Impact of the gut microbiota on the outcome after experimental stroke

Datum der Promotion: 10.03.2017
"Knowledge is like a sphere, the greater its volume, the larger its contact with the unknown"

Blaise Pascal
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Abstract (English)

Stroke is the second-leading cause of death worldwide and tremendous efforts are being made to fully characterize the pathophysiology of this disease and to identify new therapeutic targets. Importantly, stroke is associated with a high rate of complications contributing to the worsening of outcome.

In the recent years, microbiota, particularly the commensal bacterial population of the gut, gathered attention of neuroscientists. Gut microbiota not only affects the development of the immune system and plays an important role in host metabolic processes, but also contributes to the physiological function of the central nervous system. We hypothesized that gut microbiota may impact the outcome of cerebral ischemia and tested this hypothesis in an experimental stroke model using microbiota-depleted mice after broad-spectrum antibiotic pretreatment. The broad-spectrum antibiotics, implemented in our study, are often used in clinical practice for treating post-stroke infections.

We subjected microbiota-depleted C57BL/6J mice with and without continuous antibiotic treatment, microbiota-depleted mice recolonized with conventional microbiota and conventionally colonized animals to experimental stroke (middle cerebral artery occlusion, MCAo) and sham operation. We monitored general health parameters, assessed infarct size using magnetic resonance imaging and histology, investigated intestinal samples histologically and measured main immune parameters.

We observed significantly increased mortality in microbiota-depleted MCAo mice when antibiotic treatment was stopped before operation, as compared to sham-operated animals and conventionally colonized mice. All microbiota-depleted animals developed severe colitis. We showed that continuous antibiotic treatment or recolonization with conventional microbiota before surgery prevents the development of this phenotype. We did not find any differences in infarct volumes between either of the investigated groups. In line with previous studies, MCAo animals developed immunosuppression on day 5 after experimental stroke.

In summary, this is the first report showing that complex intestinal microbiota protects the host from severe lethal complications after experimental stroke. In this study, we took the advantage of an extreme model of microbiota depletion, nevertheless our results may have direct clinical implications, what needs to be tested in the clinical...
setting. Antibiotics used in treatment of severe post-stroke pneumonia in combination with post-stroke immunosuppression may disturb commensal microbiota, as well as host immunological barriers. This in turn may have similar effects, as in the mouse model, but also systemic, proinflammatory and in consequence negative effects on the ischemic brain.
Abstract (German)

Der Schlaganfall ist die zweithäufigste Todesursache weltweit und enorme Anstrengungen werden unternommen, um die Pathophysiologie dieser Erkrankung besser zu verstehen und um neue therapeutische Ansätze zu entwickeln. Schlaganfälle sind mit einer hohen Rate von Komplikationen verbunden, die zur Verschlechterung der Prognose beitragen.


Affidavit

I, Katarzyna Winek, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Impact of the gut microbiota on the outcome after experimental stroke". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date __________________________ Signature __________________________

Detailed Declaration of Contribution

Katarzyna Winek had the following share in the following publication:

Depletion of cultivatable microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke


- idea and design of the study (together with Ulrich Dirnagl and Andreas Meisel)
- project management and coordination
- all *in vivo* procedures with support from co-authors: MCAo and sham surgery,
handling, clinical examination and extensive monitoring, recolonization, infarct volume measurement by MRI, behavioural tests [data not shown], pathological examination, samples collection and preservation
- design of FACS investigations (together with Claudia Dames and Caterina Curato)
- direct laboratory supervision of Priscilla Koduah, MSc, during her master thesis (contribution to experiment II)
- data interpretation with support from co-authors
- statistical analysis of acquired data
- preparation of figures
- writing the publication (with support from co-authors)
- coordination of the review-process and writing responses to the reviewers
(edited/complemented by Ulrich Dirnagl, Andreas Meisel, Markus Heimesaat, André Fischer, Stefan Bereswill)

Signature, date and stamp of the supervising University teacher

Prof. Ulrich Dirnagl

Signature of the doctoral candidate

_________________________
Katarzyna Winek
Excerpt of the Journal Summary List (ISI Web of Knowledge℠)

ISI Web of Knowledge℠
Journal Citation Reports℠

2014 JCR Science Edition

Journals from: Subject categories CLINICAL NEUROLOGY

Journals 1 - 20 (of 192)

Ranking is based on your journal and sort selections.

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STROKE - rank 14/192 in CLINICAL NEUROLOGY
Depletion of Cultivable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke

Katarzyna Winek, MD; Odilo Engel, DVM; Priscilla Koduah, MSc; Markus M. Heimesaat, MD; André Fischer, PhD; Stefan Bereswill, PhD; Claudia Dames, MSc; Olivia Kershaw, PhD; Achim D. Gruber, PhD; Caterina Curato, PhD; Naoki Oyama, MD; Christian Meisel, MD; Andreas Meisel, MD; Ulrich Dirnagl, MD

Background and Purpose—Antibiotics disturbing microbiota are often used in treatment of poststroke infections. A bidirectional brain–gut microbiota axis was recently suggested as a modulator of nervous system diseases. We hypothesized that gut microbiota may be an important player in the course of stroke.

