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1. Einleitung

1.1. Bedeutung der Pneumokokkenmeningitis

Die bakterielle Meningitis ist eine der am schwersten wiegenden Infektionserkrankungen des Menschen. Die jährliche Inzidenz wird in Industrieländern zwischen 2.5 und 4.5 je 100 000 Einwohner angegeben (Faustini 2000, Ross 2001). Im Mittel aller Altersgruppen sind Pneumokokken (*Streptococcus pneumoniae*) der häufigste Erreger und zeichnen sich durch eine besonders ungünstige Prognose aus (van de Beek 2004). Auch bei Verfügbarkeit von effektiver antibiotischer Therapie und moderner Intensivmedizin liegt die Mortalität der Pneumokokken-Meningitis weiterhin bei bis zu 30 % (Durand 1993, Schuchat 1997, van de Beek 2004). Etwa zwei Drittel der Todesfälle resultieren aus frühen intrakraniellen Komplikationen (Pfister 1993). Zudem weisen bis zu einem Drittel der überlebenden Patienten Folgeschäden auf, darunter insbesondere neuropsychologische Störungen (Bohr 1983, Pomeroy 1990, de Louvois 2007, Hoogman 2007).

1.2. Pathogenese der Pneumokokkenmeningitis

Pneumokokken sind fakultativ pathogene grampositive Diplokokken, die den Nasen-Rachenraum von bis zu 30 % Gesunder über eine Tröpfcheninfektion zeitweilig kolonisieren (Bridy-Pappas 2005). Einzig bekannter Wirt und somit alleiniges Reservoir dieser Bakterien ist der Mensch. Ein sehr großer Teil des Genoms, das inzwischen von drei Pneumokokkenstämmen (TIGR4, D39, R6) vollständig sequenziert ist (Tettelin 2001, Hoskins 2001, Lanie 2007), kodiert für Virulenzfaktoren, die die Kolonisation ermöglichen und das Wachstum konkurrierender Bakterien hemmen können (Hava 2003, Pericone 2000, Regev-Yochay 2006). Veränderungen des umgebenden Milieus, z.B. durch koinzidente Infektionen des Respirationstraktes (McCullers 2006), können zu einer Veränderung des Expressionsprofils und Ausbildung eines invasiven Phänotyps führen (Hava 2003, Paterson

2006). Pneumokokken stellen den wichtigsten Erreger ambulant erworbener Pneumonien dar. Weitere häufig durch Pneumokokken ausgelöste Erkrankungen sind Sinusitis und Otitis media. Deutlich seltener werden Sepsis und Meningitis verursacht. Für die bakterielle Meningitis im allgemeinen wird ein hämatogener Infektionsmodus angenommen (Scheld 1979), wobei nach gängiger Ansicht zirkulierende Bakterien die Blut-Liquor-Schranke in erster Linie im Bereich des Plexus choroideus überwinden können (Engel 1944, Tuomanen 1993, Leib 1999), ohne dass hierfür allerdings sichere experimentelle Evidenz bestünde. Daneben wurde auch ein über den PAF-Rezeptor vermittelter aktiver transzellulärer Transport in Endothelien der Bluthirnschranke nachgewiesen (Ring 1998). Im Liquor selbst können die Bakterien sich zunächst nahezu ungehemmt vermehren, da optimale Nährstoffkonzentrationen vorliegen und zelluläre wie humorale Bestandteile des Immunsystems weitestgehend fehlen.

1.3. Komponenten und akute Komplikationen der Immunantwort

Im Verlauf weniger Stunden nach Eindringen der Bakterien in den Liquorraum kommt es wirtsseitig zu einer konstitutiven Immunantwort. Nach heutigem Verständnis ist zu ihrer Auslösung die Interaktion von bakteriellen Bestandteilen mit sogenannten *pattern-recognition*-Rezeptoren (PRRs) erforderlich, die bestimmte konservierte Motive (sogenannte *pathogen-associated molecular patterns*, PAMPs) erkennen (Akira 2006). Die wichtigste Familie dieser PRRs sind die Toll-like-Rezeptoren (TLRs). Sie werden auf einer Vielzahl immunkompetenter Zellen konstitutiv exprimiert. Im Falle von Pneumokokken stellen Peptidoglycan und Lipoteichonsäure (Schröder 2003) sowie bakterielle Lipopeptide (Takeda 2002) als wichtigste Bestandteile der Zellwand Liganden von TLR2 dar. Daneben werden das Exotoxin Pneumolysin von TLR4 (Malley 2003) und CpG-haltige bakterielle DNA von TLR9 (Albiger 2007) erkannt. Nach Erkennung von Bakterienbestandteilen durch perivaskuläre und meningeale Makrophagen sowie durch Endothelzellen werden über eine Aktivierung proinflammatorischer Signalkaskaden chemotaktische Faktoren freigesetzt (Täuber 1999,

Zwijnenburg 2006). In den Endothelzellen der postkapillären Venulen werden Adhäsionsmoleküle hochreguliert (Quagliarello 1992, Freyer 1995, Turowski 2005). Als Folge werden zirkulierende Granulozyten und Monozyten aus dem Blutstrom über die Blut-Liquor-Schranke rekrutiert. Im Liquor findet sich eine massive Zunahme der Leukozytenzahl (Pleozytose). Die eingewanderten Leukozyten produzieren proinflammatorische Zytokine und verstärken somit die Inflammation (Zysk 1997). Im Krankheitsverlauf beteiligt sich auch Glia an der Entzündungsantwort (Kreutzberg 1996). Zeitgleich mit der Rekrutierung von Leukozyten kommt es zu einer graduellen Zunahme der Durchblutung in pialen Gefäßen (Berkowitz 1993), die durch vasodilatierende Mediatoren wie Stickstoffmonoxid (NO), Kinine und Neuropeptide vermittelt wird (Pfister 1995, Lorenzl 1996, Paul 1997). Durch die Öffnung der Blut-Hirn-Schranke kommt es außerdem zur Ausbildung eines vasogenen Hirnödems (Quagliarello 1992, Leib 1999, Koedel 2002). Gemeinsam führen diese Faktoren bereits früh zu einem graduellen Anstieg des intrakraniellen Druckes (ICP), während später im Krankheitsverlauf Störungen von Liquorresorption und Liquorzirkulation als weitere hirndrucksteigernde Faktoren hinzutreten können (Scheld 1980, Pfister 1993).

1.4. Neuronaler Schaden

In Tiermodellen, aber auch in Sektionspräparaten verstorbener Patienten mit Meningitis sind neuronale Zellverluste nachweisbar, die als Ursache der neurologischen Residuen bei Überlebenden der Erkrankung betrachtet werden (Nau 2002a). Zu unterscheiden sind einerseits ischämische Läsionen, deren Entstehung auf vaskuläre Komplikationen der Meningitis (Vaskulitis, lokale Vasospasmen) zurückgeführt wird (Pfister 1992). Diese Läsionen betreffen oft den Cortex und weisen histologisch hypoxisch geschädigte oder nekrotische Neurone auf. Pathogenetisch wurden freie Radikale als Ursache von Gefäßwandschäden (McKnight 1992, Pfister 1992, Kastenbauer 2002) sowie Endotheline als wichtige Vermittler des Vasospasmus (Pfister 2000) während Meningitis identifiziert.

Andererseits finden sich apoptotisch untergegangene Neurone, die bei Menschen und Nagetieren vorwiegend in hippokampalen Strukturen mit Betonung des Gyrus dentatus angetroffen werden (Nau 1999, Nau 2002a) und in einer hippokampalen Atrophie bei Überlebenden resultieren können (Free 1996). Über die Genese dieser Schädigungsform während bakterieller Meningitis liegen keine abschließenden Erkenntnisse vor. Nach derzeitigem Verständnis (Nau 2002a) tragen wirtsseitig von immunkompetenten Zellen gebildete neurotoxische Faktoren, darunter Stickstoffmonoxid, freie Sauerstoffradikale und proapoptotische Zytokine (Koedel 1999, Bogdan 1997) sowie bakterielle Stoffwechselprodukte zu den neuronalen Verlusten im Gyrus dentatus bei. Eine besondere Rolle spielen dabei Pneumolysin und H_2O_2 als zwei von Pneumokokken gebildete Exotoxine (Braun 2002). H_2O_2 entsteht im oxidativen Stoffwechsel aller Zellen, wird aber üblicherweise durch Catalase detoxifiziert. Pneumokokken verfügen über keine funktionale Catalase, so dass sie H_2O_2 in größerem Umfang in die Umgebung freisetzen (Spellerberg 1996). In vitro lösen Pneumokokken zwei Typen von Apoptose aus, wobei eine frühe, Caspase-unabhängige Form von einer späteren Caspase-vermittelten Toxizität abgegrenzt werden kann (Mitchell 2004, Braun 1999).

1.5. Herleitung der Fragestellungen

Aus dem oben Dargestellten ist zu entnehmen, dass die wirtsseitige Immunantwort erheblich zur ungünstigen Prognose der bakteriellen Meningitis beiträgt. Dies gilt sowohl für die durch Zytokine und vasoaktive Substanzen vermittelten frühen intrakraniellen Komplikationen (Hyperämie, Hirnödem, intrakranielle Hypertension) als auch für die verzögert auftretenden neuronalen Schäden durch Einwirkung proapoptotischer Zytokine und freier Radikale. Andererseits kann die Immunantwort keinen wirksamen Beitrag dazu leisten, bakterielles Wachstum im Liquorraum und die Freisetzung von bakteriellen Toxinen einzudämmen. Im Kontext einer antibiotischen Therapie ist es daher wünschenswert, die Entzündungsreaktion

zu begrenzen (Van der Flier, 2003). Dies ist umso bedeutender, da durch bakterizide Antibiotika, wie sie zur kalkulierten Therapie der Meningitis eingesetzt werden, eine massive Freisetzung bakterieller Zellwandbestandteile ausgelöst wird, die eine weitere Verstärkung der Immunantwort zur Folge hat (Nau 2002b). Aufgrund aktueller Empfehlungen wird daher unmittelbar vor Beginn der antibakteriellen Behandlung einer Meningitis bei Kindern und Erwachsenen Dexamethason zur Immunmodulation verabreicht (Fitch 2007). Allerdings konnte bisher vorwiegend ein günstiger Einfluss dieser Therapie auf systemische Komplikationen und Mortalität gezeigt werden (van de Beek 2004), während experimentelle Befunde eher auf eine Verstärkung des neuronalen Zellverlustes hindeuten (Leib 2003). Hieraus ergibt sich der Bedarf für gezieltere Interventionen zur Begrenzung der Inflammation. Angesichts der Vielzahl nachgeschalteter Signalwege ist ein möglichst früher Ansatzpunkt anzustreben, idealerweise auf der Ebene der Erkennung von Bakterien durch immunkompetente Zellen. Eine hierauf basierende Therapie sollte unmittelbar vor Beginn der antibiotischen Chemotherapie appliziert werden, um die Verstärkung der Inflammation durch freigesetzte Bakterienzellwand zu unterbinden. Wie dargestellt wird davon ausgegangen, dass Pneumokokken vorwiegend über die Rezeptoren TLR2, TLR4 und TLR9 erkannt werden, wobei TLR2 die größte Bedeutung zugeschrieben wurde. Allerdings haben TLR2-knockout-Mäuse während experimenteller Pneumokokkenmeningitis keinen antiinflammatorischen Phänotyp gezeigt (Koedel 2003). Dieser fehlende Effekt konnte später auf eine redundante Erkennung über TLR4 zurückgeführt werden, da das Fehlen des gemeinsamen Adapterproteins MyD88, welches für die Signaltransduktion von TLR2 und TLR4 erforderlich ist, eine verminderte Inflammation zur Folge hatte (Koedel 2004). Aufgrund der bisherigen Erkenntnisse konnte somit die Rolle des TLR2-Systems in der Pneumokokkenmeningitis nicht eindeutig definiert werden. Die unter 2.1 aufgeführte Arbeit bewegt sich in diesem Kontext und hatte die Induktion einer akuten Meningitis durch alleiniges TLR2-Signaling zum Gegenstand.

Weiterhin wurde dargelegt, dass intrakranielle Komplikationen eine wesentliche Grundlage der frühen Mortalität der bakteriellen Meningitis bilden. Da die Entwicklung einer zerebralen Hyperperfusion einen wesentlichen Beitrag zur potentiell lebensbedrohlichen intrakraniellen Hypertension leistet, stellt die Regulation des zerebralen Blutflusses einen wichtigen Forschungsgegenstand in der bakteriellen Meningitis dar. Die unter 2.2 aufgeführte Studie befasste sich mit der Wirkung der Neuropeptidfreisetzung aus meningealen perivaskulären C-Fasern des Nervus trigeminus (trigeminovaskuläres System) auf die Blutflussregulation in der Meningitis. In der Studie 2.3 wurde die Bedeutung des von Pneumokokken gebildeten H_2O_2 für die frühe Hyperämie untersucht.

Für das Outcome nach bakterieller Meningitis stellt das Ausmaß neuronaler Schäden eine wichtige Determinante dar. Wie dargelegt werden in der Pneumokokkenmeningitis bakterielle und wirtsseitige Faktoren gleichermaßen für die Neurotoxizität verantwortlich gemacht. Eine besondere Bedeutung hat dabei die Schädigung von Zellen durch oxidativen Stress (Koedel 1999). Auf Wirtsseite stellen die einwandernden Leukozyten den wesentlichen Produzenten von reaktiven Sauerstoffspezies und Stickstoffmonoxid dar (Babior 2002, Bogdan 2000). Diese Moleküle dienen in erster Linie der Bekämpfung des infektiösen Pathogens. Andererseits können sie auch im eukaryonten Organismus durch Peroxidation von Zellmembranen, Störung des mitochondrialen Energiestoffwechsels und Schädigung der DNA zum Zelltod führen. Peroxynitrit als das Reaktionsprodukt von NO und dem Superoxidanion O_2^- ist eine besonders toxische Verbindung (Szabo 2003). Alternativ kann diese Verbindung unter bestimmten Bedingungen aber auch aus der Reaktion von H_2O_2 und NO hervorgehen (McBride 1999). Auf die Produktion von H_2O_2 durch Pneumokokken wurde bereits hingewiesen. In der unter 2.4 angeführten Arbeit wurde daher untersucht, welchen Effekt wirtsseitig synthetisiertes NO und bakterielles H_2O_2 auf die Entstehung neuronaler Zellverluste im Gyrus dentatus haben, und ob die Bildung von Peroxynitrit aus bakteriellem H_2O_2 und wirtsseitigem NO einen wesentlichen neurotoxischen Mechanismus darstellt.

Die zentrale Rolle der Immunantwort für den neuronalen Zelltod in der Meningitis sowie das hohe Interesse an antiinflammatorischen Therapieansätzen wurden bereits dargestellt. In diesem Zusammenhang wurde in der Arbeit 2.3 die Hemmung der Neuropeptidfreisetzung aus dem trigeminovaskulären System neben den Effekten auf Blutflussregulation und Ödementstehung auch im Hinblick auf eine Modulation der Rekrutierung von Leukozyten in den Liquorraum untersucht. In der Arbeit 2.5 wurde schließlich untersucht, welchen Einfluß das proapoptotische Zytokin *tumor necrosis factor alpha-related apoptosis-inducing ligand* (TRAIL) auf den Inflammationsverlauf und den neuronalen Schaden in der experimentellen Meningitis nimmt. TRAIL gehört zusammen mit CD95/CD95L und TNF alpha zu einer Familie von extrinsischen Todessignalen. In der experimentellen Pneumokokkenmeningitis konnte zuvor bei neugeborenen Ratten ein neurotoxischer Effekt von TNF alpha nachgewiesen werden (Bogdan 1997), während kein sicherer Einfluss des CD95-System gezeigt werden konnte (Paul 2004). Das TRAIL–System wurde erstmals im Kontext der Meningitis untersucht, einerseits im Hinblick auf eine mögliche Neurotoxizität von TRAIL selbst, wie sie bei der experimentellen Autoimmunenzephalitis (EAE) beschrieben wurde (Aktas 2005), andererseits im Hinblick auf regulierende Effekte an den Leukozyten, wie sie frühere ex-vivo Befunde vermuten ließen (Renshaw 2003).

2. Eigene wissenschaftliche Arbeiten

2.1. **Olaf Hoffmann**, Johann S. Braun, Doreen Becker, Annett Halle, Dorette Freyer, Emilie Dagand, Seija Lehnardt, Jörg R. Weber. TLR2 mediates neuroinflammation and neuronal damage. J Immunol 2007; 178:6476-81.

Die Gegenwart von Krankheitserregern wird vom angeborenen Immunsystem über so genannte *pattern-recognition* Rezeptoren detektiert. Nach herrschender Meinung stellt der Toll-like Rezeptor 2 (TLR2) einen sehr wichtigen Sensor für grampositive Erreger dar. In der bakteriellen Meningitis ist die Rolle des TLR2-Systems allerdings bislang nicht eindeutig geklärt. In dieser Studie wurde das synthetische bakterielle Lipopeptid Pam_3CysSK_4 - ein selektiver Agonist an heterodimeren TLR2/1-Rezeptoren - eingesetzt, um bei Ratten und Mäusen eine experimentelle Meningitis auszulösen. Im Rattenmodell kam es innerhalb von 6 h nach intrathekaler Applikation zum Einstrom von Leukozyten in den Liquor sowie zu einer deutlichen Zunahme des regionalen zerebralen Blutflusses und des intrakraniellen Druckes. In Wildtyp-Mäusen (C57Bl/6) war nach 24 h ebenfalls eine Pleozytose sowie eine Zunahme apoptotischer Neurone im Gyrus dentatus festzustellen. Diese Effekte waren in TLR2-knockout-Mäusen nicht nachweisbar. In Zellkulturuntersuchungen an primären Rattenneuronen zeigte Pam_3CysSK_4 keine Toxizität. Dagegen induzierte die Substanz in kultivierter Mikroglia aus Wildtyp-Mäusen – nicht jedoch in TLR2-defizienter Mikroglia - die Freisetzung von neurotoxischen Molekülen in den Überstand. Zusammengefasst konnte in dieser Studie gezeigt werden, dass durch selektive Stimulation von TLR2-Rezeptoren die typischen wirtsvermittelten Aspekte einer akuten bakteriellen Meningitis ausgelöst werden können. Synthetische bakterielle Lipopeptide können genutzt werden, um TLR2-abhängige Neurodegeneration in vivo zu untersuchen.

O. Hoffmann, J. S. Braun, D. Becker, A. Halle, D. Freyer, E. Dagand, S. Lehnardt, J.R. Weber. TLR2 mediates neuroinflammation and neuronal damage. J Immunol. 2007;178(10):6476-81.

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2.2. **Olaf Hoffmann**, Nikolas Keilwerth, Margarethe Bastholm Bille, Uwe Reuter, Klemens Angstwurm, Ralf R. Schumann, Ulrich Dirnagl, Joerg R. Weber. Triptans Reduce the Inflammatory Response in Bacterial Meningitis. J Cereb Blood Flow Metab 2002;22:988-96.

