Stroke Propagates Bacterial Aspiration to Pneumonia in a Model of Cerebral Ischemia

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Background and Purpose—Bacterial pneumonia is the most common cause of death in patients sustaining acute stroke and is believed to result from an increased aspiration. Recently, stroke-induced immunodeficiency was described in a mouse model of cerebral ischemia, which is primarily caused by overactivation of sympathetic nervous system. We tested if stroke-induced immunodeficiency increases the risk of pneumonia after aspiration in a newly developed model of poststroke pneumonia.

Methods—Experimental stroke in mice was induced by occlusion of the middle cerebral artery (MCAO) for 60 minutes. Aspiration pneumonia was induced by intranasal application of 20 μL of a defined suspension of Streptococcus pneumoniae in phosphate-buffered saline 4 or 14 days after MCAO. Treatment comprised moxifloxacin (100 mg/kg body weight, six times every 2 hours after operation) or propranolol (30 mg/kg body weight, immediately before as well as 4 and 8 hours after MCAO). Readout was lung histology and bacterial counts in lung and blood.

Results—Nasal inoculation of only 200 colony-forming units of S pneumoniae caused severe pneumonia and bacteremia after experimental stroke, whereas 200 000 colony-forming units are needed to induce comparable disease in sham animals. Aspiration pneumonia in stroke animals outlasted acute stroke state but was preventable by β-adrenoreceptor blockade.

Conclusions—Experimental stroke propagates bacterial aspiration from harmless intranasal colonization to harmful pneumonia. Prevention of infections by β-adrenoreceptor blockade suggests that immunodepression by sympathetic hyperactivity is essential for progression of bacterial aspiration to pneumonia. (Stroke. 2006;37:2607-2612.)

Key Words: aspiration ▪ mice ▪ pneumonia ▪ Streptococcus pneumoniae ▪ stroke

The prognosis of stroke depends mainly on the incidence of complications.1 Bacterial pneumonia is the most frequent severe complication1–3 and the most common cause of death in patients sustaining a stroke.4 Furthermore, its incidence correlates with the severity of stroke5 and is associated with poor outcome.6 However, even minor strokes, without clinical symptoms, may be complicated by pneumonia.7 The risk for pneumonia is highest in the acute state of stroke, but it remains increased for up to several months during rehabilitation.8

Reduction of bulbar reflexes, drowsiness, the bedridden patient’s state, and subsequent aspiration are considered to account for the high incidence of bacterial pneumonia after stroke. However, aspiration alone cannot explain the high incidence of stroke-associated pneumonia.9 Aspiration occurs in healthy adults during sleep without inducing pneumonia.10 The reasons for the high incidence of pneumonia in patients sustaining a stroke are still incompletely understood.

Recently, we described stroke-induced immunodeficiency in a mouse model of cerebral ischemia,11 which is mainly caused by sympathetic nervous system overactivation and results in spontaneous bacterial infections resulting from impaired cell-mediated immune responses. In a newly developed model of poststroke pneumonia, we tested whether stroke-induced immunodeficiency increases the risk of pneumonia after aspiration. To induce pneumonia, we used Streptococcus pneumoniae as a result of its clinical relevance. Elderly and persons with coexisting diseases are of particular risk for pneumococcal infections.

Materials and Methods

Bacterial Cell Culture

D39 capsular type 2 S pneumoniae (Rockefeller University) was grown in C+Y medium12 to an OD (620 nm) of 0.4 to 0.6, pelleted, and resuspended in sterile phosphate-buffered saline (PBS). The bacterial inoculum was prepared by adjusting the bacterial concentration to various colony-forming units (CFU)/mL using a photometer. Bacteria were plated on blood agar plates to confirm quantity and viability before and after each experiment.

Received March 20, 2006; final revision received June 22, 2006; accepted July 6, 2006.

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Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000240409.68739.2b
Model of Experimental Murine Stroke
Stroke was induced by middle cerebral artery occlusion (MCAO) in pathogen-free 129S6SvEv mice (aged 9–13 weeks, mixed gender) as described previously. Briefly, a monofilament was inserted into the common carotid artery during halothane anesthesia and advanced to the origin of the middle cerebral artery. It was left there for 60 minutes until reperfusion. In sham-operated animals, the monofilament was also inserted and advanced to the origin of the middle cerebral artery, but withdrawn immediately by 2 mm, thereby avoiding ischemia. All animal experiments were performed according to Charité and governmental guidelines.

