Intrathecal Treatment with the Anti-Phosphorylcholine Monoclonal Antibody TEPC-15 Decreases Neuronal Damage in Experimental Pneumococcal Meningitis

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Abstract
Background: Neuronal injury in pneumococcal meningitis is a consequence of microglial activation and direct toxicity by bacterial products and systemic inflammation. Methods: The treatment effect of the TEPC-15 antibody recognizing teichoic and lipoteichoic acids was investigated in murine microglial cells and in a rabbit model of pneumococcal meningitis. Results: In vitro, the TEPC-15 antibody recognizing teichoic and lipoteichoic acids increased Streptococcus pneumoniae phagocytosis by murine microglial cells. In rabbit ceftriaxone-treated S. pneumoniae meningitis, intracisternal TEPC-15 reduced the density of apoptotic neurons in the hippocampal dentate gyrus (116 ± 70 vs. 221 ± 132/mm²; p = 0.03). Cerebrospinal fluid inflammatory parameters (protein, lactate, leukocytes, prostaglandins) were not reduced in TEPC-15-treated rabbits. Conclusion: Intracisternal treatment with the TEPC-15 antibody reduced neuronal damage probably by promoting rapid phagocytosis of bacterial products.

Introduction
Neuronal injury in bacterial meningitis is a consequence of leukocyte invasion into the central nervous system, stimulation of microglia and resident macrophages, and direct toxicity of bacterial components [1]. Pneumococcal cell wall components attract leukocytes into the central nervous system, which release reactive oxygen species and proteolytic enzymes [2]. Bacterial cell walls of Streptococcus pneumoniae and group B streptococci induce nitric oxide production in glial cells and neurotoxicity [3]. Upon activation, microglial cells do not only adopt a proinflammatory phenotype causing neurotoxicity but also can increase their ability to clear pathogens after invasion of the central nervous system. We have recently demonstrated the ability of primary cultures of microglial cells to phagocyte and intracellularly kill pathogens causing bacterial meningitis, including encapsulated pathogenic S. pneumoniae [4].

Teichoic and lipoteichoic acids (LTA) are the most potent proinflammatory constituents of the membrane and the cell wall of S. pneumoniae. They are composed of repetitive oligosaccharide units conjugated to phosphorylcholine. LTA cause profound meningeal inflammation when injected into the subarachnoid space [5]. Treatment
strategies with the focus on decreasing the concentrations of bacterial compounds in the cerebrospinal fluid (CSF) have been reported beneficial in the past: treatment with the non-bacteriolytic antibiotic rifampin reduced the release of LTAs, neuronal damage and mortality in experimental models of bacterial meningitis [6–8]. Similarly, the non-bacteriolytic antibiotic daptomycin reduced CSF inflammation and brain damage [9, 10].

Therapies which are able to lower the CSF concentrations of proinflammatory bacterial products are therefore promising with respect to minimizing cerebral complications in bacterial meningitis. Since bacterial cell wall components induce microglial activation, stimulate the systemic inflammation and contribute to neuronal injury, we studied the efficacy of the anti-phosphorylcholine monoclonal mouse IgA antibody TEPC-15 recognizing LTAs to modulate the inflammatory response and to decrease neuronal damage in bacterial meningitis.

Materials and Methods

Primary Mouse Microglia Cell Culture

Primary cultures of microglial cells were prepared from brains of newborn C57Bl/6N mice (1–2 days) as previously described [4]. Microglial cells were plated in 96-well plates at a density of 20,000 cells/well. Cells were treated with either Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml streptomycin/penicillin (unstimulated group), with the Toll-like receptor (TLR) 2 agonist tripalmitoyl-S-(bis(palmitoyloxy)propyl)-Cys-Ser-(Lys)3-Lys (Pam3CSK4; EMC Microcollections, Tübingen, Germany) or with the TLR9 agonist CpG oligodeoxynucleotide 1668 (TIB Molbiol, Berlin, Germany) was used to count dentate granule cells labeled by the in-situ tailing reaction and to measure the area of the granular cell layer of the dentate gyrus by a blinded observer (J.G.). Adjacent sections stained by hematoxylin-eosin showed morphological features of apoptosis in the same neurons. The density of apoptotic neurons was expressed as the number of marked cells per mm² of the granular cell layer. The in-situ tailing of 5μCi of 3H-thymidine (NECIS, Munich, Germany) was used to count dentate granule cells labeled by the in-situ tailing reaction and to measure the area of the granular cell layer of the dentate gyrus by a blinded observer (J.G.). Adjacent sections stained by hematoxylin-eosin showed morphological features of apoptosis in the same neurons. The density of apoptotic neurons was expressed as the number of marked cells per mm² of the granular cell layer.