Kopfschmerzen und Meningismus belegen eine Aktivierung trigeminaler Afferenzen in den Meningen während bakterieller Meningitis. $5HT_{1B/D/F}$-Rezeptorantagonisten (Triptane) werden in der Behandlung der Migräne eingesetzt, um die Freisetzung von proinflammatorischen Neuropeptiden (Substanz P, CGRP) aus aktivierten perivaskulären Fasern des N. trigeminus (Neuroinflammation) zu inhibieren. In der vorliegenden Studie wurde das antiinflammatorische Potential von Triptanen während experimenteller Meningitis evaluiert. In einem Rattenmodell der Pneumokokkenmeningitis (Dauer 6 h) konnten durch Zolmitriptan und Naratriptan der Leukozyteneinstrom in den Liquor, die Zunahme des regionalen Blutflusses und des Hirndruckes sowie die Ödembildung signifikant reduziert werden. Eine partielle Umkehr dieser Effekte durch $5HT_{1D}$ und $5HT_{1B}$ Rezeptorantagonisten belegt die Spezifität der Intervention. Histologisch konnte gezeigt werden, dass durch die Behandlung mit Triptanen die Neuropeptidfreisetzung aus perivaskulären Nervenfasern in der Dura mater während der Meningitis blockiert wird. Patienten mit bakterieller Meningitis zeigten eine deutlich erhöhte Konzentation von CGRP im Liquor. Zusammenfassend weisen die Ergebnisse darauf hin, dass meningeale Nervenfasern neben ihrer nozizeptiven Funktion auch proinflammatorische Wirkungen in der Frühphase der Meningitis vermitteln. Triptane könnten daher eine neuartige Interventionsmöglichkeit in der bakteriellen Meningitis darstellen.

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Triptans Reduce the Inflammatory Response in Bacterial Meningitis

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Summary: Severe headache and meningism provide clear evidence for the activation of trigeminal neurotransmission in meningitis. The authors assessed the antiinflammatory potential of $5HT_{1B/D/F}$ receptor agonists (triptans), which inhibit the release of proinflammatory neuropeptides from perivascular nerve fibers. In a 6-hour rat model of pneumococcal meningitis, zolmitriptan and naratriptan reduced the influx of leukocytes into the cerebrospinal fluid, and attenuated the increase of regional cerebral blood flow. Elevated intracranial pressure as well as the brain water content at 6 hours was reduced by triptans. These effects were partially reversed by a specific $5HT_{1D}$ as well as by a specific $5HT_{1B}$ receptor antagonist. Meningitis caused a depletion of calcitonin gene-related peptide (CGRP) and substance P from meningeal nerve fibers, which was prevented by zolmitriptan and naratriptan. In line with these findings, patients with bacterial meningitis had significantly elevated CGRP levels in the cerebrospinal fluid. In a mouse model of pneumococcal meningitis, survival and clinical score at 24 hours were significantly improved by triptan treatment. The findings suggest that, besides mediating meningeal nociception, meningeal nerve fibers contribute to the inflammatory cascade in the early phase of bacterial meningitis. Adjunctive treatment with triptans may open a new therapeutic approach in the acute phase of bacterial meningitis.
Key Words: Meningitis—Trigeminovascular system—Neuropeptides—Triptans—Pneumococci.

The early pathophysiologic events of bacterial meningitis are the influx of leukocytes into the cerebrospinal fluid (CSF) the breakdown of the blood–brain and blood–CSF barrier, brain edema, increased CSF outflow resistance, and cerebrovascular complications, all contributing to the disastrous outcome (Quagliarello and Scheld, 1992). The combination of the acute inflammatory response, secondary effects of the altered physiology, and bacterial factors that may cause neuronal injury (Braun et al., 1999) results in 28% mortality (Durand et al., 1993) and permanent sequelae in up to 50% of the survivors (Bohr et al., 1984). Consequently, adjunctive treatment strategies to improve the outcome of bacterial meningitis are aimed at reducing the inflammatory response.

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Headache and meningism are the leading clinical features of bacterial meningitis and provide clear evidence for the activation of sensory nerve fibers. These symptoms are mediated through sensory nerve fibers that originate from the trigeminal nerve and innervate the meninges. In addition to their ability to mediate pain, sensory nerve fibers can release vasoactive factors including the proinflammatory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP). Apart from their role in afferent nociception, trigeminal nerve fibers participate in the vasomotor innervation of meningeal blood vessels (Suzuki et al., 1989), forming the so-called trigeminovascular system (TVS) (May and Goadsby, 1999). Unmyelinated sensory C-fibers release vasoactive neuropeptides from perivascular terminals in response to nociceptive stimuli (Edvinsson et al., 1990), including the presence of an inflammatory environment (Ebersberger et al., 1999). Of these neuropeptides, CGRP and SP are potent dilators of cerebral arteries (Edvinsson et al., 1987; McCulloch et al., 1986), and SP leads to increased vascular permeability and plasma protein extravasation (Pernow, 1985; O'Shaughnessy and Connor, 1993). In the dura mater, these phenomena have been summarized as neurogenic inflammation. Recent

anatomic studies have revealed the presence of nerve fibers immunoreactive for several neuropeptides including CGRP and SP also within the leptomeninx (Fricke et al., 1997), arguing that the proinflammatory role of neuropeptide release is not confined to the dura mater, but may affect the subarachnoid space and possibly the adjacent cortical tissue. Thus, modulation of neuropeptide release may offer a novel path to decrease meningeal inflammation.

Calcitonin gene-related peptide is a potent vasodilator in the cerebral circulation and has been linked to hyperemia during central nervous system inflammation (Brian et al., 1995). Both SP and CGRP promote neutrophil adherence to endothelial cells *in vitro* (Sung et al., 1992; Zimmerman et al., 1992). Increased blood flow, activation of endothelial cells and neutrophil adhesion to the endothelium are initial features of bacterial meningitis.

Sectioning the nasociliary nerve 2 weeks before meningitis induction significantly reduced the rise in regional cerebral blood flow (rCBF) on the denervated side (Weber et al., 1996). These experiments suggest a critical role for the TVS, most likely due to the depletion of neuropeptides otherwise released locally from the trigeminal nerve into the perivascular space (Suzuki et al., 1989; Weber et al., 1996). Similarly, early pial vasodilation in experimental meningitis of the rat was reduced by local treatment with an SP antagonist (Pfister et al., 1995), as was the inflammatory response in murine meningoencephalitis caused by *Trypanosoma brucei* (Kennedy et al., 1997).

Neuropeptide release is inhibited by agonist action on presynaptic $5HT_{1B}$ and $5HT_{1D}$ receptors coexpressed on sensory neurons (Arvieu et al., 1996; Durham and Russo, 1999). Stimulation of these receptors by specific agonists (triptans) effectively blocks vasodilation and protein extravasation from meningeal blood vessels in response to nociceptive stimuli (Buzzi et al., 1991; Buzzi and Moskowitz, 1990, 1991; Connor et al., 1997; Huang et al., 1993; Martin et al., 1997; Messlinger et al., 1997). In the present study, we hypothesized a potential proinflammatory role of the trigeminovascular system in bacterial meningitis. Here we demonstrate that the $5HT_{1B/D/F}$ receptor agonists zolmitriptan and naratriptan reduce leukocyte influx, the increase of cerebral blood flow and intracranial pressure, brain edema, and prolong survival in experimental pneumococcal meningitis. We conclude that besides mediating pain, sensory nerve fibers of the meninges add to the early inflammatory host response.

MATERIALS AND METHODS

All animal experimental designs were reviewed and approved by the Senate of Berlin.

Rat experiments

Table 1 outlines the experimental group design used in the study. The general experimental procedure was as described earlier (Weber et al., 1996). Experiments were performed on 51 male Wistar rats (280 to 330 g) anesthetized with intraperitoneal sodium thiopental (100 and 20 mg/kg every 2 hours; Trapanal, Byk Gulden, Konstanz, Germany). Animals were tracheotomized and mechanically ventilated (AP-10; K. Effenberger, Paffing, Germany). End-tidal CO_2 was monitored continuously (Artema MM204; Heyer, Bad Ems, Germany). Body temperature was measured by a rectal probe and maintained at 37.8°C ± 0.4°C using a heating pad. A transducer (Statham P109 EZ, Spectramed, Oxnard, CA, U.S.A.) connected to a catheter placed in the left femoral artery measured mean arterial blood pressure (MABP) continuously. From this catheter, arterial blood samples were analyzed for Pa_{O_2}, Pa_{CO_2}, and pH at 0, 2, 4, and 6 hours. The left femoral vein was cannulated for infusion of triptans or saline. A 3 × 3-mm area of the parietal bone lateral to the sagittal suture was thinned to allow laser Doppler flow (LDF) measurements (Periflux 4001 Master, Järfälla, Sweden) in cortical blood vessels (Lindauer et al., 1993; Weber et al., 1996). A catheter was placed into the cisterna magna through an occipital burr hole and connected to a pressure transducer (Statham P109 EZ, Spectramed) for continuous intracranial pressure (ICP) measurement. One hundred microliters CSF was removed from this catheter and replaced by 100 μL saline in controls or 100 μL pneumococcal cell wall (PCW) suspension. Instillation of PCW induces an inflammatory response similar to bacterial meningitis (Pfister et al., 1992). At the end of the experiment, CSF samples were obtained to determine the CSF leukocyte count. Animals were then killed by exsanguination. Brains were removed and heat dehydrated for 18 hours at 180°C. Wet and dry brain weights

TABLE 1. *Experimental groups and parameters of inflammation at 2, 4, and 6 hours after the beginning of the experiment*

			Laser Doppler flow (% of baseline)			ΔICP (cm H_2O)			CSF (leukocytes/μL)	Brain water (%)
IC injection	IV treatment	*n*	2 h	4 h	6 h	2 h	4 h	6 h	6 h	6 h
Saline	Saline	2	94.0 ± 3.0	101.9 ± 2.6	110.5 ± 4.0	−0.3 ± 1.1	−0.8 ± 1.1	−0.5 ± 1.4	20 ± 21	80.99 ± 0.63
	Naratriptan	2	105.2 ± 1.5	111.4 ± 8.2	103.6 ± 8.0	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.0	46 ± 8	81.28 ± 0.36
	Zolmitriptan	2	97.8 ± 9.3	87.7 ± 1.7	91.4 ± 10.7	−0.4 ± 0.5	0.2 ± 2.0	−0.2 ± 1.7	29 ± 13	80.88 ± 0.37
PCW	Saline	6	129.6 ± 12.8	152.3 ± 15.7†	194.4 ± 28.2†	6.4 ± 2.6†	12.3 ± 5.8†	17.0 ± 6.1†	13,028 ± 6,317†	83.05 ± 0.69†
	Naratriptan	5	109.3 ± 28.6	127.5 ± 29.0	126.4 ± 21.6*	−0.2 ± 0.7*	2.3 ± 2.7*	3.2 ± 5.1*	2,998 ± 1,725*	81.29 ± 0.42*
	Zolmitriptan	7	111.6 ± 14.4	117.7 ± 14.5*	123.2 ± 16.4*	0.9 ± 3.2*	3.9 ± 1.4*	4.7 ± 1.3*	3,646 ± 1,555*	81.51 ± 0.37*
	Zolmitriptan (posttreated)	5	106.8 ± 17.6	123.2 ± 18.6	143.8 ± 15.0*	4.5 ± 0.9	6.0 ± 2.6*	7.3 ± 2.5*	4,366 ± 2,105*	81.15 ± 0.15*

Data are given as mean ± SD. In the posttreated zolmitriptan group, IV treatment was started 2 hours after PCW instillation.

IC, intracisternal; IV, intravenous, ΔICP, increase of intracranial pressure compared with baseline; CSF, cerebrospinal fluid; PCW, pneumococcal cell wall suspension.

* Indicates a significant difference ($P < 0.05$) compared with untreated meningitis; † indicates a significant difference compared with animals treated with saline intracisternally (ANOVA, Duncan *post hoc* analysis).

were compared to calculate the water content as an indicator of the presence of brain edema.

Triptan treatment of rats. Zolmitriptan (a generous gift from Zeneca Pharmaceuticals, Macclesfield, U.K.) and naratriptan (a generous gift from Glaxo Wellcome UK Ltd., Uxbridge, U.K.) were obtained in pure form without auxiliary substances. For intravenous application, zolmitriptan was dissolved in DMSO and diluted with 0.9% saline to a final concentration of 0.5 mg/mL. All substances were given intravenously as an initial bolus at the time of meningitis induction followed by a continuous infusion for the remainder of the experiment. Naratriptan was applied as a bolus of 7.5 mg/kg followed by 2.5 mg $\cdot$ $kg^{-1} \cdot h^{-1}$; for zolmitriptan, dosages were 3 mg/kg and 1 mg $\cdot$ $kg^{-1} \cdot h^{-1}$, respectively. These doses were selected as effective doses from a limited dose-finding series. In a delayed-treatment group, zolmitriptan was started 2 hours after the induction of meningitis using the same dosages. In animals not receiving triptans, saline infusions were given instead. A further series of zolmitriptan-treated animals were given the selective $5HT_{1B}$ antagonist SB 216641 (Biotrend GmbH, Köln, Germany; n = 4), the selective $5HT_{1D}$ antagonist BRL 15572 (Biotrend GmbH; n = 4), or both (n = 5) prior to induction of meningitis in an attempt to restore inflammation and confirm the specificity of the observed effects. For this purpose, SB 216641 (10 mg/kg) was dissolved in purified water; BRL 15572 (10 mg/kg) was dissolved in DMSO. Both substances were given intraperitoneally 10 minutes before the beginning of zolmitriptan infusion.

Immunochemistry of rat dura mater. For demonstration of CGRP and SP within meningeal nerve fibers, immunochemistry was performed in rats injected intracisternally with saline (n = 2), animals with untreated meningitis (n = 2), and animals with naratriptan- or zolmitriptan-treated meningitis (n = 5). Skulls were immersed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for 2 hours immediately after removal of the brains. After overnight incubation in 20% sucrose, dura mater was removed from the bone and immunostained using a free-floating technique. Following repeated washes in PBS, specimens were blocked with 2% normal goat serum and 0.2% Triton X in PBS and then incubated overnight with a polyclonal rabbit antibody against rat CGRP (1:1,000 in blocking solution) or Substance P (1:500) at 4°C. For visualization, an antirabbit antibody conjugated to Texas red was used (1:200, 1 hour). Omission of the primary antibody was used to control for unspecific binding of the secondary antibody. All antibodies were purchased from Sigma (Sigma-Aldrich, Deisenhofen, Germany).

Mouse model of meningitis

Mice experiments were conducted using a modification of a previously published model (Tang et al., 1996). In brief, male 129S6 mice (20 g) were anesthetized with intraperitoneal ketamine (100 mg/kg; Ketanest, Parke-Davis GmbH, Freiburg, Germany) and xylazine (20 mg/kg; Rompun, Bayer AG, Leverkusen, Germany). A skin incision was made exposing the lumbar spine. Using a 30-gauge needle and a Hamilton syringe, 50 μL of a suspension containing 5×10^5 colony-forming units (cfu) live pneumococci in sterile PBS were slowly injected into the spinal canal at the level of L2 or L3. The skin incision was closed using dermal clips. Immediately after inoculation, animals received either zolmitriptan (3 mg/kg, n = 10) or vehicle (n = 10) intraperitoneally. Animals were then allowed to wake up and given free access to food and water. Absence of pareses and adequate waking were verified. Mice were kept under constant surveillance beginning at 12 hours after inoculation. At this time, intraperitoneal injections of zolmitriptan or vehicle were repeated. The duration of the experiment was limited to 24 hours. Primary end points were time of death and a clinical score at 24 hours in the surviving animals. The score was defined as follows: normal activity, 5 points; inactivity, but normal locomotion after stimulation, 4 points; ataxic gait, 3 points; delayed righting, 2 points; unable to right, 1 point; dead, 0 points. Immediately at the time of death or at the termination of the experiment at 24 hours, CSF was collected for determination of bacterial titers. Using a modification of previously described methods (Carp et al., 1971; Meyding-Lamade et al., 1996) a skin incision was made over the head and neck. After dissection of the suboccipital muscles under a preparation microscope, the cisterna magna was punctured and CSF withdrawn using a 27-gauge butterfly cannula connected to a Luer lock Hamilton syringe. For the determination of bacterial titers, serial dilutions of the CSF in sterile PBS were plated on sheep blood agar and grown overnight at 37°C with 5% CO_2.

Bacterial preparations

For the preparation of live bacteria, encapsulated pneumococci from a clinical isolate (strain D39, kindly provided by Dr. E. Tuomanen, St. Jude Children's Research Hospital, Memphis, TN, U.S.A.) were grown in standard C+Y culture medium (Lacks and Hotchkiss, 1960) overnight at 37°C with 5% CO_2. After centrifugation at 10,000 *g* for 2 minutes, the pellet was resuspended in sterile PBS. Using a standard curve, the number of cfu per milliliter in this preparation was determined photometrically. Aliquots for the inoculation of animals were then prepared using adequate dilutions in sterile PBS. Correctness of cfu calculations was verified by plating serial dilutions of the inoculate.

Pneumococcal cell walls, which permit study of the inflammatory host response in the absence of bacterial metabolic effects, were prepared as follows. Unencapsulated pneumococci (Strain PnR 527, Jena, Germany) were cultivated overnight on Columbia agar plates, suspended in pyrogen-free saline, and heat inactivated. After disintegration by ultrasound, pneumococcal cell walls were produced and modified as described earlier (Tuomanen et al., 1985; Weber et al., 1995). The concentration of PCW corresponded to 10^7 cfu/mL. Absence of lipopolysaccharides was ensured using a chromogenic limulus amebocyte lysate test (BioWhittaker, Walkersville, MD, U.S.A.).

Measurement of calcitonin gene-related peptide concentration in human cerebrospinal fluid

Diagnostic CSF samples from 10 patients with bacterial meningitis and 14 samples from controls (i.e., patients in whom CSF studies revealed no inflammatory changes) were included in the analysis. For a diagnosis of bacterial meningitis, CSF pleocytosis of >1,000 cells/μL with >90% neutrophils was required. The CGRP concentrations were measured using an enzyme-linked immunoassay (human CGRP EIA kit, SPI-BIO, Massy, France) according to the manufacturer's instructions.

Tumor necrosis factor-α bioassay

The tumor necrosis factor-α (TNF-α) bioactivity was measured as described previously (Freyer et al., 1999). In brief, a modified L 929 cytotoxicity assay (Flick and Gifford, 1984) was performed by adding 100 μL CSF per well to the culture medium in the presence of 1 μg/mL actinomycin D. After incubation at 37°C for 20 hours, cell viability was quantified by the uptake of crystal violet in living cells, which was determined spectrophotometrically (595 nm) using an ELISA reader (Dynatech, Denkendorf, Germany). Equivalent concentrations

of rat TNF-α (a gift from Dr. P. Scholz, Schering AG, Berlin, Germany) were used as a standard.

Statistical analysis

For descriptive statistics, data are expressed as mean values and standard deviations. For the rat experiments, comparisons between groups were performed using one-way ANOVA with *post hoc* testing by the Duncan multiple-range test. In the mice experiments, a log-rank test was performed to test for significance of the difference in survival time between meningitis treated with vehicle or zolmitriptan, while nonparametric Mann-Whitney U tests were used to test for a statistically significant difference in clinical score and CSF bacterial titers at the time of death between the two groups. Mann-Whitney U tests were also used to test for a statistically significant difference of CSF CGRP concentrations between patients and 12 controls and of TNF-α bioactivity in rat CSF between PCW-induced meningitis and naratriptan-treated meningitis. Statistical tests were performed using SPSS 10 statistical software (SPSS Inc., Chicago, Illinois, U.S.A.).

RESULTS

Rat experiments

Values of MABP, Pa_{O_2}, Pa_{CO_2}, and pH were within normal ranges during the entire experiment, and no biologically relevant differences were found between the experimental groups (Table 2). In animals intracisternally injected with saline, there was no significant change in rCBF or ICP during the 6-hour experimental period (Table 1, Figs. 1A and 2). Brain water content at the end of the experiment was 80.99% ± 0.63% and leukocyte count in the CSF was 20 ± 21 cells/μL. In animals treated with intracisternal saline, no significant effect of triptan treatment could be demonstrated on ICP, brain water content, or CSF leukocyte count.

