Minocycline delays but does not attenuate the course of experimental autoimmune encephalomyelitis in Streptococcus pneumoniae-infected mice

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Objectives: Experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), can be aggravated by a mild Streptococcus pneumoniae infection. This study was performed to assess whether treatment with antibiotics inhibiting bacterial protein synthesis reduces the detrimental effect of infection on the course of EAE.

Methods: In vitro, release of proinflammatory pneumococcal products was studied by enzyme immunoassay and western blot. Seven days after induction of EAE (prior to the onset of symptoms) mice were infected intraperitoneally with S. pneumoniae and treated either with the inhibitors of bacterial protein synthesis minocycline or rifampicin, or with the β-lactam ceftriaxone.

Results: During bacterial killing in vitro, minocycline and rifampicin released lower quantities of proinflammatory bacterial products from S. pneumoniae than ceftriaxone. Mice treated with minocycline developed symptoms of EAE 1 day later than mice treated with ceftriaxone. Neither minocycline nor rifampicin therapy, however, reduced the severity of EAE in comparison with ceftriaxone treatment.

Conclusions: Although statistically significant (P = 0.04), a delay of 1 day in the onset of symptoms of EAE after minocycline treatment is of minor clinical relevance. These data do not support the hypothesis of superiority of a bacterial protein synthesis inhibitor over a β-lactam antibiotic for the treatment of concomitant infections during the latent phase of EAE or MS.

Keywords: rifampicin, ceftriaxone, lipoteichoic acids, pneumolysin

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the CNS, which is characterized by perivascular inflammatory lesions, demyelination and axonal damage. The aetiology of MS remains unknown. Infections probably contribute to the pathogenesis of this disease. In MS patients infections of the respiratory tract can cause aggravation or relapse.1-3

Experimental autoimmune encephalomyelitis (EAE), an animal model that mimics many aspects of MS, is induced by subcutaneous injection of myelin proteins. Similar to the findings in humans, viral and bacterial infections were found to be important triggering factors for EAE in mice.4,6 Moreover, bacterial components such as peptidoglycans (PGN), lipopolysaccharide (LPS) and DNA-containing unmethylated cytosine–guanosine motifs (CpG) can be co-stimulatory factors for disease development in EAE.7-9 Recently, we showed that EAE mice challenged intraperitoneally with live Streptococcus pneumoniae and treated with ceftriaxone during the latent period between immunization and onset of clinical symptoms had a more severe course of EAE compared with uninfected mice.10 Aggravation of EAE by S. pneumoniae was mediated by Toll-like receptor 2 (TLR2): the severity of EAE was not increased in S. pneumoniae-infected TLR2−/− mice.10 TLRs can recognize specific patterns of microbial components and regulate the activation of both innate and adaptive...
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Immunity. Lipoteichoic acid (LTA) and teichoic acid (TA), major constituents of the cell wall of S. pneumoniae, act via TLR2. The pneumococcal haemolysin pneumolysin, in addition to its pore-forming activity in biological membranes, is an agonist of TLR4. These components are released during autolysis and lysis of pneumococci by antibiotic therapy. Depending on the mechanism of action, classes of antibacterials differ with respect to the release of proinflammatory compounds from S. pneumoniae. β-Lactam antibiotics act by inhibition of bacterial cell wall synthesis leading to lysis, whereas rifampicin and minocycline inhibit bacterial RNA and protein synthesis. During treatment with β-lactam antibiotics higher amounts of LTA/TA pneumolysin and pneumococcal DNA are set free than during therapy with antibiotics inhibiting pneumococcal RNA synthesis.

Since ceftriaxone released greater quantities of proinflammatory pneumococcal components than inhibitors of bacterial protein synthesis and an S. pneumoniae infection treated with ceftriaxone aggravated the course of EAE, we assumed a milder course of EAE after an S. pneumoniae infection treated with antibiotics inhibiting bacterial protein synthesis than after a ceftriaxone-treated pneumococcal infection. For this reason, we conducted two randomized studies comparing the impact of either rifampicin or minocycline with the standard therapy ceftriaxone on EAE complicated by an intraperitoneal pneumococcal infection.

Material and methods

In vitro experiments

The S. pneumoniae type 3 strain used for the in vitro and in vivo studies was originally isolated from a patient with meningitis (gift of Professor Martin G. Täuber, University of Bern, Switzerland). It grows well in vitro, is highly susceptible to ceftriaxone (MIC 0.03 mg/L, MBC 0.06 mg/L), rifampicin (MIC 0.008 mg/L, MBC 0.06 mg/L) and minocycline (MIC 0.06 mg/L, MBC 0.12 mg/L), and produces a uniform course of infection in mice.

