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Rifampin Followed by Ceftriaxone for Experimental Meningitis Decreases Lipoteichoic Acid Concentrations in Cerebrospinal Fluid and Reduces Neuronal Damage in Comparison to Ceftriaxone Alone

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Rifampin (RIF) releases smaller quantities of lipoteichoic acids (LTAs) from *Streptococcus pneumoniae* than ceftriaxone (CRO). Due to the rapid development of resistance, RIF cannot be used as a single agent for therapy of bacterial meningitis. For this reason, we compared the effect of treatment with RIF followed by treatment with CRO (RIF-CRO) or the effect of treatment with clindamycin (CLI) followed by treatment with CRO (CLI-CRO) to that of CRO alone on the concentrations of LTAs and teichoic acids in vitro. The effects of RIF-CRO on LTA concentrations in cerebrospinal fluid (CSF) and on neuronal injury were investigated in a rabbit model of *S. pneumoniae* meningitis. In vitro, bacterial titers were effectively reduced by CRO, RIF-CRO, and CLI-CRO when each drug was used at 10 μg/ml. The levels of release of LTAs after the initiation of therapy were lower in RIF-CRO- and CLI-CRO-treated cultures than in cultures treated with CRO alone ($P < 0.05$ from 3 to 12 h after initiation of treatment). Similarly, in rabbits, the increase in the amount of LTAs in CSF was lower in RIF-CRO-treated animals than in CRO-treated animals ($P = 0.02$). The density of dentate apoptotic granular cells was lower after RIF-CRO therapy than after CRO therapy (medians, 58.4 and 145.6/mm^2, respectively; 25th quartiles, 36.3 and 81.7/mm^2, respectively; 75th quartiles, 100.7 and 152.3/mm^2, respectively; $P = 0.03$). Therefore, initiation of therapy with a protein synthesis-inhibiting antibacterial and continuation of therapy with a combination that includes a β-lactam may be a strategy to decrease neuronal injury in bacterial meningitis.

Neuronal injury in bacterial meningitis is a consequence of leukocyte invasion into central nervous system compartments, stimulation of microglia and resident macrophages, and the direct toxicity of bacterial components on the cerebral endothelium and neuronal cells. Pneumococcal cell wall components attract leukocytes into the central nervous system and stimulate the release of proinflammatory cytokines (9, 25). Teichoic acids and lipoteichoic acids (LTAs) are the most potent proinflammatory constituents of the membrane and cell wall of *Streptococcus pneumoniae*. When injected into the subarachnoid space, LTAs cause profound meningeal inflammation (25).

In the initial phase of treatment with β-lactam antibiotics, a brisk increase in the level of meningeal inflammation occurs (13). Several bactericidal antibiotics without a primary influence upon cell wall synthesis (trovafloxacin, rifampin [RIF], rifabutin, and quinupristin-dalfopristin) delayed and/or decreased the release of LTAs in comparison with the time of release after treatment with ceftriaxone (CRO) (15, 22, 26). In this respect, bacterial protein synthesis inhibitors were more effective than quinolones (22, 23). In humans, adverse outcomes after *S. pneumoniae* meningitis correlated with the LTA concentration in cerebrospinal fluid (CSF) upon hospital admission (20). Consequently, in a mouse model of *S. pneumoniae* meningitis, RIF reduced the concentrations of LTAs in serum and CSF and lowered the overall mortality rate from 49 to 26% compared with the concentrations and mortality rates noted after treatment with CRO. Kaplan-Meyer survival analysis revealed a substantial reduction in the rate of mortality during the first 24 h in mice receiving RIF (16). In rabbit *S. pneumoniae* meningitis, RIF inhibited the free radical production of leukocytes at the onset of therapy and reduced neuronal damage in the dentate gyrus of the hippocampal formation compared to the effects of CRO (2). Due to the rapid development of bacterial resistance, however, RIF cannot be used as a single agent for the treatment of bacterial meningitis. One strategy to overcome this problem is to start treatment with a nonbacteriolytic protein synthesis inhibitor and to add a β-lactam antibiotic later. For this reason, we studied in vitro whether pretreatment with RIF or clindamycin (CLI) is able to inhibit the release of LTAs caused by CRO therapy alone. Furthermore, we evaluated in a rabbit model of experimental *S. pneumoniae* meningitis if this strategy has a beneficial effect on neuronal damage in vivo.

