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Doxycycline Reduces Mortality and Injury to the Brain and Cochlea in Experimental Pneumococcal Meningitis

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Bacterial meningitis is characterized by an inflammatory reaction to the invading pathogens that can ultimately lead to sensorineural hearing loss, permanent brain injury, or death. The matrix metalloproteinases (MMPs) and tumor necrosis factor alpha-converting enzyme (TACE) are key mediators that promote inflammation, blood-brain barrier disruption, and brain injury in bacterial meningitis. Doxycycline is a clinically used antibiotic with anti-inflammatory effects that lead to reduced cytokine release and the inhibition of MMPs. Here, doxycycline inhibited TACE with a 50% inhibitory dose of 74 μM in vitro and reduced the amount of tumor necrosis factor alpha released into the cerebrospinal fluid by 90% in vivo. In an infant rat model of pneumococcal meningitis, a single dose of doxycycline (30 mg/kg) given as adjuvant therapy in addition to ceftriaxone 18 h after infection significantly reduced the mortality, the blood-brain barrier disruption, and the extent of cortical brain injury. Adjuvant doxycycline (30 mg/kg given subcutaneously once daily for 4 days) also attenuated hearing loss, as assessed by auditory brainstem response audiometry, and neuronal death in the cochlear spiral ganglion at 3 weeks after infection. Thus, doxycycline, probably as a result of its anti-inflammatory properties, had broad beneficial effects in the brain and the cochlea and improved survival in this model of pneumococcal meningitis in infant rats.

Pneumococcal meningitis has a high level of mortality (up to 30%), and brain and/or cochlear damage occurs in up to 50% of the survivors (2). Inflammatory mediators, such as tumor necrosis factor alpha (TNF-α) and matrix metalloproteinases (MMPs), are produced during the host response to bacteria and contribute to the pathophysiology that can ultimately lead to death, brain damage, and hearing impairment (23, 26, 29, 36). The role of cytokines and MMPs in the pathophysiology of the cochlear damage associated with pneumococcal meningitis is still unknown. However, it has been demonstrated that MMPs are constitutively expressed at high levels in the cochlea (8) and that in meningitis pneumococci and polymorphonuclear leukocytes (PMNs) extend from the cerebrospinal fluid (CSF) to the perilymph via the cochlear aqueduct (5).

Tetracyclines are bacteriostatic agents with broad-spectrum antimicrobial activity (3). Doxycycline is a semisynthetic, long-acting, second-generation tetracycline which is absorbed rapidly and penetrates well into the brain and CSF (48). Doxycycline has been shown to have anti-inflammatory effects that are separate and distinct from its antimicrobial action (13, 16, 35, 43). These effects include the reduction of cytokine release and the inhibition of MMPs (7, 38). Experimental and clinical studies have indicated that treatment with doxycycline may be beneficial in inflammatory diseases associated with excessive MMP activity (7, 13, 35).

In pneumococcal meningitis massive subarachnoid and ventricular space inflammation is triggered by the presence of bacteria in the CSF space (7, 13, 35). Cytokines, such as TNF-α, trigger a cascade of inflammatory mediators, including MMPs. In turn, MMPs and related metalloproteinases can act as sheddases or convertases as they transform membrane-bound cytokines, cytokine receptors, and adhesion molecules into their soluble forms (27). TNF-α-converting enzyme (TACE), a membrane protein that contains a disintegrin and a metalloproteinase domain, is a highly efficient sheddase of TNF-α and TNF receptors and plays an integral part in the inflammatory network of MMPs and cytokines in bacterial meningitis (17, 23).

Pharmacologic inhibition of MMPs and TACE has beneficial effects in experimental models of bacterial meningitis. The hydroxamic acid-based MMP inhibitor BB-94 prevented the extravasation of Evans blue, an indicator of blood-brain barrier (BBB) permeability, in a model of meningococcal meningitis (36). Another MMP inhibitor, GM-6001, reduced cortical neuronal injury in an infant rat model of pneumococcal meningitis (24). Treatment with BB-1101, an inhibitor of both MMPs and TACE, reduced the concentration of TNF-α in the CSF, reduced mortality, and attenuated brain injury in experimental pneumococcal meningitis (23).

While these drugs have not been tested in clinical studies, doxycycline is a relatively inexpensive drug with a well-characterized clinical safety profile. Here we demonstrate that doxycycline potently inhibits TACE in vitro and reduces TNF-α release into the CSF in a model of sterile meningitis. Given as an adjuvant with a beta-lactam antibiotic for pneumococcal meningitis, doxycycline reduced mortality, protected the BBB, and reduced injury in the cerebral cortex. Doxycycline also

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attenuated neuronal death in the cochlear spinal ganglion, which correlated with attenuation of hearing loss.

MATERIALS AND METHODS

Model of sterile meningitis. A model of sterile meningitis was used to exclude the effects of antimicrobial mechanisms on inflammatory parameters. The nonencapsulated *Streptococcus pneumoniae* mutant R6 was grown at 37°C in brain heart infusion (BHI) to the mid-logarithmic phase, washed with sterile saline, and resuspended in saline at a concentration of 5×10^{10} CFU/ml. The bacteria were then heat inactivated at 80°C for 20 min, and 10 μl of the suspension containing heat-inactivated R6 pneumococci (hiR6) was injected intracisternally into nursing Wistar rats ($n = 14$) on postnatal day 11 using a 32-gauge needle. At 3, 6, 12, and 24 h after induction of sterile meningitis, CSF was obtained by puncture of the cisterna magna to determine inflammatory parameters, as described below.

Model of meningitis with live pneumococci. Nursing Wistar rats were injected intracisternally on postnatal day 11 with 10 μl of saline containing $2.5 \times 10^6 \pm 1.5 \times 10^6$ CFU/ml *S. pneumonia*e as described previously (23). At 18, 24, and 40 h after infection, all animals were assessed clinically as described elsewhere (23), and CSF was obtained from the cisterna magna to document infection and to determine the number of bacteria by plating serial dilutions of CSF on blood agar plates. If incubation of 5 μl of undiluted CSF did not result in growth of bacteria, the CSF was considered to be sterile. The detection limit for bacteria in the CSF was 200 CFU/ml. Immediately after CSF was removed 18 h after infection, antibiotic treatment with 100 mg/kg ceftriaxone (Roche Pharma, Reinach, Switzerland) given subcutaneously (s.c.) was initiated.

