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Central Nervous System and the Colonic Bioreactor: Analysis of Colonic Microbiota in Patients with Stroke Unravels Unknown Mechanisms of the Host Defense after Brain Injury

Alexander Swidsinski, Vera Loening-Baucke, Monika Krüger[1], Steffen Kirsch[2]

Humboldt University, Charité Hospital, Campus Charité Mitte, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms and Department of Medicine, Section of Gastroenterology, Charité Universitätsmedizin Berlin, Berlin, Universität Leipzig, Veterinärmedizinische Fakultät, Institut für Bakteriologie und Mykologie[1], Leipzig, Asklepios Fachklinikum Teupitz[2], Teupitz, Germany

Background/Aims: Stroke is accompanied by septic complications due to quickly changing polymicrobials of unclear origin. This study was aimed to find the source of stroke-associated-infections. **Methods:** We investigated the biostructure of the colonic microbiota in patients hospitalized in two stroke units using fluorescence in situ hybridization in order to find the source of stroke-associated-infections. Non-stroke subjects and animals were used as controls. **Results:** Typical for stroke was a leukocyte migration into the mucus between day 1-3, in numbers that are otherwise characteristic for active ulcerative colitis (CAI ≥6); subsequent abrupt "decontamination" of the main fermentative *Roseburia, Bacteroides* and *Faecalibacterium prausnitzii* groups and disappearance of leukocytes in the stool; arrest of bacterial fermentation between day 3 to 7 in extents exceeding the effects of any presently know antibiotics. Then resetting in which *Enterobacteriaceae, Bifidobacteriaceae* and *Clostridium difficile* temporarily outnumber *Bacteroides, Roseburia* and *Faecalibacterium prausnitzii*, and after that decline with normalization of these bacteria to initial values. **Conclusions:** The colon is a bioreactor containing many potential pathogens. The mucus barrier shields the host from bacteria. The events following stroke stress the pivotal role of the brain in maintaining this shield and indicate an existence of emergency brakes that temporary terminate the biofermentation. **(Intest Res 2012;10:332-342)**

Key Words: Microbiota; Stroke; Colitis; *Clostridium difficile*; *Escherichia coli*

INTRODUCTION

Stroke is an explosion of polymorbidity, accompanied by life threatening septic complications.[1,2] The origin of the quickly changing polymicrobial infectious events is the endogenous flora from the skin, oro-pharynx, uro-genital region and colon. The colon is the largest reservoir for bacteria. The colon is physiologically seen a bioreactor. Here, bacteria ferment waste products of digestion, recycle water and electrolytes, and win additional energy via short fatty acids and lactate. Bacterial growth is actively promoted, leading to concentrations of 10^{12} bacteria per mL of stool. The optimal growth conditions attract, besides fermentative strains, all microorganisms that are able to exist in this environment, leading to an unprecedented diversity of more than 5,000 species in each healthy colon.[3] Most species are probably harmless wanderers brought into the colon by accident,

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Correspondence to Alexander Swidsinski, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Charité Universitätsmedizin Berlin, Campus Mitte, 10098 Berlin, Germany. Tel: +49-30-450-514-003, Fax: +49-30-450-514-933, E-mail: alexander.swidsinski@charite.de
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some are important to fermentation, many however, are clear infectious agents such as *Clostridium perfringens* that causes gas gangrene, enterococci that cause endocarditis, *Eschericia coli* that cause sepsis and *Bacteroides*, which are involved in abscesses. Potential risks from these and many other pathogens that are highly concentrated in the colon are minimized by a mucus barrier that completely separates the microbial compartment from the colonic wall in the healthy person. Water absorption makes the mucus layer, which contacts the columnar epithelium, increasingly viscous and completely impenetrable to bacteria.

The maintenance of the mucus barrier is a joint effort of different systems including peristalsis, mucus production, efflux, dehydration, regulation of electrolytes absorption and humoral and immune responses. All of them are cerebrally controlled and critically impaired in stroke. While an abundant literature exists about neurological and vascular pathology that accompanies stroke, little is known about the involvement of indigenous microbiota.

Colonic microbiota can be investigated in terms of composition and structure. Methods, such as polymerase chain reaction (cloning/sequencing) or culture, identify isolated bacteria or bacterial sequences within a mix of individual bacterial cells without their reference to each other or spatial distribution. The rich diversity of colonic microbiota, high inter-individual variance, and heterogeneity of bacterial distribution in the stool, make a comprehensive investigation of microbial composition to an elaborate and costly matter with low relevance. Fluorescence *in situ* hybridization (FISH) captures the structural composition of intestinal microbiota in relation to colonic anatomy and function. We have previously developed the method of structure functional analysis based on FISH of punched-out stool cylinders. The differences in disease specific responses of microbiota in mucus, germinal compartment and working compartment of the colonic bioreactor were used for diagnosis and evaluation.[4,5]

While analyzing randomly selected stool samples from patients admitted to the stroke unit, we found in some of these samples leukocytes in numbers that are otherwise characteristic for active UC. A coincidence of stroke and colitis is thus far unknown. Therefore, to better understand the interactions between the host, colonic microbiota and post-stroke infections, we extended our studies and investigated stool from patients admitted to two stroke units and used data from healthy persons, patients with IBS, patients with UC and patients from cardiac emergency units as controls. In addition, we investigated stools and intestines from animals with massive brain damage from slaughter facilities.

METHODS

1. Patients

Intensive care patients:

1) Patients with stroke or transient ischemic attack (TIA, clinical diagnosis confirmed by CT) admitted to the stroke units of the Charité Hospital in Berlin and the Asklepios Fachkliniken in Teupitz (stroke: 46 patients, mean age 68±14 years, 314 samples; TIA: 35 patients, mean age 68±23 years, 110 samples).

2) Non-stroke controls admitted to the intensive care units for myocardial infarction or severe episode of hypertension at the Charité and Bad Saarow Hospitals (29 patients, mean age 59±18 years, 55 samples).