Methods—We investigated the outcome of focal cerebral ischemia in C57BL/6J mice after an 8-week decontamination with quintuple broad-spectrum antibiotic cocktail. These microbiota-depleted animals were subjected to 60 minutes middle cerebral artery occlusion or sham operation. Infarct volume was measured using magnetic resonance imaging, and mice were monitored clinically throughout the whole experiment. At the end point, tissues were preserved for further analysis, comprising histology and immunologic investigations using flow cytometry.

Results—We found significantly decreased survival in the middle cerebral artery occlusion microbiota-depleted mice when the antibiotic cocktail was stopped 3 days before surgery (compared with middle cerebral artery occlusion specific pathogen-free and sham-operated microbiota-depleted mice). Moreover, all microbiota-depleted animals in which antibiotic treatment was terminated developed severe acute colitis. This phenotype was rescued by continuous antibiotic treatment or colonization with specific pathogen-free microbiota before surgery. Further, infarct volumes on day one did not differ between any of the experimental groups.

Conclusions—Conventional microbiota ensures intestinal protection in the mouse model of experimental stroke and prevents development of acute and severe colitis in microbiota-depleted mice not given antibiotic protection after cerebral ischemia. Our experiments raise the clinically important question as to whether microbial colonization or specific microbiota are crucial for stroke outcome. (Stroke. 2016;47:1354-1363. DOI: 10.1161/STROKEAHA.115.011800.)

Key Words: animal model ■ antibiotic ■ brain–gut microbiota axis ■ ischemic stroke ■ microbiota depletion

Stroke is the second leading cause of death worldwide and the most frequent cause of long-term disability in adults in developed countries. Despite progress in understanding the pathophysiology of damage of this devastating disease, all efforts to establish pharmacological brain-protective strategies based on this knowledge have been futile. This has led stroke researchers to shift focus from neuroprotection to other modifiable determinants of outcome, in particular complications. Taken together, complications, such as infections, increased intracranial pressure, and sarcopenia explain at least 20% of the overall outcome of stroke patients. Infections, particularly pneumonia, are the most common complication after stroke, contributing to increased mortality and worsening the neurological outcome. A substantial number of stroke patients are treated with antibiotics, often including combinations of broad-spectrum antimicrobial agents. Effects of this treatment on commensal microbiota have been neglected and the impact of microbiota on stroke outcome has not been investigated to date.

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Commensal microbiota, in particular that of the gut, has recently entered center stage in biomedicine due to the advances in DNA sequencing and bioinformatics. Gut microbiota not only defends the host from invading pathogens, but also stimulates angiogenesis,1 regulates fat storage2 and controls gut permeability.3 This was demonstrated by sequencing whole microbiotic genomes (microbiomes), by using germ-free (ie, abiotic) animal models and selectively colonizing with specific microorganisms. Specific microbiota is required for proper organ development, including the immune system and brain, and in many other physiological functions. Consequently, germ-free mice develop altered structures of the brain, blood–brain barrier, and immune organs,6,7 and are resistant to diet-induced obesity because of changes in metabolic profiles.4 Disturbances in the gut microbiota have been linked to several pathologies, including inflammatory bowel disease,8 obesity,9 type I diabetes mellitus,10,11 as well as neurological conditions, such as multiple sclerosis, Guillain–Barré syndrome, nociceptive pain or stress, anxiety,12 and neurodevelopmental disorders.13

Little to nothing is known, however, about the modulating impact of gut microbiota on acute central nervous system (CNS) injury in stroke or, in reverse, the impact of stroke on the composition or functional profile of microbiota. This is surprising, because there are important reasons to suspect a relevant interplay between the lesioned brain in stroke and the microbiome, particularly the gut microbiome. Stroke causes immunodepression, which renders the organism susceptible to bacterial infections.14–16 Indeed, infections after stroke have a major impact on stroke outcome. Pneumonia, for example, is the most frequent cause of death in acute stroke.1 Immunodepression after stroke leads to a breakdown of epithelial barriers,17,18 making the gut an important potential source of invading bacteria. Stroke modulates the activity of the autonomic nervous system, which contributes to immunodepression, but also affects gut motility and permeability.19,20 Further, because of their increased susceptibility to infection, many stroke patients are treated with potent combinations of antibiotics, with possibly drastic consequences for the commensal bacterial microbiota.