All animal studies were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland, and were performed using National Institutes of Health guidelines for animal experiments.

Treatment regimens. For sterile meningitis, animals received doxycycline (30 mg/kg given s.c.; $n = 7$; Vibravenös; Pfizer AG, Zurich, Switzerland) or an equal volume of saline (250 μl; $n = 7$) at the time of intracisternal injection of the hiR6 (pretreatment study). This dose has been shown previously to suppress cerebral MMP-9 activity in rodents and to reduce ischemic brain damage (9, 22).

To study the histopathological outcome posttreatment, we used a model with live bacteria. Beginning 18 h after infection, all animals ($n = 132$) received ceftriaxone (100 mg/kg given intraperitoneally [i.p.] every 12 h) and were randomized for treatment with a single dose of doxycycline (30 mg/kg given s.c.; $n = 67$) or with the same volume of saline (250 μl; $n = 65$). Treatments with ceftriaxone and doxycycline were initiated within 1 min of each other. The additives used in the formulation of doxycycline (polyvinylpyrrolidone and 2-amionethanol) are ingredients that are commonly used for preparation of pharmaceutical products, and no neuroprotective effects of these compounds have been reported. At 18, 24, and 40 h after infection, all animals were assessed clinically, and CSF was obtained from the cisterna magna to quantify the bacteria. At 40 h after infection, the surviving animals were sacrificed, and brain damage and BBB integrity were assessed as described below.

To assess cochlear damage, animals were infected ($n = 34$) and randomized for treatment with doxycycline (30 mg/kg given s.c. every 24 h; $n = 20$) or with the same volume of saline ($n = 14$). Mock-infected animals ($n = 9$) that received doxycycline were included in the study. All animals ($n = 43$) were treated with ceftriaxone (100 mg/kg given i.p. every 12 h). This treatment schedule was maintained for 4 days, starting 18 h after infection. At 3 weeks after infection, animals were sacrificed, and the density of type I neurons in the spiral ganglion was assessed by histomorphological analysis of cochlear sections (one randomly chosen cochlea per animal). The auditory brainstem responses (ABR) were measured in both ears of a subset of randomly chosen animals just before they were sacrificed ($n = 16$, $n = 13$, and $n = 5$ for the doxycycline, saline, and mock-infected groups, respectively).

Pharmacologic studies. The pharmacokinetics and the penetration of doxycycline into the CSF in animals with bacterial meningitis were studied by determining drug levels in the plasma and in the CSF 2, 4, 6, 8, and 10 h after s.c. injection of a single dose of doxycycline (30 mg/kg) given 18 h after infection of a separate group of animals ($n = 15$). Drug concentrations were determined by the agar diffusion method (12). The standard curve for plasma was generated with rat plasma, while the standard curve for CSF was generated with saline containing 10% rat plasma. *Escherichia coli* ATCC 10536 was used as the indicator strain.

Histopathology. To assess the effect of doxycycline on brain damage in animals with pneumococcal meningitis, the brains were evaluated histomorphologically 40 h after infection as described previously (23). Briefly, animals were perfused with 4% paraformaldehyde, and the brains were removed from the skulls and postfixed for 4 h in paraformaldehyde. The extent of cortical brain damage was assessed quantitatively, and the results were expressed as the percentage of the total cortical area, as described in detail previously (24). Neurons in the dentate granule layer of the hippocampus with a nuclear morphology typical of apoptosis were counted and scored, and the average score per animal was calculated from the data obtained for the sections evaluated (24). Animals that died before 40 h were excluded from the analysis. The evaluations were performed by an investigator blinded to the clinical, microbiological, and treatment data for the animals.

Histopathology of the cochlea. To determine the density of type I neurons in the spiral ganglion, the region of the temporal bone containing the cochlea was dissected from the skull and decalcified for 10 days in phosphate-buffered saline containing 120 mM EDTA and 0.1% sodium azide. One cochlea of each animal was embedded in paraffin, and the six most mid-modiolar sections (thickness, 10 μm) were mounted on a glass slide and stained with toluidine blue. The specimens were analyzed with a light microscope, and the images were digitized. The outline of the Rosenthal's canal was traced using the NIH Image program (version 1.61; U.S. National Institutes of Health, Bethesda, MD). The type I neurons in 10 profiles of the Rosenthal's canal, from the base to the apex of the cochlea, were counted. The results were expressed as the density of type I neurons per 10^4 μm^2 (40).

ABR measurements. Using inactin-anesthetized animals, ABR were recorded in an audiometric room (model AC-1 chamber; Industrial Acoustics Company, Niederkrüchten, Germany) for both ears in response to click stimulation (presentation rate, 20 clicks/s). Subdermal needle electrodes were placed in the mastoid of the ear tested (active), at the vertex (reference), and in the cervical neck muscles (ground). The sound stimulus consisted of 100-μs square wave impulses synthesized digitally using the SigGen32 software (System II; Tucker-Davis Technologies, Alachua, FL), fed into a programmable attenuator, and after D/A conversion transduced by a speaker (2405H ultra-high-frequency driver; JBL Professional, Northridge, CA) located 4 cm from the pinna. The system was calibrated with a sound level meter (2231 modular precision sound level meter; Brüel & Kjær, Nærum, Denmark) by measuring with a 0.5-in. microphone (type 4189; Brüel & Kjær, Nærum, Denmark) the maximum peak level (expressed in dB SPL; reference pressure, 20 μPa) emitted by the speaker when it was driven by a square wave impulse. ABR were amplified (10^3), band-pass filtered between 100 Hz and 3 kHz (model 1201low-noise preamplifier; Ithaco, Ithaca, NY), and averaged ($n = 500$) during a 12-ms window with the data acquisition software BioSig32 (System II; Tucker-Davis Technologies, Alachua, FL). The intensity of the click stimulus was reduced from 110 to 0 dB SPL in 10-dB steps. The hearing threshold was defined as the lowest intensity which induced the appearance of a visually detectable first peak.

In vitro time kill assay for pneumococci. Pneumococci were cultured in BHI to an optical density at 590 nm of 0.3 and then diluted 40-fold in BHI. The effects of doxycycline (1× MIC), ceftriaxone (4× MIC), and a combination of doxycycline (1× MIC) and ceftriaxone (4× MIC) were then assessed by quantitative determination of bacterial numbers by culturing serial dilutions obtained at 2, 4, and 6 h.