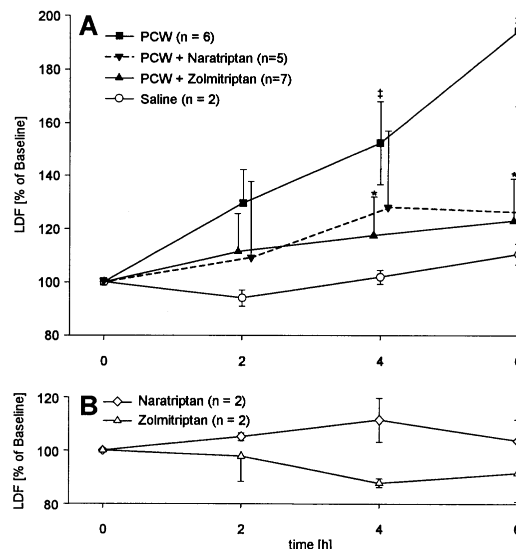

FIG. 1. (A) Pneumococcal cell walls (PCW) induce an increase of regional cerebral blood flow that is inhibited by zolmitriptan. *Indicates a significant difference (P < 0.05) compared with untreated meningitis. ‡Indicates a significant difference (P < 0.05) compared with animals treated with saline intracisternally (ANOVA, Duncan *post hoc* test). **(B)** In animals treated with saline intracisternally, triptans have no significant effect on laser Doppler flow (LDF).

TABLE 2. *Physiologic parameters at 2, 4, and 6 hours after intracisternal challenge*

IC injection	IV treatment		MABP (mm Hg)	pH	Pa_{CO_2} (mm Hg)	Pa_{O_2} (mm Hg)
Saline	Saline	0 h	117 ± 0	7.422 ± 0.031	42.8 ± 2.7	272.1 ± 62.3
		2 h	119 ± 6	7.391 ± 0.037	41.0 ± 1.4	252.0 ± 31.7
		4 h	113 ± 11	7.379 ± 0.032	40.0 ± 1.6	234.4 ± 50.4
		6 h	98 ± 28	7.373 ± 0.076	38.9 ± 2.1	212.6 ± 44.3
	Naratriptan	0 h	119 ± 14	7.411 ± 0.061	41.8 ± 0.1	169.0 ± 28.6
		2 h	117 ± 22	7.405 ± 0.004	38.5 ± 2.8	159.6 ± 20.6
		4 h	107 ± 19	7.420 ± 0.021	37.1 ± 3.3	155.2 ± 13.6
		6 h	101 ± 11	7.417 ± 0.003	34.6 ± 7.4	148.7 ± 19.2
	Zolmitriptan	0 h	117 ± 11	7.426 ± 0.010	41.7 ± 0.2	200.8 ± 7.8
		2 h	111 ± 3	7.436 ± 0.048	42.6 ± 0.9	176.3 ± 19.7
		4 h	98 ± 7	7.393 ± 0.037	40.7 ± 2.1	141.1 ± 33.9
		6 h	90 ± 6	7.390 ± 0.044	41.8 ± 3.2	208.1 ± 49.5
PCW	Saline	0 h	111 ± 15	7.426 ± 0.047	43.5 ± 3.6	162.2 ± 31.0
		2 h	113 ± 11	7.426 ± 0.028	42.1 ± 4.0	166.8 ± 39.8
		4 h	105 ± 10	7.402 ± 0.044	40.5 ± 4.8	171.6 ± 23.3
		6 h	97 ± 14	7.409 ± 0.039	39.4 ± 3.5	171.3 ± 24.1
	Naratriptan	0 h	107 ± 21	7.391 ± 0.029	41.7 ± 1.4	262.1 ± 128.6
		2 h	108 ± 20	7.368 ± 0.032	42.6 ± 3.7	195.0 ± 51.0
		4 h	103 ± 19	7.396 ± 0.041	36.7 ± 6.7	159.4 ± 30.8
		6 h	101 ± 23	7.394 ± 0.006	37.0 ± 4.0	172.1 ± 20.1
	Zolmitriptan	0 h	106 ± 7	7.428 ± 0.029	40.8 ± 3.0	213.0 ± 163.2
		2 h	113 ± 4	7.416 ± 0.056	38.4 ± 3.1	263.2 ± 155.6
		4 h	107 ± 9	7.415 ± 0.058	37.9 ± 3.5	181.5 ± 48.5
		6 h	107 ± 6	7.432 ± 0.040	36.6 ± 3.3	232.5 ± 125.2
	Zolmitriptan	0 h	124 ± 5	7.428 ± 0.043	39.6 ± 3.7	162.1 ± 34.9
	(posttreated)	2 h	108 ± 16	7.417 ± 0.054	40.1 ± 3.4	155.5 ± 53.5
		4 h	106 ± 20	7.394 ± 0.064	39.0 ± 6.9	187.4 ± 48.9
		6 h	97 ± 13	7.394 ± 0.024	38.0 ± 2.0	179.2 ± 29.1

IC, intracisternal; IV, intravenous; MABP, mean arterial blood pressure; PCW, pneumococcal cell wall suspension.

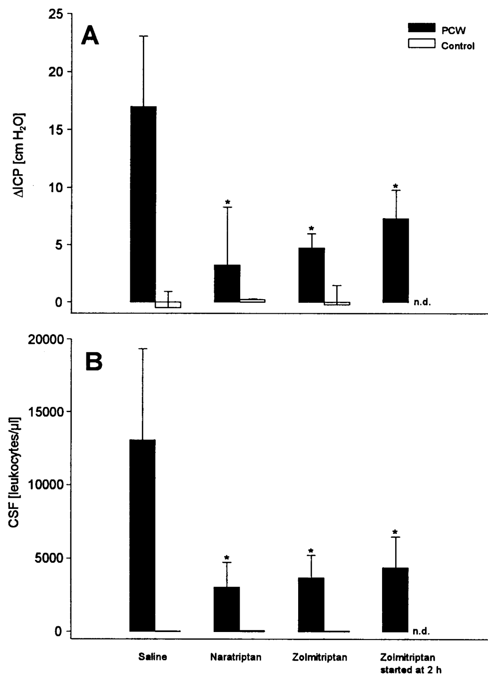

FIG. 2. (A) Intracranial pressure (ICP) is significantly increased in pneumococcal cell wall (PCW)-induced meningitis. Increase is inhibited by naratriptan and zolmitriptan started at 0 hours or by zolmitriptan started at 2 hours. **(B)** Influx of leukocytes into the cerebrospinal fluid (CSF) associated with PCW-induced meningitis is reduced by triptan treatment. *Indicates a significant difference ($P < 0.05$) compared to untreated meningitis (ANOVA, Duncan *post hoc* test).

In the untreated meningitis group, a significant increase in rCBF as well as an increase in ICP compared to animals intracisternally injected with saline was observed (Table 1, Figs. 1 and 2B). These changes were accompanied by a significant influx of leukocytes into the CSF and raised brain water content (Table 1, Fig. 2B). Treatment of PCW-challenged animals with zolmitriptan or naratriptan significantly reduced the increase in blood flow, ICP, and the formation of brain edema, as well as the leukocytes in the CSF (Table 1, Figs. 1 and 2). Zolmitriptan treatment 2 hours after meningitis induction still significantly reduced LDF and ICP increase, brain edema, and CSF leukocytosis. The inhibition of the inflammatory response in zolmitriptan-treated meningitis was partially reversed by pretreatment with the $5HT_{1B}$ antagonist SB 216641, the $5HT_{1D}$ antagonist BRL 15572, or a combination of these antagonists. Data for cell count and intracranial pressure are summarized in Figs. 3A and 3B. Increase in rCBF at 6 hours in PCW-induced meningitis, which was reduced from 196% ± 16% at baseline to 121% ± 21% with zolmitriptan, was restored to 142% ± 45% with SB 216641, to 315% ± 98% with BRL 15572, and to 206% ± 29% with a combination of both antagonists. Zolmitriptan led to a decrease in brain water content from 83.06% ± 0.31% in untreated meningitis to 81.40% ± 0.45% ($P < 0.05$), which was reversed by BRL 15572 14 to 83.40% ± 0.44% ($P < 0.05$), by SB 216641 to 82.57% ± 0.37% ($P < 0.05$), and by the addition of both substances to 82.35% ± 0.44% ($P < 0.05$). In animals treated with saline intracisternally and saline intravenously, no significant effect of SB 216641 or BRL 15572 on LDF, ICP, leukocyte influx, or brain water content after was observed after 6 hours (data not shown).

Immunochemistry of rat dura mater. In rats treated with saline intracisternally, immunostaining for CGRP and SP was observed in nerve fibers of different caliber (Figs. 4A and 4D). In comparison, immunoreactivity for both neuropeptides was markedly reduced in the untreated-meningitis group (Fig. 4B, E). Depletion of CGRP and SP was inhibited in zolmitriptan-treated meningitis (Fig. 4C, F). Neuropeptide release was also attenuated by naratriptan (data not shown).

Tumor necrosis factor-α bioactivity in rat cerebrospinal fluid. At 6 hours after intracisternal instillation of PCW, TNF-α bioactivity in the CSF of rats receiving intravenous saline was equivalent to 899 ± 447 pg/mL compared to 115 ± 61 pg/mL in naratriptan-treated meningitis ($P = 0.045$).

Mouse experiments

At 24 hours after inoculation, 7 out of 10 mice with untreated meningitis had died compared to 2 out of 10

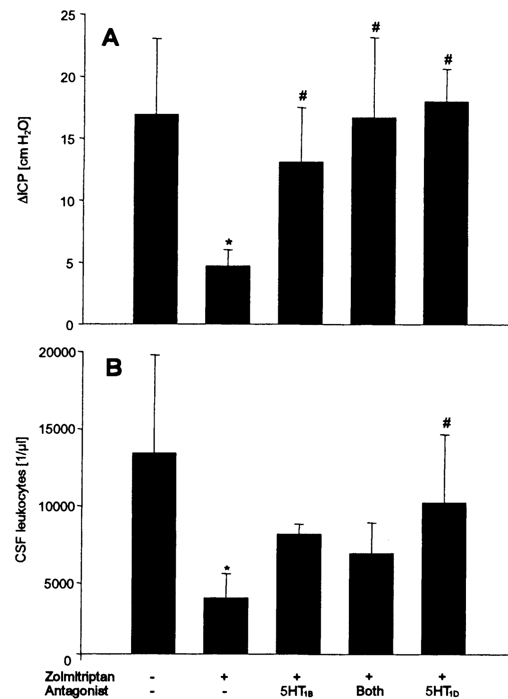

FIG. 3. The inhibition of intracranial pressure (ICP) increase **(A)** and leukocyte influx into the cerebrospinal fluid (CSF) **(B)** in zolmitriptan-treated meningitis is partially reversed by pretreatment with the selective $5HT_{1B}$ receptor antagonist SB 216641 (n = 4), the $5HT_{1D}$ receptor antagonist BRL 15572 (n = 4), or both (n = 4). *Indicates a significant difference compared to untreated meningitis; #indicates a significant difference compared to zolmitriptan treated meningitis ($P < 0.05$; ANOVA, Duncan *post hoc* test).

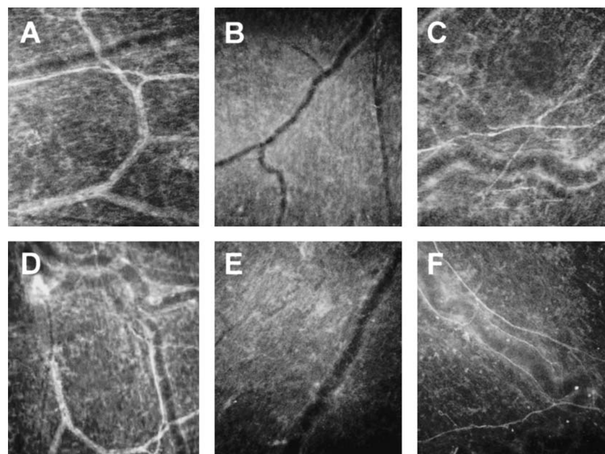

FIG. 4. Immunochemistry results for calcitonin gene-related peptide **(A–C)** and substance P **(D–F)** from a representative study in which n = 2 or n = 3 each. Immunoreactivity is present in dural whole-mount preparations of animals treated with saline intracisternally **(A** and **D)**, but is largely absent after 6 hours of meningitis induced by pneumococcal cell wall (PCW) intracisternally **(B** and **E)**. Immunoreactivity is preserved in PCW-induced meningitis treated with zolmitriptan intravenously **(C** and **F)**. Original magnification, ×100.

mice with zolmitriptan-treated meningitis. Kaplan-Meier survival curves for these animals are shown in Fig. 5A. This difference in survival was statistically significant ($P = 0.02$, log-rank test). The clinical score at 24 hours (Fig. 5B) was 0.8 ± 1.4 in untreated meningitis compared to 3.3 ± 1.9 in zolmitriptan-treated meningitis ($P < 0.01$, Mann-Whitney U test). Mean CSF bacterial titers were 7×10^8 cfu/mL (range, $4 \times 10^7 - 3 \times 10^9$) in animals receiving vehicle IP compared to 3×10^9 cfu/mL (range, $3 \times 10^7 - 2 \times 10^{10}$) in animals given zolmitriptan IP ($P = 0.931$, Mann-Whitney U test). Similar effects were observed with naratriptan treatment (data not shown).

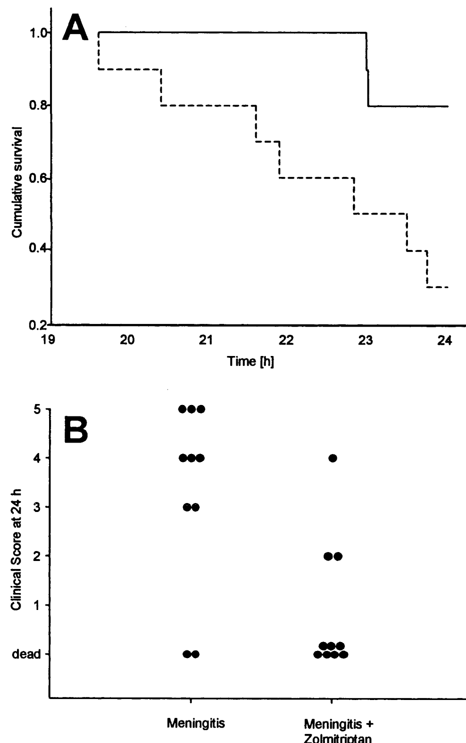

FIG. 5. (A) Kaplan-Meier survival curves of 129S6 mice intrathecally infected with 5×10^5 colony-forming units (cfu) live encapsulated pneumococci (strain D39). Solid line: mice treated with 3-mg/kg zolmitriptan intraperitoneally (IP) at 0 and 12 hours (n = 10). Broken line: mice treated with vehicle IP (n = 10). The difference in survival time was statistically significant ($P = 0.02$, log-rank test). **(B)** Clinical score at 24 hours (5 = best, 0 = dead). The score difference was statistically significant ($P < 0.01$, Mann-Whitney U test).

Calcitonin gene-related peptide concentrations in human cerebrospinal fluid samples

The CGRP concentration in controls was 27.1 ± 1.7 (range, 25.3–31.74) pg/mL. The concentration of CGRP in CSF of patients with bacterial meningitis was 67.9 ± 87.5 (range, 29.2–266.1) pg/mL (Fig. 6). The difference between the groups was statistically significant ($P < 0.01$, Mann-Whitney U test).

DISCUSSION

We have shown for the first time that pharmacologic intervention with triptans has potent antiinflammatory effects in bacterial meningitis in the rat. Zolmitriptan and naratriptan significantly reduced the influx of leukocytes into the CSF as well as the increase of regional cerebral blood flow and ICP and the formation of brain edema. Triptans also significantly improved survival and clinical score at 24 hours in mice infected intrathecally with live pneumococci. Our experiments demonstrate that in the early phase of bacterial meningitis, inflammation mediated by the trigeminovascular system contributes to bacterially induced host responses.

Using two animal models, we investigated the effect of triptan treatment on different aspects of bacterial meningitis. In particular, intrathecal infection of mice with live encapsulated pneumococci allowed us to study the

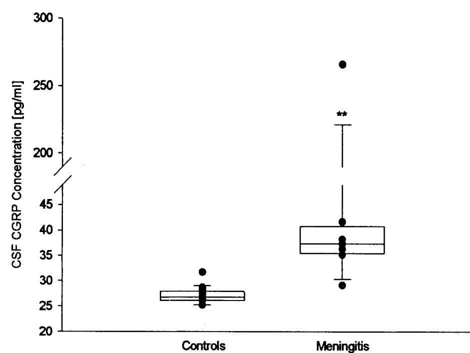

FIG. 6. The calcitonin gene-related peptide (CGRP) concentrations in the cerebrospinal fluid (CSF) of patients with bacterial meningitis (n = 10) are significantly higher than those found in controls. $^{**}P = 0.001$ (Mann-Whitney U test).

effects of triptans in untreated meningitis, whereas PCW-induced meningitis in rats was used to model the host response caused by bacterial lysis. Antibiotic-induced lysis of pneumococci triggers the release of PCW. The concentration of cell walls, which consist mainly of teichoic acid (Höltje and Tomasz, 1976), is related to the clinical outcome in human pneumococcal meningitis (Schneider et al., 1999).

Two findings argue that the antiinflammatory effects observed in our rat experiments are related to typical agonist action of the triptans at serotonergic receptors on perivascular nerve fibers. First, triptan treatment inhibited the depletion of CGRP and SP from these fibers during meningitis as demonstrated by immunochemistry. Second, the antiinflammatory effects were reversed to a large extent by coapplication of $5HT_{1B}$ and $5HT_{1D}$ receptor antagonists. The fact that inflammation was not restored completely may be explained by additional action of triptans on other serotonin receptors, such as the $5HT_{1F}$ receptor (Martin et al., 1997).

All of the antiinflammatory effects of triptans that were observed during our experiments may be linked to the inhibition of neuropeptide release. Both SP and CGRP have been shown to promote the activation of neutrophils and microvascular endothelial cells, which is required for the invasion of neutrophils into the CSF space. In detail, SP and CGRP upregulate the expression of the β_2-integrins CD11 and CD18 on neutrophils and promote their adherence to endothelial cells *in vitro* (Zimmerman et al., 1992). Furthermore, SP enhances the production of leukotrienes, TNF-α secretion, and regulates LFA-1 and ICAM-1 (Saban et al., 1997). Activating effects on human neutrophils exceeding those of SP were also demonstrated for CGRP (Richter et al., 1992). Endothelial cells may be activated through signaling by compounds of the bacterial cell wall (Freyer et al., 1999), but they are also a source as well as a target of proinflammatory cytokines (Freyer et al., 1999). Both CGRP and SP stimulate the production and release of these cytokines from immune competent cells (Sakuta et al., 1995). In our study, we found a decrease of TNF-α in the CSF of animals with triptan-treated meningitis. Induction of the adhesion molecules ICAM-1 and VCAM-1 on the endothelial surface by SP was previously demonstrated (Quinlan et al., 1998). Thus, reduced CSF leukocyte counts in triptan-treated meningitis may be related to decreased release of neuropeptides otherwise promoting endothelial and leukocyte activation. Additional inhibition might be mediated by direct action of triptans on endothelial $5HT_{1B}$ receptors (Riad et al., 1998).

Decreased endothelial activation may exert a protective effect on the blood–brain barrier by reducing the production of inducible NO (Freyer et al., 1999), a mediator of trigeminal nociceptive transmission (Hoskin et al., 1999). Since activation of the vascular endothelium is a prerequisite for the transcytosis of pneumococci through cerebral endothelial cells (Ring et al., 1998), triptans might theoretically also decrease bacterial invasion into the CNS. These effects could not be studied in our mouse model due to the intrathecal mode of infection. On the other hand, presence of identical bacterial concentrations supports the conclusion that improved survival and clinical score of the triptan-treated mice was related to influences on the inflammatory host response rather than to bacterial mechanisms.

