Clindamycin is neuroprotective in experimental Streptococcus pneumoniae meningitis compared with ceftriaxone

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Abstract
In animal models of Streptococcus pneumoniae meningitis, rifampin is neuroprotective in comparison to ceftriaxone. So far it is not clear whether this can be generalized for other protein synthesis-inhibiting antimicrobial agents. We examined the effects of the bactericidal protein synthesis-inhibiting clindamycin (n = 12) on the release of proinflammatory bacterial components, the formation of neurotoxic compounds and neuronal injury compared with the standard therapy with ceftriaxone (n = 12) in a rabbit model of pneumococcal meningitis. Analysis of the CSF and histological evaluation were combined with microdialysis from the hippocampal formation and the neocortex. Compared with ceftriaxone, clindamycin reduced the release of lipoteichoic acids from the bacteria (p = 0.004) into the CSF and the CSF leucocyte count (p = 0.011). This led to lower extracellular concentrations of hydroxyl radicals (p = 0.034) and glutamate (p = 0.016) in the hippocampal formation and a subsequent reduction of extracellular glycerol levels (p = 0.018) and neuronal apoptosis in the dentate gyrus (p = 0.008). The present data document beneficial effects of clindamycin compared with ceftriaxone on various parameters linked with the pathophysiology of pneumococcal meningitis and development of neuronal injury. This study suggests neuroprotection to be a group effect of bactericidal protein synthesis-inhibiting antimicrobial agents compared with the standard therapy with β-lactam antibiotics in meningitis.

Keywords: ceftriaxone, clindamycin, microdialysis, neuroprotection, Streptococcus pneumoniae meningitis.


In the last four decades, mortality from community-acquired bacterial meningitis has remained unchanged (5–10% in children and 25% in adults) (Roos et al. 1997). Long-term neurological sequelae and death in bacterial meningitis are caused jointly by several factors: (i) the systemic inflammatory response of the host, leading to leucocyte extravasation into the subarachnoid space, vasculitis, brain oedema and secondary ischaemia; (ii) stimulation of resident microglia within the CNS by bacterial compounds and (iii) direct toxicity of bacterial haemolysins on neurones (Braun et al. 2002; Nau and Brück 2002; Stringaris et al. 2002). Of the various adjunctive therapeutic approaches effective in animals, only dexamethasone therapy has been shown to decrease hearing loss, overall neurologic sequelae and mortality in children with Haemophilus influenzae type B meningitis and adults with Streptococcus pneumoniae meningitis.
meningitis (O dio et al. 1991; De Gans and Van de Beek 2002). However, in animal experiments, dexamethasone increased neuronal damage in the dentate gyrus of the hippocampal formation (Zysk et al. 1996; Leib et al. 2003) and dexamethasone was not beneficial under the conditions of developing countries (Qazi et al. 1996; Molyneux et al. 2002). Several approaches interfering selectively with the mechanisms of neuronal injury are effective in animal models: inhibition of leucocyte migration into the subarachnoid space by antibodies against the CD18 epitope (Tuomanen et al. 1989; Zysk et al. 1996; Braun et al. 1999), matrix metalloproteinase antagonists (Leib et al. 2000), antagonists of excitatory amino acids (Leib et al. 1996b), inhibitors of transcription factors (Koedel et al. 2000) or caspas (B raun et al. 1999) or free radical scavengers (Leib et al. 1996a; Koedel and Pfister 1999). The first event accessible for therapeutic intervention, so far, is the release of proinflammatory or toxic bacterial components during initiation of antibiotic therapy. Rifampin, an antibiotic inhibiting bacterial protein synthesis, reduces mortality and neuronal injury in pneumococcal meningitis compared with the β-lactam antibiotic ceftriaxone (Nau et al. 1999; Böttcher et al. 2000). Rifampin, however, was also neuroprotective in animal models of permanent and transient focal cerebral ischaemia (Yulug et al. 2004). It is still a matter of debate whether or not rifampin activates the glucocorticoid receptor (Calleja et al. 1998; Herr et al. 2000) and whether the beneficial effect of rifampin in bacterial meningitis is caused by the inhibition of the release of proinflammatory/toxic bacterial products or by direct immunosuppression.

The aim of this study was to compare the effects of clindamycin, a bactericidal protein synthesis-inhibiting antibiotic with no immunosuppressive activity on the pathophysiology and the severity of neuronal injury in Streptococcus pneumoniae meningitis, with those of the standard therapy consisting of the β-lactam antibiotic ceftriaxone. This study should answer the question whether the neuroprotection by rifampin, observed in earlier experiments, is a group effect of protein synthesis-inhibiting antibiotics or is restricted to single compounds with additional properties. Therefore, we combined microdialysis, a potent approach to detect changes in the biochemical composition of the extracellular fluid in various tissues (Ungerstedt and Pycock 1974) in two different brain regions (i.e. frontal cortex and hippocampal formation), with the analysis of CSF and histological examination of the brain.