TACE inhibition assay. Inhibition of TACE by doxycycline was assessed using recombinant human TACE and a fluorescence-quenched peptide substrate (21).

Inflammatory parameters. Soluble TNF-α in the CSF was measured by a rat TNF-α immunoassay (Quantikine; R&D Systems, Minneapolis, MN). CSF samples were centrifuged, the supernatants were diluted 1:50 in dilution buffer, and the assay was performed by following the manufacturer's instructions. PMNs in the CSF were counted in a hematocytometer chamber after 10-fold dilution in 0.4% Türk's solution.

Assessment of BBB integrity. To quantify changes in BBB integrity, we assessed the levels of extravasated serum albumin in the cerebral cortex in a subset of animals. At 40 h after infection, doxycycline-treated animals ($n = 14$) and saline-treated animals ($n = 14$) were perfused with phosphate-buffered saline. The frontal pole of the brain was removed and homogenized (1:6, wt/vol) in 20 mM HEPES buffer (pH 7.4) containing 1 mM acetic acid, 0.1 mM dithiothreitol, 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Protein concentrations were determined by the Pierce bicinchoninic acid assay (Pierce, Rockford, IL). Albumin concentrations in cortex homogenates were determined with a rat-specific albumin enzyme immunoassay kit (Nephrat; Exocell Inc., Philadelphia, PA).

Statistics. Normally distributed variables were expressed as means ± standard deviations unless indicated otherwise and were compared using one-way analysis of variance, followed by the unpaired Student t test. Variables that were not normally distributed were compared by using the Kruskal-Wallis test. When the latter test yielded a statistically significant value ($P < 0.05$), a pairwise comparison was done using the two-tailed nonparametric Mann-Whitney U test. Sur-

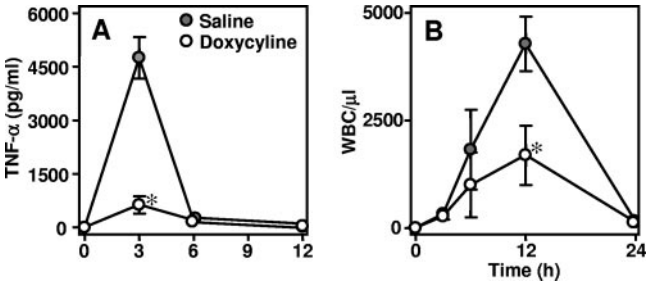

FIG. 1. Anti-inflammatory effect of doxycycline in animals with sterile meningitis. (A) Intracisternal injection of hiR6 resulted in release of large amounts of TNF-α into the CSF after 3 h (gray circles). Pretreatment with doxycycline reduced the amount of TNF-α released by 90% (open circles) (the asterisk indicates that the *P* value is <0.03 for a comparison with saline-treated animals at 3 h after infection [n = 3 for each group]). (B) Influx of PMNs into the CSF after injection of hiR6. Pretreatment with doxycycline significantly reduced the number of PMNs in CSF by 56% (the asterisk indicates that the *P* value is <0.05 for a comparison with saline-treated animals at 12 h after infection [n = 4 for each group]). WBC, white blood cells.

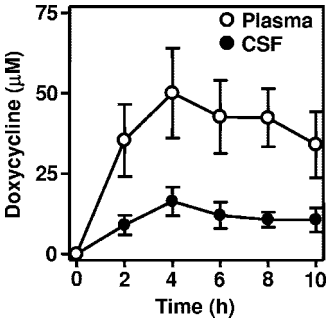

FIG. 2. Pharmacokinetics of doxycycline. Doxycycline at a dose of 30 mg/kg was administered 18 h after infection.

vival curves were analyzed by Kaplan-Meier analysis. The correlation between ABR thresholds and the density of type I neurons in the spiral ganglion was tested by assuming Gaussian populations (Pearson).

RESULTS

Inhibition of TNF-α release by doxycycline. Doxycycline inhibited recombinant human TACE, and the 50% inhibitory concentration (IC_{50}) was 74 ± 4 μM in vitro. In animals with sterile meningitis, intracisternal injection of hiR6 resulted in strong release of TNF-α into the CSF after 3 h (Fig. 1A). The TNF-α concentrations were comparable to those in meningitis models obtained by using live pneumococci (23). The TNF-α levels normalized within 12 h after injection of hiR6. Pretreatment of the animals with 30 mg/kg doxycycline administered i.p. reduced the amount of TNF-α released by 90% (Fig. 1A). The release of TNF-α caused by hiR6 was followed by an influx of PMNs into the CSF, which peaked 12 h after injection of hiR6 and disappeared by 24 h (Fig. 1B). Pretreatment with 30 mg/kg doxycycline reduced the number of PMNs in CSF by 56% (Fig. 1B). We concluded that reduction of the inflammation in the CSF resulting from doxycycline treatment is modulated by strong inhibition of early TNF-α release, probably as a result of the inhibitory effect of doxycycline on the activity of TACE.

Pharmacokinetic study. The concentrations of doxycycline after s.c. administration of 30 mg/kg peaked in plasma and CSF 4 h after administration (50 ± 24 μM in plasma and 16 ± 8 μM in CSF). The ratio of the area under the curve for CSF to the area under the curve for plasma 10 h after administration was 28.4% (Fig. 2).

Effect of doxycycline on bacterial killing by ceftriaxone. One concern regarding the use of doxycycline as adjuvant therapy for pneumococcal meningitis is the potential antagonistic effect with bactericidal antibiotics. Indeed, in vitro the killing of pneumococci by ceftriaxone at a concentration that was four times the MIC was partially antagonized by administration along with doxycycline (1× MIC) (Fig. 3A). In animals with experimental meningitis, however, combined therapy with doxycycline (30 mg/kg) and ceftriaxone (100 mg/kg) and therapy with ceftriaxone (100 mg/kg) alone did not result in significantly different bacterial titers in the CSF 6 h after the initiation of antibiotic treatment (CSF was sterile in 6/23 and 7/21 animals, respectively [not significantly different]; $8.0 \times 10^5 \pm 15.0 \times 10^5$ CFU/ml [n = 23] and $3.2 \times 10^5 \pm 6.7 \times 10^5$ CFU/ml [n = 21], respectively [not significantly different]). Amounts of CSF sufficient for assessment of quantitative bacterial titers were not obtained from all animals. At 40 h, CSF samples from animals in the two treatment groups were sterile (Fig. 3B). Thus, the antagonism observed in vitro seems to have little relevance for bacterial clearance in vivo.