Drug Administration
Propranolol (Sigma-Aldrich) was dissolved in 0.9% sodium chloride at 6 mg/mL and administered intraperitoneally (30 mg/kg body weight) immediately before as well as 4 and 8 hours after MCAO. Moxifloxacin (Bayer Vital GmbH) was dissolved in a mixture of saline and HCl and adjusted to pH 7.0 with NaOH. Mice were injected intraperitoneally with moxifloxacin 100 mg/kg body weight in 100 μL PBS (vehicle) six times every 2 hours after operation.

Model of Experimental Murine Pneumonia
Mice were anesthetized by intraperitoneal injection of 100 μL of a mixture of 20 mg/mL ketamine and 4 mg/mL xylazine 4 days or 14 days after MCAO or sham operation. Subsequently, 20 μL of a defined pneumococcal suspension in PBS was given intranasal with simultaneous blockage of the mouth to ensure aspiration. Maximal bacterial aspiration was ensured by fixing mice head up in a vertical position for 15 minutes. The 4-day time point was chosen because most aspects of stroke-induced immunodepression are worst at the fourth day after stroke. In our model, stroke is invariably associated to aspiration pneumonia characterized by lobar consolidation, edema, necrosis and neutrophilic infiltrates.

Results
Stroke Increases Susceptibility to Aspiration-Induced Pneumonia With S pneumoniae
MCAO consistently induced large cerebral infarctions of the middle cerebral artery territory (Figure 1A) comprising striatum and cerebral cortex. Intranasal application of 200 CFU of S pneumoniae 4 days after MCAO or the sham procedure resulted in severe pneumonia exclusively in animals with dysphagia in MCAO mice.

Histology
Lungs were immersed in 4% PFA for 48 hours at 4°C and embedded in paraffin wax. Ten-micrometer thick sections were obtained by microtome dissection and stained with hematoxylin and eosin. Twenty representative sections of lungs were chosen per animal and evaluated by two investigators, who were blind to the treatment groups. Lung sections were evaluated according to the following histopathologic criteria: distension of alveolar units, thickening of the alveolar septa, tissue consolidation perivascular and peribronchial edema, as well as peri- and intraalveolar leukocyte infiltration. Brains were snap-frozen in 2-methylbutane and placed on dry ice and stained with hematoxylin and eosin.

Figure 1. Aspiration pneumonia in stroke but not in sham animals. (A) Histologic examination of the brain. Coronal sections of a hematoxylin and eosin-stained mouse brain 72 hours after middle cerebral artery occlusion showing the infarction area. Lung histology of (B–D) sham and (E–G) stroke mice 18 hours after intranasal application of S pneumoniae. Representative 12-μm sections of hematoxylin and eosin-stained lungs from (E–G) middle cerebral artery occlusion but not from (B–D) sham animals revealed signs of a severe bacterial pneumonia characterized by (E and F) lobar consolidation, edema, necrosis and neutrophilic infiltrates (E and F). Magnifications=(B and E) 25-fold, (C and F) 100-fold, or (D and G) 400-fold. Slides are representative of seven mice per group.
stroke and only minimal pulmonary bacterial load in sham mice 24 hours after inoculation. Histologic examination of the lungs from stroke animals revealed typical signs of bacterial pneumonia showing massive infiltration of granulocytes and purulent debris (Figure 1B through 1E).

To test whether susceptibility to aspiration-induced pneumonia was dependent on the number of inoculated bacteria, serial dilutions of *S. pneumoniae* suspensions containing up to $10^5$ CFU/mL were prepared. Compared with stroke animals, at least a three order of magnitude higher number of bacteria was required to induce pneumonia (with a bacterial burden in the lung similar to stroke animals) in sham mice (Figure 2A). Despite the increase in lung bacterial burden, sham mice did not become bacteremic, even with the highest number of bacteria inoculated. In contrast, stroke mice readily developed bacteremia already with a bacterial inoculum as low as 200 CFU (Figure 2).