Materials and methods

Stereotaxic surgery, microdialysis and model of meningitis

After i.m. induction of anaesthesia with ketamine (25 mg/kg) and xylazine (5 mg/kg), 35 male New Zealand White rabbits (2.4–3.0 kg; Charles River, Sulzfeld, Germany) were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) equipped with an appropriate rabbit adaptor. Anaesthesia was maintained intravenously with ketamine (17 mg/kg/h) and xylazine (3.4 mg/kg/h) during placement of the probes and, thereafter, by i.v. urethane for the entire duration of the experiment (27 h). After calibrating the position of the rabbit head (Patterson 1977), the skull was exposed by a midline incision. Two 2-mm diameter holes were drilled, the dura mater was incised and microdialysis probes (molecular weight cut-off 100 kDa; membrane length 2 mm; CMA 12; CMA/Micdialysis AB, Solna, Sweden) were inserted into the right frontal cortex (coordinates relative to bregma: A, +4 mm; L, +5.8 mm and V, from pial surface 5.5 mm) and the right hippocampal formation (coordinates relative to bregma: A, −6 mm; L, +8 mm and V, from pial surface 6 mm following a line with a 20° angle compared with the sagittal level). The major part of the hippocampal probe was located in the dentate gyrus. The probes were perfused with CNS perfusion medium (CMA/Micdialysis AB) supplemented with 5 mM L-phenylalanine (Sigma, Deisenhofen, Germany) at a constant flow rate of 1 μL/min using a precision pump (Harvard 22; Harvard Apparatus Inc., Holliston, MA, USA). Samples were collected every 90 min using a microfraction collector (Univentor 820; Univentor Ltd, Zejtun, Malta). A minimum of 90 min of equilibration was allowed before sampling was started to achieve stable levels of all substances to be measured.

After two sampling periods (i.e. 3 h) rabbits were inoculated intracisternally with 10⁶ colony-forming units (CFU) of a Streptococcus pneumoniae type 3 (SP3) strain (kind gift of Prof. M. Täuber, University of Bern, Switzerland). All animal experiments were approved by the Animal Care Committee of the District Government of Braunschweig (Lower Saxony, Germany).

Experimental protocol

Antibiotic treatment was started 15 h after infection, either with ceftriaxone (Rocephin™; Hoffmann-LaRoche, Grenzach-Wyhlen, Germany; n = 12; 20 mg/kg i.v. loading, 10 mg/kg/h maintenance dose) or clindamycin (Clindamycin™; Azupharma, Gerlingen, Germany; n = 12; 20 mg/kg i.v. loading, 10 mg/kg/h maintenance dose). Eleven additionally examined animals received continuous i.v. injection of sterile saline to serve as untreated controls. According to previously published data, a single i.v. dose of 20 mg/kg clindamycin or 20 mg/kg ceftriaxone in the rabbit yields initial serum concentrations of approximately 15 or 100 mg/L, respectively (Schmidt et al. 1997; Trostdorf et al. 1999; Teixeira et al. 2000). The elimination half-life of both antibiotics in rabbits is approximately equal (approximately 200 min; estimates from concentration–time curves published in Teixeira et al. 2000). With respect to the elimination half-lives of both drugs, the maintenance doses used (10 mg/kg/h) lead to a moderate increase of the antibiotic serum concentrations. This has been documented for the ceftriaxone regimen used in the present study (Schmidt et al. 1997; Trostdorf et al. 1999). During the antibiotic regimen chosen, the ceftriaxone CSF levels in meningitic rabbits range from 2 to 20 μg/mL (Schmidt et al. 1997; Trostdorf et al. 1999). A dose of 20 mg/kg clindamycin leads to maximum CSF concentrations of approximately 1 μg/mL in rabbits with Streptococcus pneumoniae meningitis (Paris et al. 1996).

CSF (0.3 mL) was drawn by intracisternal puncture before and at 15, 18, 21 and 24 h after infection. Leucocyte densities and pneumococcal CSF titres were determined (Nau et al. 1997). Bacterial titres (CFU/mL) at 15, 18, 21 and 24 h served for log-linear regression analysis (log CFU/mL/h). The remaining CSF was
centrifuged at 3000 g for 10 min and the supernatant fluid was
stored at −70°C for further analysis.

The body temperature of all animals was measured before and at
15, 18, 21 and 24 h after infection. At the end of each experiment,
the animals were killed with an overdose of i.v. thiopental
(Trapanal®; Byk Gulden, Konstanz, Germany). The microdialysis
probes were then perfused with cresyl violet (Merck, Darmstadt,
Germany) to stain the positions of the microdialysis membranes and
brains were removed and fixed in 4% formaldehyde. On the
following day, the position of the probes in the frontal cortex and the
hippocampal formation was verified macroscopically on coronal
sections of the fixed brains. The microdialysate was analysed only in
animals with correctly positioned probes and no visible superficial
injury caused by the surgical procedure (n = 9 in each group) for
lactate, glutamate, formation of hydroxyl radicals (m-tyrosine), lipid
peroxidation [malondialdehyde (MDA)] and cell membrane degra-
dation products (glycerol). Brains were embedded in paraffin for
histological examination and quantification of neuronal apoptosis by
in situ tailing. After the experiment, in vitro recovery of each probe
was determined for glucose to assure full functionality of the probes.
The probes were perfused with CNS perfusion medium while
suspected in a standard glucose solution (5.0 mM) at room
temperature (22°C) and on a magnetic stirrer.

Detection of lipoteichoic acid in CSF
Pneumococcal lipoteichoic acid (LTA) in CSF (samples drawn at
15 h and compiled samples from 18 and 21 h after infection) was
measured by an enzyme immunoassay as described previously
(Stueertz et al. 1998). The assay uses the TEPC-15 monoclonal
antibody directed against the phosphorylcholine moiety of teichoic
and LTA (Sigma) as capture antibody and a polyclonal antibody
raised against purified LTA as detector antibody.