We speculate that gut microbiota may be an important direct or indirect modulating factor for stroke outcome by (1) causing infection, (2) modulating the immune or autonomic nervous system and thus other complications (eg, sarcopenia), or (3) exerting metabolic or humoral effects on the brain. As a first step to test this hypothesis, we choose to study the effect of extensively depleting commensal microbiota on stroke outcome in a well-characterized murine model of focal cerebral ischemia, in a clinically relevant setting, specifically by using combinations of potent antibiotics. These broad-spectrum antibiotic-treated animals were termed as microbiota-depleted, and they did not harbor any bacteria cultivatable on standard microbiological media.21 We decided to choose this previously established model to avoid confounders present in germ-free animals, such as deficiencies in the immune system or altered brain physiology, biochemistry, and anatomy.7,22,23 In our study, we found no effect of microbiota depletion on the brain lesion (infarct volume), but we did find a protective effect of gut microbiota on survival.

Materials and Methods
Detailed description of materials and methods can be found in the online-only Data Supplement.

Animals and Housing
Female C57BL/6J mice (Forschungseinrichtung für Experimentelle Medizin, FEM, Charité Berlin, Germany) harboring a conventional microbiota were transferred to autoclaved cages and treated with quintuple antibiotic cocktail consisting of ampicillin (1 g/L; Ratiopharm), vancomycin (500 mg/L; Cell Pharm), ciprofloxacin (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and metronidazole (1 g/L; Fresenius) in the drinking water available ad libitum according to the previously published protocol.21 The microbiological status of mice was controlled every week as described previously.23 Cultural and molecular methods revealed that the intestinal microbiota was virtually depleted 8 weeks after broad-spectrum antibiotic treatment.21,24

Experimental Stroke (Middle Cerebral Artery Occlusion)
Surgical procedures were conducted under microorganism-reducing conditions (sterile gown, gloves, instruments, surgical hand wash before operations, nonsterile helpers during surgery). Middle cerebral artery occlusion (MCAo; experimental focal cerebral ischemia) was performed according to the standard operating procedure from the Department of Experimental Neurology, Charité Berlin, Germany.22

Magnetic Resonance Imaging
Infarct volume was assessed using magnetic resonance imaging (Bruker 7T PharmaScan 70/16) on day one after MCAo.

Flow Cytometric Analysis of Spleens, Mesenteric Lymph Nodes, and Peyer’s Patches
Cells were phenotyped on LSR Fortessa flow cytometer with FACSDiva software (BD Biosciences, Heidelberg, Germany) using antibodies specified in the online-only Data Supplement. Data were analyzed with FlowJo software (Tree Star Inc, Ashland, OR).
Microbiological Investigation of Fecal Samples

Cultural analyses of aerobic, microaerophilic, and anaerobic bacterial species abundant in the fecal samples were performed as described previously.21

Hematoxylin and Eosin Staining of Intestinal Samples

Swiss rolls of intestinal segments were isolated during necropsy,26 immersion-fixed in 4% paraformaldehyde, and embedded in
paraffin. 5-μm-thick sections were cut, dewaxed, stained with hematoxylin and eosin following standard protocols.

Methods to Prevent Bias and Exclusion Criteria
Cages with animals were randomly assigned to experimental groups. Operations, and daily health examination were performed unblinded because of the microorganism-reducing conditions required during surgery and handling. The exclusion criteria were (1) unsuccessful stroke, based on magnetic resonance imaging investigation in the experiments I and II or histological assessment in the experiment III, and (2) death on day of the surgery (Table I in the online-only Data Supplement).

Statistics
Statistics were performed using SPSS Statistics (IBM SPSS Statistics for Macintosh, Version 20.0.; IBM Corp, Armonk, NY). Sample size calculations (G*Power 3.1.7) assumed effect size Cohen’s $d=1$ for comparison primarily between conventionally colonized MCAo animals and microbiota-depleted MCAo mice using $t$ test. For the calculation, we implemented $\alpha=0.05$ and $\beta=0.8$ for all experiments and drop-out rate of 10% because of MCAo and 5% to the sham surgery. Total sample sizes were $n=57$ in experiment I, $n=46$ in experiment II, and $n=27$ in experiment III. The original data of this study is available online: http://dx.doi.org/10.6084/m9.figshare.1476224.

Results
Extensive Depletion of Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Does Not Affect Volume of the Ischemic Brain Lesion 1 Day After Experimental Stroke
In 3 series of experiments, we used microbiota-depleted mice generated by an 8-week broad-spectrum antibiotic regimen.24 In the first experimental set, we aimed to assess long-term outcome of focal cerebral ischemia (MCAo) using this model (Figure 1A). Considering the immunomodulatory properties of antibiotics26,29 and their possible neuroprotective or neurotoxic effects,30 we stopped the antibiotic treatment in the AB(+/+) groups 72 hours before surgical intervention. Additionally, we introduced a specific pathogen-free (SPF) AB(−/−) control group treated with the quintuple antibiotic cocktail only for 48 hours up to 72 hours before operation and conventionally colonized mice without any antibiotic treatment SPF AB(+/−). To further characterize effects of the antibiotic regime and extensive depletion of commercial microbiota on the outcome of focal cerebral ischemia in the next experimental series, we additionally investigated the AB(+/+) groups, in which the treatment with antibiotic cocktail was continued up to the end of the experiments, and the groups recolonized with intestinal microbiota derived from SPF AB(−/−) littersmates. We performed microbiological investigations of fecal samples in all experimental series and did not find any cultivatable microorganisms in samples from microbiota-depleted AB(+/+) and microbiota-depleted AB(+/+) mice at the time point of surgery.