Nonhospitalized controls: Ambulatory controls were randomly selected from the gastroenterological outpatient clinic of the Charité Hospital and included:

1) Twenty patients with active UC (CAI ≥6, mean age 44±10 years)

2) Non-IBD controls: 20 patients with IBS (mean age 48±13 years) and 20 healthy controls (students, laboratory personal and relatives; mean age 34±7 years).

Patients receiving antibiotics two month prior to sample collection were excluded from all groups.

2. Collection of Stool Samples

The collection of punched-out fecal cylinders, fixation, embedding, cutting and mounting on glass slides were described previously.[4,5]

Stroke unit: Stool samples from disabled patients in the stroke and cardiac units were collected by medical staff immediately after defecation by inserting pieces of pre-cut drinking straws into the stool. In 10 of the stroke patients, the sampling was performed from all available stools during their hospital stay or when readmitted resulting in 154 samples.

In the other 36 stroke patients, the samples were collected randomly at different days post stroke resulting in one to 4 samples per patient and together 160 stool samples.

The stool samples from the outpatients and healthy controls were collected weekly by the patients themselves.[4] The first 3 delivered stool cylinders of multiple samples available from outpatients and healthy controls were used for evaluation resulting in 60 stool samples for each non-IBD control group.

Acute brain damage in animals: Since all modern slaughter facilities induce brain damage to anesthetized animals, we used the animals of these facilities to obtain filled colon segments at different time points. Slaughter of animals was processed according to the German state animal welfare.

Rabbits: Because of their small body size, we investigated short term changes in 9 rabbits stunned by blow to the neck. After exsanguination, the viscera were removed prior to be-

heading and skinning. Sections of colon were obtained within 5, 30 and 60 minutes after the blow. For each time point, 3 animals were sacrificed. The slaughtered animals were then processes as usual.

Piglets: Piglets were stunned by a bolt pistol. The bolt penetrates the skull, enters the cranium and destroys brain matter. The stunned animals are holstered onto an overhead rail and exsanguinated. Evisceration, sampling and fixation were performed within 30 to 90 minutes. Four piglets each after 30 and 90 minutes were investigated.

3. FISH

All tissue materials from animals were fixated in Carnoy solution and then embedded into paraffin using standard techniques. Sections of 4 μm thickness were placed on SuperFrost Plus slides (R. Langenbrinck, Emmendingen, Germany).

Microscopy was performed using a Nikon e600 fluorescence microscope (Nikon, Tokyo, Japan). The images were photo documented using a Nikon DXM 1200F color camera and software (Nikon). Hybridizations were performed in multicolor FISH according to previously described protocols for evaluation of tissue specimens and the criteria for identification of bacteria.[4,6]

4. Leukocytes in the Mucus

The sections from the fecal cylinder were at least 3 mm thick. The leukocytes were enumerated in three non-overlapping microscopic fields of the same sample with highest concentrations of DAPI positive nuclei at magnification of ×100. A raster of 1,000×1,000 μm was used to count the numbers. Using approximation that at least one third of each microscopic field with leukocytes was occupied by mucus and rounding a thickness of sections from 4 μm to 5 μm (200 possible batches in 1 mm) the sum of leukocytes within 3 fields multiplied with 200 will correspond to the number of leukocytes in 1 μL. The number of leukocytes in whole section was multiplied with the number of all non-overlapping fields ×200 at magnification of ×100.

5. Bacterial Concentrations in Stool

Bacteria were counted within a 100 μm^2 area of the microscopic field (×1,000). Bacteria with uneven distribution or overall low concentrations were enumerated within larger areas of 100×100 μm^2 or within all microscopic fields. The conversion of the numbers within a microscopic field to concentrations of bacteria per mL was based on the calculation that a 10 μL sample with a cell concentration of 10^7 cells per mL has 40 cells per average microscopic field at a magnification of 1,000.[7] A preserved fecal cylinder is necessary for the quantitative evaluation of mucus and the gradient in distribution of bacteria. The fixation of stool to a cylinder is not possible if the stool is watery. Since the changes of microbiota normally need days or weeks even in such diseases as active Crohn's disease and UC, the collection of intact stool cylinder samples is usually not difficult. In stroke patients however, we were confronted with massive changes in concentrations of microbiota, which could occur even within hours. To avoid dispensing of stool samples of which only incomplete cylinders were won or only sediments of stools were available; we restricted the evaluation in the present study to assessment of leukocyte concentrations and to the dynamics of bacterial concentrations within the biofermentative compartment. Complete disappearance of bacteria from the stool sample, implied its disappearance from all compartments.

6. FISH Probes

Oligonucleotide probes were synthesized with a fluorescein isothiocyanate, Cy3- or Cy5- reactive fluorescent dye at the 5´end (MWG Biotech, Ebersberg, Germany).

We selected 12 group-specific FISH probes, and the universal for all bacteria Eub 338 probe for our study, including: **Erec482** (*Clostridium* group XIVa/*Roseburia* group),[8] **Bac303** (*Bacteroides*/*Prevotella* group),[9] **Fprau** (*Faecalibacterium prausnitzii* group),[10] **Ebac1790** (*Enterobacteriaceae*),[11] **Bif164** (*Bifidobacteriaceae*),[12] **Chis150** (*Clostridium histoliticum*),[8] **Clit135** (*Clostridium difficile*, *Clostridium lituseburense* group),[8] **Veil223** (*Veilonella*),[13] **Ecyl387** (*Eubacterium cylindroides*),[13] **Ehal1469** (*Eubacterium hallii*),[13] **Ato291** (*Atopobium*)[14] and **Lab158** (*Lactobacillus*).[15]

7. Statistics

All statistical analyses were performed using the statistical software package SPSS version 15.0 (SPSS Inc., Chicago, IL, USA), with $P<0.05$ considered significant.