Detection of CSF protein and lactate content
CSF protein and lactate concentrations were determined by
colourimetric assays (BCA Protein Test; Pierce, Rockford, IL,
USA; Greiner, Flacht, Germany).

Detection of lipid peroxidation in CSF and microdialysate
Lipid peroxidation in CSF and microdialysate was assessed by
measuring MDA as the thiobarbituric acid adduct by HPLC with
fluorescence detection. Derivatization was carried out as previously
described (Lykkesfeldt 2001) with the inclusion of 70 μM butylated
hydroxytoluene as chain-breaking antioxidant. The reaction mixture
was separated on a Zorbax Eclipse C8 column (particle size 5 μm;
150 × 4.6 mm i.d.; Agilent Technologies, Mountain View, CA,
USA) fitted with a SecurityGuard C8 guard column (Phenomenex,
Torrance, CA, USA) using a mobile phase consisting of 30% (v/v)
methanol in 50 mM potassium dihydrogen phosphate buffer
(pH 7.0) and 0.02% sodium azide at a flow rate of 1 mL/min.
Signals were monitored at 515/553 nm (excitation/emission).
The MDA-thiobarbituric acid adduct eluted at 3.75 min and was
quantified by comparison with a standard curve prepared from
tetramethoxypropane (Lykkesfeldt 2001).

Determination of interstitial fluid hydroxyl radical formation
To determine non-enzymatic hydroxylation of n-phenylalanine
added to the microdialysis perfusion fluid as an index of interstitial
fluid hydroxyl radical formation, m-tyrosine was measured in
microdialysate fractions by HPLC with fluorescence detection
(Themann et al. 2001). Samples (10 μL) were injected onto a
250 × 4.6 mm Nucleosil C18 column (5 μm particle size; Supelco,
Bellefonte, PA, USA) using a cooled autosampler (L-7250; Hitachi,
Tokyo, Japan) and separated by a mobile phase consisting of 50 mM
KH2PO4 and 8% methanol adjusted to pH 4.0 with phosphoric
acid delivered by a gradient pump (L-7100; Hitachi) at a flow rate
of 1.0 mL/min. After 15 min the column was flushed with
methanol/water using a gradient programme. Tyrosine isomers and
n-phenylalanine were detected using a fluorescence detector
(F-1080; Hitachi) at set 275 nm (excitation) and 310 nm (emission).
Data were recorded and analysed by an HPLC computer system
(HSM 4.1; Hitachi). The concentration of m-tyrosine and
n-phenylalanine was calculated by comparing the integrated peak
areas with those of authentic standards (Sigma/Fluka, Buchs,
Switzerland) prepared in perfusion solution. m-Tyrosine was adjusted
for small changes in n-phenylalanine concentration that occurred
over the entire length of each given microdialysis experiment.

Detection of glycerol and lactate concentrations in
microdialysate
Microdialysate glycerol and lactate concentrations were measured
using a microdialysis analyser (CMA 600; CMA/Microdialysis AB).
This analysis is based on the enzymatic formation of hydrogen
peroxide which is subsequently derivatized to a colourimetric
product. The analyser settings were adapted to preclinical sample
measurement.

Measurement of amino acids in microdialysate
Amino acids were analysed by reversed-phase HPLC with fluores-
cence detection. The microdialysis perfusate (10 μL) was incubated
with 10 μL of a reagent consisting of o-phthalaldehyde (Sigma;
40 mM) and mercaptoethanol (Carl Roth, Karlsruhe, Germany; 0.4% w/v) in boric acid (Merck; 0.4M), pH 10.4, for 60 s at 4°C in an
HPLC autoinjector (CMA 200/240; CMA/Microdialysis AB). This
solution (15 μL) was then injected onto a 4 × 60 mm Nucleosil-100
C18 (particle size 5 μm) column (Knauer, Berlin, Germany) and
eluted with a sodium acetate buffer (Riedel de Haen, Seelze,
Germany; 70 mM, pH 6.95) containing 5% methanol (Riedel de
Haen) and 3% tetrahydrofuran (Merck) at a flow rate of 1 mL/min.
An isocratic elution was first maintained for 8 min, a gradient of
methanol was then applied to elute the more hydrophobic
derivatives and finally the column was regenerated. Detection was
achieved with a fluorescence detector (CMA/280; CMA/Microdial-
ysis AB) using excitation and emission bands of 350 and 495 nm,
respectively. The concentrations were calculated with a SP 4290
integrator (Spectra Physics, Mountain View, CA, USA) against
freshly prepared standard solutions.