A culture of S. pneumoniae grown for 10 h at 37°C in tryptic soy broth was divided into fractions of 100 mL. Then ceftriaxone and minocycline were added for 12 h at 37°C. Control cultures were grown without antibiotics. The experiment was performed six times on different days. Bacterial counts were determined at 0, 1, 2, 3, 6, 9 and 12 h by plating 10 µL samples of 10-fold dilutions of bacteria on blood agar plates, and the bactericidal rate was determined by log-linear regression. The concentrations of ceftriaxone and minocycline were chosen according to the unbound plasma concentrations observed in humans: ceftriaxone, 10 mg/L; minocycline, 1 mg/L. Samples (1 mL) for the detection of pneumolysin and LTA were collected immediately prior to the addition of the antibiotics and 12 h later. Supernatants were separated from bacterial cells by centrifugation (10000 rpm, 10 min) and stored at –80°C. For the comparison of the release of LTA/TA pneumolysin during treatment with rifampicin (10 and 3 mg/L, respectively) and ceftriaxone therapy (10 and 8 mg/L, respectively) previously published data were re-evaluated.

Detection and quantification of proinflammatory bacterial products

Pneumolysin was detected in the supernatants after 12 h of growth of S. pneumoniae using quantitative immunoblotting. Pneumococcal LTA/TA were measured after 12 h of growth by a previously described sandwich ELISA.

Mice

Female C57 BL/6 mice (age 6–8 weeks) were purchased from Charles River GmbH (Sulzfeld, Germany). All animal experiments were approved by the Animal Care Committee of the University Hospital Göttingen and by the District Government of Braunschweig, Lower Saxony. Mice were housed in pathogen-free conditions and received water and food ad libitum.

Induction of EAE

C57BL/6 mice were immunized subcutaneously in both flanks with 200 µg of myelin oligodendrocyte glycoprotein 35–55 peptide (MOG35-55) in 50 µL of phosphate-buffered saline (PBS) and 100 µL of incomplete Freund’s adjuvant (Sigma-Aldrich, Deisenhofen, Germany) containing 1 mg of desiccated Mycobacterium tuberculosis (H37Rv; Difco Laboratories, Detroit, MI, USA) on day 0 in the ceftriaxone/minocycline experiment and on day 0 and day 7 in the ceftriaxone/rifampicin experiment. Additionally, mice were injected intraperitoneally with Bordetella pertussis toxin in 300 µL of PBS on day 0 and 2 (Sigma-Aldrich, Deisenhofen, Germany) (150 ng in the ceftriaxone/minocycline experiment, 100 ng in the ceftriaxone/rifampicin experiment).

Infection with S. pneumoniae and antibiotic therapy

To examine the effect of S. pneumoniae infection on the course of EAE, 7 days after the immunization with MOG35-55 mice were infected intraperitoneally with 6.8 ± 0.6 log cfu of S. pneumoniae type 3 in 0.5 mL of 0.9% NaCl in the minocycline/ceftriaxone group and with 5.9 ± 0.1 log cfu in the rifampicin/ceftriaxone group. Starting 12 h after infection, all mice received subcutaneous antibiotic treatment with either ceftriaxone (Rocephin, Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) or rifampicin (Grüenthal, Stolberg, Germany) or minocycline (Sigma-Aldrich, Deisenhofen, Germany) at a dose of 100 mg/kg twice daily. The short interval between S. pneumoniae application and antibiotic therapy was necessary to ensure mild infection. In the experiments where ceftriaxone and minocycline were compared, treatment was given for 3 days. This short treatment course was chosen because the injection of minocycline appeared painful, probably because of its low pH. In the experiments where ceftriaxone and rifampicin were compared treatment was given for 5 days.

Clinical evaluation

Mice were weighed and starting from day 7 scored daily for clinical EAE signs according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, complete tail paralysis and mild hind limb weakness; 2, complete tail paralysis and strong hind limb weakness; 2.5, unilateral hind limb paralysis; 3, complete hind limb paralysis; 3.5, complete hind limb paralysis and forelimb weakness; 4, tetraplegia; 5, death of EAE. Mice were killed by cervical dislocation when the EAE score was higher than 3.0 or loss of weight was more than 20% of the maximum weight. An EAE score of 4.0 was given when a mouse had to be killed because of the severity of EAE. In the ceftriaxone/minocycline experiments 11 mice of the ceftriaxone group and 9 mice of the minocycline group had to be killed because of severe EAE symptoms. Three mice of the minocycline group died of infection on day 9 and 10 prior to the development of EAE symptoms and were excluded from statistical analysis.
analysis. In the ceftriaxone/rifampicin experiment three mice of each group had to be killed owing to the severity of EAE. The mild pneumococcal infection used in this study caused mild lethargy and a temporary weight loss below 10%, but no limb weakness. All animals that did not die subsequent to failure of antibiotic therapy fully recovered prior to the onset of symptoms of EAE.