Reversibility of Poststroke Pneumonia and Bacteremia

We have shown previously that a catecholamine-mediated strong inhibition of cell-mediated immune responses is the major cause of spontaneous systemic bacterial infections in experimental stroke. We therefore tested whether pharmacological blockade of sympathetic activation abrogates the increased susceptibility to aspiration-induced pneumococcal pneumonia and bacteremia. The β-adrenoreceptor antagonist propranolol, administered immediately before and twice after MCAO, followed by intranasal administration of *S. pneumoniae* at 4 days after stroke drastically reduced pneumonia and completely blocked bacteremia (Figure 3).

Infectious Susceptibility Persists Beyond the Acute Phase of Stroke

*S. pneumoniae* was applied intranasally 14 days after induction of stroke to determine whether the increased susceptibility to aspiration-induced pneumonia also extends to the postacute phase of stroke. Severity of aspiration-induced pneumonia at 14 days after stroke was decreased only slightly

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**Figure 2.** Experimental stroke increases susceptibility to aspiration-induced pneumonia. (A) Experimental setup: to prevent spontaneous poststroke infections, MCAO mice were treated with moxifloxacin within the first 12 hours after the surgical procedure. Sham-operated animals, which do not develop spontaneous infections, also received moxifloxacin as a control. All sham animals (*n=7* in each group) survived, whereas one of eight mice in the MCAO group died within 24 hours after surgery. Surviving animals (*n=7* in each group) were inoculated with *S. pneumoniae* at day 4 and killed at day 5. (B) Intranasal inoculation with 200 colony-forming units *S. pneumoniae* resulted in a high bacterial load in lung tissue as well as in bacteremia 24 hour after bacterial challenge in MCAO but not in sham animals. The bacterial inoculum that was required to induce pneumonia with similar bacterial burden in sham animals was a thousand times larger compared with stroke animals. In contrast to MCAO mice, sham controls did not develop bacteremia. Data are shown as box plots of colony-forming units/mL (log10) *S. pneumoniae* in blood or lung tissue homogenate. *P*<0.05, Mann-Whitney U test.

**Figure 3.** Reversibility of poststroke aspiration pneumonia and bacteremia by propranolol. (A) Experimental setup. The β-adrenoreceptor antagonist propranolol was administered immediately before and twice after MCAO. Surviving animals in the sham group (seven from seven), MCAO group (seven from eight), and propranolol-treated MCAO group (seven from 10) were inoculated with *S. pneumoniae* at day 4 and killed at day 5. (B) Stroke animals (MCAO) developed high titer pneumonia and bacteremia 24 hours after bacterial challenge. In contrast, treatment with propranolol greatly reduced pneumonia and prevented bacteremia. Data are shown as boxplots of colony-forming units/mL (log10) *S. pneumoniae* in blood or lung tissue homogenate. *P*<0.05, Mann-Whitney U test.
creased susceptibility to infections after cerebral ischemia. Immunoinhibitory effects, contribute to the long-lasting infections, in addition to stress mediator-induced bacteremia (Figure 4). These data indicate that early mice at day 14 after stroke resulted in both severe pneumonia and bacteremia after antibiotic therapy, one group of mice did not receive moxifloxacin (MXFX groups) or vehicle within the first 12 hours after surgical procedure. There was no mortality in both sham groups (n = 6 per group), whereas only six of 14 mice in the MCAO group and six of seven mice in the MCAO + MXFX group survived until day 14. Surviving animals were exposed to intranasal instillation of 200 colony-forming units \( S \) pneumoniae at day 14 and killed at day 15. (B) MCAO animals not treated with moxifloxacin immediately after stroke developed high bacterial titers in both lung and blood after bacterial challenge compared with sham mice. In contrast, early postoperative treatment of MCAO animals with moxifloxacin (MCAO + MXFX), which effectively prevented early spontaneous bacteremia and pneumonia (data not shown), completely prevented bacteremia and reduced lung bacterial burden 24 hours after bacterial challenge at day 14. Data are shown as box plots of colony-forming units/mL (log10) \( S \) pneumoniae in blood or lung tissue homogenate. *P < 0.05, Mann-Whitney U test.