Quantification of apoptosis
Paraffin sections (1 μm) of the left brain hemispheres were
deparaffinized and stained with haematoxylin and eosin. Adjacent
deparaffinized and hydrated sections were used for visualization of
DNA fragmentation by in situ tailing. For this purpose they were
-treated with 100 μg/mL proteinase K (Sigma-Aldrich, Steinheim,
Germany) for 15 min at 37°C followed by incubation with the
tailing mixture for 60 min at 37°C. After blocking with 10% fetal
calf serum for 15 min at room temperature (22°C) the sections were incubated with alkaline phosphatase-labelled anti-digoxigenin antibody (1:250 diluted in 10% fetal calf serum) for 60 min at room temperature. The reaction was visualized with 4-nitroblue-tetrazolium-chloride/5-bromo-4-chloro-3-indoly-l-phosphate and counterstained with nuclear fast red-aluminium hydroxide. All reagents were purchased from Boehringer-Mannheim (Mannheim, Germany). To quantify the density of apoptotic neurones, haematoxylin and eosin-stained sections were used to measure the area of the granular cell layer of the dentate gyrus with an imaging analysis system (BX51; Olympus, Hamburg, Germany; software AnalySIS® 3.2; Soft Imaging System GmbH, Münster, Germany). The number of apoptotic cells was counted in the adjacent section stained with the in situ tailing reaction and expressed per mm² of the granular cell layer (Zysk et al. 1996).

In vitro studies
To exclude a direct neuroprotective activity of ceftriaxone we tested the effects of this antibiotic on organotypic hippocampal slice cultures (OTC) challenged with SP3 and on pneumolysin-induced neurotoxicity in a neuroblastoma cell line (SH-SY5Y). In the latter system clindamycin was also examined.

Organotypic hippocampal cultures
NMRI mice (6–8 days old) bred at the animal care facility of the Max-Planck Institute for Biophysical Chemistry (Göttingen, Germany) were decapitated. The hippocampal formation was prepared and cut transversally with a McIlwain tissue chopper into 400-μm thick slices under sterile conditions. Slices were kept in Grey’s balanced salt solution supplemented with 36 mM D-glucose at 4°C for 30 min. Thereafter, slices were embedded in plasma clots on glass coverslips, which were then coagulated by the addition of thrombin. Coverslips were transferred to plastic culture tubes containing culture medium composed of 50% Hanks’ basal medium, 25% Hanks’ balanced salt solution, 25% heat-inactivated horse serum supplemented with glutamine (1 mM) and D-glucose (36 mM). Culture tubes were placed in a roller device rotating at 10 revolutions/h in an air-ventilated incubator at 36°C. After 12 days, cultures were challenged with approximately 10⁸ CFU/mL of heat-inactivated SP3 for 48 h. OTC (n = 14 each group) were treated with (i) SP3 alone or (ii) SP3 together with ceftriaxone at concentrations of 1 or 100 μg/mL. Negative control cultures were not exposed to pneumococci. To determine cell damage in OTC the vital dye propidium iodide (PI; Sigma-Aldrich) was used. With loss of cell membrane integrity PI enters the cell and binds to DNA. PI fluorescence is therefore related to necrotic or late apoptotic cell death. After incubation with SP3, the medium was replaced by PI (25 μg/mL dissolved in medium) followed by incubation for 1 h at 36°C. Thereafter, OTC were examined by dark-field fluorescence microscopy (Axiophot; Zeiss, Oberkochen, Germany). Fluorescence intensity was recorded by a charge coupled device (CCD) camera (Axiocam MRm; Zeiss). Subsequently, cultures were fixed with 4% buffered formaldehyde in order to induce maximum membrane damage, briefly washed in 0.1 M phosphate buffer, restained with PI-containing medium and again examined by dark-field fluorescence microscopy. Light intensity was quantified offline with the image analysis software Sigma Scan® Pro 5.0 (Jandel Scientific Software, San Rafael, CA, USA). Cell damage induced in the dentate gyrus was calculated as the ratio of light intensity before fixation to light intensity following fixation × 100 [PI uptake (%)].

SH-SY5Y neuroblastoma cell culture
SH-SY5Y human neuroblastoma cells (a gift from Dr D. Isbrandt, Centre of Molecular Medicine, University of Hamburg, Germany) were maintained in RPMI medium (Biochrom AG, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO₂ in ambient air. SH-SY5Y cells were seeded into 96-well plates at a density of 100 000/cm² and cultures treated with medium containing 0.5 μg/mL pneumolysin and ceftriaxone or clindamycin at concentrations of 1, 10 and 100 μg/mL.

The viability of SH-SY5Y cells was determined 4 h later using the WST-1-Cell Proliferation Reagent (Roche Applied Science, Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt WST-1 by active mitochondria producing a soluble formazan. This conversion only occurs in viable cells. Cells were incubated with WST-1 for 2 h and the formazan dye formed was then quantified by measuring the optical density at 490 nm using a Genios multiplate reader (Tecan, Crailsheim, Germany). The absorbance directly correlated with the number of metabolically active cells.

Statistics
Data are presented as medians (25th/75th percentiles). Different time points within the same treatment group were compared by a Wilcoxon signed rank test. In the presence of three groups, post-tests were only performed when the overall test indicated the presence of statistically significant differences (principle of closed testing). Under this precondition, post-tests can be performed without p-value adjustment for repeated testing (Bauer 1991). In the presence of more than three groups, p-values were adjusted for repeated testing as indicated below. Comparisons between two groups at single time points (before and after initiation of treatment) were performed by a Mann–Whitney U-test. An extension of the Wilcoxon–Mann–Whitney test to factorial designs with longitudinal data was used to compare the time profiles of different treatment groups (Brunner et al. 2001). To determine bactericidal activity (log CFU/mL/h) of the antibiotic agents used, a log-linear regression analysis of the bacterial titres at 15, 18, 21 and 24 h was performed. In vitro effects of ceftriaxone and clindamycin were examined with Kruskal–Wallis non-parametric ANOVA, followed by Dunn’s multiple comparison test. p-values less than 0.05 were considered statistically significant.