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MATERIALS AND METHODS

Pathogen. A penicillin-sensitive *S. pneumoniae* type 3 strain originally isolated from an adult with meningitis (gift of M. G. Täuber, University of Bern, Bern, Switzerland) was used in the in vivo and in vitro experiments. After several passages in rabbits, infected CSF was cultured for 24 h on blood agar plates, and the resulting bacteria were suspended in sterile saline. Aliquots were kept at −70°C. MICs and minimal bactericidal concentrations, determined by the broth macrodilution method, were 0.03 and 0.06 μg/ml, respectively, for CRO; 0.008

and 0.06 μg/ml, respectively, for RIF; and 0.015 and 0.125 μg/ml, respectively, for CLI.

In vitro studies. Bacteria were grown overnight in tryptic soy broth (TSB) to an optical density at 578 nm of approximately 0.1. Bacteria were collected by centrifugation and were resuspended in fresh TSB to a final concentration of approximately 10^6 to 10^7 CFU/ml. The resuspension in fresh TSB ensured growth in the logarithmic phase for at least 3 h; i.e., drugs were added during the logarithmic phase of growth. Bacterial counts were determined at 0, 3, 6, 9, and 12 h by plating 10-μl samples of 10-fold dilutions onto blood agar plates. In the presence of antibacterial agents, the bactericidal rates (the change in the log number of CFU per milliliter per hour) were determined by log-linear regression analysis of bacterial titers versus time. Control cultures were grown after resuspension without antibiotics. For antibiotic treatment (with each antibiotic used at a concentration of 10 μg/ml), (i) CRO, (ii) RIF, (iii) CLI, (iv) RIF followed by CRO after 6 h (RIF-CRO), and (v) CLI followed by CRO after 6 h (CLI-CRO) (n = 5 for each group) were used.

Rabbit model. After intramuscular anesthesia with ketamine (25 mg/kg of body weight) and xylazine (5 mg/kg), New Zealand White rabbits were inoculated intracisternally with approximately 10^6 CFU of an *S. pneumoniae* type 3 strain. Anesthesia was maintained with intravenous urethane for the duration of the experiment (24 h).

Intravenous antibiotic treatment was initiated 12 h after infection with either CRO (Rocephin; Hoffmann-La Roche, Grenzach-Wyhlen, Germany; n = 11; loading dose, 20 mg/kg; maintenance dose, 10 mg/kg/h) or RIF (Rifa; Grünenthal, Stolberg, Germany; n = 11; loading dose, 10 mg/kg; maintenance dose, 5 mg/kg/h), followed 6 h later by treatment with CRO (loading dose, 20 mg/kg; maintenance dose, 10 mg/kg/h). Blood and CSF were drawn at 12, 14, 18, 20, and 24 h. At 24 h after infection, the rabbits were killed by intravenous injection of 75 mg of thiopental (Trapanal; Byk Gulden, Konstanz, Germany). The brains were removed and fixed in 4% paraformaldehyde for 24 h. Two animals in the CRO group and one animal in the CRO-RIF group died before the end of the experiment at 24 h after infection. Data for these animals were not included in the evaluation.

Antibiotics were administered continuously to rapidly produce steady-state concentrations in serum and to eliminate fluctuations in concentrations in CSF. CRO levels in CSF were similar (approximately 5 μg/ml) after the administration of a single dose (125 mg/kg) and after continuous administration of CRO at 10 mg/kg/h (5, 14).