The Ethics Committee of the Charité Hospital approved this study, and Ethics Committee of the Regierungspräsidium Leipzig: TVV-Nr. 2/07 approved the animal study.

RESULTS

1. Migration of Leukocytes into the Mucus Post Stroke

One to 7 leukocytes were found in 4/60 samples from healthy controls and in 3/60 samples from IBS patient. Leukocytes were found in concentrations of mean/max 17±60/460×10^5/μL bacteria/ml in all patients (58/60 samples) with active UC (CAI ≥6). High concentrations (mean/max 7±12/48×10^5/μL) of leukocytes were also observed in stool samples from 33 of the 46 stroke patients at days 1 to 3 post stroke (Table 1).

Leukocytes were present in three consecutive weekly stool samples in 18 of the 20 patients with UC. This constancy in

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Table 1. Occurrence and Concentrations of Leukocytes in Stools of Different Patient Groups

	Out patients			Intensive care patients						*P*-value
	Healthy (n=20)	IBS (n=20)	UC/CAI≥6 (n=20)	Heart attack, all patients (n=29)	TIA, all patients (n=35)	Stroke patients (n=46)				
	A	B	C	D	E	F	G	H		
						Stroke Day 1-3	Stroke Day 7-8	Stroke Other days		
Occurrence in at least one of the samples of patient/all samples	4/20 20%	3/20 15%	20/20 100%	4/29 14%	9/35 26%	33/46 72%	4/40 10%	6/36 17%		
Patients with occurrence in three consecutive samples	0	0	90% 58/60	0	0	0	0	0		
Mean concentration/max concentration ($\times 10^5/\mu L$)	0.0006±0.0025/ 0.014	0.0004±0.0019 0.012	**17±60/460**	0.01±0.06/ 0.3	0.4±1.6 10	**7±12** **48**	0.8±0.4 20	0.04±0.06 12	C/F ns; C and F/all other *P*<0.001	

CAI, colitis activity index; TIA, transient ischemic attack; ns, not significant.

leukocyte presence was observed in none of the other investigated groups (Table 1).

Leukocytes in healthy controls and IBS patients, if at all, were found only in one of the three consecutive weekly stool samples. Similarly, the leukocytes in stool of patients with stroke and TIA were observed mainly during days 1 to 3 post stroke (72% or 33 of 46 patients). Leukocytes were found in samples of single stroke patients at days 7 to 8 (4/40) and between days 14 to 18 (5/36 patients; Table 1). Most of the stool samples between days 1-3 and partially 14-18 were free of leukocytes. The change from high concentrations of leukocytes to no leukocytes in stool of the same patient was abrupt. In patients with multiple defecations a day, the findings could switch from zero to extremely high leukocyte counts and back within the same day. The minimum of time between positive and negative for leukocyte in stools was 6 hours.

In patients with UC the leukocytes were condensed to a dense layer covering the stool surface or incorporated within stool masses (Fig. 1).

Typically no space was seen between leukocytes. The appearance of leukocytes in stroke patients was different. The leukocytes were loosely spread in thick mucus covering stool in "onion-like" manner (Fig. 1B). The appearance of leukocytes in stool of TIA patients was similar to that observed in stroke patients, in them, the leukocytes were mainly observed from days 1 to 3 post-stroke (7/35) and disappeared quickly thereafter, however the mean concentrations of leukocytes were significantly lower in TIA than stroke patients (P<0.0.001). The differences in the mean numbers of leukocytes in patients with UC and stroke day 1-3 and all other intensive care or ambulant control patient groups (Table 1) were highly significant (P<0.001).

Colonic microbiota post stroke: The concentrations of the habitual bacterial groups, *Roseburia, Bacteroides* and *Faecalibacterium prausnitzii* were significantly reduced in patients with IBS as compared to healthy controls (P<0.001), while the concentrations of occasional bacterial groups, *Bifidobacteriaceae, Enterobacteriacae* and *C. difficile* were increased (P<0.001). The results in the patients admitted to the cardiac emergency unit took an intermediate position between healthy controls and IBS patients (Table 2). The trend in reduction of habitual bacteria and increase in occasional bacteria was much more pronounced in patients with stroke. The mean concentrations of habitual bacteria such as *Roseburia, Bacteroides* and *Faecalibacterium prausnitzii* in stools of stroke patients were already significantly reduced in the first 2 days after admission as compared to healthy controls and IBS patients (P<0.01-0.001), while concentrations of *Bifidobacteriaceae* and *Enterobacteriaceae* were increased. The mean concentrations of *C. difficile* were in the first three days higher than in healthy controls and patients from the cardiac emergency unit (P<0.001), but not statistically significant compared to IBS patients. The concentrations of bacteria from the first days after the stroke depended on the pres-

