CONCISE COMMUNICATION

Rifampin Reduces Production of Reactive Oxygen Species of Cerebrospinal Fluid Phagocytes and Hippocampal Neuronal Apoptosis in Experimental Streptococcus pneumoniae Meningitis

Tobias Böttcher,1 Joachim Gerber,2 Andreas Wellmer,2
Alexander V. Smirnov;2 Fariba Fakhrjanali,2
Eilhard Mix,1 Jürgen Pilz,3 Uwe Klaus Zettl,1
and Roland Nau2

Bacterial compounds induce the production of reactive oxygen species (ROS) in meningitis. Rifampin releases smaller quantities of proinflammatory compounds from Streptococcus pneumoniae than do β-lactam antibiotics. Therefore, rabbits infected intracisternally with S. pneumoniae were treated intravenously either with rifampin 5 mg/kg/h or ceftriaxone 10 mg/kg/h (n = 9 each). Before initiation of antibiotic treatment, a strong positive correlation between ROS production of cerebrospinal fluid (CSF) phagocyte populations and bacterial CSF titers was observed (granulocytes: r = .90, P < .0001; monocytes: r = .81, P < .0001). CSF leukocytes from rifampin-treated rabbits produced less ROS (monocytes at 2 h after initiation of treatment: P = .045; at 5 h: P = .014; granulocytes at 5 h: P = .036) than did leukocytes from animals receiving ceftriaxone. The CSF malondialdehyde concentrations and the density of apoptotic neurons in the dentate gyrus were lower in rifampin- than in ceftriaxone-treated animals (P = .002 and .005). The use of rifampin to reduce the release of ROS and to decrease secondary brain injury appears promising.

An intense inflammatory reaction of the host is considered to be one cause of secondary brain damage in meningitis. Reactive oxygen species (ROS) released into the central nervous compartments during inflammation are of dual character. Although essential for the inactivation of pathogens, they can also cause injury to the host. ROS can inactivate a variety of enzymes and ion transporters and induce lipid peroxidation with consecutive release of peroxidation byproducts and may trigger DNA cleavage. This may eventually result in loss of membrane function, DNA damage, and cell death [1, 2].

In meningitis, bacterial lysis by β-lactam antibiotics causes a burst of meningeal inflammation immediately after the initiation of antibiotic therapy [3, 4]. This is due to the release of proinflammatory components of the bacterial cell wall (peptidoglycans, teichoic [TA] and lipoteichoic acids [LTA], and endotoxin) into the cerebrospinal fluid (CSF) [3, 5–7]. The inflammatory host response after the initiation of therapy with antibiotics currently used for meningitis may contribute to early mortality and long-term sequelae in bacterial meningitis. Antibiotics that act by inhibiting protein synthesis, such as rifamycins, release smaller quantities of LTA/TA than do β-lactam antibiotics both in vitro and in vivo [6, 7]. Rifampin, as compared with ceftriaxone, reduced early mortality in a mouse model of Streptococcus pneumoniae meningitis [7]. For these reasons, we compared the effect of rifampin versus ceftriaxone on ROS production of CSF phagocytes, on CSF malondialdehyde (MDA) concentrations, and on neuronal damage in a rabbit model of pneumococcal meningitis.

Materials and Methods

Model of meningitis. After intramuscular induction of anesthesia with ketamine (25 mg/kg) and xylazine (5 mg/kg), 18 New Zealand White rabbits (weight ~2.5 kg) were inoculated intracisternally with 106 colony-forming units (CFU) of a S. pneumoniae type 3 strain [4]. Anesthesia was maintained by intravenous urethane for the entire duration of the experiment (24 h).

Experimental protocol. Antibiotic treatment was started 16 h after infection, either with ceftriaxone (Rocephin, Hoffmann-LaRoche, Grenzach-Wyhlen, Germany; n = 9; 20 mg/kg intravenously loading, 10 mg/kg/h maintenance dose) or rifampin (Rifa, Grünenthal, Stolberg, Germany; n = 9; 10 mg/kg intravenously loading, 5 mg/kg/h maintenance dose).

CSF (0.3 mL) was drawn by intracisternal puncture at 16, 18,
21, and 24 h after infection. Leukocyte densities and pneumococcal CSF titers were determined [4]. Bacterial titers at 16, 18, 21, and 24 h served for log-linear regression analysis (log CFU/mL/h).