In situ tailing. Deparaffinized and hydrated 1-μm sections were treated with 50 μg of proteinase K (Sigma, Deisenhofen, Germany) per ml for 15 min at 37°C in a reaction mixture containing 10 μl of 5× tailing buffer, 1 μl of digoxigenin DNA labeling mix, 2 μl of cobalt chloride, 12.5 U of terminal transferase, and the necessary amount of distilled water to give a volume of 50 μl. After the sections were washed, they were incubated with 10% fetal calf serum (FCS) for 15 min at room temperature and then washed again. A solution of alkaline phosphatase-labeled antidigoxigenin 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-indolylphosphate (27). The sections were counterstained with nuclear fast red-aluminum hydroxide (all reagents were from Roche Diagnostics, Mannheim, Germany).

Quantification of apoptotic neurons. Hematoxylin-eosin-stained sections were used to measure the area of the granule cell layer of the dentate gyrus in the hippocampal formation with a Contron Videoplan computer (Grundig, Nürnberg, Germany). A blinded observer counted the numbers of apoptotic cells in the adjacent section stained by the in situ tailing reaction. Hematoxylin-eosin-stained sections and sections stained by the in situ tailing reaction showed morphological features of apoptosis in the same neurons. The density of apoptotic neurons was expressed as the number of marked neurons per square millimeter of the hippocampal granule cell layer.

Enzyme immunoassay for quantifying LTAs and teichoic acids. An enzyme immunoassay was used to measure the release of LTAs during antibiotic treatment (22). Briefly, the purified LTAs used for immunization and construction of the standard curve were prepared from unencapsulated strain *S. pneumoniae* R6. Polyclonal antibodies were raised in New Zealand White rabbits immunized subcutaneously with 500 μg of LTAs mixed with an equal volume of incomplete Freund's adjuvant. The commercially available monoclonal antibody TEPC-15 (Sigma) was used as the capture antibody, and the polyconal rabbit antiserum raised against LTAs was used as the detector antibody. CRO, RIF, and CLI did not interfere with the LTA assay. The LTA concentrations were measured in CSF samples taken at 12, 14, 18, 20, and 24 h after infection. LTA concentrations in supernatants of bacterial cultures in TSB were determined at 0, 3, 6, 9, and 12 h after the start of antibiotic treatment.