Statistical analysis
Statistical evaluation and graphical presentation were performed using GraphPad Instat 3.05 (GraphPad Software, San Diego, CA, USA) for the paired Wilcoxon test and the Fisher’s exact test. For the unpaired Student’s t-test GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) was used. Data were expressed as means ± SEM. Differences were considered statistically significant, when P was <0.05.

Results
Reduced release of LTA and pneumolysin into the supernatants of the bacterial cultures after the addition of minocycline compared with ceftriaxone
The bacterial concentrations prior to the addition of ceftriaxone or minocycline were 6.6 ± 0.6 log cfu/mL. Bacterial concentrations in cultures not treated by antibiotics rose to 8.6 ± 0.5 log cfu/mL after 3 h and were 5.8 ± 0.3 log cfu/mL after 12 h subsequent to spontaneous autolysis of S. pneumoniae. There was no bacterial growth after exposure to 1 mg/L minocycline or 10 mg/L ceftriaxone for 12 h. Ceftriaxone reduced the bacterial concentration at a rate of –2.2 ± 0.5 log cfu/mL/h, and minocycline at a rate of –0.8 ± 0.06 log cfu/mL/h (P = 0.02). In the supernatants of cultures treated with ceftriaxone, 12 h after initiation of therapy LTA/TA and pneumolysin concentrations were higher than those in the supernatants of cultures treated with minocycline (pneumolysin, 11.5 ± 8.6 μg/L versus 1.7 ± 0.9 μg/L, P = 0.03; LTA, 1409 ± 915 μg/L versus 127 ± 59 μg/L, P = 0.03, Wilcoxon matched-pairs signed-ranks test) (each group n = 6). Similarly, in previous experiments comparing the release of LTA/TA and pneumolysin, ceftriaxone released higher amounts of proinflammatory bacterial compounds from S. pneumoniae than rifampicin (pneumolysin, 11.6 ± 2.1 versus 4.6 ± 1.2 μg/L, P = 0.03; LTA, 684 ± 277 versus 239 ± 105 μg/L, P = 0.03, Wilcoxon matched-pairs signed-ranks test, each group n = 6) (see Figure 1a and b).

Delayed onset of EAE in S. pneumoniae-infected mice after therapy with minocycline compared with ceftriaxone
Infection of EAE mice with S. pneumoniae in the latency period between immunization and first clinical symptoms aggravates the course of EAE.10 In animals treated with minocycline, the onset of symptoms of EAE was delayed by 1 day (minocycline: day 13.1 ± 0.4) compared with mice treated with ceftriaxone (day 12.0 ± 0.3; P = 0.04). Minocycline, however, did not influence other parameters of the course of EAE, i.e. the mean cumulative score, the mean maximum score and the incidence (see Table 1 and Figure 2a) (n = 30 in the minocycline group, n = 36 in the ceftriaxone group).

Discussion
Key proinflammatory compounds of the important human pathogen S. pneumoniae are LTA/TA and pneumolysin. Pneumolysin, in addition to its ability to stimulate the innate immune system, at higher concentrations damages eukaryotic cells by forming pores in the cell membranes.14,24–26 LTA/TA and pneumolysin contribute to the development of septic shock and
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Table 1. Delayed disease onset after therapy with minocycline compared with ceftriaxone; clinical parameters of active EAE in S. pneumoniae-infected mice with different antibiotic treatment (the table shows the total number of mice studied)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of S. pneumoniae infection</th>
<th>Incidence (%)</th>
<th>Mean day of disease onset (±SEM)</th>
<th>Mean maximum score (±SEM)</th>
<th>Mean cumulative score (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minocycline</td>
<td>7</td>
<td>30/30 (100)</td>
<td>13.1 ± 0.4</td>
<td>3.1 ± 0.1</td>
<td>57.7 ± 3.1</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>7</td>
<td>36/36 (100)</td>
<td>12.0 ± 0.3</td>
<td>3.2 ± 0.1</td>
<td>61.8 ± 3.0</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.04</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Figure 2. Course of EAE after treatment of S. pneumoniae peritonitis with minocycline, rifampicin or ceftriaxone. MOG35–55 immunized mice received intraperitoneal injections of 6.8 ± 0.6 log cfu S. pneumoniae 7 days after immunization in the rifampicin/ceftriaxone experiment. Twelve hours after infection, treatment with ceftriaxone, rifampicin or minocycline, respectively, was started twice daily for 3 days (minocycline versus ceftriaxone) or 5 days (rifampicin versus ceftriaxone). Mice were weighed and scored for signs of EAE every day. Graphs show mean clinical scores of the animals ± SEM. (a) S. pneumoniae-infected EAE mice showed a delayed disease onset when treated with minocycline compared with ceftriaxone (P = 0.04). (b) No differences in the course of EAE after S. pneumoniae infection and treatment with rifampicin compared with ceftriaxone were found.