Results
Body temperature
The body temperature of all animals was controlled before and 15, 18, 21 and 24 h after infection. At 15 h the body temperature was 39.6 (39.0/39.9)°C in control animals, 40.0 (39.2/40.1)°C in animals randomized to receive ceftriaxone and 39.9 (38.6/40.4)°C in rabbits randomized to receive clindamycin (p = 0.71). At the end of the study the body temperature was 36.7 (36.5/37.8)°C in untreated controls,
38.3 (37.2/38.7) °C in ceftriaxone-treated rabbits and 38.2 (36.7/39.7) °C ($p = 0.48$) in the clindamycin group.

**Bacterial titres in CSF**

Bacterial titres in the CSF (log CFU/mL) at initiation of treatment did not differ between the control and/or the two treatment groups [control, 8.00 (7.40/8.00); clindamycin, 7.78 (6.87/8.15); ceftriaxone, 8.15 (7.75/8.53); $p = 0.43$]. In untreated animals, the median bacterial titre did not change substantially until the end of the study [7.89 (7.60/8.21)]. Both treatment regimens decreased bacterial titres significantly compared with untreated controls ($p < 0.0001$). Compared with clindamycin, ceftriaxone treatment led to a faster elimination of viable bacteria from the CSF ($p < 0.0001$) (Table 1).

**Release of lipoteichoic acid in CSF**

The LTA concentrations in the CSF did not differ between the animal groups before therapy was started [control, 23.07 (17.55/25.45) ng/mL; clindamycin, 24.64 (18.54/28.28) ng/mL; ceftriaxone, 26.85 (19.98/33.28) ng/mL; $p = 0.64$]. During antibiotic treatment LTA concentrations in clindamycin-treated animals decreased [21.64 (15.86/30.41) ng/mL] whereas they rose in the ceftriaxone group [38.38 (34.09/44.43) ng/mL; $p = 0.006$]. LTA concentrations in the CSF of untreated controls did not change during the study [24.16 (18.05/33.84) ng/mL] and were significantly lower compared with ceftriaxone-treated animals ($p = 0.023$), while no difference was noted compared with clindamycin treatment ($p = 0.49$) (Fig. 1).

**Leucocyte count, protein content and lactate concentration in CSF**

Before initiation of antibiotic treatment (15 h after infection) animals of all groups did not differ significantly in leucocyte count [control, 1520 (411/1901) million particles (Mpt)/L; clindamycin, 1952 (1008/3003) Mpt/L; ceftriaxone, 2603 (838/5476) Mpt/L; $p = 0.54$], protein content [control, 1487 (1478/1503) mg/L; clindamycin, 1943 (1090/3226) mg/L; ceftriaxone, 2981 (910/3386) mg/L; $p = 0.93$] or lactate concentration [control, 8.20 (5.02/11.41) mM; clindamycin, 8.32 (6.96/12.53) mM; $p = 0.84$]. At the end of the experiment (after 9 h of antibiotic treatment) neither lactate nor protein levels ($p = 0.65$ and 0.41, respectively) differed between the three experimental groups. Substantial differences were noted in the number of leucocytes in the CSF 24 h after infection. It was reduced in untreated controls and clindamycin-treated animals compared with those treated with ceftriaxone ($p = 0.01$ and 0.011, respectively) (Table 1).

**Lactate concentration in microdialysate**

Lactate concentration in the microdialysate increased until initiation of antibiotic therapy in the frontal cortex [from 0.12 (1184/2334) mg/L; clindamycin, 1943 (1090/3226) mg/L; ceftriaxone, 2981 (910/3386) mg/L; $p = 0.93$] or lactate concentration [control, 10.79 (7.36/11.31) mM; clindamycin, 8.20 (5.02/11.41) mM; ceftriaxone, 8.32 (6.96/12.53) mM; $p = 0.84$]. At the end of the experiment (after 9 h of antibiotic treatment) neither lactate nor protein levels ($p = 0.65$ and 0.41, respectively) differed between the three experimental groups. Substantial differences were noted in the number of leucocytes in the CSF 24 h after infection. It was reduced in untreated controls and clindamycin-treated animals compared with those treated with ceftriaxone ($p = 0.01$ and 0.011, respectively) (Table 1).

<table>
<thead>
<tr>
<th>Control (n = 11)</th>
<th>Ceftriaxone (n = 12)</th>
<th>Clindamycin (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactericidal activity (delta log CFU/mL/h)</td>
<td>0.00 (–0.006/0.02)</td>
<td>0.50 (–0.59/–0.37)</td>
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<tr>
<td>Leucocyte count (Mpt/L) 24 h postinfection</td>
<td>971 (328/1728)</td>
<td>2592 (1957/3902)</td>
</tr>
<tr>
<td>Protein concentration (mg/L) 24 h postinfection</td>
<td>2646 (1478/3716)</td>
<td>1925 (1343/3803)</td>
</tr>
<tr>
<td>Lactate concentration (mM) 24 h postinfection</td>
<td>14.73 (10.87/20.26)</td>
<td>12.58 (10.05/13.10)</td>
</tr>
</tbody>
</table>