Rabbit Model of Experimental Meningitis

A penicillin-sensitive S. pneumoniae type 3 strain originally isolated from an adult patient with meningitis (MIC/MBC of ceftriaxone 0.03/0.06 μg/ml) was used (gift from M.G. Täuber, University of Bern, Switzerland). After intramuscular anesthesia with ketamine (25 mg/kg bodyweight) and xylazine (5 mg/kg bodyweight), New Zealand white rabbits were inoculated intracisternally by suboccipital puncture with 10⁶ CFU of S. pneumoniae. Anesthesia was maintained by intravenous urethane for the entire duration of the experiment (24 h).

Animals received injections of the anti-phosphorylcholine monoclonal antibody TEPC-15 (n = 12) recognizing teichoic acid and LTA at 12 h (0.03 mg), 14 h (0.01 mg) and 17 h (0.01 mg). Rabbits of the control group (n = 9) were treated with the same amount of saline intrathecally. In practice, a needle was cautiously moved into the subarachnid space, a small amount of CSF was withdrawn, and then 100 μl of TEPC-15 solution (or in controls an equal amount of saline) was given. Thereafter, the needle was removed promptly.

All animals were treated with ceftriaxone (Rocephin®, Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) which was administered intravenously as a continuous infusion from 12 to 24 h after infection (20 mg/kg bodyweight loading, 10 mg/kg bodyweight/h maintenance dose).

Blood and CSF were drawn at 12, 14, 17, 20 and 24 h. Pneumococcal CSF titers were determined by plating 10 μl of undiluted and serial 10-fold dilutions on blood agar plates. CSF leukocytes (WBC) were counted in a Fuchs-Rosenthal hemocytometer. Protein content and lactate concentrations in CSF were measured by colorimetric assays (BCA Protein test, Pierce, Rockford, Ill., USA; Lactate PAP test, Greiner Biochemica, Flacht, Germany). Prostaglandin E2 (PGE₂) was quantified using an enzyme immunoassay (R&D Systems, Minneapolis, Minn., USA).

At 24 h after infection, rabbits were sacrificed by intracardial injection of 3 ml potassium-chloride 7.45%. Brains were removed and fixed in 4% formalin for immunohistochemical analysis. In situ Tailing

Deparaffinized and hydrated 1-μm-thick sections were treated with 50 μg/ml proteinase K (Sigma, Deisenhofen, Germany) for 15 min at 37 °C in a reaction mixture containing 10 μl of 5X tailing buffer, 1 μl digoxigenin DNA labeling mix, 2 μl cobalt chloride, 12.5 U terminal transferase and the necessary amount of distilled water to give a volume of 50 μl. After washing, the sections were incubated with 10% FCS for 15 min at room temperature and then washed again. A solution of alkaline phosphatase-labeled anti-digoxigenin antibody in 10% FCS (1:250) was placed on the sections for 60 min at 37 °C. The color reaction (black) was developed with 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate (NBT/BCIP). The sections were counterstained with nuclear fast red-aluminium hydroxide (reagents from Roche, Mannheim, Germany).

Quantification of Apoptotic Neurons

An imaging system (BX51, Olympus, Hamburg, Germany; software AnalySIS® 3.2, Soft Imaging System GmbH, Münster, Germany) was used to count dentate granule cells labeled by the in-situ tailing reaction and to measure the area of the granular cell layer of the dentate gyrus by a blinded observer (J.G.). Adjacent sections stained by hematoxylin-eosin showed morphological features of apoptosis in the same neurons. The density of apoptotic neurons was expressed as the number of marked cells per mm² of the granular cell layer.

Statistics

Data were expressed as means ± standard deviation and compared by t test, or ANOVA followed by Bonferroni’s multiple comparisons test. Bacterial titers in CSF served for log-linear regression analysis. p < 0.05 was considered statistically significant.
Results

**TEPC-15 Stimulated the Phagocytosis of S. pneumoniae D39 by Microglial Cells in vitro**

Opsonization of bacteria with TEPC-15 alone stimulated phagocytosis when tested at high concentrations (10 μg/ml). Pre-stimulation of microglial cells with Pam3CSK4 and opsonization of bacteria with TEPC-15 at low and high concentrations had an additive effect on bacterial phagocytosis, whereas TEPC-15 and CpG were only additive at high TEPC-15 concentrations (fig. 1).