Figure 4. Infectious susceptibility persists beyond the acute phase of stroke and depends on early-onset poststroke infections. (A) Experimental setup. MCAO and sham mice were either treated with moxifloxacin (MXFX groups) or vehicle within the first 12 hours after surgical procedure. There was no mortality in both sham groups (n = 6 per group), whereas only six of 14 mice in the MCAO group and six of seven mice in the MCAO + MXFX group survived until day 14. Surviving animals were exposed to intranasal instillation of 200 colony-forming units \( S \) pneumoniae at day 14 and killed at day 15. (B) MCAO animals not treated with moxifloxacin immediately after stroke developed high bacterial titers in both lung and blood after bacterial challenge compared with sham mice. In contrast, early postoperative treatment of MCAO animals with moxifloxacin (MCAO + MXFX), which effectively prevented early spontaneous bacteremia and pneumonia (data not shown), completely prevented bacteremia and reduced lung bacterial burden 24 hours after bacterial challenge at day 14. Data are shown as box plots of colony-forming units/mL (log10) \( S \) pneumoniae in blood or lung tissue homogenate. *P < 0.05, Mann-Whitney U test.

compared with that at 4 days after ischemia (Figures 2 and 4). In contrast, susceptibility to bacteremia was almost abolished at day 14 after ischemia (Figure 4).

To determine whether the protection from developing bacte- remia at day 14 after stroke was the result of early preventive antibiotic therapy, one group of mice did not receive moxifloxaci- cin at day 1 after stroke. These mice developed spontaneous bacteremia and a high mortality (60% at day 5) as described previously. Intranasal bacterial inoculation in surviving mice at day 14 after stroke resulted in both severe pneumonia and bacteremia (Figure 4). These data indicate that early spontaneous infections, in addition to stress mediator-induced immunoinhibitory effects, contribute to the long-lasting increased susceptibility to infections after cerebral ischemia.

**Discussion**

We demonstrate that otherwise harmless minor bacterial aspiration leads to severe pneumonia and bacteremia after experimental stroke. The deleterious combination of stroke-facilitated aspiration and stroke-induced immunodeficiency increases susceptibility to infection. This increase of infection susceptibility lasts for at least 14 days after stroke onset.

Apart from an increase of intracranial pressure, pneumonia is the most common and most severe life-threatening complication of stroke. Currently, poststroke pneumonia is thought to develop mainly as a consequence of aspiration as a result of dysphagia and immobilization. Forty percent to 70% of patients sustaining stroke develop dysphagia of which approximately 40% aspirate. Although poststroke aspiration increases the risk of developing pneumonia seven-fold, aspiration alone is not sufficient to explain the high incidence of pneumonia in acute stroke. Our recent murine stroke data indicate that stress mediators induce a rapid and long-lasting suppression of cell-mediated immunity, which may explain the high incidence of infections in patients sustaining a stroke. In stroke models, additional factors such as exposure to anesthetics and surgery may impair leukocyte functions and, thus, may contribute to the increased risk of infection. However, in contrast to MCAO mice, sham-operated animals neither showed a relevant attenuation of immune responsiveness nor developed spontaneous bacterial infections indicating that the surgical procedures and anesthetics do not play a major role in the impaired antibacterial defenses after stroke in this model.

The increased susceptibility to aspiration-induced pneumonia and bacteremia in animals with stroke was abrogated by pharmacological blockade of the sympathetic nervous system with propranolol, which is known to prevent suppression of cellular immune responses after experimental stroke. Susceptibility to infection persisted beyond the acute phase of stroke. In addition, early bacterial infections contributed to the long-lasting increased susceptibility to infections after stroke. Thus, our data suggest that stroke-induced impaired antibacterial defense, but not aspiration itself, is a critical factor for the high incidence of pneumonia in patients sustaining a stroke in the acute and early rehabilitation phase.

Our murine model of stroke-associated aspiration pneumonia reflects a key clinical problem: aspiration of \( S \) pneumoniae causes lobar pneumonia, mimicking the clinical, histopathologic, and microbiologic characteristics of the disease in humans. Lobar pneumonia is almost invariably caused by \( S \) pneumoniae and occurs most often in elderly patients with coexisting diseases. In approximately 50% of cases, pneumococcal pneumonia is accompanied by bacteremia or septicemia. Pneumococcal pneumonia is the most prevalent microorganism in community-acquired pneumonia and a leading cause of morbidity and mortality worldwide. Stroke-associated pneumonia in the hospital setting is often caused by several bacterial specimens, including Gram-positive and -negative bacteria. In two recent studies, we demonstrated that mice develop spontaneous Gram-negative (mainly \( Escherichia coli \)) pneumonia and bacteremia after cerebral ischemia. In this study, we extend our previous results demonstrating that experimental stroke is also associated with an increased susceptibility to Gram-positive (\( S \) pneumoniae) bacterial infections. Further studies in this model addressing the susceptibility to aspiration...
pneumonia induced by other clinically relevant pathogens (e.g., Staphylococcus aureus and Proteus mirabilis), particularly in a polymicrobial approach would be of clinical and therapeutical interest.