Reduction in mortality, BBB leakage, cortical brain injury, and neuronal loss in the cochlear spiral ganglion. In the posttreatment study, which more closely reflected the clinical situation, the combined treatment with doxycycline and ceftriaxone significantly improved survival within 40 h after infection compared with treatment with ceftriaxone alone; the mortality rates were 20% for doxycycline plus ceftriaxone and 48% for saline plus ceftriaxone ($P < 0.02$) (Fig. 4A). Furthermore,

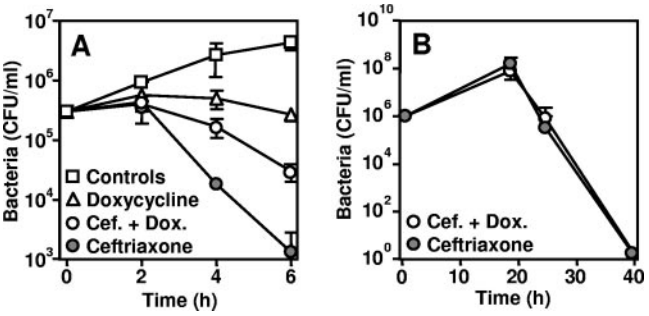

FIG. 3. Interaction of ceftriaxone and doxycycline in vitro and in vivo. (A) In vitro time kill curves for *S. pneumoniae*. The bacteriostatic antibiotic doxycycline (1× MIC) inhibited growth of pneumococci. The bactericidal antibiotic ceftriaxone (4× MIC) rapidly killed the pneumococci. The combination of doxycycline (1× MIC) and ceftriaxone (4× MIC) (Cef. + Dox.) exhibited in vitro antagonism. (B) Bacterial CSF titers in vivo at 0, 18, 24, and 40 h after infection with live pneumococci. At 24 h (6 h after the initiation of antibiotic treatment) combined therapy with doxycycline (30 mg/kg) and ceftriaxone (100 mg/kg) (Cef. + Dox.) and therapy with ceftriaxone (100 mg/kg) alone did not result in significantly different bacterial titers in the CSF ($8.0 \times 10^5 \pm 15.0 \times 10^5$ CFU/ml [n = 23] and $3.2 \times 10^5 \pm 6.7 \times 10^5$ CFU/ml [n = 21], respectively [not significantly different]). At 40 h after infection, CSF samples from all animals were sterile.

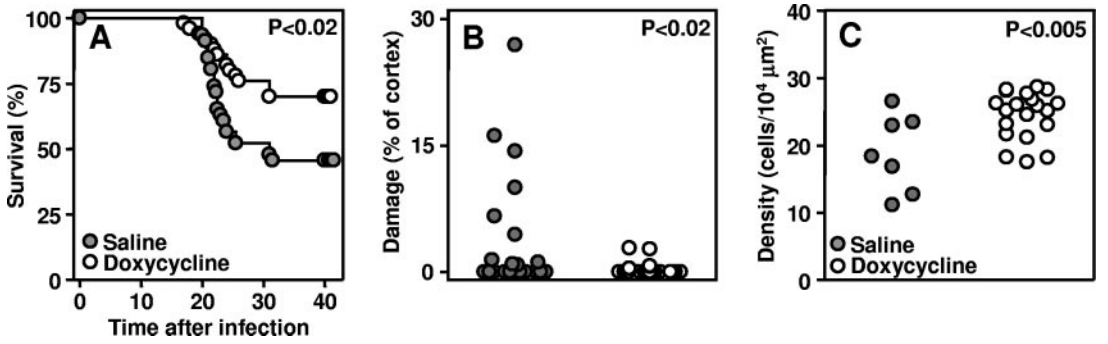

FIG. 4. Effect of doxycycline on survival and neuronal damage in the cortex and the cochlear spinal ganglion. (A) When doxycycline was given 18 h after infection in addition to the antibiotic ceftriaxone ($n = 67$), it significantly improved the survival compared to treatment with antibiotics alone (Saline) ($n = 65$). (B) Adjuvant doxycycline ($n = 67$) (open circles) significantly reduced the extent of neuronal injury in the cerebral cortex compared with the extent of neuronal injury with saline ($n = 65$) (gray circles). (C) Density of type I neurons in the Rosenthal's canal at 3 weeks after the infection. Adjuvant doxycycline (30 mg/kg given s.c. every 24 h for 4 days; $n = 20$) significantly reduced the neuronal loss in the cochlear spiral ganglion compared to the neuronal loss with saline ($n = 14$).

adjuvant doxycycline reduced the amount of albumin extravasated into the cerebral cortex (7.9 ± 0.9 μg cortical albumin/mg total protein for doxycycline plus ceftriaxone versus 12.7 ± 2.0 μg cortical albumin/mg total protein for ceftriaxone plus saline[$P < 0.04$]), indicating that there was significant reduction of the BBB disruption associated with bacterial meningitis. Also, adjuvant doxycycline significantly reduced the extent of cortical necrosis observed 40 h after infection (0 to 2.8% of the cortex damaged for doxycycline plus ceftriaxone versus 0 to 26.9% of the cortex damaged for saline plus ceftri-