Reduction of rCBF increase in response to triptans may reflect vasoconstriction mediated by $5HT_{1B}$ receptors on perivascular myocytes. However, no significant effect of triptans on rCBF was demonstrated in nonmeningitic animals, and earlier studies have shown that the TVS is not essential for CBF autoregulation under physiologic conditions (Sakas et al., 1989; Vraamark et al., 1998). More likely, reduced hyperemia again is an effect of reduced levels of vasoactive neuropeptides. This mechanism is in keeping with the findings after chronic nasociliary nerve sectioning in PCW-induced meningitis (Weber et al., 1996).

Increased ICP in the acute phase of bacterial meningitis may be attributed to at least three mechanisms (Quagliarello and Scheld, 1992). First, blood–brain barrier disruption causes vasogenic edema due to extravasation of plasma compounds and hyperemia. Second, cytotoxic compounds released from activated leukocytes, astrocytes (Freyer et al., 1996), microglia (Draheim et al., 1999), endothelial cells (Freyer et al., 1999), and bacteria cause cytotoxic brain edema. Finally, the increased ICP may be due to an increase of CSF outflow resistance (Scheld et al., 1980). Lower ICP and reduced brain edema in the triptan group may thus result from attenuated hyperemia as well as better preservation of the blood–brain barrier secondary to reduced leukocyte recruitment.

Relevance of these experimental findings for bacterial meningitis in humans is suggested by the clinically observed activation of the trigeminovascular system, and by our demonstration of significantly increased CGRP concentrations in the CSF of patients with bacterial meningitis. Our results may open a new approach in the design of pharmacologic interventions to treat pain and reduce life-threatening intracranial complications during the early phase of bacterial meningitis.

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2.3. **Olaf M. Hoffmann**, Doreen Becker, Joerg R. Weber. Bacterial hydrogen peroxide contributes to cerebral hyperemia during early stages of experimental pneumococcal meningitis. J Cereb Blood Flow Metab 2007; 27:1792-7.

Störungen der Blutflußregulation treten als wichtige Komplikationen invasiver Infektionen wie Sepsis und bakterieller Meningitis auf. Streptokokken, darunter insbesondere Pneumokokken, zeichnen sich durch eine fehlende Catalasewirkung aus, so dass sie große Mengen von Wasserstoffperoxid (H_2O_2) an die Umgebung abgeben. In einem Rattenmodell der Pneumokokkenmeningitis wurde die Auswirkung der bakteriellen H_2O_2-Produktion auf den regionalen zerebralen Blutfluss (rCBF) und den intrakraniellen Druck (ICP) untersucht. Verglichen mit Wildtyp-Pneumokokken (Stamm D39) verursachte die H_2O_2-defiziente Mutante *SpxB*$^-$ einen deutlich geringeren Anstieg des rCBF während sechsstündiger Meningitis. Zudem konnte der von D39 verursachte Blutflußanstieg durch enzymatischen H_2O_2-Abbau mittels Catalase oder durch Blockade Calcium-sensitiver Kaliumkanäle reduziert werden, die als Mediatoren einer H_2O_2-induzierten Vasodilation betrachtet werden. Der verbleibende Blutflussanstieg bei Meningitis durch *SpxB*$^-$ wurde durch Catalase nicht beeinflusst, was eine bedeutendere Rolle des bakteriellen H_2O_2 im Vergleich zu endogenen Quellen nahelegt. Zusammenfassend belegen die Ergebnisse, dass neben wirtsseitigen Mediatoren auch bakteriell produziertes H_2O_2 einen starken Vasodilatator darstellt, der zur frühen Hyperperfusion während Pneumokokkenmeningitis beiträgt.

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Brief Communication

Bacterial hydrogen peroxide contributes to cerebral hyperemia during early stages of experimental pneumococcal meningitis

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Alterations of blood flow contribute to major clinical complications in invasive infections such as sepsis and bacterial meningitis. As a unique feature streptococci – in particular, *Streptococcus pneumoniae*, the most frequent pathogen in bacterial meningitis – release hydrogen peroxide (H_2O_2) because of the absence of functional catalase. In a 6 h rat model of experimental meningitis, we studied the impact of bacterial H_2O_2 production on regional cerebral blood flow (rCBF) and intracranial pressure (ICP). Compared to wild-type D39 pneumococci, the increase of rCBF was diminished in meningitis induced by the H_2O_2 defective *SpxB*⁻ mutant (maximum increase, 135%± 17% versus 217%±23% of the individual baseline; $P<0.01$) or after treatment of D39-induced meningitis with H_2O_2-degrading catalase or with tetraethylammonium (TEA), a blocker of calcium-sensitive potassium channels, which mediate H_2O_2-induced vasodilation. Catalase did not significantly reduce the remaining rCBF increase caused by *SpxB*⁻, supporting the predominant role of bacterial H_2O_2. We conclude that in addition to host-sided mediators, bacterial-derived H_2O_2 acts as a potent vasodilator, which accounts for a certain proportion of the early cerebral hyperperfusion in pneumococcal meningitis.
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Keywords: bacterial meningitis; hydrogen peroxide; *streptococcus pneumoniae*; vasodilation

Introduction

Bacterial meningitis is still a life-threatening disease even with modern antibiotic treatment. Mortality is as high as 30%, and long-term neurological deficits are frequently found in survivors (de Gans *et al*, 2002). The negative outcome is largely determined by early complications, such as formation of brain edema, vascular alterations, raised intracranial pressure, and increased outflow resistance of the cerebrospinal fluid (CSF) (Scheld *et al*, 1980; Tuomanen *et al*, 1989). Although bacterial and host-sided factors are thought to contribute to blood–brain barrier breakdown, cerebral hyperemia is usually interpreted as an effect of vasoactive substances released by the host during the inflammatory response (Pfister *et al*, 1995; Lorenzl *et al*, 1996; Paul *et al*, 1997; Angstwurm *et al*, 1998; Hoffmann *et al*, 2002). In other invasive bacterial infections such as sepsis, vasodilatation contributes to systemic hypotension and organ dysfunction (Hotchkiss and Karl, 2003).

Streptococcus pneumoniae is the most frequent and disastrous pathogen in bacterial meningitis and an important cause of pneumonia and sepsis worldwide. A distinctive feature of streptococci, in particular *S. pneumoniae*, resides in the absence of catalase, a hydrogen peroxide (H_2O_2) degrading enzyme. As a result, pneumococci release significant amounts of H_2O_2 as a byproduct of oxidative metabolism. H_2O_2 limits the growth of other competing bacteria and is regarded as an additional virulence factor of pneumococci (Spellerberg *et al*, 1996; Pericone *et al*, 2002). Moreover, H_2O_2 acts as an exotoxin and may cause cytotoxic damage to host tissue (Braun *et al*, 2002; Bermpohl *et al*, 2005; Hoffmann *et al*, 2006).

In the eukaryotic organism, H_2O_2 is commonly appreciated as a cytotoxic product, which is

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synthesized by the nicotinamide adenosine dinucleotide phosphate oxidase of activated phagocytes. H_2O_2 is a potent vasodilator, which acts via the opening of calcium-activated potassium channels (K_{Ca}; Sobey *et al*, 1997). In the present study, we asked whether the release of H_2O_2 by pneumococci exerts direct vasodilator effects in early meningitis and may thus contribute to early disturbances of blood flow and intracranial pressure (ICP).

Materials and methods

Bacterial Strains and Culture

D39, an encapsulated strain of *S. pneumoniae* serotype 2 originally derived from a clinical isolate, was used as the wild type. Additionally, we used the previously published mutant *SpxB*⁻ (Spellerberg *et al*, 1996). These bacteria lack pyruvate oxidase, resulting in a 95% reduction of H_2O_2 production (Spellerberg *et al*, 1996; Pericone *et al*, 2002). For liquid cultures, the strains were grown in standard casein plus yeast medium with 5% CO_2 at 37°C. Erythromycin (Sigma, Tavfkirchen, Germany) was added to *SpxB*⁻ at a final concentration of 1 μg/mL. During log-phase growth, bacteria were pelleted by centrifugation and resuspended in pyrogen-free phosphate-buffered saline (PBS); 0.1 mol/L. Colony-forming units (CFU) per milliliter were then determined photometrically (absorption at 620 nm) using a standard curve. Adequate dilutions in pyrogen-free PBS were used to produce defined inocula. Colony-forming units calculations were verified by plating of serial dilutions. Pneumococcal cell wall (PCW) was prepared as published previously (Bermpohl *et al*, 2005).

Rat Experiments

Table 1 outlines the experimental group design. The general experimental procedure was as described earlier (Hoffmann *et al*, 2002). Experiments were performed on male Wistar rats (280 to 330 g) anesthetized with intraperitoneal sodium thiopental (100 mg/kg initially and 20 mg/kg every 2 h; Trapanal, Byk Gulden, Konstanz,

Table 1 Experimental group design and basic physiology

Challenge	*Treatment*		*MAP (mmHg)*	*CPP (mmHg)*	*pH*	*pCO₂ (mmHg)*	*pO₂ (mmHg)*
PBS		0 h	84±9	82±8	7.41±0.01	42±2	114±6
	—	2 h	86±2	84±3	7.39±0.03	39±3	99±9
	$n=3$	4 h	79±9	76±10	7.40±0.02	39±1	96±19
		6 h	84±9	81±9	7.40±0.04	39±1	115±11
D39		0 h	91±4	87±4	7.42±0.03	38±5	132±28
	—	2 h	92±12	86±10	7.39±0.04	39±4	117±20
	$n=9$	4 h	86±10	77±5	7.37±0.03	40±3	111±8
		6 h	87±11	75±11	7.38±0.04	38±4	119±13
		0 h	92±3	89±4	7.36±0.10	43±4	161±50
	Catalase	2 h	97±4	91±5	7.37±0.04	42±4	146±6
	$n=3$	4 h	95±6	86±6	7.36±0.05	40±1	117±39
		6 h	98±5	85±4	7.37±0.07	36±1	118±34
		0 h	79±11	77±12	7.46±0.09	32±1	150±50
	TEA	2 h	80±6	76±6	7.41±0.07	33±3	124±13
	$n=3$	4 h	82±4	74±3	7.39±0.03	36±3	119±9
		6 h	79±16	67±15[a]	7.30±0.03	38±5	97±3
SpxB⁻		0h	94±10	91±10	7.42±0.05	37±7	136±36
	—	2 h	87±8	82±7	7.39±0.04	39±5	137±31
	$n=8$	4 h	90±4	81±5	7.36±0.05	39±4	130±37
		6 h	88±8	77±8	7.34±0.03	38±3	140±48
		0 h	95±7	93±7	7.43±0.05	37±4	173±38
	Catalase	2 h	95±6	90±6	7.39±0.05	38±3	146±29
	$n=4$	4 h	97±8	87±8	7.37±0.07	36±3	133±34
		6 h	89±9	80±8	7.34±0.10	39±5	111±10
		0 h	77±16	76±15	7.40±0.03	32±6	109±14
	TEA	2 h	76±3	73±2	7.39±0.02	33±4	95±22
	$n=2$	4 h	70±4	65±5	7.28±0.02	38±2	93±4
		6 h	66±10	60±9[a]	7.34±0.04	41±8	102±5
PCW		0 h	92±8	89±8	7.40±0.02	35±8	114±15
	—	2 h	93±6	89±7	7.37±0.01	37±6	111±17
	$n=5$	4 h	84±15	78±14	7.37±0.02	36±3	119±17
		6 h	87±30	76±29	7.34±0.04	37±7	137±21
		0 h	88±0	85±0	7.38±0.03	36±2	118±4
	Catalase	2 h	89±9	84±10	7.29±0.04	41±8	101±30
	$n=3$	4 h	90±7	83±7	7.36±0.06	33±0	111±29
		6 h	86±13	75±12	7.32±0.00	40±0	106±22

MAP, mean arterial pressure; CPP, cerebral perfusion pressure.
[a]Measurements of cerebral blood flow and intracranial pressure at 6 h were censored in two TEA-treated animals challenged with D39 ($n=1$) and *SpxB*⁻ ($n=1$) due to a low CPP of less than 60 mm Hg.

Germany). Animals were tracheotomized and ventilated mechanically (AP-10; K Effenberger, Paffing, Germany) with air supplemented with O_2. End-tidal CO_2 was monitored continuously (Artema MM204; Heyer, Bad Ems, Germany). Body temperature was measured by a rectal probe and maintained at 37.8°C±0.4°C using a heating pad. A transducer (Statham P109 EZ, Spectramed, Oxnard, CA, USA) connected to a catheter placed in the left femoral artery measured mean arterial blood pressure (MAP) continuously. From this catheter, arterial blood samples were analyzed for P_aO_2, P_aCO_2, and pH at 0, 2, 4, and 6 h. The left femoral vein was cannulated for infusion of drugs or saline. A $3 \times 3\,mm^2$ area of the parietal bone lateral to the sagittal suture was thinned to allow laser Doppler flow (LDF) measurements (Periflux 4001 Master, Järfälla, Sweden) in cortical blood vessels. Although LDF is not a direct measure of the absolute regional cerebral blood flow (rCBF), a strong correlation between relative changes of LDF and relative changes of rCBF has been demonstrated previously. A catheter was placed into the cisterna magna through an occipital burr hole and connected to a pressure transducer (Statham P109 EZ, Spectramed, Oxnard, CA, USA) for continuous ICP measurement. Cerebral perfusion pressure (CPP) was calculated as MAP−ICP. A CPP of 60 mm Hg was considered the lower threshold of autoregulation. For challenge (0 h time point), 100 μL of CSF were withdrawn from this catheter and replaced with bacterial suspension, PCW preparation, or sterile PBS. At the end of the experiment, CSF samples were obtained to determine the CSF leukocyte count and the bacterial concentration by plating of serial dilutions. Animals were then killed by injection of 3 mol/L KCl.

Pharmacological Interventions

We used two strategies to determine the impact of H_2O_2 on cerebral blood flow increase. In a subset of experiments, catalase (EC 1.11.16; Sigma) was applied both as a continuous infusion via the femoral vein catheter (50.000 U/kg per hour) starting 15 mins before meningitis induction and as a single intrathecal injection (15.000 iE) via the cisterna magna catheter at the time of meningitis induction. In further experiments, tetraethylammonium (TEA; Sigma), a blocker predominantly of calcium-dependent potassium channels, was injected as a single bolus of 1 mg per animal via the femoral vein catheter 15 mins before meningitis induction.

Statistical Methods

Data are presented as means±standard deviations (s.d.). Intraindividual changes of LDF and ICP were examined with paired *t*-tests after testing for normal distribution, using the absolute values. To compare effects between two groups of animals, LDF was normalized by defining the individual baseline as 100%, whereas ICP was transformed into the ICP increase relative to the individual baseline. In samples with normal distribution and equal variance, Student's *t*-tests were performed; otherwise, Mann–Whitney *U*-tests were used. Multiple comparisons were performed with Kruskal–Wallis analysis of variance followed by Dunn's *post hoc* testing.

Results

Basic Physiology and Cerebrospinal Fluid Findings

Basic physiological parameters are shown in Table 1. Although there were no relevant differences in MAP and blood gas analyses between the remaining experimental groups, TEA treatment was consistently associated with a significantly reduced MAP (Acheson and Moe, 1946). At the time of intrathecal challenge (0 h time point; 15 mins after application of TEA), MAP of all animals receiving TEA was 78±11 mm Hg compared with 92±7 mm Hg in animals not receiving TEA ($P<0.01$, Mann–Whitney *U*-test). At the end of the experiment (6 h), TEA-treated animals also had a lower MAP of 75±11 mm Hg compared with 89±14 mm Hg in the remaining animals ($P=0.03$, Mann–Whitney *U*-test). Two animals treated with TEA (one challenged with D39 and one challenged with $SpxB^-$) displayed severe hypotension toward the end of the experiment, resulting in a CPP below the presumed lower level of at 6 h after challenge. To rule out a confounding effect of the low CPP, the corresponding measurements of LDF and ICP at 6 h in these two animals were censored from the statistical evaluation, while earlier measurements up to 5 h were maintained due to the presence of normal CPP.

In control animals, no CSF pleocytosis was observed at the end of the experiment (19±11 leukocytes/μL). By comparison, all animals challenged intrathecally with live bacteria or PCW developed intrathecal inflammation as evidenced by the influx of leukocytes into the CSF. Cell counts at the end of the experiment 6 h after meningitis induction were 3435±2001/μL with D39, 5771±196/μL with $SpxB^-$ and 5125±391/μL with PCW, the differences not being statistically significant (Figure 1A). Moreover, plating revealed the presence of biologically equivalent bacterial concentrations in the CSF between the two bacterial strains (D39, $5.4 \pm 1.6 \times 10^8$ CFU/mL versus $SpxB^-$ $3.0 \pm 2.4 \times 10^8$ CFU/mL; Figure 1B).

Effects on Regional Cerebral Blood Flow

In control animals receiving intrathecal PBS, the rCBF as measured by laser Doppler did not change significantly during the experimental period (maximum increase, 105%±7% of baseline; $P=0.25$ versus baseline; Figure 1C). After intrathecal infection with live D39 pneumococci, we observed a gradual increase of the rCBF to a maximum of 217%±23% of baseline ($P<0.01$ versus individual baseline and $P<0.01$ versus maximum increase in PBS-challenged controls). Infection with $SpxB^-$ caused only a minor increase