Because bacterial metabolites, products, and antigens may have contributed directly or via interactions with the immune system to the development of the ischemic lesion, we assessed the infarct volume by magnetic resonance imaging on day one after focal cerebral ischemia in the first 2 series of experiments (Figure 1A). We did not find any statistically significant differences in stroke volume 24 hours after MCAo between any of the groups under investigation (Figure 1B).
AB(+/−) MCAo group was significantly lower than that of SPF AB(−/−) mice. It was also lower than that in microbiota-depleted sham-operated animals (Figure 2A) that showed intestinal symptoms similar to those in the MCAo mice (weight loss, diarrhea, crouched position; Figure IA in the online-only Data Supplement). We were able to reproduce this finding in the second set of experiments, in which symptoms in the microbiota-depleted AB(+/−) MCAo group started 6 days after surgery (Figure 2B and Figure IC in the online-only Data Supplement). We strictly monitored all mice in the experiment, checking general well-being every 4 hours. Within ≈4 hours after onset of diarrhea, affected mice displayed symptoms resembling shock: crouched position, rough fur, lethargy, and difficulties in breathing.31 These symptoms are indicative of an acute progression of systemic sequelae, and the affected animals were euthanized in compliance with the humane end points in our experiments.

Non-Neurological Symptoms in Microbiota-Depleted Mice Undergoing Surgery Are Linked to Severe Colitis

We next examined histopathologic changes in hematoxylin and eosin–stained intestinal samples derived from microbiota-depleted and SPF animals. Remarkably, we observed acute multifocal to segmental erosive–ulcerative and necrotizing colitis in those microbiota-depleted animals, which during the experiment had displayed apparent clinical colitis symptoms, such as weight loss and diarrhea.32 Conversely, we did not find any histopathologic abnormalities in intestinal samples from SPF AB(−/−), SPF mice after short antibiotic treatment =SPF AB(+/−), or in samples from animals recolonized with conventional microbiota (Figure 3). Although during the experiments microbiota-depleted mice were kept in individually ventilated cages with autoclaved equipment and were handled under microorganism-reducing conditions (until day 5 in the experiment I because of behavioral testing planned from day 5, data not shown, and during the entire experiment II and III), we suspected possible microbial contamination and spontaneous recolonization of microbiota-depleted animals during experimental procedures. To empirically test this right from the first experiment, we divided surviving animals into 2 groups: with antibiotic treatment starting on day 16 (vancomycin 5 g/L in the drinking water ad libitum, n=5) and without antibiotic intervention (n=5). As early as one day after starting the treatment, we noted an increase in body weight in the treated group and incipient resolution of symptoms (Figure IB in the online-only Data Supplement). In some stool samples collected from microbiota-depleted mice on different days of the experiment (day 4–17), several microorganism such as Clostridium species (spp.), Bacillus spp., and Staphylococci were detected, whereas other samples were culture-negative (Table II in the online-only Data Supplement).

Figure 3. Histopathologic examination of intestinal samples. Depletion of microbiota before experimental stroke leads to the development of acute and severe colitis in the first week after cerebral ischemia. Histopathology of representative intestinal samples (hematoxylin and eosin staining; bar =100 μm) showed severe, acute erosive to ulcerative colitis present in microbiota-depleted AB(+/−) mice subjected to MCAo (A) and sham operation (B). In mice with continuous antibiotic treatment (C, microbiota-depleted AB(+/+) sham mouse) or recolonization with SPF microbiota (D, microbiota-depleted recolonized AB(+/−) MCAo mouse), colitis was prevented. MCAo indicates middle cerebral artery occlusion; and SPF, specific pathogen-free.
Continuous Antibiotic Treatment or Recolonization With Microbiota From SPF Littermates Improves the Outcome After Cerebral Ischemia

To elucidate potential pathogenic mechanisms of colitis development after stroke in microbiota-depleted AB(+/-) mice, we next investigated whether continuous preventative antibiotic treatment or restoration of commensal microbiota in microbiota-depleted mice could rescue the observed phenotype. We recolonized 2 groups of microbiota-depleted mice slated for MCAo or sham operation with intestinal microbiota from SPF AB(-/-) littermates. We observed that this intervention restored main bacterial groups of the gut microbiota. Bacteroides/Prevotella spp. and Enterococcus appeared to recolonize the intestinal niche earlier than did other bacterial families and increased total bacterial load. Furthermore, recolonized animals had lower counts of the Enterobacteriaceae and Staphylococci, albeit the differences were <2 log and thus not considered to be biologically relevant (Figure II in the online-only Data Supplement). We did not observe bacterial growth in samples from microbiota-depleted AB(+/+) or in the microbiota-depleted AB(+/-) mice: only 2 samples were positive for Staphylococci at the end point (Figure 4A–4C). When comparing survival rates among microbiota-depleted mice, we observed that antibiotic treatment during the experiment, as well as recolonization with intestinal microbiota from SPF AB(-/-) littermates protected the mice from colitis and improved survival rate. Mortality rate in the recolonized MCAo group was still higher than in the SPF AB(-/-) MCAo group; nevertheless, recolonized animals did not develop symptoms of colitis (Figures 2B and 3 and Figure IC in the online-only Data Supplement). Furthermore, in our experiments, mortality on the first day after stroke appeared to be linked primarily to the severity of ischemic damage.