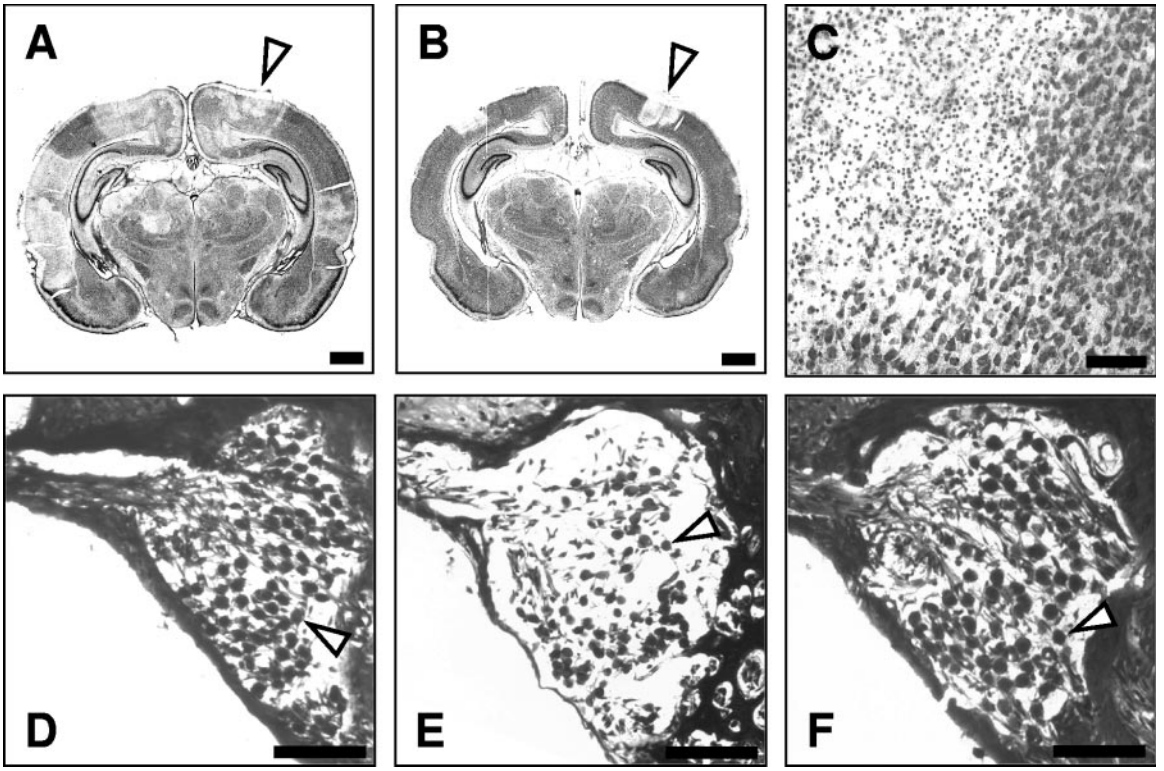

FIG. 5. (A to C) Brain histology of infant rats with pneumococcal meningitis 40 h after infection. (A and B) Whole-brain sections of the animals with the most cortical injury in the group treated with ceftriaxone alone (A) and the group treated with ceftriaxone and doxycycline (B). Areas where there was markedly reduced neuronal density were wedge shaped (arrowheads). The sections were stained with cresyl violet. Original magnification, ×5; scale bar = 1 mm. (C) Focus of cortical neuronal loss (upper left) with morphological features of necrosis, including cell swelling and fading of the cytoarchitecture. There was sharp demarcation from preserved neurons (lower right). The section was stained with cresyl violet. Original magnification, ×200; scale bar = 50 μm. (D to F) Histology of the Rosenthal's canal in the middle turn of the cochlea 3 weeks after infection. (D) In a mock-infected animal type I neurons (arrowhead) were present in the cochlear spiral ganglion. (E) In an animal that recovered from experimental pneumococcal meningitis type I neurons (arrowhead) were reduced. (F) Adjuvant doxycycline (30 mg/kg given s.c. every 24 h for 4 days) attenuated the loss of type I neurons. Scale bar = 100 μm.

axone ($P < 0.02$) (Fig. 4B and 5A to C). Apoptosis in the dentate gyrus of the hippocampus was not affected by doxycycline treatment (data not shown). Pneumococcal meningitis caused significant loss of type I neurons in the spiral ganglion, as determined 3 weeks after the infection (results are expressed as the density of type I neurons per 10^4 μm^2: 30.66 ± 2.19 for mock-infected animals versus 15.02 ± 1.87 for animals that were infected and treated with ceftriaxone plus saline [$P < 0.0001$]) (data not shown). Adjuvant doxycycline significantly attenuated the loss of type I neurons in infected animals (22.42 ± 1.66 for animals that were treated with doxycycline plus ceftriaxone versus 15.02 ± 1.87 for animals that were treated with saline plus ceftriaxone [$P < 0.005$]) (Fig. 4C and 5D to F), although there was a significant difference compared to mock-infected animals ($P < 0.01$) (data not shown).

ABR measurements. Bacterial meningitis caused a reproducible increase in the click-induced ABR thresholds (about 60 dB SPL) determined 3 weeks after infection (103.1 ± 2.47 for saline-treated animals versus 39.0 ± 3.48 for mock-infected animals [$P < 0.0001$]). Adjunctive doxycycline slightly but significantly attenuated the increase in the ABR thresholds in infected animals (94.06 ± 2.87 for doxycycline-treated animals versus 103.1 ± 2.47 for saline-treated animals [$P = 0.02$]).

DISCUSSION

In the present study performed in a rat model of pneumococcal meningitis, when the antibiotic doxycycline was given in addition to ceftriaxone, it decreased the mortality and reduced the damage to the brains and cochleas of the surviving animals.

In pneumococcal meningitis, bacteriolytic antimicrobial agents (e.g., ceftriaxone) cause the release of bacterial cell wall products, which are highly proinflammatory (44). Antibiotic therapies using less bacteriolytic agents for bacterial meningitis have been proposed previously (31, 42). Indeed, treatment with rifampin followed by ceftriaxone decreased lipoteichoic acid concentrations in cerebrospinal fluid and reduced neuronal damage compared with the results obtained with treatment with ceftriaxone alone (14).

In our study, doxycycline moderately antagonized the bactericidal effect of ceftriaxone in vitro, while it had no measurable effect on bacterial clearance from CSF in vivo. Therefore, it appears more likely that the marked protective effect of doxycycline on several parameters in this model was a result of its anti-inflammatory activity rather than interference with bacteriolysis induced by the beta-lactam antibiotic. The anti-inflammatory effects of doxycycline include reduction of cytokine release and inhibition of MMPs (7, 18, 38). We found that doxycycline also inhibits TACE, whose metalloproteinase domain exhibits considerable homology with the MMPs (17). In bacterial meningitis, MMPs and TACE have multiple roles, including extracellular matrix digestion and processing of the proinflammatory cytokine TNF-α from its inactive membrane-bound form into its active soluble form (23). Administration of TNF-α into the CSF results in pathophysiologic changes characteristic of bacterial meningitis, including breakdown of the blood-brain barrier, recruitment of PMNs, and generation of meningeal inflammation (39). A model of sterile meningitis using hiR6 allowed us to study the effect of doxycycline independent of an effect on the killing of live bacteria. In line with its TACE-inhibiting activity in vitro, doxycycline reduced TNF-α release and reduced the influx of PMNs into the CSF. Furthermore, in an experimental inflammatory disease model of the eye, doxycycline has been shown to reduce interleukin-converting enzyme and to decrease the bioactivity of the inflammatory cytokine interleukin-1 beta (41). Thus, the beneficial effect of doxycycline may at least in part be the result of attenuated inflammation.