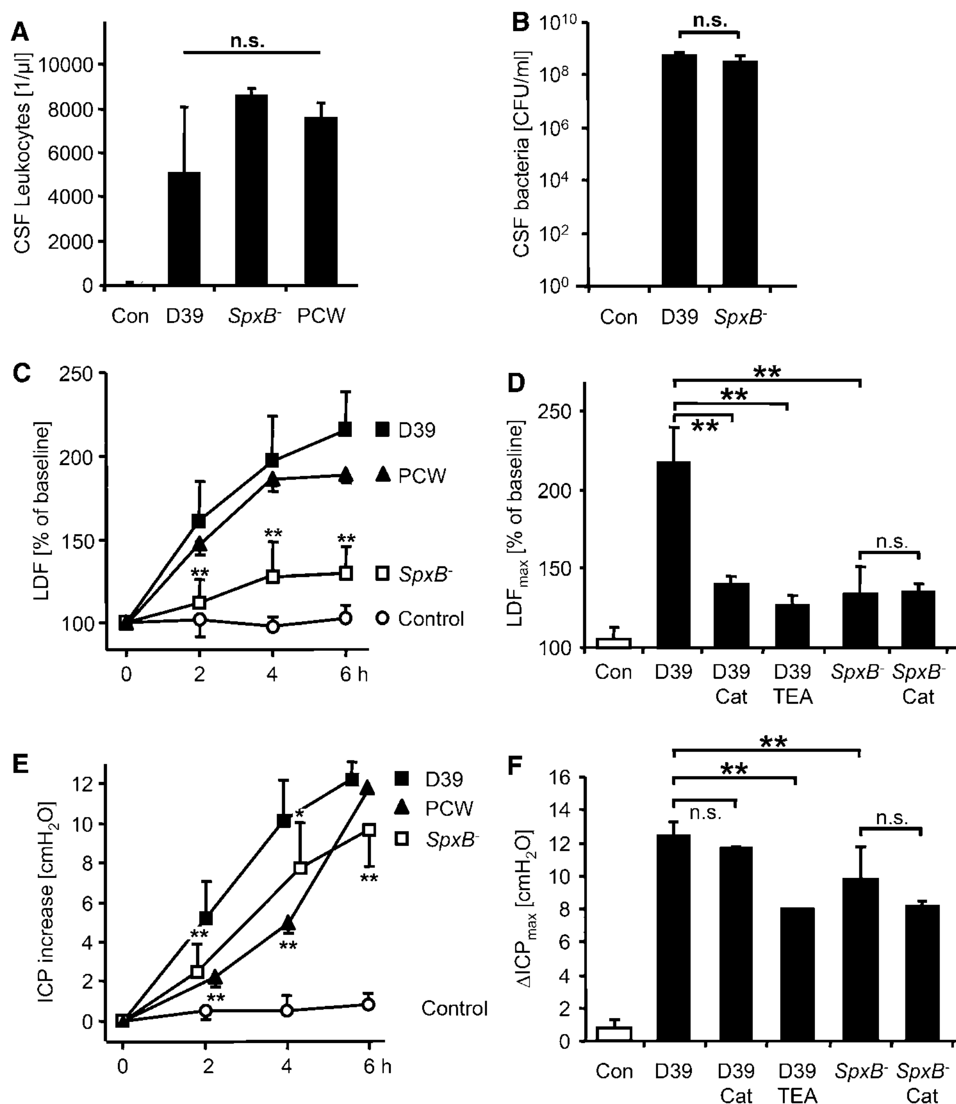

Figure 1 Impact of bacterial H_2O_2 release in early pneumococcal meningitis. (**A**) Leukocyte concentrations in the CSF 6 h after challenge are not increased in PBS-injected control rats (Con). Intrathecal infection with wild-type pneumococci (D39), H_2O_2-deficient mutants (*SpxB*$^-$), or intrathecal challenge with pneumococcal cell walls (PCW) cause a comparable influx of leukocytes into the CSF (P = n.s.; Kruskal–Wallis analysis of variance). (**B**) Plating of CSF reveals equivalent bacterial concentrations of D39 and *SpxB*$^-$ 6 h after meningitis induction. (**C**) During the experimental period of 6 h, no relevant change in rCBF as measured by Laser Doppler flow (LDF) is observed in PBS-challenged controls (open circles). Infection with wild-type D39 (closed boxes) or challenge with bacterial cell walls (PCW, closed triangles) induces a marked, gradual increase of the rCBF, which is strongly diminished with H_2O_2-deficient *SpxB*$^-$ (open boxes). $^{**}P<0.01$ versus D39-infected animals; Student's *t*-tests. (**D**) In meningitis caused by wild-type D39, treatment with catalase (Cat) or tetraethylammonium (TEA) attenuates the increase in LDF (LDF_{max}, maximum LDF increase during the 6-h experimental period). H_2O_2-deficient *SpxB*$^-$ cause significantly less hyperemia, which is not further reduced by catalase. $^{**}P<0.01$; Student's *t*-tests. (**E**) In controls (open circles), no increase of the ICP is observed during 6 h after intracisternal injection of PBS, whereas a marked, gradual increase is observed after infection with D39. The ICP increase is modestly lower with *SpxB*$^-$ than with D39. Challenge with PCW results in a similar ICP increase as D39. $^{*}P<0.05$; $^{**}P<0.01$ versus D39; Student's *t*-tests. (**F**) In meningitis induced by D39, treatment with TEA, but not catalase yields a moderate reduction of the maximum ICP increase (ΔICP_{max}). H_2O_2-deficient *SpxB*$^-$ causes modestly less intracranial hypertension than wild-type D39, which is not further reduced by catalase. $^{**}P<0.01$; Student's *t*-tests.

in LDF (maximum, 135% ± 17% of baseline; $P<0.01$ versus individual baseline and $P=0.03$ versus maximum increase in PBS-challenged controls). The LDF increase with *SpxB*$^-$ was significantly lower than with D39 ($P<0.01$ at 2, 4, and 6 h after infection). Challenge with PCW resulted in an increase of LDF to a maximum of 192% ± 5% of baseline ($P<0.01$ versus maximum increase in PBS controls and $P=0.04$ versus maximum increase in D39-infected animals).

Treatment of D39-induced meningitis with systemic catalase partially abrogated the blood flow

increase (maximum increase, 142% ± 4.0% of baseline; $P<0.01$ versus maximum increase in PBS controls and $P<0.01$ versus maximum increase with D39 alone; Figure 1E) as did systemic application of the K_{Ca} blocker TEA (maximum increase, 128% ± 6% of baseline, $P=0.02$ versus baseline and $P<0.01$ versus D39 alone). Neither intervention led to a significant change of the systemic blood pressure or of the cerebral blood flow during a 15 mins observation period before meningitis induction. Treatment of *SpxB*⁻-induced meningitis with catalase did not lead to a further reduction of the LDF increase (maximum LDF increase, 135% ± 14% of baseline; $P=0.96$ versus maximum increase with *SpxB*⁻ alone). Also, no significant effect of TEA was observed in *SpxB*⁻-induced meningitis (maximum LDF increase, 130% ± 2% of baseline, $P=0.69$ versus maximum increase with *SpxB*⁻ alone). In meningitis induced by PCW, treatment with catalase was not effective (maximum LDF increase, 181% ± 22% of baseline; $P=0.27$ versus PCW alone).

Effects on Intracranial Pressure

In control rats challenged with intrathecal PBS, no relevant change of the ICP was observed during the experimental period (maximum ICP increase, 0.8 ± 0.5 cm H_2O; $P=0.14$ versus baseline; Figure 1E). Intracisternal infection with D39 led to a gradual increase of the ICP (maximum ICP increase, 12.4 ± 0.9 cm H_2O; $P<0.01$ versus baseline and $P<0.01$ versus PBS-challenged controls). With *SpxB*⁻, we observed a slightly lower increase of the ICP (maximum increase, 9.8 ± 2.0 cm H_2O; $P<0.01$ versus baseline and $P<0.01$ versus maximum increase with D39). Starting at 60 mins, the ICP was significantly higher than in controls ($P<0.01$) but remained lower than in the D39-treated animals ($P<0.01$ at 2 and 6 h and $P=0.02$ at 4 h). After challenge with PCW, the maximum ICP increase was 11.9 ± 0.1 cm H_2O ($P=0.49$ versus maximum increase with D39). However, the increase of ICP was slower with PCW than with D39 ($P<0.01$ at 2 h and at 4 h).

Treatment of D39-infected rats with catalase did not lead to a significant reduction of the maximum ICP increase (11.7 ± 0.1 cm H_2O, $P=0.20$ versus D39 alone; Figure 1F). Conversely, TEA treatment of D39-induced meningitis resulted in a modest reduction (maximum ICP increase, 7.8 ± 0 cm H_2O; $P<0.01$ versus D39). No relevant effect of catalase was observed in rats challenged with *SpxB*⁻ (maximum ICP increase, 8.2 ± 0.3 cm H_2O, $P=0.13$ versus *SpxB*⁻ alone) or PCW (maximum ICP increase, 10.0 ± 1.2 cm H_2O, $P=0.10$ versus PCW alone).

Discussion

The major finding of this study is that in pneumococcal meningitis, a substantial proportion of early cerebral hyperemia is directly caused by the infectious pathogen through the release of bacterial H_2O_2. Compared to wild-type pneumococci, meningitis-associated increase of rCBF was markedly reduced with H_2O_2-deficient pneumococci, by scavenging of H_2O_2 using catalase, or on pharmacological blockade of K_{Ca}.

Compared to the significant vasodilator effects of D39, we found a residual blood flow increase in *SpxB*⁻-induced meningitis of approximately 30%, which seems to be independent of the bacterial H_2O_2 production. In these experiments, biologically equivalent bacterial and leukocyte concentrations in the CSF argue against decreased virulence of *SpxB*⁻ (Spellerberg *et al*, 1996). A role of host-derived H_2O_2 can be ruled out given the inability of catalase to reduce the blood flow increase in *SpxB*⁻ or PCW-induced meningitis. As a likely H_2O_2-independent mechanism, host-derived nitric oxide—a well-known, potent vasodilator released during the inflamatory response—has been shown to mediate part of the pial vasodilation in meningitis (Paul *et al*, 1997). Although catalase has previously been reported to reduce the blood flow increase in pneumococcal meningitis (Pfister *et al*, 1992), our findings provide significant new insight by identifying bacteria as the relevant source of vasoactive H_2O_2.

Compared to *SpxB*⁻, a more substantial blood flow increase was induced by PCW. Because both of these challenges are unable to release H_2O_2, this points to a different stimulatory effect on the host immune response and host-mediated vasodilation. Detection of invading pathogens by the innate immune system is affected by binding of pathogen-associated molecular patterns (PAMP) to pattern recognition receptors, most prominently of the toll-like receptor (TLR) family (Akira *et al*, 2006). In the case of pneumococci, recognition of teichoicated peptidoglycans and bacterial lipopeptides from the bacterial cell wall by the TLR2 receptor constitute the most potent signal (Weber *et al*, 2003). In patients, the clinical severity of pneumococcal meningitis correlates with the CSF concentration of major bacterial cell wall components (Schneider *et al*, 1999). It may therefore be argued that the stimulatory effect of mechanically disintegrated cell walls (PCW) on the immune system is stronger and more immediate than that of live bacteria, which do not expose pathogen-associated molecular patterns of the inner cell wall before lysis occurs. Along the same lines, our findings clearly establish a different pathophysiological basis for the equally pronounced blood flow effects of PCW and D39. Since catalase and TEA abrogated the blood flow increase caused by D39, bacterial H_2O_2 rather than the inflammatory host response (as with PCW) is the major vasodilator during early D39-induced meningitis.

Intracranial hypertension during meningitis has been explained as the result of several pathophysiological changes, that is, hyperemia, formation of

brain edema after breakdown of the blood–brain barrier, and—later in the course of the disease—increased outflow resistance of the CSF (Scheld *et al*, 1980; Tuomanen *et al*, 1989). In our experiments, the increase of ICP observed with D39 was not significantly reduced by treatment with catalase, and only a very modest reduction was achieved by TEA treatment or with H_2O_2-deficient bacteria, despite the fact that all of these strategies were effective in reducing hyperemia. It appears that H_2O_2-induced hyperemia plays only a minor role in the evolution of intracranial hypertension during the early phase of pneumococcal meningitis. Together with the strong increase of ICP after intracisternal challenge with PCW, these results support a central role for the inflammatory host response in the generation of intracranial hypertension (Tuomanen *et al*, 1989; Weber *et al*, 1995). Other bacterial factors to consider include the pneumococcal toxin pneumolysin (Braun *et al*, 2002).

In conclusion, our findings identify bacterial-derived H_2O_2 as a major vasodilator during early pneumococcal meningitis, which acts in concert with the vasodilator effects of host-derived inflammatory mediators. Bacterially induced vasodilation represents a new concept for blood flow regulation in meningitis and may be of importance in other invasive infections such as sepsis.

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2.4 **Olaf Hoffmann**, Janine Zweigner, Shannon H. Smith, Dorette Freyer, Cordula Mahrhofer, Emilie Dagand, Elaine I. Tuomanen, Joerg R. Weber. Interplay of Pneumococcal Hydrogen Peroxide and Host-Derived Nitric Oxide. Infect Immun 2006;74:5058-66.

Oxidative Schädigung von Geweben des Wirtsorganismus während Infektionen wird auf die Produktion reaktiver Sauerstoff- und Stickstoffverbindungen durch immunkompetente Zellen zurückgeführt. Daneben produzieren bestimmte Pathogene, darunter *Streptococcus pneumoniae*, zytotoxisches Wasserstoff-Peroxid (H_2O_2) sowie vermutlich auch Stickstoffradikale, wenngleich zu letzteren keine umfassenden Untersuchungen vorliegen. In dieser Arbeit wurde der relative Beitrag bakterieller und wirtsseitig produzierter Sauerstoff- und Stickstoffradikale zum zellulären Schaden während der Pneumokokken-Meningitis untersucht. Besonderes Augenmerk lag auf Peroxynitrit als einem gemeinsamen Produkt, welches aus der Reaktion von H_2O_2 und NO hervorgehen kann.
Um die Bildung von 3-Nitrotyrosin (Gewebemarker für nitrosativen Stress) in infizierten Mikrogliakulturen zu reduzieren, waren zugleich eine H_2O_2-Defizienz der Pneumokokken (Mutanten *SpxB*$^-$ oder *CarB*$^-$) als auch eine Hemmung der wirtsseitigen NO-Synthese mittels Aminoguanidin erforderlich. In einem Mausmodell der Pneumokokkenmeningitis zeigten sich reduzierte Bildung von 3-Nitrotyrosin und verminderter neuronaler Schaden im Gyrus dentatus sowohl bei Infektion mit den bakteriellen Mutanten *SpxB*$^-$ oder *CarB*$^-$, andererseits aber auch nach Infektion von iNOS-knockout Mäusen mit Wildtyp-Bakterien. In der Schlussfolgerung sprechen die Befunde dafür, dass das Zusammenspiel von prokaryotischem H_2O_2 und eukaryotischem NO wesentlich zum zellulären Schaden in der Pneumokokkenmeningitis beiträgt.

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Interplay of Pneumococcal Hydrogen Peroxide and Host-Derived Nitric Oxide

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Reactive oxygen and nitrogen species are released by immune-competent cells and contribute to cellular damage. On the other hand, certain pathogens, including *Streptococcus pneumoniae*, are known to produce hydrogen peroxide (H_2O_2), while production of nitrogen radicals by bacteria presumably occurs but has been poorly studied. We determined the relative contributions of bacterial versus host-derived oxygen and nitrogen radicals to cellular damage in pneumococcal infection. A special focus was placed on peroxynitrite as a hypothetical common product formed by the reaction of H_2O_2 and NO. In microglial cultures, reduction of the formation of 3-nitrotyrosine and cellular damage required H_2O_2-deficient (Δ*spxB* or Δ*carB*) pneumococci and inhibition of host NO synthesis with aminoguanidine. In infected C57BL/6 mice, neuronal loss and immunopositivity for nitrotyrosine in the dentate gyrus were markedly reduced with Δ*spxB* or Δ*carB* bacterial mutants and in inducible nitric oxide synthase knockout mice. We conclude that although host and bacteria both produce oxygen and nitrogen radicals, the interplay of prokaryotic H_2O_2 and eukaryotic NO is a major contributor to cellular damage in pneumococcal meningitis.

The host response to invading bacteria involves not only immune responses but also release of nonspecific and chemically highly reactive molecules. Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) can damage membrane structures and DNA of prokaryotic as well as eukaryotic cells. Considerable quantities of ROI are produced by macrophages and polymorphonuclear leukocytes in response to different bacterial stimuli. Additionally, macrophages are significant sources of RNI such as nitric oxide (NO). As a joint product of ROI and RNI, peroxynitrite is a particularly destructive molecule that exerts antimicrobial effects but also initiates host cell damage (6, 7).

Reactive oxygen species (ROS) are generated by oxidative metabolism of all aerobic cells, but particularly efficient production is achieved by the NADPH (phagocyte) oxidase of neutrophilic and eosinophilic granulocytes and mononuclear phagocytes (3). This enzyme complex produces superoxide (O_2^-) from oxygen, most of which is then converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) (22). Further reduction yields hydroxyl radicals (OH) and ultimately H_2O. In the host, NO is generated by a family of NO synthases (1). Of these enzymes, the calcium-dependent neuronal and endothelial isoforms are constitutively active and produce nanomolar amounts of NO as a strictly local neurotransmitter and modulator of vascular tone. Conversely, a calcium-independent, inducible isoform (inducible nitric oxide synthase [iNOS]) is inactive in most resting cells and is induced in cells with phagocytic capacities under pathological conditions, e.g., in infection, trauma, or ischemia (11).

While the production of ROS and RNI in the host has been extensively studied, it is less well appreciated that bacteria also produce these compounds. In particular, *Streptococcus pneumoniae* releases large amounts of H_2O_2 due to the absence of catalase to neutralize H_2O_2 produced by pyruvate oxidase (SpxB) (pyruvate plus O_2 plus P_i yields acetyl phosphate plus H_2O_2 plus CO_2) (2). The antimicrobial effect of H_2O_2 provides pneumococci with a significant advantage over other, non-H_2O_2-producing bacteria. Streptococci also produce nitrogen radicals, for instance, through the metabolism of arginine by carbamoyl-phosphate synthase (CarB) (13). Pneumococci are the leading cause of invasive infections such as community-acquired pneumonia and meningitis (15, 32). Pneumococcal meningitis is associated with 34% mortality (14) and with persistent neurological sequelae in 30 to 50% of survivors (8, 35). Apoptotic loss of neurons during meningitis may contribute to this particularly poor outcome (10, 25). While the mechanisms of host toxicity are not resolved in detail, pneumococcal H_2O_2 has been identified as one important apoptosis-inducing pneumococcal toxin (5, 9).

Reactive oxygen and reactive nitrogen compounds converge to form peroxynitrite ($ONOO^-$), an extremely toxic oxidant. Reaction of O_2^- with NO is regarded as the classical path for $ONOO^-$ formation, occurring at near-diffusion-limited rates in aqueous solution. At elevated concentrations, NO may compete with superoxide dismutase for O_2^-, leading to increased production of $ONOO^-$ (4). In addition to O_2^-, H_2O_2 may also be used for the generation of $ONOO^-$. In contrast to its normal O_2^--detoxifying function, Cu^{2+}-containing SOD-1 may become a peroxidase in the presence of elevated H_2O_2 concentrations, catalyzing the formation of O_2^- (18, 20). Moreover, SOD-1 will catalyze the formation of $ONOO^-$

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TABLE 1. Targeted genes in pneumococcal mutant strains

Gene	Function of protein	Amplified fragment size (position)	Primer 1	Primer 2
carB (SP1275)	Carbamoyl-phosphate synthase	376 bp (61–437)	5′-CACCGGAATTCGGTCAGGCTGCTGAGTTTGAC-3′	5′-TCGCGGATCCGATTCTGGAATGGGTTGTTC-3′
nox (SP1323)	NADH oxidase	304 bp (38–342)	5′-CACCGGAATTCGTACAGCATGTATCAATACC-3′	5′-GTCGCGGATCCTCGATTGGTGGCAAGATTGG-3′
nex (SP1215)	Putative transporter of H^+ and nitrite in the FNT family	346 bp (123–469)	5′-CACCGGAATTCCAGTACTGCTGCAGGTGC-3′	5′-GTCGCGGATCCCCAATTCATTTGAGCGGCC-3′
spxB (SP0642)	Pyruvate oxidase		See reference 33	See reference 33

when both H_2O_2 and NO are present (23). Notably, bacterial manganese-containing SOD (MnSOD) has been identified as a virulence factor of pneumococci in experimental pneumonia (37). $ONOO^-$ causes damage to cells in various ways, including lipid peroxidation (30), DNA breakage (31), and modification of proteins through nitration or oxidation of aromatic or thiol residues. Ultimately, $ONOO^-$-induced toxicity results in cell death (34).

The present study is based on the hypothesis that in pneumococcal infections, an interplay between eukaryotically and prokaryotically derived oxidants contributes to detrimental neurotoxicity.

MATERIALS AND METHODS

Bacterial strains and growth. D39, an encapsulated strain of *S. pneumoniae* serotype 2, was used as the wild type in all experiments. For liquid cultures, the strains were grown in standard casein plus yeast (C+Y) medium (21) or in microglial culture medium (9, 28). Mutant bacteria were grown in the presence of 1 μg/ml erythromycin to maintain the chromosomally integrated plasmid pJDC9 (see below). After centrifugation and resuspension in pyrogen-free 0.1 M phosphate-buffered saline (PBS), CFU per milliliter was determined photometrically (by absorption at 620 nm) using a standard curve. The correctness of CFU calculations was verified by plating of serial dilutions.

Recombinant DNA methods. Pneumococcal mutants were made by insertion-duplication mutagenesis (26). Table 1 shows a synopsis of targeted genes, primers used to amplify a 300- to 400-bp internal region of the gene of interest, and amplified fragments. After amplification, the resulting fragment was digested with EcoRI and BamHI and then ligated into pJDC9 (12), and the resulting plasmid was then transformed into *Escherichia coli*. Positive clones were selected on agar plates containing 1 μg/ml erythromycin. Plasmid DNA from these colonies was purified using a QIAGEN (Valencia, CA) Miniprep kit according to the manufacturer's recommendations and verified by sequencing. Insertion-duplication was accomplished by natural transformation, and transformants were selected on blood agar plates containing 1 μg/ml erythromycin. Mutations were confirmed by PCR.