Our data may have implications for patients with acute central nervous system lesions other than stroke such as brain trauma, because these patients are also compromised by severe bacterial infections as a result of immunodeficiency. In our murine stroke model, we have shown that an impaired early T- and NK-cell response, in particular decreased IFN-γ production—mediated by an overactivation of the sympathetic nervous system—is the critical stroke-induced defect in antibacterial defense against Gram-negative pathogens, mainly E coli. In a murine sepsis model, it has been demonstrated that ablation of the sympathetic nervous system decreases Gram-negative but promotes Gram-positive bacterial dissemination indicating contrasting effects of the sympathetic nerve activation on host immune responses to different pathogens. However, our data suggest that inhibition of increased sympathetic activity after cerebral ischemia using the β-adrenergic receptor blocker propranolol not only protects against spontaneous Gram-negative bacterial infections, but also preserves effector immune responses against Gram-positive pathogens. That notwithstanding, propranolol may have other antiinfective effects beyond preventing stroke-induced immune cell dysfunctions.

Although extrapolation of our experimental results to the clinical situation must be performed with caution, we believe that our growing insight into these mechanisms may open new therapeutic avenues in the treatment of patients sustaining a stroke. Screening all patients sustaining a stroke for fever, but also significantly improves survival and neurologic outcome in our experimental model. Although these data strongly suggest that the beneficial effects of moxifloxacin treatment on stroke outcome are the result of the prevention of early poststroke bacterial infections, we cannot exclude additional putative properties of moxifloxacin (e.g., antiexcitotoxic, immunomodulatory, modulation of the sympathetic response) in addition to its antibacterial effects. The concept of preventative antibacterial therapy has been tested successfully in other clinical conditions such as in patients with neutropenic cancer treated by chemotherapy. However, a proof of concept trial (ESPIAS) in patients with acute stroke using the fluoroquinolone levofloxacin failed in prevention of poststroke infections. Although several reasons may account for the negative result of the ESPIAS trial, we propose that the low dose of levofloxacin used in that trial (500 mg instead of 1000 mg daily) may be responsible for the lack of efficacy to prevent infectious complications. The results from the ESPIAS trial support current stroke guidelines that advise prompt treatment of infection but warn against antibiotic prophylaxis. That notwithstanding, further trials of preventive antiinfective therapy, which may also include antibiotics with neuroprotective effects, are needed to evaluate the effects of such therapy on stroke outcome. In a still (since 2 years) ongoing proof of concept trial (PANTHERIS), we are currently investigating the effectiveness of preventive antibacterial therapy using moxifloxacin in patients sustaining a stroke (www.controlled-trials.com/isrctn/trial//0/74386719.html). Interestingly, both ESPIAS and PANTHERIS used fourth-generation fluoroquinolones, which have a well-documented efficacy in respiratory bacterial infections and a broad antibacterial spectrum against pathogens relevant in early-onset stroke-associated pneumonia.

In conclusion, this study suggests that stroke shifts harmless aspiration to severe potentially life-threatening infection. Consequently, poststroke preventive antiinfective or immunomodulatory strategies might be promising approaches to improve poststroke outcome.

Acknowledgments
We thank Sabine Cho and Claudia Muselmann for excellent assistance with the stroke and microbiologic experiments.

Sources of Funding
This work was supported by the Hermann and Lilly Schilling Stiftung, Deutsche Forschungsgemeinschaft, the Charité Althoff fellowship (A.M.), and the Pusch heritage.

Disclosures
A patent application on preventive antiinfective therapy in stroke has been filed to the European Patent Office (PCT/EP03/02246); patent owner: Charité Universitätsmedizin Berlin; patent inventors: Andreas Meisel, Christian Meisel, Konstantin Prass, and Ulrich D intriguing.


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Stoke. 2006;37:2607-2612; originally published online August 31, 2006;
doi: 10.1161/01.STR.0000240409.68739.2b
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/37/10/2607