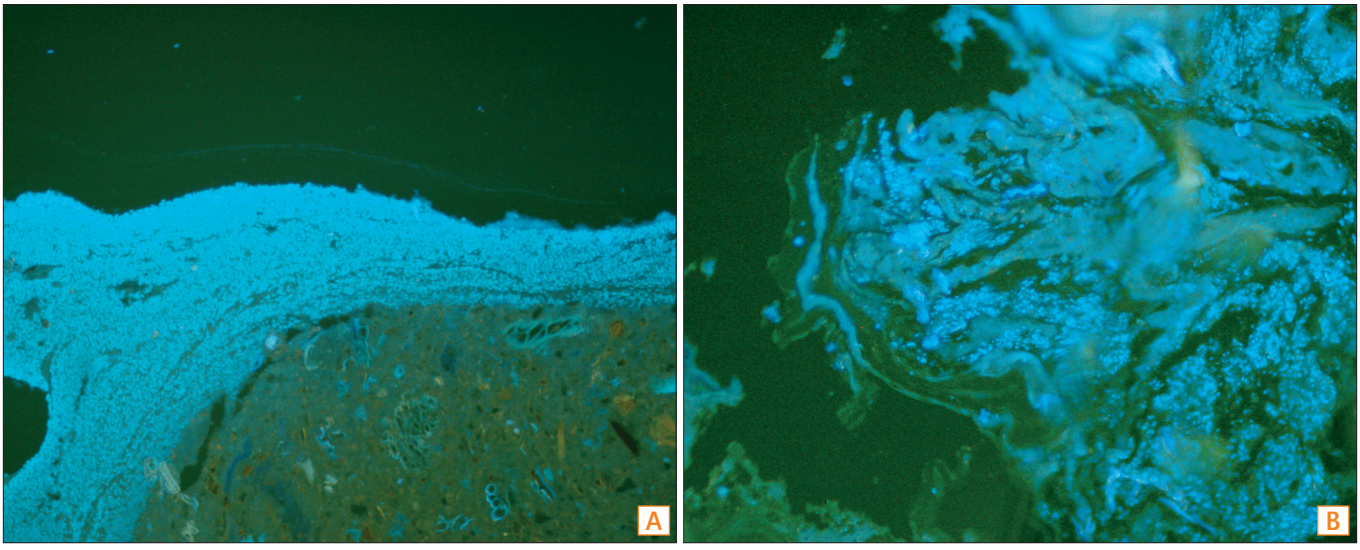

Fig. 1. Leukocyte migration into the mucus post stroke (DAPI FISH counter stain, ×100). (A) Patient with UC, the blue nuclei of leukocytes are tightly packed to a dense layer covering the surface of the stool with nearly no spaces between leukocytes. (B) Second day after stroke, the nuclei of leukocytes are loosely accumulated in the massively thickened mucus.

ence of leukocytes in the mucus. Concentrations of habitual bacteria in stools positive for leukocytes were higher and statistically not different from concentrations observed in IBS patients (Subgroups B and E; Table 2). In all longitudinally investigated cases, where leukocytes were initially present and disappeared thereafter, the fall in concentrations of habitual bacterial groups was observed immediately after the disappearance of the leukocytes. The leukocytes had disappeared nearly completely from the stool of all stroke patients in 3 days. This disappearance was followed by a massive fall in the mean concentrations of the habitual bacterial groups, resulting in the lowest means in the period between 3 to 7 days as compared to all other control groups or time periods post stroke. *Roseburia, Bacteroides* and *Faecalibacterium prausnitzii* were not detectable in 59%, 56% and 51% of stool samples between days 3 and 7 post stroke. The mean concentrations and occurrence of occasional bacterial groups between days 3 to 7 post stroke were slightly but not significantly lower than in the days 1 to 2.

Between days 8 to 14 and 15 to 21, the mean concentrations of habitual bacteria increased steadily reaching values in the fourth week that were higher than on admission and were statistically not different from levels observed in IBS patients. The occasional bacteria increased likewise, starting with the second week post stroke, however, their concentrations were initially much higher than that of the habitual bacteria. Between days 8 to 14, the mean concentrations of occasional bacterial groups achieved their maximum. This increase did not last. After reaching the maximal concentration and with the normalization of the concentrations of the habitual bacterial groups in week 3 post stroke, the concentrations of occasional bacteria started to decline reaching their lowest concentration after day 22 post stroke (Table 2).

The severity, course and outcome of the stroke, the duration of stay in the stroke unit and the dynamics of recovery differed from patient to patient. The pooling of individual measurements as performed in Table 2 suggests the gradual and smooth change in bacterial concentrations. This impression is biased. Table 3 shows the longitudinal daily measurements in concentrations of 12 bacterial groups in a 68-year old man with media infarct and re-stroke at day 7.

The fluctuations in bacterial concentrations are abrupt, with specific bacterial groups completely disappearing or suddenly emerging and increasing to very high levels. During this study, five more patients remained >20 days in the stroke unit in the Teupitz Hospital, providing sufficient amount of stool samples for the longitudinal evaluation. Fig. 2 illustrates daily dynamics in concentrations of three habitual (*Roseburia, Bacteroides* and *Faecalibacterium prausnitzii*) and three occasional (*Enterobacteriaceae, Bifidobacteriaceae* and *C. difficile*) groups for each of these patients $\times 10^9$/mL.

Despite large intra-individual differences, typical for all these patients was a nearly complete disappearance of both habitual and occasional bacterial groups from the colon following the stroke, lasting for one or two weeks. The bacterial concentrations started to increase at the end of the second or third week post stroke. The most profound increase was initially observed for the occasional bacterial groups *Bifidobacteriaceae, Enterobacteriaceae* and *C. difficile*. The occasional bacterial groups reached concentrations higher than the habitual bacterial groups, and made up most of the mass of the colonic microbiota for a short period of time. The dominance of occasional bacteria was short. With the increasing normalization of habitual bacteria after day 20 post stroke, the abso-

Table 2. Concentrations of Colonic Microbiota ($10^5/\mu L$) in Controls and Different Time Periods Following Stroke (mean+SD)

	Heart			Stroke						*P*-value
	Healthy controls	IBS controls	Cardiac emergency unit	Days 1-2 all samples	Days 1-2 with leukocytes	Days 3-7	Days 8-14	Days 15-21	Days >22	
Samples	60	60	55	53	(35)	86	72	61	42	
	A	B	C	D	E	F	G	H	I	
Erec	25.0±7.6	17±8	19±8	11±10	17±7	**1.7±4.2**	4.5±5.9	6.0±6.3	12.3±8.8	F/all *P*<0.001; I/ABCD ns; I/GH *P*<0.0001; D/ABC *P*<0.01-0.001 B/E ns
Bac	19±6	14±9	16±7	8±7	13±6	**2.2±4.7**	5.3±6.0	5.9±7.2	11.0±7.6	
Fprau	15±6	13±7	15±7	9±8	14±6	**1.4±2.6**	3.0±4.4	5.2±5.6	9.0±7.7	
Ebac	0.2±0.4	0.7±1.5	0.4±1.0	1.2±2.8	1.6±3.4	1.9±3.6	**3.4±4.8**	**4.7±5.6**	1.4±3.2	GH/ABCDFI; *P*<0.05-0.001
Bif	0.7±2.0	1.4±2.1	1.0±2.1	2.4±3.3	3.3±3.6	2.3±3.7	**4.2±5.3**	**3.6±5.6**	1.5±2.7	GH/ABCI; *P*<0.05
Clit	0.1±0.6	0.1±0.4	0.1±0.4	0.2±0.6	0.1±0.4	0.1±2.5	1.2±4.0	**2.2±4.2**	0.4±0.9	H/ABCDFI; *P*<0.05-0.001;G/H ns; D/AC *P*<0.05-0.001 B/E ns