Data are expressed as median values (25th/75th percentiles). Statistical significance was tested by a Mann–Whitney U-test (*$p < 0.05$; **$p < 0.001$ compared with untreated controls; †$p < 0.05$; ††$p < 0.001$ compared with ceftriaxone treatment). CFU, colony-forming units.
(0.06/0.16) mM at 0 h to 0.25 (0.14/0.47) mM at 15 h] as well as in the hippocampal formation [from 0.28 (0.20/0.43) mM at 0 h to 0.40 (0.34/0.61) mM at 15 h]. In untreated controls lactate concentration in the microdialysate further increased in both locations during the experiment [frontal cortex 24 h, 0.62 (0.27/0.75) mM; hippocampal formation 24 h, 0.52 (0.40/0.73) mM], while no further increase was observed in both treatment groups, independent of the catheter location. The differences between the control and/or treatment groups were not significant.

Lipid peroxidation in CSF
Before initiation of treatment, MDA in the CSF did not change substantially [0 h, 0.123 (0.107/0.140) µM; 15 h, 0.134 (0.096/0.190) µM]. At the end of the experiment, MDA in the CSF had increased in all three groups [control, 0.305 (0.239/0.409) µM; clindamycin, 0.195 (0.171/0.312) µM; ceftriaxone, 0.203 (0.112/0.248) µM], with no significant differences between control and treated animals (p = 0.06 and 0.24, respectively) or between the two treatment groups (p = 0.72).

Formation of m-tyrosine in microdialysate
m-Tyrosine, a marker of hydroxyl radical formation, increased before initiation of antibiotic treatment in both microdialysis probe locations [frontal cortex: 0 h, 0.57 (0.51/0.66) µM; 15 h, 0.71 (0.60/0.89) µM, p = 0.004; hippocampal formation: 0 h, 0.58 (0.53/0.63) µM; 15 h, 0.75 (0.69/0.90) µM, p = 0.0001]. In the antibiotic-treated animals, m-tyrosine concentrations continued to slightly increase in the frontal cortex independently of the mode of treatment while, in untreated controls, the m-tyrosine levels remained unchanged. In contrast, compared with ceftriaxone-treated animals, hippocampal m-tyrosine formation declined in the control group (p = 0.028) and during clindamycin therapy (p = 0.034), while it further increased during ceftriaxone treatment (Fig. 2).

Lipid peroxidation in microdialysate
Despite significant increases in interstitial hydroxyl radical formation, microdialysate MDA concentrations were rather low and did not change substantially prior to initiation of therapy [frontal cortex: 0 h, 0.10 (0.06/0.17) µM; 15 h, 0.09 (0.08/0.14) µM; p = 0.16; hippocampal formation: 0 h, 0.06 (0.05/0.19) µM; 15 h, 0.05 (0.04/0.18) µM; p = 0.29]. As a consequence, the therapeutic regimens had no influence on the course of MDA production in the interstitial fluid.

Glycerol concentrations in microdialysate
The glycerol concentration in the microdialysates did not change substantially from preinfection levels until 15 h after infection [frontal cortex: 0 h, 2.83 (2.00/5.34) µM; 15 h, 2.82 (2.00/6.24) µM; hippocampal formation: 0 h, 2.66 (2.00/5.53) µM; 15 h, 3.10 (2.00/4.93) µM]. Similar to hydroxyl radical formation, glycerol concentrations in the frontal cortex increased during antibiotic therapy without differences between the experimental groups (p = 0.35). In contrast, in the hippocampal formation glycerol levels in the microdialysate fell during clindamycin treatment while ceftriaxone therapy led to an increase (p = 0.018). In untreated controls, glycerol levels slightly rose towards the end of the experiment (p > 0.05) (Fig. 3).

Glutamate concentrations in microdialysate
Glutamate increased in both brain regions prior to treatment of meningitis [frontal cortex: 0 h, 1.15 (0.54/3.44) µM; 15 h, 8.70 (2.87/22.48) µM, p < 0.0001; hippocampal formation: 0 h, 1.32 (0.73/2.26) µM; 15 h, 9.36 (2.43/20.14) µM, p < 0.0001]. In the neocortex, control conditions or antibiotic treatment did not influence the further rise of glutamate concentration in the extracellular space. In contrast, in the hippocampal formation untreated controls and clindamycin-treated animals demonstrated a reduced increase in extracellular glutamate concentration compared with ceftriaxone therapy (p = 0.011 and 0.016, respectively) (Fig. 4).

In situ tailing
In untreated animals 125.8 (99.3/208.5) apoptotic neurons/mm² were counted in the dentate gyrus. Compared with
Compared with ceftriaxone (apy, glycerol levels remained unchanged or even slightly decreased an increase of glycerol in the microdialysate. With clindamycin treatment and, to a lesser extent, control conditions led to an increase of glycerol in the microdialysate. With clindamycin therapy, glycerol levels remained unchanged or even slightly decreased compared with ceftriaxone ($p = 0.018$). Data are median values (25th/75th percentiles). Statistical significance was tested by an extension of the Wilcoxon–Mann–Whitney test to factorial designs with longitudinal data to compare the time profiles of the three experimental groups (*$p < 0.05$).

Ceftriaxone-treated animals [183.2 (129.3/212.8)$/mm^2$] clindamycin therapy reduced the density of apoptotic neurones in the dentate gyrus [114.7 (72.2/144.2)$/mm^2$; $p = 0.008$] (Fig. 5).