The remaining CSF was immediately centrifuged at 250 g for 10 min to isolate the leukocyte pellet (1). Supernatant (1) was centrifuged a second time at 3000 g for 10 min, and supernatant (2) was stored at −70°C for protein, lactate, rifampin, ceftriaxone, and MDA determination. Pellet (2) was discarded. CSF protein content and lactate were determined by colorimetric assays (BCA-protein-test, Pierce, Rockford, IL; Greiner, Flacht, Germany). Antibiotic and lactate were determined by colorimetric assays (BCA-protein-test, Pierce, Rockford, IL; Greiner, Flacht, Germany). Antibiotic and lactate were determined by colorimetric assays (BCA-protein-test, Pierce, Rockford, IL; Greiner, Flacht, Germany). Antibiotic and lactate were determined by colorimetric assays (BCA-protein-test, Pierce, Rockford, IL; Greiner, Flacht, Germany).

Detection of ROS. ROS production of CSF phagocytes was measured by flow cytometry using an EPICS cytometer and XL-2 software (Coulter Immunotech, Miami, FL) with linearly amplified forward scatter (FS) and side scatter (SS) and logarithmically amplified fluorescence. Intracellular H₂O₂, serving as a pli¬ed forward scatter (FS) and side scatter (SS) and logarithmi-

curves were constructed in drug-free 1 : 20 diluted rabbit serum. Escherichia coli rifampin) and stored at 70°C on agar using Bacillus subtilis ATCC 6633 spores (Difco, Detroit; rifampin) and Escherichia coli ATCC 25922 (ceftriaxone). Standard curves were constructed in drug-free 1 : 20 diluted rabbit serum.

Detection of apoptosis. Rabbits were sacrificed by intravenous injection of 75 mg thiopental (Trapanal, Byk Gulden, Konstanz, Germany). One-micrometer sections containing the hippocampal formation were stained with hematoxylin and eosin (HE) and by in situ tailing to visualize DNA fragmentation. The number of apoptotic neurons per mm² of dentate granular cell layer was quantified [9].

Statistics. Data were expressed as mean ± SD. Unpaired 2-tailed t test was performed for the comparison between rifampin- and ceftriaxone-treated animals and paired 2-tailed t test for the detection of changes within one group during the course of the experiment and for stimulation experiments. The correlation between CSF leukocyte densities, bacterial titers, and ROS production before antibiotic treatment was studied by Spearman rank correlation. A P value <.05 was considered significant.

Results

Rifampin was almost as effective as ceftriaxone in reducing bacterial CSF titers in vivo (titers at 16 h: 7.67 ± 1.41 vs. 7.89 ± 1.00; Δlog CFU/mL/h −0.26 ± 0.08 vs. −0.30 ± 0.11, difference not significant). The antibacterial CSF concentrations at 24 h (rifampin: 2.18 ± 0.76 μg/mL; ceftriaxone: 4.98 ± 3.19 μg/mL) were ~270 times above the MIC of 0.008 μg/mL (rifampin) and ~170 times above the MIC of 0.03 μg/mL (ceftriaxone).

CSF lactate and protein concentrations increased during the course of the experiment in both ceftriaxone- and rifampin-treated animals (no significant differences). CSF white blood cell counts (16 h: 3723 ± 5628 vs. 3348 ± 2753/mL; 18 h: 3390 ± 2884 vs. 3986 ± 2830/μL; 21 h: 4301 ± 2951 vs. 3777 ± 3024/μL; 24 h: 3263 ± 2311 vs. 5925 ± 3032/μL) were not significantly different in ceftriaxone- and rifampin-treated animals at any time point.

At 16 h, bacterial CSF titers were correlated with the ROS production of CSF phagocyte populations (PMN r = .90, P < .0001; MN r = .81, P < .0001). ROS production of PMNs or MNs and CSF white blood cell count were not positively correlated (PMN r = −.35, P = .15; MN r = −.32, P = .19). Similarly, there was no correlation between the bacterial CSF titers and the CSF white blood cell count (r = −.32, P = .19).

Prior to antibiotic treatment, PMN and MN ROS production did not differ significantly among animals assigned for either ceftriaxone or rifampin treatment. At 18 h, MNs from rifampin-treated rabbits produced less ROS than MNs from ceftriaxone-treated animals (P = .045). At 21 h after infection, both PMNs

samples were injected onto the HPLC system. The MDA DNPH derivative was separated from interfering compounds at 30°C on a reversed-phase column (Nucleosil C18 3 × 125 mm, 3 μm particle size, SepServ, Berlin, Germany; mobile phase 38% acetonitrile, 61.8 % water, 0.2 % [v/v] acetic acid, flow rate 0.6 mL/min) and quanti¬fied with an UV detector at 310 nm. Peak areas were compared with the peak areas of the MDA standard curve (10, 5, 2.5, 1.25, and 0.625 nmol/mL) with a Merck D 2000 Chromato-Integrator (Merck).