Bold numbers mark significantly and excessively decreased (Erec, Bac, Fprau) or increased mean concentrations (Ebac, Bif, Clit) when compared to other groups or time periods.
Erec, *Roseburia*; Bac, *Bacteroides*; Fprau, *Faecalibacterium prausnitzii*; Ebac, *Enterobacteriaceae*; Bif, *Bifidobacteriaceae*; Clit, *Clostridium difficile*; ns, not significant.

Table 3. Concentrations of Microbiota ($10^5/\mu L$) in All Stools of a 68-year-old Male Stroke Patient

Day after stroke	2	4	5	8	11	14	15	17	18	18	20	21	22	24	26	29	31	31	32
Leukocytes in mucus $\times 10^5$ μL	64	0	0	8	0	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0
Erec	22	3	1	4	0	2	0	2	2	8	16	11	6	6	5	15	4	12	18
Bac	18	0	0	5	0.5	1	0	0	0.1	1	0	1	11	15	3	12	11	5	12
Fprau	15	0. 1	0	1	0	0	0	0	0	0.2	0	0	12	7	5	5	8	8	9
Clit	0	0		0	0.4	0	0	0	0	8	11	6	8	2	4	4	0	1	0
Chis	0	0	0	0.1	0	0	0	0	1	4	2	3	1	0	0	0	0	0	0
Ebac	0.1	0	0	0.1	0	0	0	0.2	1	8	4	1	4	0.2	1	0	0	0	0
Veil	0	0	0	0	0	0	0	0	0	0.4	0.4	0	0	0	0	0	0	0	0
Ecyl	4	1	0	0	0	0	0	0	0	6	4	3	2	0	0	0	0	0	0
Ehal	0.1	0	0	0	0	0	0	0	0	0	2	0	0.2	0	0	0	0	0	1
Bif	0	0	0	0	0	0	0	0	0	1	0	0	0.1	0	0	0	0.1	0	0
Ato	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0.1	0
Lab	0.1	0	0	0	0	0	0	0	0	12	8	0	0	0.2	1	0	0	0	0.1

Three habitual and 9 occasional bacterial groups are enumerated in all available stool samples using FISH. Zero indicates that no bacteria were detectable.
Erec, *Roseburia*; Bac, *Bacteroides*; Fprau, *Faecalibacterium prausnitzii*; Clit, *Clostridium difficile*; Chis, *Clostridium histoliticum*; Ebac, *Enterobacteriaceae*; Veil, *Veilonella*; Ecyl, *Eubacterium cylindroides*; Ehal, *Eubacterium halili*; Bif, *Bifidobacteriaceae*; Ato, *Atopobium*; Lab, *Lactobacillus*.

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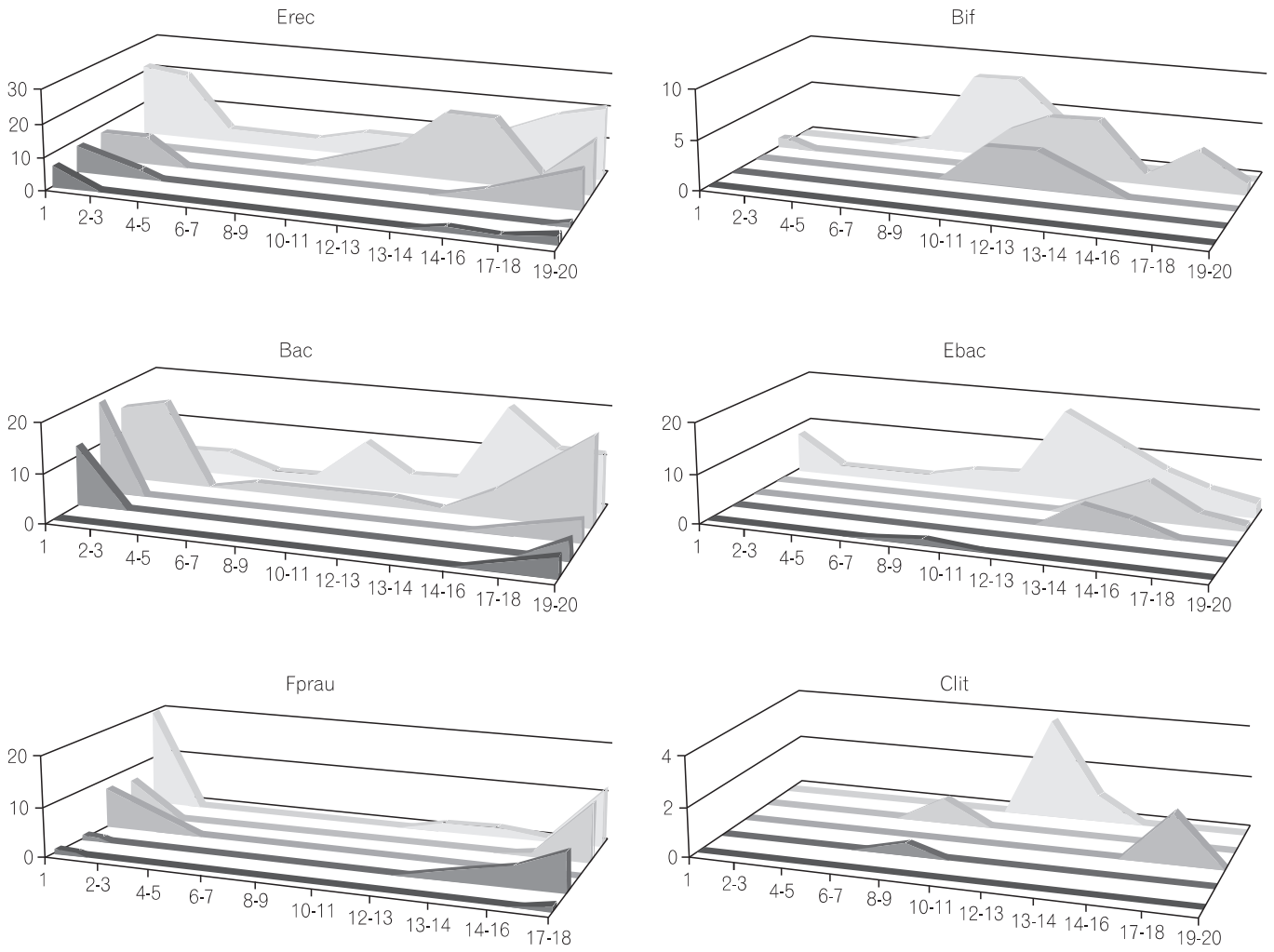