Microbiota-Depleted MCAo Mice Show Systemic Immunodepression on Day 5 After Cerebral Ischemia

In a third set of experiments, we investigated whether depletion of the intestinal microbiota might influence immune parameters after focal cerebral ischemia. We assessed main immune cell populations and percentages of interferon gamma (IFNγ) and interleukin 17 (IL-17) secreting cells after ex vivo stimulation with phorbol 12-myristate...
13-acetate (PMA) and ionomycin from spleens, mesenteric lymph nodes, and Peyer’s patches. Assessment took place on day 5 after surgery in microbiota-depleted AB(+/+) MCAo, AB(+/−) MCAo, AB(+/−) sham, and AB(+/−) naïve animals. Overall, we observed that cell counts of T cells (including CD4+ and CD8+ T cells subsets), B cells, and T cell receptor γδ+ (TCRγδ+) cells in spleens from mice subjected to cerebral ischemia tended to be lower than cell counts in naïve and sham-operated groups. In the AB(+/+) MCAo group, numbers of B cells and CD8+ cytotoxic T cells, as well as percentages of IFNγ-producing CD8+, TCRγδ+, and CD4+ helper T cells were significantly lower compared with sham-operated naïve group and higher percentages of IL-17-producing CD4+ and TCRγδ+ cells than in sham-operated animals (Figures 6 and Figure IIIC in the online-only Data Supplement).

**Discussion**

We aimed at investigating the effects of microbiota depletion with broad-spectrum antibiotics on the outcome of experimental stroke. We hypothesized that uncompromised gut microbiota acts as an important modulator of stroke outcome. We applied a well-characterized murine model of experimental stroke in animals after depletion of microbiota with broad-spectrum antibiotics frequently used in intensive care medicine. Our main findings were that the absence of culturable microbiota at the time of induction of focal cerebral ischemia (1) does not affect infarct sizes 1 day later and (2) induces excessive mortality manifesting between days 5 and 7 (3) and that this mortality was prevented by continuous antibiotic treatment or by the recolonization of microbiota-depleted mice with a complex conventional intestinal microbiota from SPF littermates. This is the first demonstration that gut microbiota can affect outcome after acute CNS injury.

Stroke may interfere with normal gut microbiota–host interaction on many levels. After a brain lesion, the CNS engages in intense signaling with the immune system, resulting in a decrease of immune cell numbers and their functionality. This is termed stroke-induced immunodepression.14–16 Altered systemic immunity after CNS lesion may lead to the breakdown of mucosal barriers and to the translocation of bacteria and their products (such as bacterial cell wall constituents or toxins) to the host blood stream or lymphatic organs, further impacting the immune system and providing costimulation in deleterious immune–brain cell interaction. Furthermore, a direct impact of brain lesions on bacterial microbiota is expected through the effects of the sympathetic nervous system, as well as the vagus nerve: acute brain lesions affect the outflow of the autonomic nervous system. This may reduce gut motility and increase its permeability via the enteric nervous system and ultimately lead to bacterial translocation, infection, and sepsis. Antibiotic therapy in patients with brain lesions further disturbs the composition or even eradicates commensal bacterial communities and produces bacterial fragments, which may act as toxins and costimulants. Moreover, changes in nutrition, which might affect the gut microbiome diversity,17 are often a consequence of acute stroke, with dysphagia and unconsciousness mandating parenteral or tube feeding.

To date, only few studies have addressed the effects of brain lesions on the gut and its microbiota. In an experimental model of stroke, stress prior MCAo induced bacterial translocation and contributed to negative outcome.17 Schulte-Herbrüggen et al demonstrated in a mouse model of stroke similar to the one used in our study that 24 hours after focal cerebral ischemia, T- and B cell counts are reduced in the Peyer’s patches.18 Swidsinski et al found signs of ulcerative...
colitis and dramatic changes in gut microbiota composition in stool samples from patients with acute stroke. Hayakawa et al described a sudden decrease in commensal organisms and an increase in potentially harmful bacteria after severe insults, as is seen in cerebral vascular disease. Karlson et al reported that patients with symptomatic atherosclerosis have altered gut metagenome with enrichment in the Collinsella genus and reduced levels of β-carotene in serum.