Cell culture experiments. A human microglial cell line exhibiting many characteristics of primary human microglia was provided by C. A. Colton (Georgetown University, Washington, DC) and grown as described previously (9, 28). For bacterial challenge a final concentration of 1×10^7 CFU/ml was added to microglia (multiplicity of infection, 10:1) for 4 h. Aminoguanidine-HCl (3 μM; Sigma-Aldrich, Munich, Germany) was added at the beginning. At 4 h, supernatants were removed, filtered, and stored at −20°C for analysis.

Cytochemistry for fluorescent microscopy. Loss of mitochondrial membrane integrity, an early marker of apoptosis, was studied by adding MitoTracker CMX-Ros (200 nM for 30 min; Invitrogen, Karlsruhe, Germany). Other wells were incubated for 5 min with 1 μg/ml propidium iodide, a nuclear stain that is excluded by healthy cells. Cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 3 min. Following repeated changes of PBS, cells were blocked with serum, incubated with a polyclonal nitrotyrosine antibody (Upstate, Waltham, MA; 1:100) at 4°C overnight, and visualized using an Alexa 488-labeled secondary antibody (Invitrogen). For a positive control, microglia were chemically nitrosylated by incubation with 1 mM H_2O_2 and 1 mM $NaNO_2$ (Sigma) in pH 5 acetate buffer for 30 min. The specificity of this protocol was ensured by a further experiment where preincubation of the primary antibody with 10 mM nitrotyrosine completely abolished the nitrotyrosine signal in chemically nitrosylated microglia.

Flow cytometry. For quantification using a fluorescence-activated cell sorter (FACS), 2.5×10^5 to 5×10^5 cells/well were challenged with bacteria. Following incubation with MitoTracker (500 nM, 30 min), cells were fixed with 4% PFA for 15 min and permeabilized with 70% ethanol at −20°C overnight. In other experiments, we assessed apoptotic cells by exposure of phosphatidylserine on the plasma membrane. For this purpose, 0.5×10^6 to 1×10^6 cells were resuspended in 100 μl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing fluorescein-conjugated annexin V (1.0 μg; Becton Dickinson, Franklin Lakes, NJ) and incubated for 15 min at 20°C. Propidium iodide was added prior to analysis in 250 μl binding buffer at a final concentration of 1.0 μg. A total of 10,000 cells were analyzed by FACS using the excitation of a 488-nm line of an argon ion laser. Nitrotyrosine labeling was performed as described above. Fluorescein isothiocyanate-labeled annexin V fluorescence was measured at 530 ± 20 nm, while MitoTracker red 580 (final concentration, 200 nM; Molecular Probes) and propidium iodide were measured at >580 nm.

H_2O_2 production and nitrite measurements. The H_2O_2 production assay was based on a published method (29) and its adaptation (16). Nitrite was assayed by the Griess reaction (17). Cell culture supernatants or bacterial supernatants were harvested at 4 h or at late-logarithmic phase.

Murine meningitis model. All experimental designs fully complied with federal and institutional guidelines and were reviewed and authorized by the hospital research boards or state authorities as applicable. Experiments were conducted on 4- to 5-week-old mice weighing about 20 g. B6 129P2-Nos2^{tm1Lau} and B6 129PF2-J mice (The Jackson Laboratory, Bar Harbor, ME) were used in experiments with live D39 bacteria. Mutants were first confirmed to exhibit wild-type rates of growth in blood prior to comparisons in the meningitis model. Meningitis was induced with 5×10^5 CFU pneumococci by using a modification of a previously described method (19, 24). Cerebrospinal fluid (CSF) leukocytes were counted and bacterial concentrations determined as described elsewhere (24). The animals were perfused transcardially with PBS followed by 4% PFA in PBS, pH 7.4; after removal, brains were postfixed in 4% PFA for 4 h and then transferred to PBS until paraffin embedding.

Histological techniques. For the evaluation of neuronal damage in the dentate gyrus, 5-μm-thick paraffin sections were stained with standard hematoxylin and eosin as well as terminal deoxynucleotidyltransferase-mediated UTP nick end labeling (TUNEL). For quantification, the number of damaged neurons in the dentate gyrus was counted and divided by the area of the dentate gyrus on multiple sections. Planimetry for this purpose was performed using Stereo Investigator software (MicroBrightfield Europe, Magdeburg, Germany). For TUNEL labeling, sections were deparaffinized and digested with 10 μg/ml proteinase K. The TUNEL reaction was performed for 60 min at 37°C using a commercially available kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Following incorporation of digoxigenin-labeled dUTP, sites of DNA single-strand breaks were visualized using a fluorescein-conjugated anti-digoxigenin Fab fragment (1:250). Additional slides were doubly stained by the TUNEL reaction as described above, followed by incubation with antibodies directed against the neuronal antigen NeuN or by incubation integrin alpha M (CD11b) as a marker of activated microglia. In these experiments, slides were deparaffinized and subjected to microwave antigen retrieval. Following TUNEL staining and blocking in 10% normal goat serum with 0.3% Triton X-100 in PBS for 1 h, slides were incubated overnight at 4°C with an anti-NeuN antibody (Chemicon; mouse monoclonal antibody; 1:500 in blocking solution) or anti-CD11b (Chemicon; rat monoclonal antibody; 1:100 in blocking solution). Hoechst 33258 (Invitrogen; 1:10,000) was used as a nuclear counterstain. For the detection of nitrotyrosine, deparaffinized

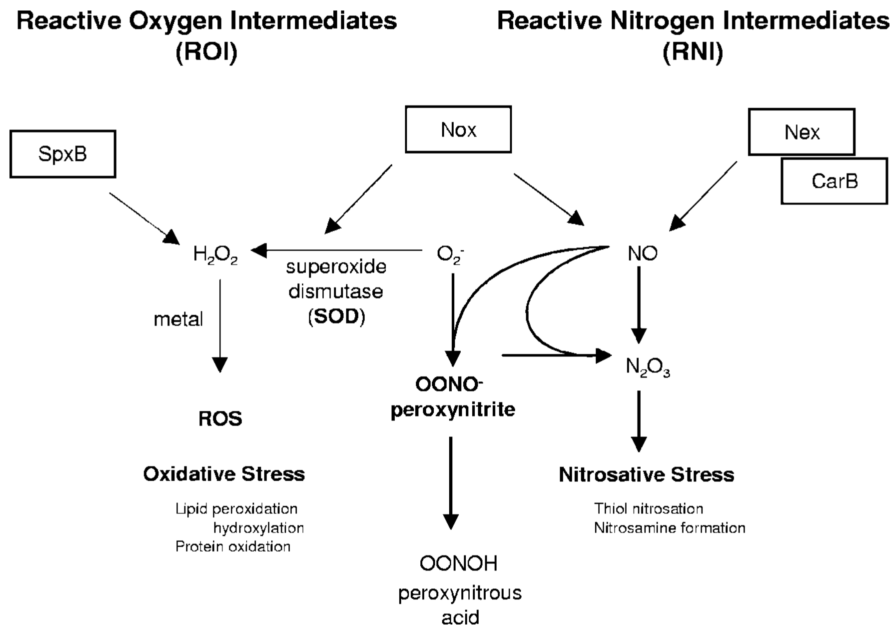

FIG. 1. Overview of the production of reactive oxygen and nitrogen species by pneumococci. Reactive oxygen species, such as H_2O_2 and O_2^-, can cause oxidative stress on their own or interact with NO to generate the potent peroxynitrite radical. NO alone can cause nitrosative stress. ROI and RNI are by-products of metabolic reactions involving the enzymes indicated (boxed). Proteins encoded by genes targeted by insertion duplication mutagenesis are boxed. For protein designations, see Table 1.

sections were blocked with serum and incubated overnight at 4°C with a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY; 1:100). The signal was visualized using an Alexa 488-conjugated secondary antibody (1:200 in blocking solution; Molecular Probes, Eugene, OR). To ensure the specificity of the signal, additional sections were incubated in blocking solution without primary antibody or with primary antibody solution competed with 10 mM 3-nitrotyrosine (Sigma).

iNOS in situ hybridization. Murine iNOS-specific antisense and sense RNA probes were synthesized from commercially available templates (Cayman Chemical, Ann Arbor, MI) using T7 and T3 RNA polymerases as appropriate (Stratagene, La Jolla, CA). The probes were labeled with digoxigenin using a commercially available kit according to the manufacturer's instructions (Roche, Mannheim, Germany). For in situ hybridization, 4-μm-thick paraffin sections were deparaffinized, rehydrated, and rinsed with Tris-buffered saline. Following incubation with 0.2 M HCl for 10 min and digestion with proteinase K (100 μg/ml in Tris-buffered saline with 2 mM $CaCl_2$) for 20 min at 37°C, the sections were acetylated with 0.5% acetic anhydride in 0.1 M Tris (pH 8.0) for 10 min. After dehydration through graded ethanol, sections were immersed in chloroform for 5 min and then rehydrated with 100% and 95% ethanol. After preincubation in a humid chamber at 60°C for 30 min, the sections were incubated with hybridization mix under glass coverslips [50% deionized formamide, 4× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 5× Denhardt's solution, 200 μg/ml salmon sperm DNA, 100 μg/ml poly(A), 25 mM sodium phosphate, 1 mM sodium pyrophosphate, and 5% dithiothreitol plus 3 ng of the RNA probe]. The sections were heated to 94°C for 4 min on a hot plate and then incubated overnight at 68°C in a humid chamber. Following repeated washing steps, sections were blocked with 10% fetal calf serum and a proprietary blocking agent (Boehringer, Ingelheim, Germany). Bound probe was then detected using an alkaline phosphatase-labeled anti-digoxigenin antibody (1:500 in blocking solution for 60 min; Boehringer) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) as the chromogenic substrate as stated by the manufacturer (Roche).

Statistical analysis. Data are presented as means ± standard deviations. Statistical analysis was performed using SigmaStat (SPSS Inc., Chicago, IL). After normal distribution and equal variance were ensured, differences between groups were evaluated using Student's *t* tests. The correlation between H_2O_2 production and viable cells was detected by Spearman's rank test.

RESULTS

Metabolic characterization of bacterial mutant strains. To study the impact of specific bacterial metabolic pathways on oxidative damage to the host during pneumococcal meningitis, we used isogenic bacterial mutants of D39 bearing functional inactivation of genes encoding key proteins of oxidative metabolism (Fig. 1; Table 1). All mutant strains exhibited growth rates similar to that of wild-type D39 in C+Y medium and in microglial tissue culture medium. Release of H_2O_2 and NO was quantified for each strain and was similar in tissue culture medium or bacterial culture medium. Whereas D39 accumulated 111 ± 61 μM H_2O_2 in the microglial tissue culture medium, reduced release of H_2O_2 was observed for the pyruvate oxidase-negative (Δ*spxB*) and carbamoyl phosphate synthase-negative (Δ*carB*) mutants (Fig. 2A), while no significant reduction was observed for bacteria lacking the formate-nitrite transporter family member (Δ*nex* mutant) or NADH oxidase (Δ*nox* mutant) (Fig. 2A). Cultivating D39 in C+Y medium revealed comparable H_2O_2 concentrations (data nor shown). The specificity of the Δ*spxB* effect was affirmed by a similar decrease in H_2O_2 accumulation in D39 in the presence of catalase. As an indication of NO release, 8.3 ± 4.4 μM nitrite was detected in the supernatant of wild-type D39 in microglial tissue culture medium (Fig. 2B). Nitrite production was significantly reduced for the Δ*carB* mutant (3.6 ± 0.7 μM), while no relevant change was found with the remaining mutants (Fig. 2B). The reduction in nitrite production was not due to H_2O_2 production, since addition of 130 μM H_2O_2 to D39 did not appreciably change nitrite accumulation (data not shown).

Effect of modulating bacterial H_2O_2 and NO on toxicity to microglia in vitro. Wild-type D39 caused pronounced cell

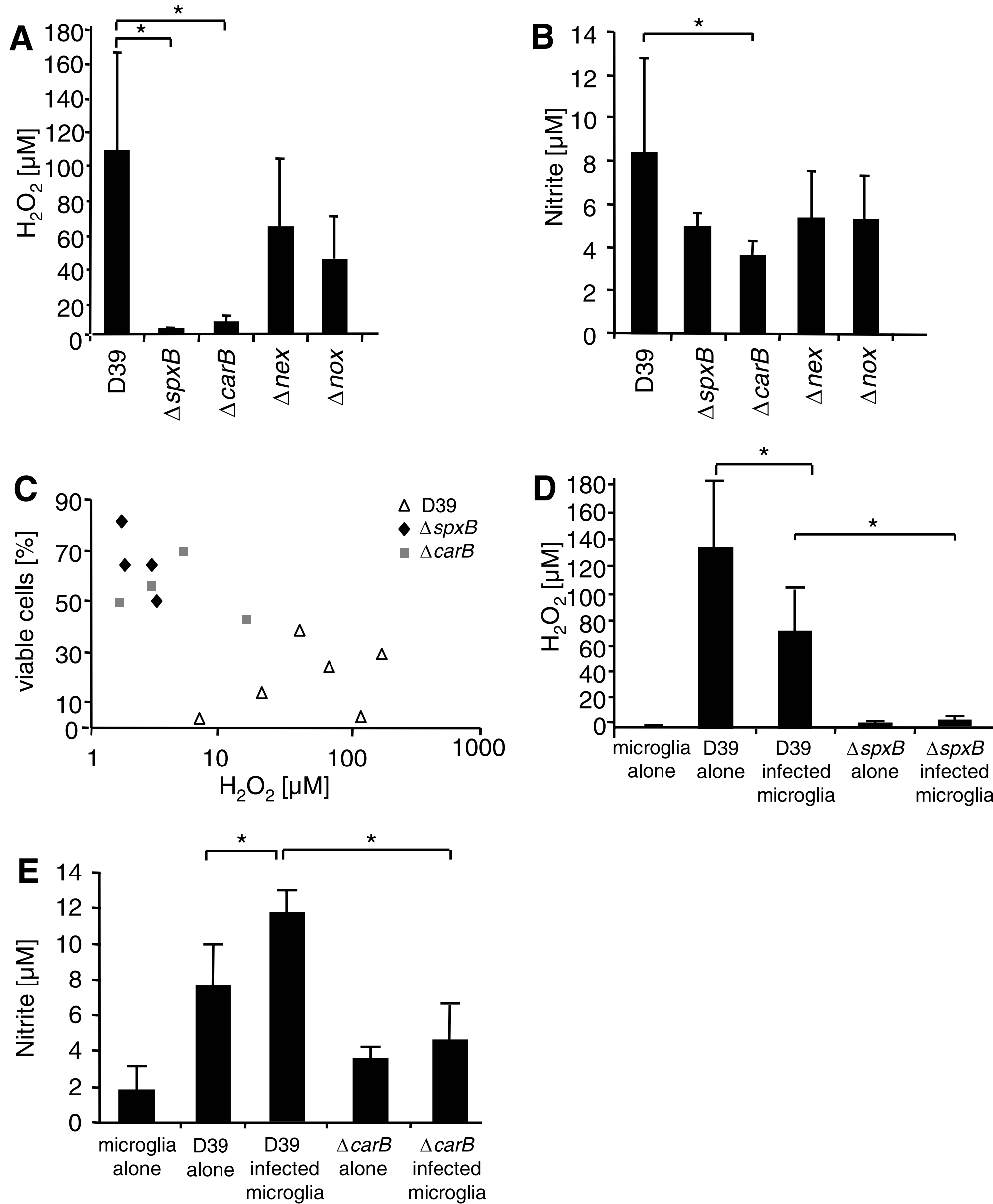

FIG. 2. Metabolic characterization and cytotoxicity of the pneumococcal mutants. (A and B) Measurement of H_2O_2 production (A) and nitrite formation (B) in the supernatant of 1×10^7/ml bacteria following incubation in microglial culture medium for 4 h at 37°C (medium alone or medium plus dead pneumococci produced no detectable H_2O_2 or nitrite). Asterisk indicates a statistically significant difference between wild-type and mutant bacteria ($P < 0.01$; $n = 4$). (C) Correlation between H_2O_2 production and viability of microglia 4 h after infection with 1×10^7 CFU/ml of D39 or the Δ*spxB* or Δ*carB* mutant (Spearman correlation coefficient [r], −0.74; $P < 0.05$). Each symbol represents an independent experiment. (D) H_2O_2 production in tissue culture medium by bacteria alone or in the presence of microglia ($n = 3$). (E) Production of nitrite by D39 bacteria in tissue culture medium in the presence and absence of microglia ($n = 3$).

death, ranging from 62 to 100% of cells (mean ± standard deviation, 80.4% ± 4.95%) (Fig. 2C). By comparison, both Δ*spxB* and Δ*carB* mutants showed significantly increased percentages of surviving cells at 4 h (38.4% ± 4.90% dead cells for the Δ*spxB* mutant [$P < 0.05$]; 47.0% ± 4.44% dead cells for the Δ*carB* mutant [$P < 0.05$]). No such protective effect was observed for the Δ*nex* (72.8% ± 10.3% dead cells) and Δ*nox* (86.5% ± 4.0% dead cells) mutants. Moreover, a positive correlation between bacterial H_2O_2 production and cell loss was demonstrable (Spearman rank order correlation, 0.74; $P <$ 0.05) (Fig. 2C). No correlation between bacterial NO production and cell damage was detected (data not shown).