Fig. 2. Longitudinal changes in the concentrations of colonic microbiota. Longitudinal changes in concentrations of colonic microbiota (10^9 bacteria per mL) over 20 days post-stroke in single stool samples from 5 different patients. Six bacterial groups including *Roseburia* (Erec), *Bacteroides* (Bac), *Faecalibacterium prausnitzii* (Fprau), *Bifidobacteriaceae* (Bif), *Enterobacteriaceae* (Ebac), *Clostridium difficile* (Clit) are given for each patient. Each patient has its own gray scale in the presentation.

Table 4. Mean Leukocytes ($\times 10^3/\mu L$) in the Mucus of the Proximal Colon Following Lethal Brain Damage and Exsanguination

Minutes post brain damage	0	15–30	30–60	90
Rabbit	0.01	56	100	
Piglet			200	310

lute concentrations of occasional bacteria started to decline often resulting in levels below detection (Fig. 2).

Slaughtered animals: Table 4 summarizes data on short term leukocyte migration into the mucus of the proximal colon after lethal brain damage. Hardly any leukocytes can be found in the mucus of rabbit intestine immediately after death, however already 15 minutes later leukocyte can be seen, and are more pronounced after 30 minutes (Fig. 3). Similarly, high concentrations of leukocytes were observed in piglets 30 minutes after lethal brain damage and increased further by 90 minutes.

DISCUSSION

The main finding of our study is a remarkable post stroke dynamic in the biostructure of colonic microbiota: massive leukocyte migration in the stool mucus on days 1 to 3, subtotal eradication of the main fermentative bacterial groups on days 4 to 7 and recovery of the colonic microbiota thereafter.

We have to recall the general principles of colonic function in order to interpret the observed changes.[4,5]

Functional structure of colonic microbiota and its diagnostic significance in health and disease: The colonic biofermentor has at least three functionally different

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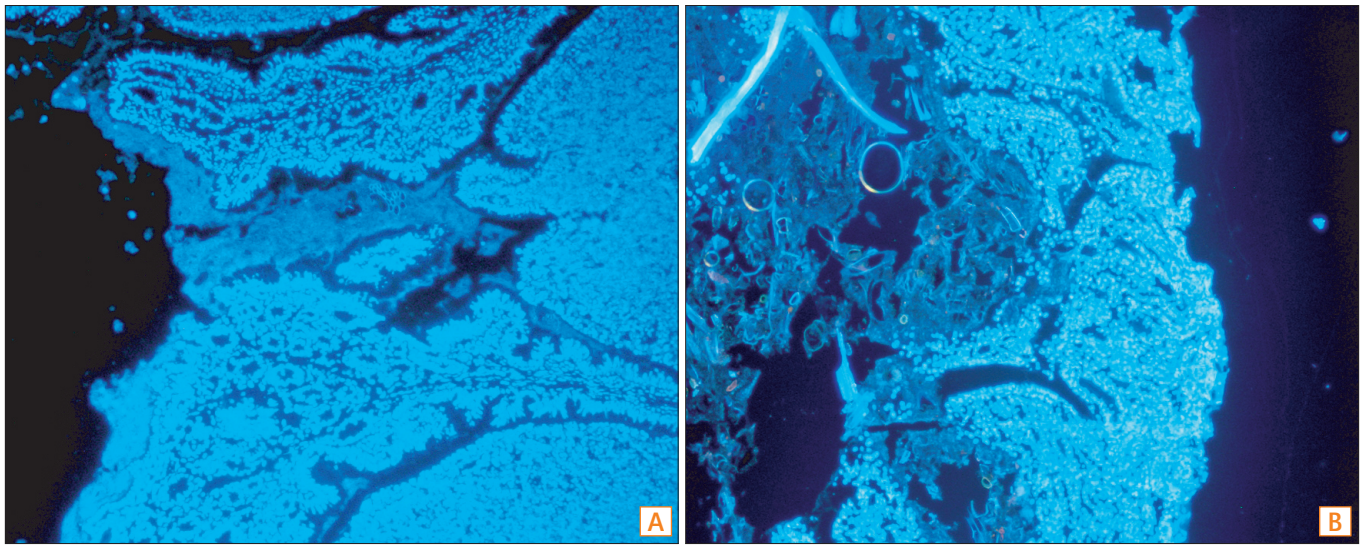

Fig. 3. Leukocyte migration (DAPI FISH counterstain, ×100). Proximal colon of a rabbit 5 minutes (A) and 30 minutes (B) after stunning with a neck blow. Only singular leukocytes can be seen immediately after stunning. A massive leukocyte migration into the mucus of the large intestine can be observed after 30 minutes. Leukocytes can be seen as large blue spots in the lumen of the intestine.

compartments: The central working or fermentative area, the outer separating mucus, and a germinal coat located between separating mucus and fermentative compartment.