**In vitro** studies

In OTC, incubation with SP3 caused significant cellular injury ($p < 0.01$). Ceftriaxone did not diminish this effect at a concentration of either 1 or 100 $\mu$g/mL (Fig. 6a).

Treatment of SH-SY5Y cells, a neuroblastoma cell line, with pneumolysin caused a substantial decrease of metabolic activity measured by the WST-1 test indicating cell destruction ($p < 0.01$). Neither ceftriaxone at concentrations of 1, 10 or 100 $\mu$g/mL nor clindamycin at the same concentrations inhibited pneumolysin-induced damage (Fig. 6b).

**Discussion**

In a well-characterized rabbit model of *Strep. pneumoniae* meningitis the present study demonstrates beneficial effects of clindamycin treatment compared with the standard therapy of ceftriaxone on the release of proinflammatory bacterial products. This was followed by reduced hydroxyl radical formation and lower concentrations of glutamate and glycerol in the interstitial fluid of the hippocampal formation and finally led to decreased neuronal injury in the dentate gyrus.

Lipoteichoic acid is a major proinflammatory component of the cell wall of Gram-positive bacteria. Injected intracerebrally, it generates an inflammatory response similar to that observed in bacterial meningitis (Tuomanen *et al.* 1985). Compared with other proinflammatory bacterial compounds
Clindamycin reduces neuronal injury

So far, microdialysis has only been used in infection models to measure amino acids, lactate, glucose, nitric oxide concentrations and the distribution of antibiotic agents in the brain extracellular fluid (Guerra-Romero et al. 1992, 1993; Perry et al. 1993; Granero et al. 1995; Destache et al. 1998). In this study, the analysis of brain interstitial fluid by microdialysis documents the presence of hydroxyl radicals in the brain parenchyma. Reactive oxygen species (ROS), such as the hydroxyl radical, are considered to be important mediators of neuronal damage in meningitis (Leib et al. 1996a) because radical scavengers have consistently exerted beneficial effects in experimental models of this disease (Pfister et al. 1992; Koedel and Pfister 1997; Loeffler et al. 2001). Although essential for the inactivation of pathogens, ROS can cause injury to the host by impairing the function of enzymes and ion transporters, inducing lipid peroxidation, or by causing DNA damage. Excitatory amino acids also contribute substantially to neuronal damage in bacterial meningitis, especially in the hippocampal formation (Leib et al. 1996b; Mawatari et al. 1996; Tumani et al. 2000). In a newborn rat model of Streptococcus Group B meningitis kynurenic acid, a broad spectrum receptor inhibitor of excitatory amino acids was moderately neuroprotective (Leib et al. 1996b).

In this study we demonstrated a decreased hydroxyl radical production (measured by the conversion of d-phenylalanine to m-tyrosine) and a reduction of increased extracellular glutamate levels in the hippocampal formation following treatment with clindamycin compared with ceftriaxone. However, the concentrations of m-tyrosine measured in the brain interstitial fluid were relatively low compared, for instance, with those induced by 6-hydroxydopamine (Ferger et al. 2001). Furthermore, interstitial free Fe²⁺ concentrations, known to increase during cerebral ischaemia, were low and did not rise during the course of infection (data not shown). The absence of extensive hydroxyl radical production and oxidative damage in the CNS interstitial fluid is in agreement with recent studies in which the cerebral vasculature and not the brain parenchyma was identified as the major target of oxidative damage in pneumococcal meningitis (Christen et al. 2001; Schaper et al. 2002). In the brain tissue, excitatory amino acids, such as glutamate, are likely to play a more important role than ROS in the pathophysiology of meningitis and the development of secondary neuronal damage. As clindamycin treatment led to a reduction of hippocampal extracellular glutamate and even small concentrations of ROS are able to augment excitotoxic damage (Pellegrini-Giampietro et al. 1990), the neuroprotective effect of clindamycin compared with ceftriaxone in this model probably relies on its action on both glutamate and ROS levels in the hippocampal extracellular fluid.

Glycerol, an integral component of cell membrane lipids, is an established parameter of the extent of membrane degradation (Marklund et al. 1997; Hillered et al. 1998). Glycerol concentrations were reduced in the hippocampal

(e.g. DNA, peptidoglycans). LTA caused the most profound overall cell damage in OTC (Schmidt et al. 2001). A reduction of LTA concentrations in the CSF compared with therapy with β-lactams, as observed in the present study during clindamycin treatment, has also been shown to occur with rifabutin, quinupristin-dalfopristin and, less pronounced, with moxifloxacin and trovafloxacin (Stuertz et al. 1999). The decreased liberation of bacterial products by clindamycin was followed by beneficial effects on the release of several potentially neurotoxic compounds in the brain tissue.