Radical production in eukaryotic cell and bacterial cocultures. To address the relative contributions of prokaryotic and eukaryotic metabolism to the production of H_2O_2 and NO, both were measured in the supernatants of microglial cultures 4 h after infection with pneumococci. In comparison to bacteria alone in tissue culture medium (132 ± 56 µM), total H_2O_2 accumulation decreased when bacteria were exposed to microglia (73 ± 46 µM) ($P < 0.05$) (Fig. 2D). Elimination of the bacte-

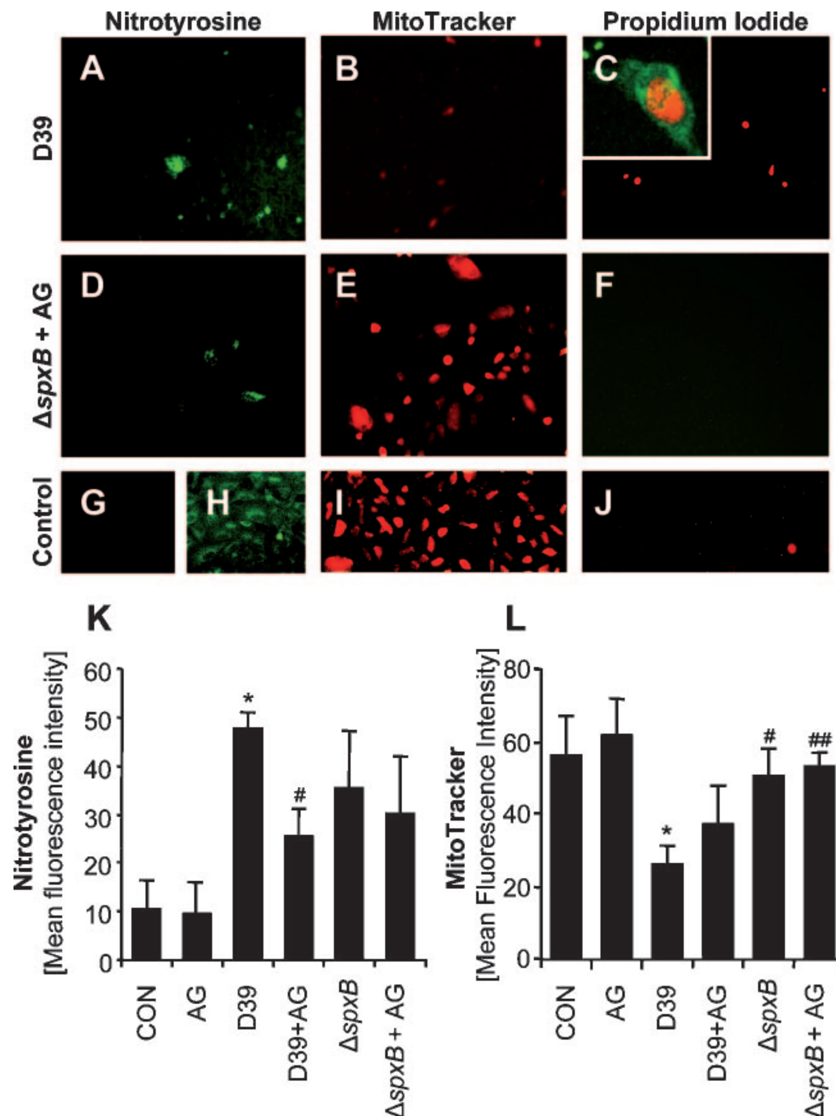

FIG. 3. Interplay of bacterial H_2O_2 and microglial NO affects nitrotyrosine formation, mitochrondrial membrane potential, and cell death. (A through J) Healthy microglia stain negative for nitrotyrosine (G), positive for mitochondrial integrity with MitoTracker (I), and negative for cell death with propidium iodide (J). Treatment of microglia with H_2O_2 and $NaNO_2$ induced positive nitrotyrosine staining (H). Infection of microglia with D39 induced the formation of nitrotyrosine (A), decreased the uptake of MitoTracker (B), and caused cell death as shown by the uptake of propidium iodide (C). The inset in panel C shows the colocalization (yellow) of propidium iodide (red) and nitrotyrosine (green). The absence of bacterial H_2O_2 (Δ*spxB*) resulted in a decrease in nitrotyrosine staining (D) and cell death (F) and restored mitochondrial function (E). (K and L) Staining was quantitated by FACS analysis comparing a control (CON) to D39 bacteria with or without the iNOS inhibitor aminoguanidine (AG). All experiments were performed at least in triplicate. Symbols indicate statistically significant ($P < 0.05$) differences between D39 and the control (*), D39 alone versus D39 plus AG (# in panel K), D39 versus the Δ*spxB* mutant (# in panel L), and D39 versus the Δ*spxB* mutant plus AG (##).

rial H_2O_2 source (Δ*spxB* mutant) nearly abolished H_2O_2 accumulation in the presence or absence of microglia (Fig. 2D), indicating bacteria, and specifically SpxB, as the primary source of this oxidant. Addition of D39 bacteria to microglia resulted in an increase in the nitrite level above that of microglia alone, an effect that was reduced for the Δ*carB* mutant (Fig. 2E). These data suggest that both the pneumococcus and microglia contribute to nitrite production and that loss of CarB function eliminates most of the NO produced by bacteria. Thus, Δ*spxB* and Δ*carB* were identified as valid tools to probe the bacterial contributions to radical-induced damage.

Interplay of bacterial H_2O_2 and microglial NO. Infection of microglial cultures with D39 led to increased nitrotyrosine immunostaining as a footprint of peroxynitrite formation (Fig. 3A and K) and to a severe loss of mitochondrial membrane and cellular integrity (Fig. 3B, L, and C). Double labeling with anti-nitrotyrosine was observed in a subset of propidium iodide-positive cells (Fig. 3C, inset). Treatment of D39-infected cells with aminoguanidine, an inhibitor of iNOS, decreased the nitrotyrosine signal (Fig. 3K) and preserved an intense MitoTracker signal (Fig. 3L). However, Δ*spxB* mutant-infected cells were able to maintain mitochondrial integrity (Fig. 3L) despite

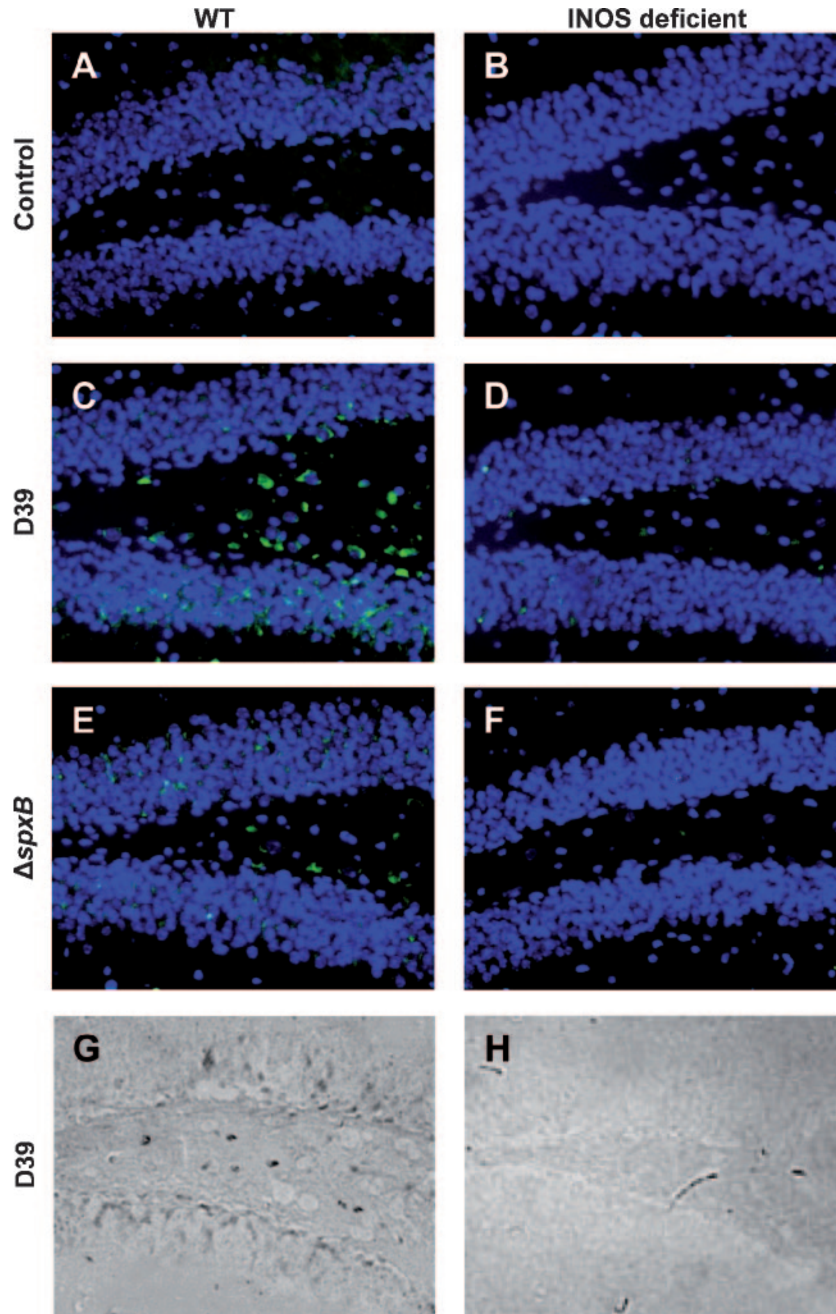

FIG. 4. Interplay of bacterial H_2O_2 and host-derived NO effects on nitrotyrosine formation in experimental meningitis. (A through F) Immunohistochemistry for nitrotyrosine in the dentate gyri of wild-type (A, C, and E) or iNOS$^{-/-}$ (B, D, and F) mice under control conditions (A and B) or 24 h after meningitis induction with D39 (C and D) or the H_2O_2-deficient (Δ*spxB*) mutant (E and F). (G and H) In situ labeling for iNOS mRNA in the dentate gyrus after induction of meningitis with D39 in wild-type (G) and iNOS$^{-/-}$ (H) mice. Data are representative of three to six animals per condition.

modest nitrotyrosine staining (Fig. 3K). The absence of bacterial H_2O_2 in conjunction with inhibition of host NO (Fig. 3E) had a similar effect as the absence of bacterial H_2O_2 alone. These results suggest that bacterial H_2O_2 is an important source of cellular damage.

Interplay of bacterial H_2O_2 and host-derived NO effects peroxynitrite formation in meningitis. All strains grew to $>10^6$ CFU/ml in the CSF, and all mice developed CSF pleocytosis and histological evidence of meningeal inflammation, which were absent in saline-treated controls.

Compared to controls, infected mice displayed strongly increased immunoreactivity for nitrotyrosine in inflammatory cells in the subarachnoid space, in the adjacent superficial cerebral cortex, and also within hippocampal structures, predominantly in the dentate gyrus and hilus region (Fig. 4A versus C). Less nitrotyrosine signal was observed in meningitis

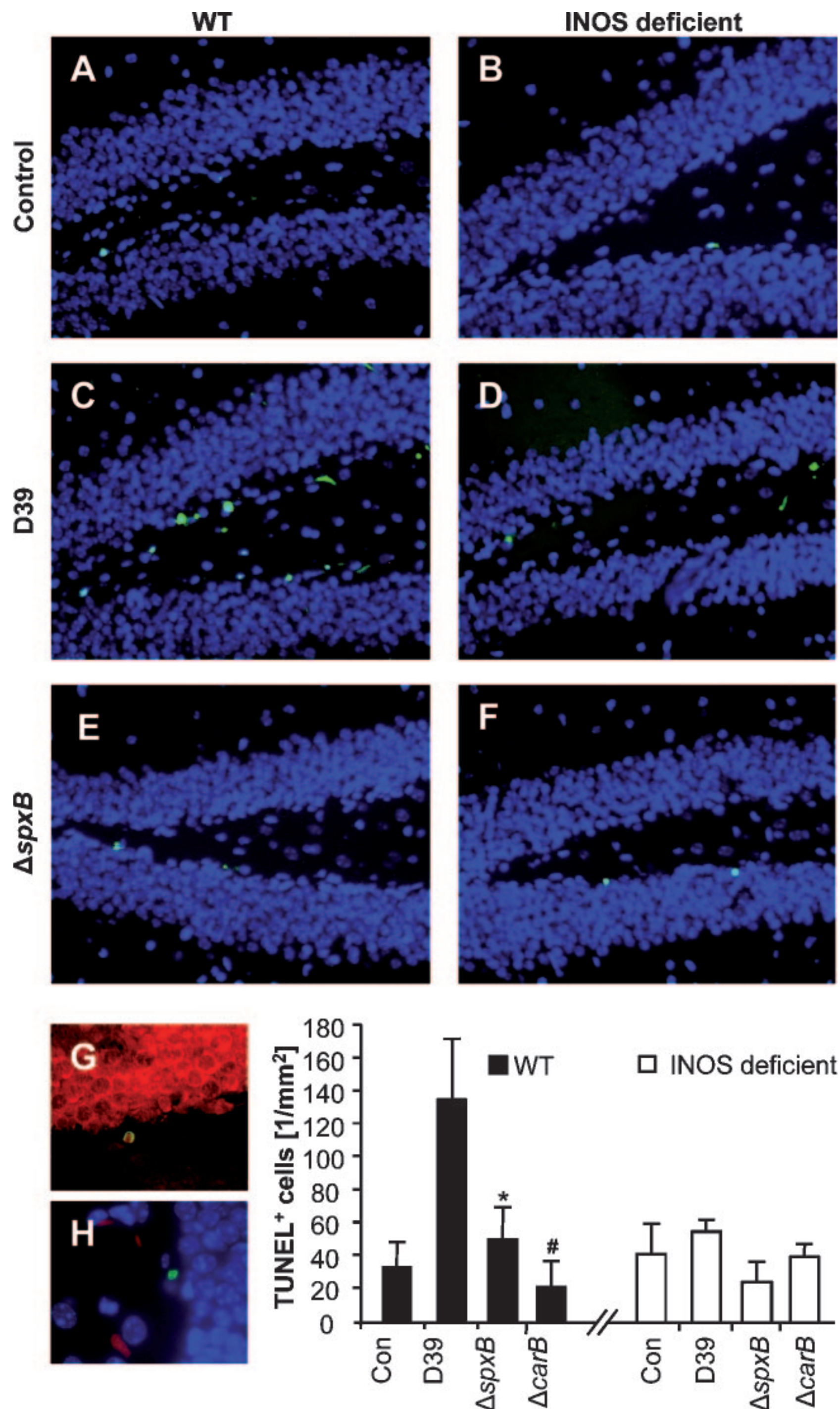

FIG. 5. Contribution of host inducible nitric oxide and bacterial H_2O_2 to neuronal loss in vivo. (A through F) Detection of apoptotic cells via the TUNEL reaction in the dentate gyrus under control conditions (A and B) or 24 h after meningitis induction with D39 (C and D) or the Δ*spxB* mutant (E and F). (G) Double labeling with the TUNEL signal (green) and an anti-NeuN antibody (red) identified most apoptotic cells as neurons. (H) No colocalization of the TUNEL signal (green) with the microglial marker CD11b (red) was observed. Hoechst 33258 was used as a nuclear counterstain (blue). (I) Quantitation of TUNEL-positive cells per square millimeter of dentate gyrus in wild-type (filled bars) and iNOS$^{-/-}$ (open bars) mice infected with D39, Δ*spxB*, or Δ*carB* bacteria. Data represent three to six animals per condition. *, $P = 0.037$ for comparison with D39 in wild-type mice; #, $P = 0.021$ for comparison with D39 in wild-type mice.

due to Δ*spxB* (Fig. 4E) or Δ*carB* (data not shown) mutants. iNOS knockout mice in all instances displayed markedly less signal than wild-type mice infected with the same bacterial strain (Fig. 4B, D, and F). In situ hybridization for iNOS mRNA was positive in the cortex, ependyma, and dentate gyrus of wild-type mice treated with D39 (Fig. 4G), but not in iNOS-deficient mice (Fig. 4H) or in saline-treated controls. These data suggest that bacterial H_2O_2 and host iNOS-derived

NO contribute to the formation of peroxynitrite in vivo but that host-derived NO production has a stronger influence.

Interplay of bacterial H_2O_2 and host-derived NO translates into neuronal damage. Meningitis induced by D39 pneumococci in wild-type mice was followed by neuronal damage. Shrunken neurons with condensed or fragmented nuclei were most abundant in the subgranular and inner granular layers of the dentate gyrus, followed by the hilar and CA3 regions. The majority of these cells were also labeled by TUNEL (Fig. 5C and I). Damage was attenuated in meningitis induced by Δ*spxB* (Fig. 5E and I) and Δ*carB* (Fig. 5I) mutants. In iNOS knockout mice, excess cell death was almost completely abolished irrespective of the bacterial strain used (Fig. 5B, D, F, and I). Double labeling with TUNEL and an anti-NeuN antibody (Fig. 5G) identified most apoptotic cells as neurons, while no colocalization of the TUNEL signal with the microglial marker CD11b was observed (Fig. 5H).

DISCUSSION

Death of hippocampal neurons in bacterial meningitis is driven in part by direct toxic effects of bacteria but also to a significant extent by specific and nonspecific immune responses. Here we report that host-derived NO and bacterium-derived H_2O_2 contribute to this damage and become most prominent in the interplay between pneumococcal oxidative and eukaryotic nitrogen intermediates, leading to the formation of peroxynitrite.

Genes for bacterial enzymes with potential influence on the generation of H_2O_2 and NO were disrupted by insertion-duplication mutagenesis. As previously reported, the disruption of pyruvate oxidase in Δ*spxB* pneumococci led to a significant reduction of H_2O_2 release (27, 33). Conversely, disruption of NADH oxidase (*nox*, an enzyme required for the complete reduction of O_2 to H_2O) had no effect on H_2O_2, suggesting that, at least under the conditions in culture, these pathways do not contribute significantly to the release of H_2O_2 by *S. pneumoniae*. NO release by *S. pneumoniae* was also detected and is a novel observation in the context of potential bacterially induced cytotoxicity. Presumably because carbamoyl phosphate is required for the synthesis of citrulline as a precursor of arginine (13), lack of carbamoyl phosphate synthase activity resulted in reduced ability of *S. pneumoniae* to release NO. Surprisingly, we also observed markedly reduced H_2O_2 release by a Δ*carB* mutant. The mechanism of this effect is unclear but does not arise from polar effects, since the gene is not in an operon.

By infecting microglial cell cultures with the mutant pneumococcal strains, we were able to demonstrate their relative contributions to radical production. A significant link between the absolute concentration of H_2O_2 and the rate of eukaryotic cell death was identified. The H_2O_2 concentration was lower in supernatants from D39-infected microglia than from bacteria cultured alone, suggesting a cellular capacity to either partially scavenge or chemically degrade bacterially derived H_2O_2. In support of the previously described role of H_2O_2 as a pneumococcal exotoxin (5, 10), microglia were not identified as a relevant source of H_2O_2 in these experiments. In contrast, eukaryotic cells were identified as the primary source of damaging NO.

H_2O_2 toxicity is generally thought to involve the generation of hydroxyl (HO) radicals by interacting with Fe^{2+} ions (Fenton's reaction), ultimately leading to peroxidation and cross-linking of cellular and mitochondrial membrane lipids. However, we hypothesized that in infected microglial cultures, bacterial H_2O_2 could interact with iNOS-derived NO to form $ONOO^-$. As a footprint of $ONOO^-$ formation, 3-nitrotyrosine was detected in D39-infected microglial cultures. Using Δ*spxB* bacteria in combination with a pharmacological inhibitor of iNOS, we found a contribution of both bacterial H_2O_2 and host NO to the formation of $ONOO^-$. In these experiments, inhibition of NO production with aminoguanidine appeared to have as strong an effect on nitrotyrosine formation as lack of bacterial pyruvate oxidase activity.

The significance of peroxynitrite as an agent of microglial toxicity was then determined by comparing the survival of cells exposed to H_2O_2 or NO individually or in combination as $ONOO^-$. Bacterial H_2O_2 had a greater impact on the loss of mitochondrial and cellular integrity than the formation of NO, and indeed, cellular damage showed no strong correlation with the detection of nitrotyrosine. Therefore under the conditions of the microglial culture system, it appears that pneumococcal H_2O_2 participates in the formation of $ONOO^-$ but that $ONOO^-$ is not a required intermediate for H_2O_2 toxicity.

To determine the influence of host NO and pneumococcal H_2O_2 on neuronal cell death in vivo, we performed experiments in C57BL/6 and $iNOS^{-/-}$ mice. Neuronal loss in the dentate gyrus has been demonstrated in human autopsy tissue after meningitis and in different experimental animals (24, 25). Our finding of increased local nitrotyrosine formation suggests a role for peroxynitrite in meningitis-induced neuronal damage in the hippocampus. Here we found additional evidence supporting an interplay of bacterial H_2O_2 and host NO, resulting in the formation of highly toxic peroxynitrite. Marked upregulation of iNOS mRNA at 24 h after meningitis induction was previously demonstrated in hippocampus-enriched brain tissue by using reverse transcription-PCR (36). As with the cell culture findings, significant neuroprotection was observed with removal of either host iNOS or pneumococcal H_2O_2. Neuronal damage was paralleled by the formation of nitrotyrosine, but apoptotic nuclei were not uniformly colocalized with the nitrotyrosine signal. From the reduction of the nitrotyrosine signal in the respective knockout experiments, bacterial H_2O_2 and host iNOS-derived NO are likely to contribute to the formation of $ONOO^-$ but do not appear to be exclusive sources. Interestingly, no nitrotyrosine signal was observed in meningitis due to the Δ*carB* mutant, pointing to a possible additional role of bacterial NO production.

We conclude that in vivo and in vitro, bacterial and eukaryotic oxidants contribute to host toxicity, that part of this toxicity is related to $ONOO^-$ formation, and that part of the nitrotyrosine signal is the consequence of an interplay between host NO and bacterial H_2O_2.

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2.5. **Olaf Hoffmann**, Josef Priller, Timour Prozorovski, Ulf Schulze Topphoff, Nevena Baeva, Jan D. Lünemann, Orhan Aktas, Cordula Mahrhofer, Sarah Stricker, Frauke Zipp, Joerg R. Weber. TRAIL limits excessive host immune responses in bacterial meningitis. J Clin Invest 2007; 117:2004-13.