Detection of apoptosis. Rabbits were sacrificed by intravenous injection of 75 mg thiopental (Trapanal, Byk Gulden, Konstanz, Germany). One-micrometer sections containing the hippocampal formation were stained with hematoxylin and eosin (HE) and by in situ tailing to visualize DNA fragmentation. The number of apoptotic neurons per mm² of dentate granular cell layer was quantified [9].

Statistics. Data were expressed as mean ± SD. Unpaired 2-tailed t test was performed for the comparison between rifampin- and ceftriaxone-treated animals and paired 2-tailed t test for the detection of changes within one group during the course of the experiment and for stimulation experiments. The correlation between CSF leukocyte densities, bacterial titers, and ROS production before antibiotic treatment was studied by Spearman rank correlation. A P value <.05 was considered significant.
and MNs from rifampin-treated rabbits showed a decreased ROS production, compared with leukocytes from ceftriaxone-treated animals \((PMN\ P=0.036; MN\ P=0.014; \text{figure 1A, 1B})\). In comparison with the pretreatment ROS production \((16\ h)\), PMNs from rifampin-treated animals produced significantly less ROS at \(21\ (P=0.017)\) and \(24\ h\ (P=0.024)\). In contrast, in PMNs from ceftriaxone-treated animals the ROS production significantly fell below the pretreatment level only at \(24\ h\ (P=0.023; \text{figure 1A})\).

Prior to antibiotic treatment, FMLP failed to stimulate PMN ROS production substantially. Once antibiotic treatment was started, FMLP stimulated the PMNs of rifampin-treated animals at \(18\ (P=0.048), 21\ (P=0.002),\) and \(24\ h\ (P=0.0002)\) after infection. In ceftriaxone-treated animals, FMLP stimulated PMN ROS production only at \(21\ (P=0.016)\) and \(24\ h\ (P=0.016; \text{table 1})\). TPA increased ROS production of both phagocyte populations at all times.

The total MDA concentrations in CSF and the densities of apoptotic neurons in the dentate gyrus of the hippocampal formation were substantially lower in rifampin- than in ceftriaxone-treated animals \((P=0.002\) and \(0.005; \text{table 1})\).

**Discussion**

ROSs are considered key mediators in the pathophysiology of bacterial meningitis \([1, 2]\). In a well-characterized rabbit model of \(S.\ pneumoniae\) meningitis, the present study demonstrates a significant attenuation of ROS production by CSF phagocytes, a lower CSF MDA concentration, and a reduced density of apoptotic neurons in the dentate gyrus after rifampin treatment in comparison to the standard therapy with ceftriaxone.

PMNs, MNs, and cerebrovascular endothelial cells, but not astrocytes and neurons, generate ROS on stimulation with \(S.\ pneumoniae\) \([1, 2, 10]\). In meningitis, the major portion of ROS is produced by leukocytes, in particular by PMNs \([2]\). Therefore, flow cytometry in the CSF can be used to study ROS production in bacterial meningitis. Assessment of ROS production by the rhodamine 123 technique is established for blood and CSF phagocytes \([8, 11]\). The method allows semiquantitative measurement of the cell-specific functional stimulation in a defined compartment. The CSF MDA concentration is an indicator of overall lipid peroxidation. It is strongly elevated in patients with bacterial meningitis, compared with patients with viral meningitis and noninflammatory neurological disorders \([10]\). Lipid peroxidation is one major mechanism linking ROS production and neuronal damage in bacterial meningitis \([1, 2]\).

In the present study, prior to antibiotic treatment, the oxidative burst activity of CSF phagocytes mainly depended on the bacterial CSF titers. This agrees well with the ability of bacterial products to directly stimulate ROS production in PMNs, MNs, and endothelial cells \([10]\). Initiation of antibiotic treatment with ceftriaxone resulted in a small increase of the oxidative burst activity of CSF phagocytes, whereas rifampin therapy immediately led to a slight decrease of the oxidative activity of the CSF leukocytes (figure 1). Statistically significant differences in the ROS production of leukocytes during therapy with rifampin and ceftriaxone, respectively, were noted \(5\) (PMNs and MNs) and \(2\ h\) (MNs) after initiation of antibiotic.