A prediction based on these previous reports would suggest a gut–brain interaction after stroke with possible negative effects on the acute outcome post ischemia. Our findings did not provide evidence for such deleterious effects, in particular on infarct volume or recovery. Instead, delayed (beyond day one) effects on infarct volume or recovery could not be ruled out in view of the high morbidity and mortality of the microbiota-depleted animals after MCAo.

In contrast to a putative deleterious role of a depleted gut microbiota after stroke, we found that a complex conventional intestinal microbiota (or continuous antibiotic treatment) protects the host, whereas a compromised physiological resistance to colonization after completed antibiotic treatment may lead to severe illness and even death. It is well known that stroke induces a long-lasting immunosuppression with lymphopenia and altered cellular immune function, impairing antibacterial defense in a conventionally colonized host. Prass et al demonstrated previously that MCAo animals have reduced counts of T, NK, and B cell subsets in spleen and reduced cytokine excretion (eg, IFNγ and TNFα) as measured by ex vivo tests as early as 12 hours after experimental stroke. After MCAo in wild-type animals not treated with antibiotics, T and B cell counts were significantly reduced in Peyer’s patches compared with sham-operated animals, while no differences were found for natural killer cells and macrophages. Moreover, no significant changes in intraepithelial and lamina propria lymphocytes subsets were observed after cerebral ischemia compared with controls. In our experiments, MCAo animals showed reduced counts of T and B cells and a reduced percentage of IFNγ secreting lymphocytes on day 5 after stroke as compared with naïve and sham mice. This was observed mainly in spleens and to lesser extent in Peyer’s patches. Because we found no significant differences between microbiota-depleted AB(+/−) and AB(+/+) MCAo...
animals in immune cell numbers or function in secondary lymphoid organs, antibiotic treatment is unlikely to have mitigated depressed immunity in the gut. Moreover, survival rates of microbiota-depleted AB(+/−) sham-operated animals were higher compared with microbiota-depleted AB(+/−) MCAo group, suggesting that stroke-induced immunodepression—mediated systemic immune response syndrome might contribute to poor stroke outcome in the microbiota-depleted state.

Although the immunodepression observed after MCAo may promote bacterial infections, it does not provide a direct mechanistic explanation for the development of severe colitis in the absence of continuous antibiotic treatment. In a murine model of chronic psychosocial stress, which is known to lead to spontaneous colitis, Reber et al recently observed that animals rapidly developed mucosal immunosuppression and epithelial barrier defects associated with increased bacterial load in intestinal tissue shortly after stress onset. Later development of colitis was associated with hyperreactivity of mucosal immune cells in the elevated presence of bacterial antigens, both of which can be prevented by prolonged antibiotic treatment before and during chronic stress. Whether similar mechanisms are operational in our model of MCAo-associated colitis needs to be further elucidated.

Our study in a murine model of stroke in which we simulated the rather unphysiological scenario of virtually depleted microbiota, nevertheless raises some intriguing clinical questions because intestinal microbiota may contribute to stroke outcome in antibiotic-treated patients. Our findings indicate that the presence of an uncompromised complex intestinal microbiota may be critical for the outcome after cerebral ischemia. In the clinical scenario, considering pooled post-stroke bacterial infection rate of 30% in the first week after stroke onset, it is probable that many stroke patients are treated with antibiotics. Representative data of large stroke populations are missing. Nevertheless, in the recently published large PASS trial investigating the effect of preventive antibiotics in acute stroke on 3-month outcome, 20% of stroke patients in the control group were treated with antibiotics. Because ischemic brain lesion in many of these patients produced an immunodepressed state, they may be prone to recolonization by facultative or obligatory bacterial pathogens. This would further increase their risk of infection (in particular pneumonia).

We speculate that understanding gut microbiota–brain cross talk will contribute to a better understanding of the pathophysiology of acute CNS disorders and related complications and may lead to the improvement of current clinical practice or entirely new treatment strategies.

**Acknowledgments**

We thank Sabine Kolodziej, Yvonne Amoneit, Silvia Schulze, and Gernot Reifenberger for excellent technical assistance and Catherine Aubel for proofreading the article.

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**Disclosures**

None.

**References**


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Depletion of Cultivatable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke

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SUPPLEMENTAL MATERIAL

Depletion of cultivatable gut microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke

Katarzyna Winek MD, Odilo Engel DVM, Priscilla Koduah MSc, Markus M. Heimesaat MD, André Fischer PhD, Stefan Bereswill PhD, Claudia Dames MSc, Olivia Kershaw PhD, Achim D. Gruber PhD, Caterina Curato PhD, Naoki Oyama MD, Christian Meisel MD, Andreas Meisel MD, Ulrich Dirnagl MD
Detailed materials and methods:

Animals and housing
During the experiments, female C57BL/6J mice (Forschungseinrichtung für Experimentelle Medizin, FEM, Charité Berlin, Germany) after microbiota depletion by quintuple antibiotic treatment\(^1\) in the Institute of Microbiology and Hygiene, (Charité Berlin, Germany) and SPF littersmates were housed in the animal facility of the Department of Experimental Neurology (Charité Berlin, Germany).