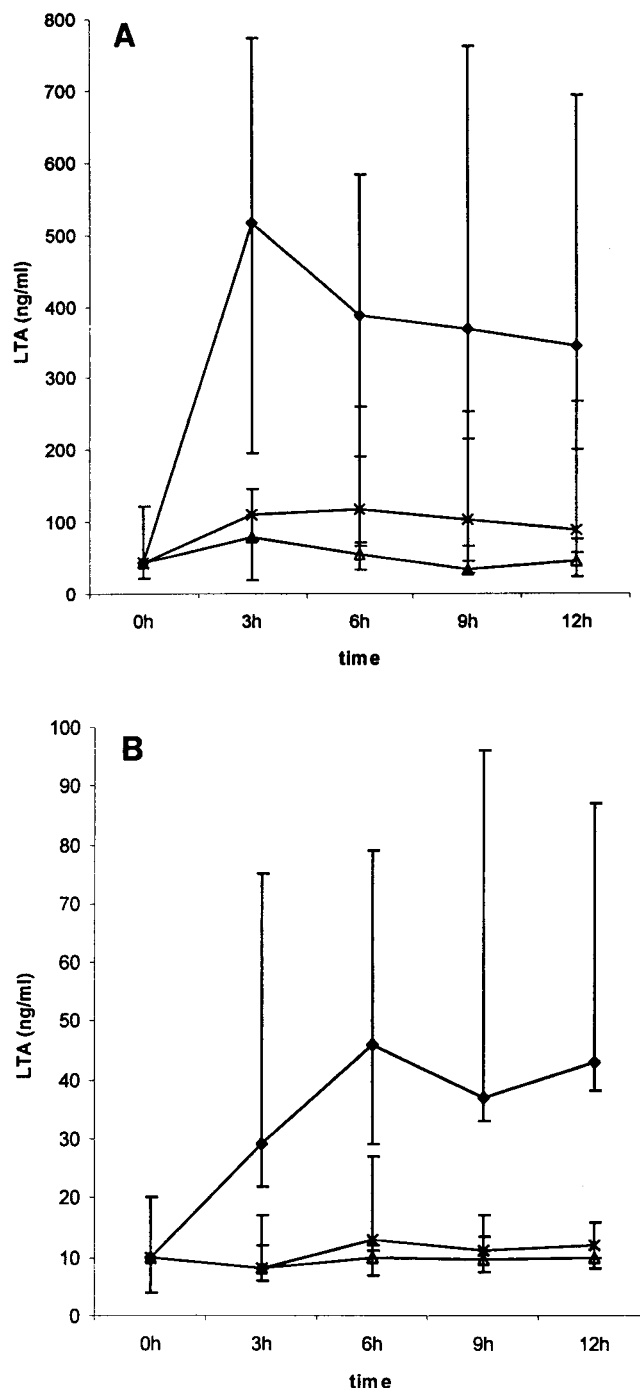

FIG. 1. Release of LTAs and teichoic acids in cultures of *S. pneumoniae* type 3 during antibiotic treatment (10 μg/ml) from 0 to 12 h after initiation of treatment (the values are the medians ± the 25th and 75th quartiles). (A) ♦, CRO; ×, RIF; △, RIF-CRO ($P < 0.05$ for all groups versus CRO from 3 to 12 h; n = 5). (B) ♦, CRO; ×, CLI; △, CLI-CRO ($P < 0.05$ for all groups versus CRO from 3 to 12 h; n = 5).

Statistical analysis. In vitro data were compared by the Friedman paired nonparametric test, and P values were adjusted for repeated testing by Dunn's multiple-comparison test. Data from the animal experiments were compared by the two-tailed Mann-Whitney U test. If not indicated otherwise, the results are expressed as medians and 25th and 75th quartiles. Bactericidal activity was

TABLE 1. Rabbit model of experimental meningitis[a]

Antibacterial regimen	Protein concn (μg/ml)	Lactate concn (mmol/liter)	White blood cell count (μl^{-1})	Apoptotic neuron count (no./mm^2)	Change in LTA concn (μg/ml)	
					Between 14 and 12 h	Between 20 and 18 h
CRO	3,894 (2,691/4,677)	5.2 (4.2/10.4)	5,611 (3,147/7,317)	145.6 (81.7/152.3)	70 (24/1,239)	5.5 (−2/19)
RIF-CRO	3,835 (2,131/4,053)	7.8 (4.3/10.7)	3,701 (2,880/7,765)	58.4 (36.3/100.7)[b]	3.5 (−119/58)[b]	−4 (−46/1)

[a] The concentrations of protein, lactate, and white blood cells in CSF were determined 24 h after induction of pneumococcal meningitis; the densities of apoptotic neurons in the dentate gyrus and the differences in the LTA concentrations in CSF were determined between 14 and 12 h (the start of antibiotic therapy was at 12 h) and 20 and 18 h (the start of CRO therapy in RIF-pretreated animals was at 18 h). The values are medians (25th quartile/75th quartile).

[b] $P < 0.05$.

evaluated by log-linear regression analysis of bacterial titers versus time. Variation was expressed as the mean ± standard deviation (SD), and groups were compared by parametric analysis of variance. $P < 0.05$ was considered significant.