Das Zytokin TRAIL (*Tumor necrosis factor-related apoptosis-inducing ligand*) hat wichtige regulatorische Funktionen im Immunsystem, unter anderem in der Abwehr von Tumorerkrankungen. In dieser Arbeit wurden entzündungshemmende Effekte von endogenem und rekombinanten TRAIL (rTRAIL) in der experimentellen bakteriellen Meningitis untersucht. Nach intrathekaler Applikation von gereinigten Pneumokokkenzellwänden kam es bei TRAIL-defizienten Mäusen im Vergleich zu Wildtypen zu einer verlängerten Inflammation, verstärkter funktioneller Beeinträchtigung und vermehrter Apoptose von Neuronen im Gyrus dentatus. Durch Injektion von rTRAIL in den Subarachnoidalraum TRAIL-defizienter Mäuse oder durch Rekonstitution der Hematopoese mit Wildtyp-Knochenmark konnten diese Effekte aufgehoben werden. Diese Befunde sprechen für eine autoregulatorische Rolle von TRAIL innerhalb der Population der infiltrierenden Leukozyten. Auch in Wildtyp-Mäusen reduzierte intrathekal gegebenes rTRAIL Inflammation and neuronale Apoptose. Im Liquor von Patienten mit bakterieller Meningitis konnte eine vermehrte intrathekale Synthese von TRAIL nachgewiesen werden. Diese Ergebnisse legen eine neuartige Rolle von TRAIL als negativer Regulator der akuten Entzündung im zentralen Nervensystem nahe. Die Fähigkeit von TRAIL, die Entzündungsantwort zu modulieren und neuronalen Zellverlust zu reduzieren, macht einen Einsatz als neuartiges entzündungshemmendes Prinzip bei invasiven Infektionen vorstellbar.

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3. DISKUSSION

3.1. Induktion, Modulation und Termination der akuten Inflammation

In der unter 2.1. dargestellten Arbeit konnte gezeigt werden, dass die alleinige Aktivierung intrathekaler Toll-like Rezeptoren vom Typ TLR2 durch bakterielles Lipopeptid zur Auslösung der wirtsseitigen Aspekte einer akuten bakteriellen Meningitis einschließlich der Induktion neuronaler Apoptose im Gyrus dentatus hinreichend ist. Dies ist in mehrfacher Hinsicht von Interesse, da Unsicherheit darüber besteht, welches der Stellenwert von TLR2 in der grampositiven Meningitis ist, welcher bakterielle Bestandteil der relevante Ligand von TLR2 ist und welche Mechanismen die Neurotoxizität bakterieller Zellwandpräparationen vermitteln. Einen wesentlichen methodischen Fortschritt bildet hierbei die Verwendung eines komplett synthetisch hergestellten TLR2-Liganden, der von Struktur und biologischer Aktivität her identisch zu natürlich vorkommenden bakteriellen Lipopeptiden ist. Zahlreiche bisherige Untersuchungen zur Erkennung von Bakterienbestandteilen durch das Immunsystems beruhten dagegen auf dem Einsatz physikalisch und chemisch aufgereinigter Bakterienpräparationen. Neben dem mehrtägigen Zeitaufwand und der Notwendigkeit, extreme Bakterienzahlen als Ausgangsmaterial heranzuzüchten, stellt insbesondere das Risiko einer Kontamination z.B. mit Lipopolysacchariden eine wesentliche Irrtumsquelle solcher Präparationen dar (Morath 2002, Weber 2003).

Ein wichtiger modulierender Einfluss auf die akute Inflammation konnte in der unter 2.2. dargestellten Arbeit für das trigeminovaskuläre System nachgewiesen werden. Kernbefund ist, dass in der Frühphase der experimentellen Meningitis nozizeptive Afferenzen die meningeale Entzündung durch Ausschüttung von proinflammatorischen Neuropeptiden verstärken. Ein Einfluß dieser Neuropeptide auf die Vasodilatation während experimenteller Meningitis sowie auf die Aktivierung von Endothelzellen und Leukozyten war bereits zuvor von anderen Autoren gezeigt worden (Pfister 1995, Zimmermann 1992, Richter 1992, Saban

1997). Die hier dargestellte Studie knüpft an eine frühere Untersuchung an, in der nach chronischer einseitiger Denervierung der Meningen eine ipsilateral reduzierte Blutflussantwort beschrieben wurde (Weber 1996). Anders als das chirurgische Verfahren konnte jetzt durch die systemische Applikation von $5HT_{1B/D/F}$-Agonisten (Triptanen) auch eine deutliche Reduktion von Liquorzellzahl, intrakraniellen Druck und Hirnödem erzielt werden. Da beide Interventionen die Freisetzung von Neuropeptiden aus perivaskulären C-Fasern des N. trigeminus in den Meningen verhindern, dürfte der geringere Effekt der chirurgischen Denervierung auf die erhaltene Innervation der nicht operierten Hemisphäre zu beziehen sein.

Aus klinischer Perspektive weisen Migräne und Meningitis-assoziierter Kopfschmerz Überschneidungen auf (Headache Classification Subcommitte 2004). Gemeinsame Eigenschaften sind eine hohe Schmerzintensität sowie das Auftreten von Photophobie, Phonophobie, Übelkeit und Erbrechen. Zudem sind $5HT_{1B/D/F}$-Agonisten (Triptane) als wirksame und gut verträgliche Substanzen zur Attackenbehandlung der Migräne bekannt (Ferrari 2001). Angesichts der hier in der experimentellen Meningitis erstmals nachgewiesenen antiinflammatorischen Wirkung von Triptanen könnte ein adjuvanter Nutzen bei Patienten mit akuter bakterieller Meningitis vermutet werden. Allerdings liegen bereits negative Erfahrungen von zwei Patienten mit vorbestehender Migräne vor, die während einer beginnenden Meningitis Triptane anwendeten (Lampl 2000). Beide Patienten sprachen hinsichtlich ihrer Kopfschmerzen nicht auf die Medikation an und zeigten später eine ausgeprägte Pleozytose im Liquor. Zur Erklärung dieser Diskrepanz kann in erster Linie der Zeitverlauf herangezogen werden. Im experimentellen Modell wurde eine hochdosierte Behandlung unmittelbar bei Meningitisinduktion und somit vor Einsetzen einer meningealen Inflammation begonnen, während die Patienten zum Zeitpunkt der Einnahme bereits erhebliche Kopfschmerzen als Ausdruck der Meningitis bemerkten (Lampl 2000). Der prophylaktische Charakter des experimentellen Ansatzes spiegelte sich auch histologisch in

den erhaltenen Neuropeptidspeichern der perivaskulären Nervenfasern wider. Entsprechend fiel im Experiment der protektive Effekt einer um 2 h verzögerten Zolmitriptan-Applikation bereits deutlich geringer aus. Ein weiterer wichtiger Unterschied liegt darin, dass zur Meningitisauslösung im Rattenmodell aufgereinigte Pneumokokkenzellwandbestandteile (PCW) eingesetzt wurden, so dass proinflammatorische und vasodilatierende Effekte (siehe 2.4.) des bakteriellen Metabolismus keine Berücksichtigung fanden.

Weiterhin konnte gezeigt werden, dass das Zytokin TRAIL die akute Inflammation in der experimentellen Pneumokokkenmeningitis durch Verkürzung der Lebensdauer von Granulozyten begrenzt und hierdurch einen neuroprotektiven Faktor in dieser Erkrankung darstellt. Die Befunde unterstützen frühere ex-vivo-Beobachtungen an menschlichen neutrophilen Granulozyten (Renshaw 2003), deren Apoptose durch Zugabe einer aktiven Form von TRAIL beschleunigt wurde. Die Autoren der älteren Studie hatten aufgrund ihrer Ergebnisse auf die Möglichkeit TRAIL-basierter Ansätze zur antiinflammatorischen Therapie hingewiesen. Der Nachweis einer verkürzten Inflammation in TRAIL-behandelten Mäusen mit experimenteller Meningitis stellt das erste erfolgreiche Beispiel einer solchen Intervention dar. Als vergleichbare Ansätze zur therapeutischen Begrenzung der Lebensdauer von Granulozyten wurden in der jüngsten Literatur die Inhibition Cyclin-dependenter Kinasen (CDKs; Rossi 2006) oder extrazellulär-signalregulierter Kinasen (ERK1/2; Sawatzky 2006) vorgeschlagen. Allerdings muss bei Blockade dieser Enzymsysteme aufgrund ihrer Ubiquität mit unerwünschten pleotropen Wirkungen wie z.B. Myelotoxizität (Song 2007) gerechnet werden. Hierin könnte ein wesentlicher Vorteil einer auf TRAIL basierenden antiinflammatorischen Therapie bestehen, da therapeutisch wirksame Dosen biologisch aktiver TRAIL-Isoformen nach aktuellem Kenntnisstand keine apoptotischen Effekte an nicht transformierten Wirtszellen haben (Ganten 2006). Auch die konstitutive Apoptose zirkulierender, nicht aktivierter Granulozyten wird offenbar nicht von TRAIL beeinflusst (Renshaw 2003). Neben der bakteriellen Meningitis stellen insbesondere Sepsis (Brown 2006)

und akute Arthritis (Eyles 2006) Erkrankungen dar, in denen eine therapeutische Begrenzung der Lebensdauer von Granulozyten wünschenswert erscheint.

3.2. Blutflußregulation

Die zerebrale Hyperämie während der Meningitis wurde bislang als Folge der wirtsseitigen Produktion vasodilatierender Faktoren im Rahmen der Entzündung gedeutet. Übereinstimmend mit dieser Ansicht zeigte sich nach Applikation von synthetischem Lipopeptid (siehe 2.1.) oder Bakterienzellwandbestandteilen (PCW; siehe 2.2) im Rattenmodell der Meningitis parallel zum Einstrom von Leukozyten eine deutliche Zunahme der Blutflußgeschwindigkeiten in der Laser-Doppler-Messung. Die Hyperämie konnte durch Blockade der wirtsseitigen Neuropeptidfreisetzung durch Triptane partiell verhindert werden. Ein neuer Aspekt in diesem Zusammenhang ist der Nachweis einer eigenständigen vasodilatierenden Wirkung von bakteriell gebildetem H_2O_2, welches in der Frühphase der experimentellen Pneumokokkenmeningitis wesentlich zur zerebralen Hyperämie beiträgt. Dieser Beitrag spiegelt sich auch in der geringeren Blutflußzunahme bei Meningitisauslösung durch synthetisches Lipopetid im Vergleich zu lebenden, H_2O_2-produzierenden Pneumokokken wider (siehe 2.1.). Der Nachweis einer direkten Modulation des Gefäßtonus durch bakterielles H_2O_2 stellt insgesamt ein Novum dar und könnte auch für hämodynamische Veränderungen bei extrazerebralen Infektionen durch Catalase-negative Streptokokken von Bedeutung sein.

3.3. Neurotoxizität

Wie eingangs dargestellt wird der Verlust von Neuronen nach gängigem Verständnis sowohl durch bakterielle Toxine als auch durch zytotoxische Produkte der Immunantwort vermittelt. In den hier vorgestellten Untersuchungen konnte im Hinblick auf wirtsseitige Faktoren die Bedeutung von TLR2 als Sensor für Bakterienbestandteile in der Pneumokokkenmeningitis

weiter aufgeklärt werden. Einerseits konnte klar gezeigt werden, dass die TLR2-abhängige Toxizität von bakteriellem Lipopeptid in der experimentellen Meningitis nicht direkt an den Neuronen selbst, sondern durch TLR2-abhängige Aktivierung immunkompetenter Zellen vermittelt wird. Außerdem wurden neue Erkenntnisse zum Stellenwert von TLR2 auch in der Meningitis durch lebende Pneumokokken gewonnen. In einer früheren Untersuchung (Koedel 2003) hatten TLR2-defiziente Mäuse im Vergleich zum Wildtyp höhere bakterielle Titer und eine mäßige Zunahme intrakranieller Komplikationen bei unveränderter Rekrutierung von Leukozyten in den Liquorraum gezeigt. Im Vergleich dazu fanden sich in der hier zu diskutierenden Arbeit 24 h nach Infektion mit lebenden Pneumokokken in beiden Genotypen identische bakterielle Titer und ein in den TLR2-defizienten Mäusen reduzierter neuronaler Schaden. Diese Befunde legen nahe, dass die Aktivierung von TLR2 einen Teil der Toxizität in der Meningitis durch lebende Pneumokokken vermittelt, wobei in der früheren Studie der protektive Effekt der TLR2-Defizienz vermutlich durch die in den TLR2-defizienten Mäuse deutlich höhere Bakteriendichte aufgehoben wurde. Ausschlaggebend kann gewesen sein, dass die Autoren der älteren Arbeit eine 20fach höhere Bakterienzahl inokulierten (Koedel 2003).

Weiterhin wurde eine neuartige Rolle von TRAIL im zentralen Nervensystem beschrieben, die fundamental von früheren Ergebnissen abweicht: Nach bisherigem Verständnis ist TRAIL in der Lage, den apoptotischen Untergang transformierter Wirtszellen auszulösen, so dass sich therapeutische Anwendungen zur Tumorbehandlung in der Entwicklung befinden (Reed 2006). Zudem induzierte die Zugabe sehr hoher TRAIL-Konzentrationen neuronalen Zelltod in explantiertem Hirngewebe von Epilepsiepatienten unter Kulturbedingungen (Nitsch 2000), und TRAIL wurde als Mediator des neuronalen Schadens in der experimentellen Autoimmunenzephalitis (EAE) beschrieben (Aktas 2005). Diese Zytotoxizität und insbesondere Neurotoxizität steht im Gegensatz zur hier beobachteten neuroprotektiven Wirkung. Grundlage dieser Diskrepanz ist offensichtlich der proapoptotische Effekt von

TRAIL auf aktivierte Granulozyten, deren wichtiger Beitrag zum Gewebsschaden während der Meningitis bereits früher (Tuomanen 1989) und erneut in der vorliegenden Studie durch Hemmung der Rekrutierung und Depletionsexperimente belegt wurde. In der zentralen Bedeutung der Granulozyten unterscheidet sich die Meningitis pathophysiologisch klar von der T-Zell-getriebenen EAE.

Für die Schädigung von Wirtszellen in vivo durch oxidativen Stress während der Meningitis wurden einerseits die wirtsseitige Produktion von Radikalen durch Entzündungszellen, andererseits direkte Toxizität u.a. von bakteriellem H_2O_2 (Braun 2002) verantwortlich gemacht. Als nicht-radikalisches Oxidans spielt H_2O_2 während Pneumokokkeninfektionen eine interessante Doppelrolle, da es sowohl von den Bakterien als auch während der Immunantwort von Phagozyten des Wirtsorganismus gebildet wird. Für sich genommen weist H_2O_2 nur mäßige Zytotoxizität auf und kann im eukaryotischen Organismus durch zelluläre und extrazelluläre Systeme (insbesondere Glutathion) leicht detoxifiziert werden. Im Kontext lokaler Inflammation können hingegen veränderte Stoffwechselwege zum Tragen kommen. So begünstigt ein niedriger pH im entzündlichen Gewebe die Bildung von Hydroxylradikalen aus H_2O_2 (Fenton-Reaktion), und bei hohen Konzentrationen von H_2O_2 kann durch Umkehr der Wirkung der Superoxid-Dismutase SOD-1 die Bildung von Superoxid-Anionen katalysiert werden (Kim 2000). In der unter 2.4. dargestellten Arbeit wurde daher erstmals die Hypothese einer indirekten Toxizität aufgestellt, bei der durch Reaktion von bakteriellem H_2O_2 mit wirtsseitigen Stoffwechselprodukten Verbindungen mit deutlich höherem Schädigungspotential entstehen können. Als ein Beispiel für diese Interaktion wurde Peroxynitrit (Szabo 2003), welches aus H_2O_2 und NO unter katalytischer Einwirkung von SOD-1 gebildet wird (McBride 1999), über sein Reaktionsprodukt 3-Nitrotyrosin nachgewiesen. Dabei konnte bestätigt werden, dass bakterielles H_2O_2 zusammen mit wirtsseitig gebildetem Stickstoffmonoxid NO eine wichtige Schädigungsursache an Wirtszellen darstellt, wobei ein Teil dieser Wirkung mit der Bildung von Peroxynitrit als

eines gemeinsamen Reaktionsprodukts einhergeht. Insbesondere bei Verursachung durch *S. pneumoniae* erscheint somit der vielfach vorgeschlagene Einsatz von Antioxidanzien zur adjuvanten Therapie der Meningitis (Nau 2002a) erwägenswert, wobei das klinisch zugelassene und nahezu nebenwirkungsfreie N-Acetylcystein als direkter Scavenger von H_2O_2 (Aruoma 1989) ein besonders sinnvoller Kandidat sein dürfte.

4. Zusammenfassung

In den hier vorgestellten Untersuchungen wurden neue Erkenntnisse zu wichtigen pathophysiologischen Aspekten der Pneumokokkenmeningitis gewonnen. Im Hinblick auf die Entstehung der Inflammationsantwort konnte gezeigt werden, dass die alleinige Erkennung von intrathekal appliziertem bakteriellem Lipopeptid durch den TLR2-Rezeptor zur Auslösung sämtlicher wirtsseitig vermittelter Aspekte der akuten bakteriellen Meningitis einschließlich neuronaler Schädigung hinreichend ist. Hinsichtlich der Blutflussregulation in der Frühphase der Erkrankung wurde ein wichtiger Beitrag des trigeminovaskulären Systems beschrieben, in dem die Stimulation afferenter C-Fasern aus den Meningen zur perivaskulären Freisetzung proinflammatorischer und vasodilatierender Neuropeptide führt. Zudem wurde ein eigenständiger vasodilatierender Effekt des bakteriellen Stoffwechselprodukts H_2O_2 erstmals beschrieben. Die additive Neurotoxizität bakterieller und wirtsseitig produzierter Oxidanzien wurde erstmals unter der Hypothese einer potenzierten Wirkung durch Bildung gemeinsamer Reaktionsprodukte untersucht, wobei Hinweise auf eine partielle Vermittlung der Toxizität durch Bildung von Peroxynitrit aus bakteriellem H_2O_2 und wirtsseitig hergestelltem NO gewonnen wurden. Schließlich wurde erstmals beschrieben, dass das Zytokin TRAIL durch Begrenzung der Lebensdauer aktivierter Granulozyten einen wichtigen und potentiell therapeutisch nutzbaren Beitrag zur Limitierung der akuten Neuroinflammation und der aus ihr resultierenden Neurotoxizität leistet.

Obwohl die Untersuchungen in in-vivo- und in-vitro-Modellen der Pneumokokkenmeningitis durchgeführt wurden, stellen die inflammationsauslösende Wirkung von bakteriellem Lipopeptid via TLR2, TLR2-vermittelte Zytotoxizität, direkte Vasodilatation durch bakterielles H_2O_2, Ineinandergreifen von bakteriellem und wirtsseitigem Stoffwechsel sowie die Termination einer granulozytär getriebenen Inflammation durch TRAIL Prinzipien von allgemeiner Bedeutung dar, die angesichts pathophysiologischer Gemeinsamkeiten etwa in

der grampositiven Sepsis von besonderem Interesse sein dürften. Im Verlauf der Arbeiten ist es somit nicht zuletzt auch gelungen, krankheitsspezifische experimentelle Verfahren zu solchen mit allgemeingültigem Modellcharakter weiterzuentwickeln.

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6. Eidesstattliche Versicherung gemäß Habilitationsordnung der Medizinischen Fakultät der Charité

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wird bzw. wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlerinnen/Wissenschaftlern und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Berlin, den 2.05.2007

Dr. med. Olaf Hoffmann