Fig. 6 (a) Cellular injury was examined in organotypic hippocampal slice cultures (OTC) incubated with medium (n = 15) as control and Streptococcus pneumoniae type 3 (SP3) alone (n = 14) or in addition to ceftriaxone (CRO) (1 µg/mL (n = 14) or 100 µg/mL (n = 14), respectively. SP3 caused a significant increase (p < 0.01) in cellular injury which could not be reversed or diminished by CRO. Data are median values (25th/75th percentiles). Statistical significance was tested with a Kruskal–Wallis non-parametric ANOVA followed by Dunn’s multiple comparison test. (b) WST test as an indicator of metabolic activity of viable cells was performed in a neuroblastoma cell line incubated with medium as control, CRO (10 µg/mL), clindamycin (CLI; 10 µg/mL) and pneumolysin (PLY; 0.5 µg/mL) alone or together with CRO (1, 10 and 100 µg/mL), respectively, or CLI at the same concentrations (n = 8 for each group). PLY caused a significant decrease (p < 0.01) in cell metabolic activity which could not be reversed or diminished by concomitant antibiotic treatment at various concentrations. Antibiotics alone had no effect on cellular metabolic activity. Data are median values (25th/75th percentiles). Statistical significance was tested with a Kruskal–Wallis non-parametric ANOVA followed by Dunn’s multiple comparison test.
formation during treatment with clindamycin compared with ceftriaxone. In agreement with this finding, a reduction of neuronal apoptosis in the dentate gyrus of the hippocampal formation by clindamycin compared with ceftriaxone was observed as measured by in situ tailing and morphology. Compared with earlier studies (Zysk et al. 1996), the overall rate of apoptosis in the present experiment was rather low. This could account for the small absolute differences observed. Although the amount of apoptosis with clindamycin therapy (median 114.7 apoptotic neurones/mm²) still appears substantial compared with the basal rate of neuronal apoptosis in uninfected rabbits (11–14 apoptotic neurones/mm²), any reduction should not be underestimated as hippocampal function is frequently impaired in survivors of bacterial meningitis (Free et al. 1996). Moreover, even an incomplete reduction of hippocampal apoptosis has been demonstrated to prevent learning impairment (Leib et al. 2001). Clindamycin exerted its beneficial properties predominantly in the hippocampal formation, the only brain region where neuronal damage (i.e. apoptosis in the dentate gyrus) occurs in this model of pneumococcal meningitis (Zysk et al. 1996). The favourable effects on m-tyrosine, glutamate and glycerol levels after initiation of antibiotic therapy were demonstrable only there and not in the frontal cortex. It is probable that the close vicinity of the hippocampal formation to the lateral ventricles makes it more vulnerable to proinflammatory and/or toxic effects of bacterial compounds released into the CSF during antibiotic treatment.

In vitro ceftriaxone itself was a scavenger of hypochlorous acid (Lapenna et al. 1995; Carreer et al. 1998). At a dose of 1 μM (equalling 0.5 μg/mL), ceftriaxone reduced irradiation-induced lactate dehydrogenase release and DNA fragmentation (Tikka et al. 2001). However, for several reasons, a substantial antioxidative action of ceftriaxone appears to be unlikely in meningitis. Ceftriaxone concentrations up to 100 μg/mL did not protect OTC, which contain neurones and glial cells, from injury caused by heat-inactivated pneumococci. Similarly, ceftriaxone concentrations from 1 to 100 μg/mL did not reduce pneumolysin-induced damage of neuroblastoma cells. Moreover, the production of ROS by CSF granulocytes and monocytes in Strep. pneumoniae meningitis was not diminished after in vivo exposure to ceftriaxone (Böttcher et al. 2000).

Antibiotic treatment is inalienable in human bacterial meningitis. Nevertheless, control conditions with sham treatment of animals with sterile saline led to interesting results. For a variety of pathophysiologically important parameters (LTA concentration and leucocyte count in the CSF, hydroxyl radical formation and glutamate levels in the extracellular fluid), during the observation period control animals performed better than ceftriaxone-treated rabbits and showed no difference compared with clindamycin treatment. This is a consequence of the burst of inflammation caused by a bacteriolytic antibiotic such as ceftriaxone in the first hours of treatment and of the relatively short observation period covering only the first 9 h of antibiotic therapy. In vitro data on the release of bacterial products and survival experiments in mice illustrate that, in the long run, any effective antibiotic therapy is more effective than no antibiotic treatment.

In conclusion, compared with ceftriaxone, clindamycin (i) reduced the amount of LTA released into the CSF; (ii) led to a lower recruitment of leucocytes into the subarachnoid space; (iii) diminished the cerebral interstitial concentration of hydroxyl radicals in the hippocampal formation, as measured by the conversion of n-phenylalanine to m-tyrosine; (iv) reduced the increase of interstitial glutamate concentrations in the hippocampal formation; (v) reduced interstitial levels of glycerol, a marker of cell membrane destruction, in the hippocampal formation and (vi) decreased the number of apoptotic neurones in the dentate gyrus. This study provides further evidence for a neuroprotective effect of protein synthesis-inhibiting antimicrobial agents compared with the standard treatment with β-lactam antibiotics in bacterial meningitis. Unlike rifampin, clindamycin possesses no additional immunosuppressive effect. This makes neuroprotection likely to be a group effect of bactericidal protein synthesis-inhibiting antimicrobial agents. So far, the clinical use of clindamycin in Strep. pneumoniae meningitis is limited due to the absence of appropriate studies in humans and can therefore not be recommended despite the favourable effects demonstrated here. Nevertheless, antibiotic treatment which reduces the release of proinflammatory/toxic bacterial products into the CSF and thereby diminishes the concentration of endogenous neurotoxic compounds in the brain tissue and alleviates neuronal damage in susceptible areas appears to be a promising therapeutic approach.

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