![Figure 1](https://academic.oup.com/jid/article-abstract/181/6/2095/2191653)

Figure 1. Reactive oxygen species (ROS) production (mean ± SD of the decadic logarithm of the mean fluorescence intensity [log mean FLi]) of cerebrospinal fluid granulocytes \((A)\) and monocytes \((B)\) during treatment of meningitis with ceftriaxone and rifampin. The asterisk (*) indicates a \(P\) value <0.05 vs. ceftriaxone (unpaired 2-tailed \(t\) test).

### Table 1. ROS production of CSF PMNs with FMLP stimulation, CSF MDA concentration, and density of apoptotic neurons in the dentate gyrus.

<table>
<thead>
<tr>
<th>Parameter, time point (h)</th>
<th>Ceftriaxone (PMNs (n=9))</th>
<th>Rifampin (PMNs (n=9))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS production stimulated by FMLP (log mean FLi)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.137 ± 0.47</td>
<td>2.135 ± 0.445</td>
</tr>
<tr>
<td>18</td>
<td>2.254 ± 0.425</td>
<td>2.055 ± 0.441*</td>
</tr>
<tr>
<td>21</td>
<td>2.0 ± 0.274a</td>
<td>1.753 ± 0.277*</td>
</tr>
<tr>
<td>24</td>
<td>1.814 ± 0.159a</td>
<td>1.735 ± 0.271*</td>
</tr>
<tr>
<td>CSF MDA concentration (nmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 and 24</td>
<td>6.87 ± 0.63</td>
<td>4.94 ± 1.01b</td>
</tr>
<tr>
<td>Apoptotic neurons (1/mm²)</td>
<td>136 ± 54</td>
<td>71 ± 33b</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD. ROS, reactive oxygen species; CSF, cerebrospinal fluid; PMN, polymorphonuclear leukocytes; FMLP, N-formyl-methionyl-leucyl-phenylalanine; MDA, malondialdehyde; FLi, fluorescence intensity.

* \(P<0.05\ vs.\) spontaneous ROS production at the same time (paired 2-tailed \(t\) test).

b \(P<0.01\ vs.\) ceftriaxone (unpaired 2-tailed \(t\) test).
activation of granulocytes during rifampin treatment: PMNs from rifampin-treated rabbits showed an earlier return of inducibility of ROS production by FMLP than PMNs from ceftriaxone-treated animals.

In addition to ROS, excitatory amino acids, especially glutamate [12, 13], appear to be involved in the generation of neuronal damage in bacterial meningitis. Oxidative injury and excitotoxicity are closely linked [14]. Both mechanisms may potentiate each other in bacterial meningitis. In animal studies, several radical scavengers attenuated early pathophysiological changes in bacterial meningitis [1]. Adjuvant therapy with α-phenyl-tert-butyl nitrate strongly reduced neuronal injury in the neocortex and hippocampal formation in experimental *Streptococcus* group B meningitis [2]. None of these approaches, however, was evaluated as an adjunct to antibiotic therapy in human bacterial meningitis.

Rifampin as an established antibacterial agent could close the gap between experimental and clinical neuroprotective approaches. The reduction of the release of proinflammatory bacterial compounds by rifampin [6, 7] decreases ROS production of CSF phagocytes and endothelial cells, reduces lipid peroxidation, and attenuates neuronal damage. The rapid induction of resistance to rifampin during monotherapy precludes its use as a single agent for *S. pneumoniae* meningitis. Nevertheless, no substantial release of LTA/TA was observed in vitro, when ceftriaxone was added 6 h after initiation of antibiotic therapy with rifampin [15]. Therefore, after initial rifampin therapy, β-lactam antibiotics may be added, when the majority of bacteria has already been killed by rifampin (i.e., 12–24 h later). The use of rifampin as an initial therapy for bacterial meningitis has as yet not been studied in humans. It would be dangerous to adapt this approach in clinical practice without first conducting an appropriately controlled clinical trial.

In conclusion, this study provides further evidence for the concept that minimizing the release of proinflammatory bacterial compounds may improve outcome in bacterial meningitis and, perhaps, other life-threatening infections. The measurement of ROS production of leukocytes by flow cytometry is a novel approach to study the intensity of the oxidative burst in the infected compartment.

Acknowledgments

We thank Prof. O. Götze and Dr. B. Pollok-Kopp, Department of Immunology, University of Göttingen, for their help in performing the flow cytometry, and Prof. V. Armstrong, Department of Clinical Chemistry, University of Göttingen, for his advice in preparing this manuscript.

References