MMP-mediated BBB disruption contributes to the development of global cerebral hypoperfusion by increasing intracranial pressure and brain ischemia (23, 24, 29, 36). In this study, doxycycline markedly reduced the BBB disruption, which may explain the beneficial effect of doxycycline therapy on cortical ischemic brain injury. Sepsis and septic shock are other complications of bacterial meningitis that contribute to the high mortality (19). In a model of sepsis, tetracycline treatment of animals reduced the 24-h mortality from 30% to 0% (28). Similarly, mortality was significantly reduced in the present study by adjuvant therapy with a single dose of doxycycline.

The development of necrotic cortical injury in pneumococcal meningitis involves MMPs and TACE, as documented in studies using hydroxamate-based inhibitors of MMPs and TACE, which are active at low (nanomolar) concentrations (23, 24, 29, 36). In contrast, doxycycline inhibits MMPs at micromolar concentrations (IC_{50} for MMP-8, 30 μM; IC_{50} for MMP-13, 1 μM), supporting the notion that the anti-inflammatory and neuroprotective effect is mediated at least in part by the inhibition of MMPs and TACE (45). In our study, the doxycycline concentrations were micromolar in the plasma and CSF after a dose of 30 mg/kg (approximately 50 μM in plasma and 15 μM in CSF). Plasma concentrations of 15 to 20 μM have been reported in humans after an intravenous dose of 200 mg doxycycline (1). Thus, the doxycycline concentrations found in the present study in infant rats are approximately two- to threefold higher than the doxycycline concentrations found in human patients after a therapeutic dose of doxycycline.

Tetracyclines have beneficial effects in other models of brain injury. In a model of focal central nervous system reperfusion injury, doxycycline (10 mg/kg every 8 h) significantly reduced the infarct volume (9). In another study, doxycycline (90 mg/kg every 12 h) and the more lipophilic compound minocycline protected hippocampal neurons of the CA1 region against global brain ischemia (49).

Apoptotic neuronal death in the hippocampus is a characteristic feature of pneumococcal meningitis in patients and corresponding animal models (6, 15, 32). Previous data suggested that MMP-TACE activity plays a role in this form of injury, since an inhibitor of MMPs and TACE (BB-1101) had a beneficial effect on both the cortex and the hippocampus (23). On the other hand, while doxycycline and several other MMP inhibitors (GM6001 and TNF484) were effective for preventing cortical injury, they failed to reduce hippocampal injury (24, 29).

Unilateral or bilateral hearing impairment is the most common neurologic sequela following meningitis and is found in up to 30% of patients (34, 47). Hearing loss results from involvement of the inner ear due to inflammation as pneumococci and leukocytes extend from the CSF to the perilymph via the cochlear aqueduct (4, 5, 11). In a model of pneumococcal meningitis the severity of permanent hearing impairment as assessed by the increase in the ABR thresholds 2 weeks after

the infection correlated significantly with the loss of type I spiral ganglion neurons (20). The results of the present study confirm and expand the correlation between the loss of type I neurons in the spiral ganglion and the degree of hearing impairment in infant rats. Use of adjunctive doxycycline also resulted in a moderate but significant decrease in the ABR thresholds of infected animals compared to the ABR thresholds of the animals that received only saline. This partial protection may reflect previous damage to the hair cells during the acute disease, probably caused by factors that adjunctive doxycycline could not attenuate, such as the release of bacterial pneumolysin in the perilymphatic and endolymphatic spaces (10, 46). If this is the case, the withdrawal of neurotrophins normally produced by the hair cells might further aggravate neuron loss in the spiral ganglion (30). This hypothesis is supported by the fact that significant differences in the neuron density of the spiral ganglion between infected and mock-infected animals can only be observed weeks after the cured infection (20, 37). Together, our results support further investigation of the beneficial effects of adjunctive doxycycline on the loss of spiral ganglion neurons caused by bacterial meningitis. This is a very important issue, since for patients, as long as the neuron density in the spiral ganglion is preserved, cochlear implants may still be able to rescue the hearing function.

There may be concerns regarding the use of a beta-lactam antibiotic in combination with a tetracycline for the treatment of pneumococcal meningitis. These concerns are based on a study published in 1951, in which patients with pneumococcal meningitis who were treated with intramuscular injections of 10^6 U of penicillin every 2 h were compared with a group of patients who were treated with a combination of penicillin (10^6 U every 2 h) plus tetracycline (500 mg every 6 h) (25). The mortality was significantly higher in the penicillin-tetracycline group (79% versus 30%). A worse outcome for patients with pneumococcal meningitis who were treated with a penicillin-tetracycline combination (85% mortality) than for patients who were treated with penicillin alone (59% mortality) was also reported in 1961 (33). In contrast, the combination of ceftriaxone with doxycycline led to an increased survival rate and had no measurable effect on bacterial clearance in the present model. Possibly, the bactericidal activity of high doses of ceftriaxone is more robust against potential antagonism by doxycycline than the bactericidal activity of moderate doses of penicillin is. Further preclinical and ultimately clinical studies are needed to assess whether the combination of ceftriaxone with doxycycline used in the present study can be used safely for bacterial meningitis therapy. Importantly, our results strengthen the concept that inhibition of cytokines and MMPs is a powerful strategy for adjunctive treatment of pneumococcal meningitis.