RESULTS

In vitro studies. Because the experiments with RIF and CLI were performed on different days, each set of experiments was provided with a separate CRO-treated control group. Antibiotic treatment at 10 μg/ml effectively reduced bacterial titers in all groups. In the first set of experiments the mean ± SD changes in bacterial loads were −0.38 ± 0.09, −0.20 ± 0.11, and −0.22 ± 0.12 log CFU/ml/h for CRO, RIF, and RIF-CRO, respectively ($P > 0.05$ for all groups versus CRO). In the second set of experiments the mean ± SD changes in bacterial loads were −0.32 ± 0.09, −0.31 ± 0.07, and −0.32 ± 0.13 log CFU/ml/h for CRO, CLI, and CLI-CRO, respectively ($P > 0.05$ for all groups versus CRO).

The bacterial load at the start of treatment was 7.02 ± 0.33 log CFU/ml (mean ± SD) in the first set of experiments and 6.14 ± 0.36 log CFU/ml in the second set of experiments and caused higher absolute concentrations of LTAs in the supernatants in the first set of experiments (Fig. 1A and B). A rapid and intense release of LTAs occurred in the first hours of exposure to 10 μg of CRO per ml. After exposure to RIF (10 μg/ml), the LTA concentrations in the supernatants were substantially lower ($P < 0.05$ for RIF versus CRO from 3 to 12 h). Pretreatment with RIF prevented the LTA release previously observed in the first hours after exposure to CRO alone ($P < 0.05$ for RIF-CRO versus CRO from 3 to 12 h) (Fig. 1A). Similarly, the amount of LTA released after treatment with CLI or CLI-CRO was lower than the amount released after treatment with CRO alone (10 μg/ml each) ($P < 0.05$ from 3 to 12 h) (Fig. 1B).

Rabbit model. Bacterial titers in vivo were effectively reduced in both groups. The mean ± SD changes in the bacterial loads were −0.42 ± 0.15 log CFU/ml/h in rabbits treated with RIF followed by CRO alone and −0.64 ± 0.16 CFU/ml/h in controls treated with CRO alone ($P = 0.003$).

Lactate and protein concentrations in CSF and white blood cell counts in CSF increased during the course of the experiment in both groups. The differences, however, were not statistically significant (Table 1).

In the initial phase of antibiotic treatment, the amount of LTA released was lower in RIF-pretreated animals. The increase in LTA concentrations in CSF, expressed as the change in concentration between 14 and 12 h, was 3.5 ng/ml (25th quartile, −119 ng/ml; 75th quartile, 58 ng/ml) in RIF-pretreated animals, whereas it was 70 ng/ml (25th quartile, 24 ng/ml; 75th quartile, 1,239 ng/ml) after initial CRO treatment ($P = 0.02$). After the initial peak in the LTA concentration in CRO-treated animals, the LTA concentrations decreased to levels comparable to those in RIF-pretreated animals. When CRO treatment was added to the RIF treatment 18 h after infection (6 h after the start of treatment with RIF), the LTA concentrations in CSF did not increase. The median change in the LTA concentration between 20 and 18 h in these rabbits was −4 ng/ml (25th quartile, −46 ng/ml; 75th quartile, 1 ng/ml) (Table 1). The density of apoptotic neurons in the dentate gyrus of the hippocampal formation was lower after therapy with RIF-CRO (Table 1). The median was 58.4/mm^2 (25th quartile, 36.3/mm^2; 75th quartile, 100.7/mm^2), whereas the median after therapy with CRO was 145.6/mm^2 (25th quartile, 81.7 mm^2; 75th quartile, 152.3 mm^2) ($P = 0.03$).