Recolonization of microbiota-depleted mice with intestinal microbiota from SPF littersmates
Two days before recolonization the antibiotic cocktail was withdrawn and replaced by autoclaved tap water. Approximately 7 fresh fecal pellets were collected from individual SPF AB(-/-) littersmates and homogenized in 10 ml sterile phosphate buffered saline (PBS). To reconstitute microbiota-depleted mice with a complex conventional microbiota, the recolonization group was challenged with 0.3 ml of the supernatant from the fecal suspension by oral gavage on two consecutive days\(^1\). To assure proper establishment of the complex microbiota, mice were recolonized four days before inclusion in stroke experiments.

Middle cerebral artery occlusion (MCAo)
Surgical interventions were performed under pathogen-reducing conditions. Mice were anaesthetized with a combination of 1.5-2% isoflurane in 70% nitrous oxide and 30% oxygen. Throughout the operation body temperature was maintained at 36.5°C ± 0.5°C using a heating plate. After a midline ventral neck incision a silicon hardener-coated nylon filament (7019PK5Re, Doccol Corp. Redlands, California USA) was introduced into the internal carotid artery (over the common carotid artery) and inserted up to the origin of the left middle cerebral artery (MCA) occluding the origin of the vessel and causing ischemic lesion in the territory supplied by MCA. The filament was left on place for 60 minutes, followed by reperfusion. Sham operations were performed by inserting the filament to shortly occlude the MCA and withdrawn immediately.

Infarct volume assessment using magnetic resonance imaging (MRI)
Mice were anaesthetized using 1.5-2% isoflurane in a 1:2 oxygen/nitrous oxide mix. Measurement was conducted using a 98/38 mm RF Coil, with an inbuilt MR-compatible physiology temperature and a monitoring unit operating on Paravision software platform (Bruker, Karlsruhe, Germany). Physiological body temperature was maintained using a heated water jacket. Axial T2 weighted images covering the region between the olfactory bulb and the cerebellum were achieved with a Turbo RARE sequence (imaging parameters: 256×256 in plane resolution, 20 slices with a thickness of 500 µm, FOV 28 mm, TR 4200 ms, TE 36 ms, acquisition time 6 min). Infarct sizes were calculated using Analyze 5.0 software (Analyze Direct, Overland Park, KS, USA).

Microbiological investigation of fecal samples
Fecal samples were collected on day 0 before MCAo and during the experiments (as shown in Fig.1). Samples were stored overnight at +4°C if analysis took place on the next day or immediately frozen and stored at -20°C for further analysis.
Flow cytometric analysis of spleens, mesenteric lymph nodes (MLN) and Peyer's patches (PP)

Single cell suspensions from PP, spleens and MLN were prepared by forcing the tissues through a fine wire mesh. To obtain single cell suspensions from PP, the patches were initially digested 30 minutes at 37°C in RPMI containing 10% FCS (Biochrom, Berlin, Germany) and 3.5mg collagenase A (Roche, Basel, Switzerland). In spleen samples RBCs were lysed with erythrocyte lysis buffer (Qiagen, Hilden, Germany). Cells were washed, resuspended in RPMI 1640 medium containing penicillin, streptomycin, 2 mM glutamine, 10% FCS (Biochrom, Berlin, Germany) and 2x10⁶ cells were stimulated for four hours with 25ng/ml PMA and 1µg/ml ionomycin. Cytokine secretion was inhibited by addition of 5µg/ml Brefeldin A after the first hour. For cell phenotyping, the following fluorescently labeled anti–mouse monoclonal antibodies (BD Bioscience, BioLegend or eBioscience, Heidelberg, Germany) were used: CD19 (6D5), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), TCRγδ (GL3), CD11b (M1/70), CD11c (N418), IFNγ (XMG1.2) and IL-17 (TC11-18H10.1). Dead cells were excluded by using LIVE/DEAD Fixable Aqua (Invitrogen, Waltham Massachusetts, USA). The following lineage markers were combined for the analysis: T cells (CD11b-CD3+CD19), T helper cells (CD11b-CD19-CD3+CD4+), cytotoxic T cells (CD11b-CD19-CD3+CD8+), B cells (CD11b-CD3-C19+) and γδ T cells (CD11b-CD19-CD3+TCRγδ+).

Statistics for supplementary figures

Statistics were performed using SPSS Statistics (IBM SPSS Statistics for Macintosh, Version 20.0. Armonk, NY: IBM Corp.). Bacterial counts were compared between recolonization groups and SPF group using Mann-Whitney U test. For immunology data: statistical analyses comparing the groups for each lymphocyte subpopulation (within one lymphatic organ) were conducted using Kruskal-Wallis Test with Dunn's post hoc.
Supplementary figures

Supplementary Figure I (SFig.I) Weight of animals from experiment I and II
A) Body weight curve from the entire experiment I. We observed acute decrease in body weight of microbiota-depleted AB (+/-) MCAo and sham animals on day 5 and 6 parallel to the onset of diarrhea. B) Empiric vancomycin treatment improved the general state and symptoms in microbiota-depleted mice. Vancomycin (5g/l in drinking water ad libitum) was implemented in the first experiment. Mice with colitis symptoms were divided into two groups - with vancomycin treatment and without. Already one day after starting the administration of vancomycin we observed increase in the body weight in the treated group and mild resolution of the colitis symptoms. C) Body weight curve from experiment II. We observed acute decrease in body weight of microbiota-depleted AB (+/-) MCAo animals on day 6, linked with the onset of colitis symptoms.