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REFERENCES

1. **Alestig, K.** 1973. Studies on doxycycline during intravenous and oral treatment with reference to renal function. Scand. J. Infect. Dis. **5:**193–198.
2. **Bedford, H., J. de Louvois, S. Halket, C. Peckham, R. Hurley, and D. Harvey.** 2001. Meningitis in infancy in England and Wales: follow up at age 5 years. BMJ **323:**533–536.
3. **Benbough, J., and G. A. Morrison.** 1965. Bacteriostatic actions of some tetracyclines. J. Pharm. Pharmacol. **17:**409–422.
4. **Bhatt, S., C. Halpin, W. Hsu, B. A. Thedinger, R. A. Levine, E. Tuomanen, and J. B. Nadol, Jr.** 1991. Hearing loss and pneumococcal meningitis: an animal model. Laryngoscope **101:**1285–1292.
5. **Bhatt, S. M., A. Lauretano, C. Cabellos, C. Halpin, R. A. Levine, W. Z. Xu, J. B. Nadol, Jr., and E. Tuomanen.** 1993. Progression of hearing loss in experimental pneumococcal meningitis: correlation with cerebrospinal fluid cytochemistry. J. Infect. Dis. **167:**675–683.
6. **Braun, J. S., R. Novak, P. J. Murray, C. M. Eischen, S. A. Susin, G. Kroemer, A. Halle, J. R. Weber, E. I. Tuomanen, and J. L. Cleveland.** 2001. Apoptosis-inducing factor mediates microglial and neuronal apoptosis caused by pneumococcus. J. Infect. Dis. **184:**1300–1309.
7. **Brown, D. L., K. K. Desai, B. A. Vakili, C. Nouneh, H. M. Lee, and L. M. Golub.** 2004. Clinical and biochemical results of the metalloproteinase inhibition with subantimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial. Arterioscler. Thromb. Vasc. Biol. **24:**733–738.
8. **Cho, Y., T. W. Gong, T. Stover, M. I. Lomax, and R. A. Altschuler.** 2002. Gene expression profiles of the rat cochlea, cochlear nucleus, and inferior colliculus. J. Assoc. Res. Otolaryngol. **3:**54–67.
9. **Clark, W. M., N. Lessov, J. D. Lauten, and K. Hazel.** 1997. Doxycycline treatment reduces ischemic brain damage in transient middle cerebral artery occlusion in the rat. J. Mol. Neurosci. **9:**103–108.
10. **Comis, S. D., M. P. Osborne, J. Stephen, M. J. Tarlow, T. L. Hayward, T. J. Mitchell, P. W. Andrew, and G. J. Boulnois.** 1993. Cytotoxic effects on hair cells of guinea pig cochlea produced by pneumolysin, the thiol activated toxin of *Streptococcus pneumoniae*. Acta Otolaryngol. **113:**152–159.
11. **Dichgans, M., L. Jager, T. Mayer, K. Schorn, and H. W. Pfister.** 1999. Bacterial meningitis in adults: demonstration of inner ear involvement using high-resolution MRI. Neurology **52:**1003–1009.
12. **Dornbusch, K.** 1978. Antibiotics in bone tissues. Methodological and practical aspects. Scand. J. Infect. Dis. Suppl. **14:**177–185.
13. **Gabler, W. L., and H. R. Creamer.** 1991. Suppression of human neutrophil functions by tetracyclines. J. Periodontal Res. **26:**52–58.
14. **Gerber, J., K. Pohl, V. Sander, S. Bunkowski, and R. Nau.** 2003. Rifampin followed by ceftriaxone for experimental meningitis decreases lipoteichoic acid concentrations in cerebrospinal fluid and reduces neuronal damage in comparison to ceftriaxone alone. Antimicrob. Agents Chemother. **47:**1313–1317.
15. **Gianinazzi, C., D. Grandgirard, H. Imboden, L. Egger, D. Meli, Y.-D. Bifrare, P. Joss, M. Täuber, C. Borner, and S. Leib.** 2003. Caspase-3 mediates hippocampal apoptosis in pneumococcal meningitis. Acta Neuropathol. (Berlin) **105:**499–507.
16. **Golub, L. M., H. M. Lee, M. E. Ryan, W. V. Giannobile, J. Payne, and T. Sorsa.** 1998. Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. Adv. Dent. Res. **12:**12–26.
17. **Gomis-Ruth, F. X., E. F. Meyer, L. F. Kress, and V. Politi.** 1998. Structures of adamalysin II with peptidic inhibitors. Implications for the design of tumor necrosis factor alpha convertase inhibitors. Protein Sci. **7:**283–292.
18. **Hurewitz, A. N., C. L. Wu, P. Mancuso, and S. Zucker.** 1993. Tetracycline and doxycycline inhibit pleural fluid metalloproteinases. A possible mechanism for chemical pleurodesis. Chest **103:**1113–1117.
19. **Kastenbauer, S., and H. W. Pfister.** 2003. Pneumococcal meningitis in adults: spectrum of complications and prognostic factors in a series of 87 cases. Brain **126:**1015–1025.
20. **Klein, M., U. Koedel, H. W. Pfister, and S. Kastenbauer.** 2003. Morphological correlates of acute and permanent hearing loss during experimental pneumococcal meningitis. Brain Pathol. **13:**123–132.
21. **Kottirsch, G., G. Koch, R. Feifel, and U. Neumann.** 2002. Beta-aryl-succinic acid hydroxamates as dual inhibitors of matrix metalloproteinases and tumor necrosis factor alpha converting enzyme. J. Med. Chem. **45:**2289–2293.
22. **Lee, C. Z., B. Xu, T. Hashimoto, C. E. McCulloch, G. Y. Yang, and W. L. Young.** 2004. Doxycycline suppresses cerebral matrix metalloproteinase-9 and angiogenesis induced by focal hyperstimulation of vascular endothelial growth factor in a mouse model. Stroke **35:**1715–1719.
23. **Leib, S. L., J. M. Clements, R. L. Lindberg, C. Heimgartner, J. M. Loeffler, L. A. Pfister, M. G. Täuber, and D. Leppert.** 2001. Inhibition of matrix metalloproteinases and tumour necrosis factor alpha converting enzyme as adjuvant therapy in pneumococcal meningitis. Brain **124:**1734–1742.
24. **Leib, S. L., D. Leppert, J. Clements, and M. G. Täuber.** 2000. Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. Infect. Immun. **68:**615–620.
25. **Lepper, M. H., and H. F. Dowling.** 1951. Treatment of pneumococcal meningitis with penicillin compared with penicillin plus aureomycin. Arch. Intern. Med. **88:**489–494.
26. **Leppert, D., S. L. Leib, C. Grygar, K. M. Miller, U. B. Schaad, and G. A. Hollander.** 2000. Matrix metalloproteinase (MMP)-8 and MMP-9 in cere-