The central working compartment contains a highly diverse and concentrated biofermentative mass. For optimal growth conditions here, the central fermentative compartment contains numerous potential pathogens. The separating mucus layer enables the safe run of the fermentation by isolating mucosa from pathogens. When the mucus separation is impaired, bacteria contact mucosa and provoke a host response. The general reaction of the host to such events is similar to what the workers in a biofermenting facility will do when its content spoiled: purging or diarrhea, disinfection or suppression of bacterial growth and resetting.

The coat of the central fermentative compartment, which faces the separating mucus layer, is the germinal area. The mucus of the separating layer is gradually softened by watery intestinal contents. Bacteria entrapped in this softened, but still immotile mucus are spaced out of the fecal stream and protected against peristalsis, purging or antibacterial substances of the lumen. These stocks stand by for resetting of the colonic bioreactor after episodes of emptying and cleansing.

Although the central fermentative area, the germinal coat and the separating mucus layer are all parts of the same fecal stream, events taking place in these compartments upon challenge are contrary and disease specific. Until now, we investigated more than 20,000 samples of fecal cylinders from different patients and intestines filled with stool from more than 400 animals, allowing the following observations.

1. Monitoring of the Current State of the Colonic Fermentation

In healthy individuals, both germinal coat and central working area are nearly undistinguishable. All bacterial groups are homogeneously distributed and for minor exceptions identically composed in fermenting compartment and germinal coat. The constantly maintained diversity and bacterial concentrations here are unprecedented in nature and could not be thus far simulated *in vitro*. They result from active promotion of growth by the host. Any intestinal dysfunction or severe disease of the host leads to impairment of the facilitation and drop in bacterial diversity and concentrations. Although all colonic bacteria are involved, groups which are always present in healthy individuals are most reliable for diagnostic purposes, since intra-individual variations can be neglected. In humans, these bacterial groups are *Roseburia, Bacteroides* and *Faecalibacterium prausnitzii*. Each of these composes 20 to 50% of the microbial biomass reaching concentrations of 10^{11} or more. We called these bacterial groups (*Roseburia, Bacteroides* and *Faecalibacterium prausnitzii*) habitual, because of their obligate and abundant presence in stools of all healthy controls and non-IBD patients.

In disease, the biofermentative output can not be achieved, the concentrations of habitual microbiota in the working area fall, while the germinal area remains uninvolved. Accordingly, the concentrations of habitual bacteria on the surface and in string like offshoots of the superficial germinal area included in stool remain high, while massive reduction of concentrations can be observed in the remainder of the stool cylinder. The impairment of the habitual bacterial groups is directly proportional to the severity of the disturbance and

can be deduced even from a single stool sample. Complete disappearance of all three habitual bacterial groups is exceptional. We have never observed such phenomenon before.

2. Long-term Monitoring of the Colonic Bioreactor

All other non-habitual bacterial groups including ubiquitous colonic bacteria such as *Enterobacteriacae* and *Bifidobacteria* are occasionally present, from the structure functional point of view. In single persons, the concentration of occasional bacteria can reach 10% of the biomass. Their presence in the stool of healthy subjects in quantities detectable by FISH is, however, not obligate and their distribution within the stool cylinder can be spatially heterogeneous with high concentrated islands surrounded by low concentrated or bacteria omitting regions. Because of the high intra-individual variability of occasional bacterial groups, their absolute concentrations and occurrence have low diagnostic relevance when isolated stool samples are used. The evaluation of occasional bacterial groups is however essential in longitudinal investigations. Since temporary dysfunction of the colonic bioreactor does not involve the germinal coat, the individual composition or "pattern" of occasional bacterial groups remains stable in each individual over long periods of time even so the concentrations of habitual bacterial groups show significant shifts. The pattern in individual composition of occasional microbiota is so typical, that healthy individuals can be recognized through their stool sample using FISH.

The time dependant changes in patterns of occasional microbiota characterize the extent of impairment of the germinal area. In acute diarrhea, the changes within working compartment can be huge, the germinal coat remains unimpaired, and the colonic microbiota restore their previous individual composition after the cessation of the event. In chronic disorders such as IBD, the germinal area is progressively destroyed: in UC by leukocytes migrating into the mucus, in Crohn's disease by substances secreted upstream from the colon, suppressing the habitual *Bacteroides* and *Roseburia* groups, and completely and persistently suppressing *Faecalibacterium prausnitzii* from both the germinal and working compartments.[4,5] The destruction of the germinal area in active IBD leads to high instability in patterns of occasional bacterial groups with massive fluctuations of them within short periods of time, without noticeable rule.

3. Post Stroke Function of the Colonic Bioreactor

The situation in post-stroke patients was unique. First, all three habitual bacterial groups representing the functional state of the fermentative area were not just diminished but in most patients completely "erased" for one or several days. Second, the changes of the biostructure of occasional colonic microbiota were even more unstable than in IBD, with concentrations of single occasional bacterial groups growing to values otherwise typical exclusive for habitual bacterial groups and falling within days to zero. Third, the dynamics in the changes of the bacterial groups were contrary to IBD in time and exactly predictable.