Supplementary Figure II (SFig.II) Microbiological analyses of microbiota-depleted recolonized AB (+/-) groups and SPF AB (-/-) MCAo animals
Main microbial groups were restored by recolonization with conventional SPF microbiota. Total load=total bacterial count and Enterob=Enterobacteriaceae, Staph=coagulase-negative Staphylococci, Ecocci=Enterococci, Lacto=Lactobacilli, Bac/Prev=Bacteroides/Prevotella spp. are presented as the colony forming units (CFU) per gram feces. Samples from animals excluded from the study (exclusion on day 1) are marked with squares. Groups recolonized with conventional microbiota were separately compared with SPF AB(-/-) group using Mann-Whitney U test. Significance levels are marked *p≤0.05; **p≤0.01; ***p≤0.001
Supplementary Figure III (SFig.III) Flow cytometric analysis of immune cell populations from Peyer's Patches, spleen and mesenteric lymph nodes (MLN) on day 5 after cerebral ischemia

Microbiota-depleted MCAo AB(+/-) n=8 (n=7 for Peyer's patches), microbiota-depleted MCAo AB(+/+) n=7, microbiota-depleted sham AB(+/-) n=6, microbiota-depleted naïve AB(+-/-) n=6. Statistical analyses for each lymphocyte subpopulation comparing the groups within one lymphatic organ were conducted using Kruskal-Wallis Test with Dunn's post hoc. Statistically significant differences were found between microbiota-depleted AB(+/+) MCAo and microbiota-depleted AB(+-/-) sham group in the percentage of cytokine producing cells:
A) CD4+ cells (IFNγ+ in spleen), B and C) TCR γδ cells (IFNγ in spleen and PP and IL17+ in MLN). Significance levels are marked as follows: Peyer's patches (PP) $p\leq0.05; $$p\leq0.01; $$$p\leq0.001; spleen *p\leq0.05; **p\leq0.01; ***p\leq0.001; mesenteric lymph nodes (MLN) #p\leq0.05; ##p\leq0.01; ###p\leq0.001.

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**Supplementary Table I (STab.I) Animals excluded from the study**
All animals excluded from the analyses with respective group and fulfilled exclusion criteria are reported in the table above.

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Depletion of gut microbiota and stroke outcome - supplement

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<td>microbiota-depleted AB(+/-) MCAo</td>
<td>d17</td>
<td>Clostridium sordelli; Clostridium perfringens</td>
</tr>
</tbody>
</table>

CFU = colony forming units, CNS = coagulase-negative staphylococci

**Supplementary Table II (STab.II)** Microbiological analysis of the stool samples from the first experimental series

In the first experimental series several recolonizing strains were found in stool samples from microbiota-depleted mice. Stool samples were collected on different time-points of the experiment (day) and stored overnight at 4°C when analysis took place next day or frozen by -20°C for further analysis. Identification of bacterial species was performed based on the assessment of growth on selective microbiological media.
References
Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.
Complete list of publications

**Original contributions:**
Depletion of cultivatable microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke.
**IF:** 5.761

Cholinergic pathway suppresses pulmonary innate immunity facilitating pneumonia after stroke.
**IF:** 5.761

Blocking stroke-induced immunodeficiency increases CNS antigen-specific autoreactivity but does not worsen functional outcome after experimental stroke.
**IF:** 6.344

**Review/Opinion articles:**
Gut microbiota as therapeutic target in CNS diseases – implications for stroke
**IF:** 5.054

Gut microbiota impact on stroke outcome: Fad or fact?
**IF:** 5.407

Impact factor according to Journal Summary List (ISI Web of KnowledgeSM) 2014

**Manuscripts in preparation:**
Exploratory investigation of intestinal function and bacterial translocation after focal cerebral ischemia in the mouse
*these authors contributed equally

Delayed autoreactivity and chronic inflammation are mediated by recruitment and activation of CD4 T cells and B cells in ischemic stroke.
Zhang T, **Winek K**, Dames C, Andrzejak E, Engel O, Dirnagl U, Meisel C, Meisel A.

Experimental stroke is accompanied by reduction in number of ciliated tracheal epithelial cells and diminished particle transport speed.

Die Bedeutung des intestinalen Mikrobioms beim ischämischen Schlaganfall
**Winek K, Dirnagl U, Meisel A.** (under revision in Aktuelle Neurologie)
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