brospinal fluid during bacterial meningitis: association with blood-brain barrier damage and neurological sequelae. Clin. Infect. Dis. **31:**80–84.
27. **Leppert, D., R. L. Lindberg, L. Kappos, and S. L. Leib.** 2001. Matrix metalloproteinases: multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis. Brain Res. Brain Res. Rev. **36:**249–257.
28. **Maitra, S. R., S. Bhaduri, P. D. Valane, T. Tervahartiala, T. Sorsa, and N. Ramamurthy.** 2003. Inhibition of matrix metalloproteinases by chemically modified tetracyclines in sepsis. Shock **20:**280–285.
29. **Meli, D. N., J. M. Loeffler, P. Baumann, U. Neumann, T. Buhl, D. Leppert, and S. L. Leib.** 2004. In pneumococcal meningitis a novel water-soluble inhibitor of matrix metalloproteinases and TNF-alpha converting enzyme attenuates seizures and injury of the cerebral cortex. J. Neuroimmunol. **151:**6–11.
30. **Miller, J. M., D. H. Chi, L. J. O'Keeffe, P. Kruszka, Y. Raphael, and R. A. Altschuler.** 1997. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. Int. J. Dev. Neurosci. **15:**631–643.
31. **Nau, R., and H. Eiffert.** 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. Clin. Microbiol. Rev. **15:**95–110.
32. **Nau, R., A. Soto, and W. Bruck.** 1999. Apoptosis of neurons in the dentate gyrus in humans suffering from bacterial meningitis. J Neuropathol Exp. Neurol. **58:**265–274.
33. **Olsson, R. A., J. C. Kirby, and M. J. Romansky.** 1961. Pneumococcal meningitis in the adult. Clinical, therapeutic, and prognostic aspects in forty-three patients. Ann. Intern. Med. **55:**545–549.
34. **Oostenbrink, R., M. Maas, K. G. Moons, and H. A. Moll.** 2002. Sequelae after bacterial meningitis in childhood. Scand. J. Infect. Dis. **34:**379–382.
35. **Pasquale, T. R., and J. S. Tan.** 2005. Nonantimicrobial effects of antibacterial agents. Clin. Infect. Dis. **40:**127–135.
36. **Paul, R., S. Lorenzl, U. Koedel, B. Sporer, U. Vogel, M. Frosch, and H. W. Pfister.** 1998. Matrix metalloproteinases contribute to the blood-brain barrier disruption during bacterial meningitis. Ann. Neurol. **44:**592–600.
37. **Rappaport, J. M., S. M. Bhatt, R. S. Kimura, A. M. Lauretano, and R. A. Levine.** 1999. Electron microscopic temporal bone histopathology in experimental pneumococcal meningitis. Ann. Otol. Rhinol. Laryngol. **108:**537–547.
38. **Roach, D. M., R. A. Fitridge, P. E. Laws, S. H. Millard, A. Varelias, and P. A. Cowled.** 2002. Up-regulation of MMP-2 and MMP-9 leads to degradation of type IV collagen during skeletal muscle reperfusion injury; protection by the MMP inhibitor, doxycycline. Eur. J. Vasc. Endovasc. Surg. **23:**260–269.
39. **Rosenberg, G. A., E. Y. Estrada, J. E. Dencoff, and W. G. Stetler-Stevenson.** 1995. Tumor necrosis factor-alpha-induced gelatinase B causes delayed opening of the blood-brain barrier: an expanded therapeutic window. Brain Res. **703:**151–155.
40. **Shinohara, T., G. Bredberg, M. Ulfendahl, I. Pyykko, N. P. Olivius, R. Kaksonen, B. Lindstrom, R. Altschuler, and J. M. Miller.** 2002. Neurotrophic factor intervention restores auditory function in deafened animals. Proc. Natl. Acad. Sci. USA **99:**1657–1660.
41. **Solomon, A., M. Rosenblatt, D. Q. Li, Z. Liu, D. Monroy, Z. Ji, B. L. Lokeshwar, and S. C. Pflugfelder.** 2000. Doxycycline inhibition of interleukin-1 in the corneal epithelium. Investig. Ophthalmol. Vis. Sci. **41:**2544–2557.
42. **Spreer, A., H. Kerstan, T. Bottcher, J. Gerber, A. Siemer, G. Zysk, T. J. Mitchell, H. Eiffert, and R. Nau.** 2003. Reduced release of pneumolysin by *Streptococcus pneumoniae* in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. Antimicrob. Agents Chemother. **47:**2649–2654.
43. **Suomalainen, K., T. Sorsa, L. M. Golub, N. Ramamurthy, H. M. Lee, V. J. Uitto, H. Saari, and Y. T. Konttinen.** 1992. Specificity of the anticollagenase action of tetracyclines: relevance to their anti-inflammatory potential. Antimicrob. Agents Chemother. **36:**227–229.
44. **Tuomanen, E., B. Hengstler, O. Zak, and A. Tomasz.** 1986. Induction of meningeal inflammation by diverse bacterial cell walls. Eur. J. Clin. Microbiol. **5:**682–684.
45. **Whittaker, M., C. D. Floyd, P. Brown, and A. J. Gearing.** 1999. Design and therapeutic application of matrix metalloproteinase inhibitors. Chem. Rev. **99:**2735–2776.
46. **Winter, A. J., S. D. Comis, M. P. Osborne, M. J. Tarlow, J. Stephen, P. W. Andrew, J. Hill, and T. J. Mitchell.** 1997. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. Infect. Immun. **65:**4411–4418.
47. **Woolley, A. L., K. A. Kirk, A. M. Neumann, Jr., S. M. McWilliams, J. Murray, D. Freind, and B. J. Wiatrak.** 1999. Risk factors for hearing loss from meningitis in children: the Children's Hospital experience. Arch. Otolaryngol. Head Neck Surg. **125:**509–514.
48. **Yim, C. W., N. M. Flynn, and F. T. Fitzgerald.** 1985. Penetration of oral doxycycline into the cerebrospinal fluid of patients with latent or neurosyphilis. Antimicrob. Agents Chemother. **28:**347–348.
49. **Yrjanheikki, J., R. Keinanen, M. Pellikka, T. Hokfelt, and J. Koistinaho.** 1998. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc. Natl. Acad. Sci. USA **95:**15769–15774.