The first event following stroke was a massive migration of leukocytes into the mucus, which could be observed within hours (if frequency of stools was high), but latest till the second or third day after the stroke. The extent of the leukocyte response post stroke was comparable with the leukocyte response in patients with active UC. Obviously the brain damage leads to loss of control of the bioreactor. The substitutive leukocyte migration neutralizes pathogen emission in the critical hours of unattended reactor flow. Interestingly, the patients admitted to the cardiac emergency unit had no comparable leukocyte reaction during their stay in the intensive care unit. The myocardial infarction is obviously insufficient to induce effects similar to stroke. The leukocyte response was, however, not ischemia specific but also observed in animals with severe brain damage. Animals with mechanical injury while slaughtered responded immediately. The leukocyte numbers in the mucus reached levels that are comparable to those observed in DSS colitis after 60 minutes.[16] Since the blood circulation at that time is already impaired, this leukocyte response must be local, mucosa driven and independent from the vascular event. Animal experiments of colonic inflammation, should consider this natural local inflammatory reaction following brain damage to avoid misinterpretations. The mode of sacrifice and the time elapsing between sacrifice, evisceration and fixation of the samples are critical for the evaluation of animal experiments especially in animals with large body mass.

The post stroke or agony induced leukocyte response is probably a provisory solution to pathogen release. The indirect confirmation of the role of bacteria is a sudden disappearance of leukocytes, which was coincident with the onset of decrease of the habitual bacterial groups during days 4 to 7. We observed in the longitudinal observation of post stroke patients that the bacterial concentrations remained high and comparable to concentrations observed in IBS patients and even healthy controls, as long as the leukocytes were present in the stool mucus. Leukocytes disappeared as soon as the concentrations of habitual bacterial fell to zero. Some of the patients admitted to the stroke unit had no leukocytes present and also no detectable habitual bacterial groups in their initial stool samples indicating that the critical events have probably taken place prior to admission to the stroke unit. In all stroke patients, however, where leukocyte reaction was observed initially and disappeared, the shutdown with suppression of the colonic microbiota was also documented, indicating that both leukocyte response and sterilization are an integral part of the same chain of events.

The fact of shutdown may appear trivial to somebody who has low experience with colonic microbiota. However it is the most astonishing observation of our study. While antibiot-

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ics may be highly efficient in culture, none of the presently known antibiotics can in clinical use cause suppression of the habitual colonic microbiota vaguely comparable to that observed after stroke. We have not performed control investigations on different groups of patients treated with antibiotics. However in the last two years, we have investigated 462 fecal cylinders obtained from different patients receiving antibiotics orally or intravenously over different periods of time. In none of these cylinders was a complete suppression of all three habitual groups observed. It appears that the human organism activates mechanisms in mortal combat, which can nearly completely suppress colonic microbiota thus reducing the potential hazards of biofermentation. The recovery begins on days 5 to 10 and is not symmetrical. It starts not with a prevailing growth of habitual bacterial groups but is overshadowed by an increase of the *Bifidobacteriaceae, Enterobacteriacea* and *C. difficile* groups. The concentrations of these bacterial groups achieved, for short periods of time in single patients, levels typical for habitual bacteria of 10^{10} and composed each up to 30% of all microbiota. Since the high concentrations of these bacterial groups are also typical for newborns patients after antibiotic treatment or patients in convalescence after severe organic diseases, it seems that their presence and dynamics in the post stroke colon are not accidental, but a regular feature of normal remodeling. *Bifidobacteriaceae, Enterobacteriaceae* and *C. difficile* are quick reacting substitutive fermenting bacteria, which, similar to pioneer plants in nature, bridge the time following devastating events. A temporary predominance of these bacterial groups in stool can be a sign of recovery of the previously impaired colonic biofermentation and must not be automatically considered a sign of their pathogenicity.

Their presence in high concentrations is transient. With the reoccupation of the colon by the main fermentative groups (days 14 to 40), the necessity for substitute fermentative groups gets lost, and their concentrations and occurrence falls to values typical for occasional bacterial groups in health.

In summary; 1. Stroke is a natural model of shut off and relaunch of the colonic bioreactor with the scope of the shifts in microbiota comparable to events occurring after birth.

2. The breakdown of neuronal control releases an inflammatory response with leukocytes migrating into the colonic mucus minutes after damage and quickly expanding in the hours afterwards. Animal experiments of colonic inflammation, should consider this natural local inflammatory reaction following brain damage to avoid misinterpretations. The mode of sacrifice and the time elapsing between sacrifice, evisceration and fixation of samples are critical for the evaluation of animal experiments especially in animals with large body mass.

3. The leukocyte response is followed by nearly complete "sterilization" of the colon with concentrations of habitual groups below detection by FISH. The leukocyte migration into the mucus ceases with the onset of "sterilization", indicating that leukocyte migration may be the response to uncontrolled release of pathogens from the bioreactor. The last assumption is also backed by the fact, that in case of restroke, the sequence of events repeats. Polymicrobial infections accompanying stroke may be a result of this pathogen release and translocation. Appearance of bacteria in blood is secondary to a defective mucus barrier, the bacterial efflux is polymicrobial and quick alternating. These limit the use of bacterial isolation, resistance testing, and antibiotic therapy based on culture data. The prophylaxis of septic complication should be considered as a measure for maintenance of the mucosal barrier.

4. The extent of microbiota suppression following stroke is unique and exceeds the effects of any presently known antibiotics, indicating existence of an extremely effective mechanisms of bacterial control in emergency. The mechanisms and substances behind this suppression have still to be explored and exploited for diagnostic and therapeutic purposes.

5. Bacteria of the *Bifidobacteriaceae, Enterobacteriaceae* and *C. difficile* groups are probably quick-response, substitute fermentative bacteria, which fill the vacuum following depletion of the main fermentative groups. A temporary predominance of these bacterial groups in stool of newborns, patients after antibiotic treatment or patients in convalescence after severe organic diseases can be a sign of recovery of the previously impaired colonic biofermentation and must not be automatically considered a sign of their pathogenicity.

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Prof. Dr. med. Matthias Endres, Klinik und Poliklinik für Neurologie Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany.

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Dr. med. Wolf V. Dörffel, Department of Cardiology, Pulmonology, Helios Klinikum Bad Saarow, Pieskower Strasse 33, 15526 Bad Saarow, Germany.

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