**Intracisternal Treatment with TEPC-15 Decreased Neuronal Damage in Experimental Pneumococcal Meningitis**

The density of apoptotic neurons in the dentate gyrus of the hippocampal formation was lower after treatment with TEPC-15 in comparison to saline-treated animals (fig. 2; 116 ± 70 vs. 221 ± 132/mm²; p = 0.03).

**CSF Parameters of Inflammation in Experimental Pneumococcal Meningitis**

Bacterial titers in CSF determined 12 h following inoculation were almost equal in TEPC-15-treated rabbits and in control animals (fig. 2; p = 0.5). The bactericidal rate expressed as Δlog CFU/ml/h was –0.68 ± 0.09 in rabbits with adjunctive TEPC-15 therapy and –0.64 ± 0.08 in control animals (p = 0.3). 24 h after infection, CSF lactate (8.6 ± 3.5 vs. 8.2 ± 1.4 mmol/l, p = 0.81), protein concentrations (4,422 ± 1,503 vs. 3,815 ± 1,451 mg/l, p = 0.43) and white blood cell counts (5,459 ± 3,455/μl vs. 7,346 ± 4,602/μl, p = 0.36) were not significantly different between TEPC-15-treated and control animals, respectively.

Twelve hours after infection, i.e. immediately before the first injection of the anti-phosphorylcholine antibody, CSF PGE₂ concentrations were not different between the TEPC-15 and the control group (298 ± 558 vs. 308 ± 458 pg/ml, p = 0.97). 14 h after infection, i.e. 2 h...
after the first TEPC-15 injection, CSF PGE$_2$ concentrations were higher in TEPC-treated rabbits than in animals receiving ceftriaxone alone ($1,256 \pm 702$ vs. $451 \pm 345$ pg/ml, $p = 0.003$).

**Discussion**

The increasing emergence of bacterial resistance and the impact of microbial diseases worldwide require new treatment strategies to adequately combat infections. One focus of research is the antimicrobial activity of novel peptides in vitro and in animal models [11, 12]. Another important approach to enhance the activity of established antiinfectives or to modulate inflammation is the use of pathogen-directed antibodies as in the present study.

Teichoic acid and LTA composed of repetitive oligosaccharide units conjugated to phosphorylcholine are considered the most potent proinflammatory constituents of the membrane and the cell wall of *S. pneumoniae* stimulating meningeal macrophages and microglia and promoting leukocyte invasion. The action of pneumococci after heat inactivation, which destroys the lytic activity of pneumolysin, has been investigated in vitro. Heat-inactivated pneumococci injured neurons cocultured with glial cells and induced cellular damage in organotypic hippocampal cultures probably by stimulation of microglia [3, 13].

In this study, the TEPC-15 antibody recognizing teichoic acid and LTA was injected into the subarachnoid space in experimental meningitis to bind LTAs. This treatment reduced the density of apoptotic neurons in the dentate gyrus of the hippocampal formation 12 h after initiation of treatment.
Inflammation in the subarachnoid space was quantified by several routine parameters and by measuring the concentration of prostaglandins in the CSF. The routine parameters of inflammation in the CSF [protein, lactate and leukocytes (WBC)] showed no significant difference between the TEPC-15 and the control group. Conversely, prostaglandin concentrations were higher in antibody-treated animals 14 h after induction of meningitis compared to controls receiving ceftriaxone alone. Probably as a consequence of the antigen-antibody reaction of teichoic acid and LTA with TEPC-15 and phagocytosis of opsonized bacterial products, antibody treatment reduced neuronal damage, although it did not decrease inflammation in the subarachnoid space. Since the interstitial space of the hippocampal formation is equilibrating with the surrounding CSF and bacterial toxins have been shown to diffuse into the hippocampal parenchyma [14–16], we hypothesize that binding of LTA and of cell wall fragments containing LTA in the CSF by TEPC-15 promoted their uptake by phagocytes in the CSF space and led to lower LTA concentrations in the brain parenchyma, thereby preventing microglial activation and protecting neuronal cells in the dentate gyrus. The observation that in primary microglial cultures the phagocytosis of an encapsulated pneumococcal D39 strain was increased after opsonization with TEPC-15 supports this hypothesis.

Concurring mechanisms, e.g. a reduced release of reactive oxygen species or cytokines, may also have been involved, and this has to be addressed in further studies.

In conclusion, intracisternal treatment with the TEPC-15 antibody recognizing LTA reduced neuronal damage without inhibiting inflammation in experimental pneumococcal meningitis. This study supports the concept of decreasing the concentration of active bacterial compounds as an effective method to protect neurons against damage in bacterial meningitis [1, 6–10], and shows for the first time that this goal cannot only be achieved by non-bacteriolytic antibiotics but also by appropriate antibodies.

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