A reduction of the release of proinflammatory and directly toxic bacterial products by inhibitors of bacterial protein synthesis decreases early mortality and neuronal injury in bacterial meningitis,31–33 and mortality in S. aureus sepsis.34 For this reason, we hypothesized that mice which suffer an S. pneumoniae infection during the early subclinical phase of EAE would also benefit from a treatment strategy aimed at minimizing the release of proinflammatory/toxic bacterial products.

Doses of 100 mg/kg ceftriaxone in mice produce maximum serum concentrations comparable to those observed in adults after doses of 2 g. The serum concentrations produced by 100 mg/kg minocycline and 100 mg/kg rifampicin in mice are higher than those observed after standard doses in humans. We chose these relatively high doses leading to serum concentrations in mice above those observed in humans receiving usual doses of rifampicin and minocycline for two reasons: (i) rifampicin at this dose lowered mortality compared with ceftriaxone at an equal dose in experimental S. pneumoniae meningitis;31 and (ii) even at 100 mg/kg twice daily, minocycline was unable to cure all mice (three mice of the minocycline group died of infection) probably because of the comparably high MIC of minocycline for S. pneumoniae. Lowering the daily minocycline dose to achieve serum concentrations comparable to those in humans during standard therapy would have resulted in a higher rate of bacteriological failure jeopardizing the purpose of this study.

The onset of the clinical symptoms of EAE was delayed by 1 day in mice treated with minocycline, but not in those treated with rifampicin, compared with the standard therapy with ceftriaxone. The severity of EAE was not substantially altered in mice receiving the inhibitors of bacterial protein synthesis rifampicin and minocycline as compared with animals receiving ceftriaxone (Figure 2a and b). Although statistically significant, a delay of 1 day in the onset of symptoms of EAE is of minor clinical relevance.

Constituents of the cell wall of Gram-positive bacteria appear to play an important role in the development and progression of MS. The CSF of MS patients showed increased levels of LTA and peptidoglycan antibodies compared with healthy controls.17,24 Macrophages are unable to completely digest the bacterial cell wall: bacterial peptidoglycan was present within antigen-presenting cells in the brain of MS patients.35 Important immunostimulatory mediators for the development of EAE are tumour necrosis factor-α and interleukin-6.36–38 A clear synthesis of these proinflammatory cytokines in human monocytes is induced by LTA concentrations from 100 to 1000 μg/L.39 Such LTA levels were measured in the culture supernatants after addition of the three antibiotics. The release of LTA and

β-Lactam antibiotics are commonly used as the standard therapy for many bacterial infections. They release larger amounts of bacterial toxins and proinflammatory products than antibiotics interfering with bacterial protein synthesis.19,20,28–31 A reduction of the release of proinflammatory and directly toxic bacterial products by inhibitors of bacterial protein synthesis decreases early mortality and neuronal injury in bacterial meningitis,31–33 and mortality in S. aureus sepsis.34 For this reason, we hypothesized that mice which suffer an S. pneumoniae infection during the early subclinical phase of EAE would also benefit from a treatment strategy aimed at minimizing the release of proinflammatory/toxic bacterial products.

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pneumolysin in vitro was lower when pneumococci were treated with minocycline or rifampicin compared with ceftriaxone.

In this study, the severity of EAE was not influenced by the mode of antibiotic treatment. One possible explanation for the inability of minocycline and rifampicin to alleviate EAE might be the short duration of the infection and the rapid elimination of live pneumococci by the antibiotics administered, which limited the intensity of the proinflammatory stimulus in the present experiment. We are, however, unaware of a chronic S. pneumoniae infection in mice with a high bacterial load and low inter-individual variation.

All three antibiotics used in this study have been described to have direct neuroprotective properties. The in vitro effects of ceftriaxone and rifampicin, however, were observed at concentrations which can hardly be reached within the CNS of humans. The potential direct neuroprotective effects of ceftriaxone and rifampicin have not been studied in EAE, whereas minocycline at doses of 25–100 mg/kg administered for several weeks alleviated EAE. The equal incidence and severity of EAE in all three treatment groups does not point to a direct neuroprotective effect of a short-duration treatment with one of these compounds in EAE.

In conclusion, bacterial infections including those caused by S. pneumoniae can aggravate the course of MS and EAE. Bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease.

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Transparency declarations
All authors have no commercial interest in this project and have no associations with the companies producing the drugs used in this study.

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