DISCUSSION

Neuronal injury in *S. pneumoniae* meningitis is not a monocausal event. Pneumococcal products are able to attract leukocytes into the central nervous system; stimulate microglial cells and resident macrophages (8, 9, 25); impair the cerebral vascular endothelium and ependyma, thereby promoting brain edema; and directly damage neurons. Pneumolysin possesses direct neurotoxicity at the concentrations encountered in CSF (3, 21). Heat-inactivated pneumococci and pneumococcal cell walls exert a cytotoxic effect in cocultures of neurons and glial cells (11), suggesting that pneumococcal components mediate damage of brain tissue during meningitis not only directly or by induction of the invasion of leukocytes but also by stimulation of glial cells. Furthermore, neuronal injury occurs subsequent to ischemia caused by brain edema and vasculitis. Reactive oxygen intermediates and excitatory amino acids are important mediators of neuronal injury. Intracellular calcium increase, energy depletion, and caspase activation are the effectors of cell death (for reviews, see references 17 and 19).

Compared to other adjunctive therapies effective in experimental meningitis, approaches that lead to reductions of the concentrations of proinflammatory or toxic bacterial compounds in CSF act at the top of the noxious cascade (for a review, see reference 18). Unlike intrathecal or intravenous application of neutralizing antibodies against defined bacterial products (e.g., LTAs and endotoxin) (10), the use of bacterial protein synthesis inhibitors decreases the release of various noxious agents originating from bacteria: LTA (22, 23, 26), peptidoglycans (26), bacterial DNA (7), and endotoxin (6, 24). Bacterial protein synthesis inhibitors also reduce the levels of toxic compounds directly released, such as pneumolysin (H.

Kerstan et al., 75th Kongr. Dtsch. Gesellschaft Neurol., abstr. V41, 2002). Therefore, inhibition of the release of bacterial products is capable of attenuating the detrimental effects of granulocyte migration and microglia and resident macrophage stimulation and the direct toxic actions of bacterial products on nervous system tissue. Principally, this approach can be combined with other strategies to reduce neuronal damage, in particular, the use of inhibitors of leukocyte migration into the central nervous system, scavengers of reactive oxygen intermediates, and excitatory amino acid antagonists. Its validity has recently been proven: treatment with RIF reduces neuronal injury and mortality in experimental *S. pneumoniae* meningitis compared to the effects of treatment with CRO (2, 16). The rapid induction of resistance to RIF during monotherapy, however, precludes its use as a single agent for meningitis. The in vitro and in vivo studies described here demonstrate that a β-lactam antibiotic can be added 6 h after initiation of RIF therapy without loosing the advantages of the initiation of therapy with RIF (i.e., lower concentrations of proinflammatory and toxic bacterial products in CSF and less neuronal injury).

The in vitro data from experiments in which therapy was started with CLI and then CRO was added, in which large quantities of LTA were not released, suggest that this concept works not only with RIF but also with other bacterial protein synthesis inhibitors. In several extracerebral experimental infections, the use of bacterial protein synthesis inhibitors has proved more beneficial than treatment with β-lactams: in a mouse model of pneumonic plague, early treatment (24 h after infection) with CRO produced 100% protection. When treatment was initiated 42 h after infection (at that time mice were bacteremic), the rate of mortality during treatment with β-lactam antibiotics was greater than 90%. Conversely, approximately 60% of mice receiving streptomycin, netilmicin, gentamicin, ciprofloxacin, and ofloxacin beginning 42 h after infection survived (4). In a mouse model of *Staphylococcus aureus* sepsis and peritonitis the protein synthesis inhibitor CLI decreased the rate of mortality compared to that achieved after CRO therapy (1).

In conclusion, sufficient evidence for the validity of the concept of modulating the release of proinflammatory bacterial compounds by antibacterials has been accumulated in vitro and in animal experiments to justify a clinical trial of the use of this regimen for bacterial meningitis. This concept may be particularly valuable for the condition in less developed countries, since adjunctive therapy with dexamethasone appears to be ineffective under these conditions (12). A properly conducted study on the possible benefit of the initiation of therapy with a bacterial protein synthesis inhibitor instead of a β-lactam antibiotic, however, will both require strict patient selection and the inclusion of a large number of patients. We hypothesize that the benefit of this approach will be greatest in patients with high bacterial loads in the central nervous system or the bloodstream.

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