–1170.

Kastenbauer, S., Koedel, U., Becker, B.F., Pfister, H.W., 2001. Experimental meningitis in the rat: protection by uric acid at human physiological blood concentrations. Eur. J. Pharmacol. 425, 149–152.

Kastenbauer, S., Koedel, U., Becker, B.F., Pfister, H.W., 2002. Oxidative stress in bacterial meningitis in humans. Neurology 58, 186–191.

Kirsch, M., de Groot, H., 2000. Ascorbate is a potent antioxidant against

peroxynitrite-induced oxidation reactions. Evidence that ascorbate acts by re-reducing substrate radicals produced by peroxynitrite. J. Biol. Chem., 16,702–16,708.

Koedel, U., Pfister, H.-W., 1999. Oxidative stress in bacterial meningitis. Brain Pathol. 9, 57–67.

Koedel, U., Bayerlein, I., Paul, R., Sporer, B., Pfister, H.W., 2000. Pharmacological interference with NF-kB activation attenuates central nervous system complications in experimental pneumococcal meningitis. J. Infect. Dis. 182, 1437–1445.

Lorenzl, S., Kodel, U., Frei, K., Pfister, H.W., 1996. Effect of the bradykinin B2 receptor antagonist Hoe140 in experimental pneumococcal meningitis in the rat. Eur. J. Pharmacol. 308, 335–341.

Patel, M., Day, B.J., 1999. Metalloporphyrin class of therapeutic catalytic antioxidants. Trends Pharmacol. Sci. 20, 359–364.

Pfister, H.W., Feiden, W., Einhaupl, K.M., 1993. Spectrum of complications during bacterial meningitis in adults. Results of a prospective clinical study. Arch. Neurol. 50, 575–581.

Rice, M.E., 2000. Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci. 23, 209–216.

Schuchat, A., Robinson, K., Wenger, J.D., Harrison, L.H., Farley, M., Reingold, A.L., Lefkowitz, L., Perkins, B.A., 1997. Bacterial meningitis in the United States in 1995. Active Surveillance Team. New Engl. J. Med. 337, 970–976.

Seo, M.Y., Lee, S.M., 2002. Protective effect of low dose of ascorbic acid on hepatobiliary function in hepatic ischemia/reperfusion in rats. J. Hepatol. 36, 72–77.

Simon, R.P., Beckman, J.S., 2002. Why pus is bad for the brain. Neurology 58, 167–168.

Tauber, M.G., Moser, B., 1999. Cytokines and chemokines in meningeal inflammation: biology and clinical implications. Clin. Infect. Dis. 28, 1–11.

Tuomanen, E., Liu, H., Hengstler, B., Zak, O., Tomasz, A., 1985. The induction of meningeal inflammation by components of the pneumococcal cell wall. J. Infect. Dis. 151, 859–868.

Tuomanen, E., Saukkonen, K., Sande, S., Cioffe, C., Wright, S.D., 1989. Reduction of inflammation, tissue damage, and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocytes. J. Exp. Med. 170, 959–968.

Wayner, D.D., Burton, G.W., Ingold, K.U., Barclay, L.R., Locke, S.J., 1987. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim. Biophys. Acta 924, 408–419.