Research article

**Microscopy of bacterial translocation during small bowel obstruction and ischemia in vivo – a new animal model**
Stephan Samel*1, Michael Keese1, Martha Kleczka1, Sybille Lanig1, Norbert Gretz2, Mathias Hafner3, Jörg Sturm1 and Stefan Post1

Address: 1Department of Surgery, University Hospital Mannheim, University of Heidelberg, Germany, 2Medical Research Center, University Hospital Mannheim, University of Heidelberg, Germany and 3Institute of Molecular Biology and Cell Culture Technology, Fachhochschule Mannheim, Germany

E-mail: Stephan Samel* - stephan.samel@chir.ma.uni-heidelberg.de; Michael Keese - michael.keese@chir.ma.uni-heidelberg.de; Martha Kleczka - stephan.samel@chir.ma.uni-heidelberg.de; Sybille Lanig - slanig@hotmail.com; Norbert Gretz - norbert.gretz@zmf.ma.uni-heidelberg.de; Mathias Hafner - m-hafner@fh-mannheim.de; Jörg Sturm - joerg.sturm@chir.ma.uni-heidelberg.de; Stefan Post - stefan.post@chir.ma.uni-heidelberg.de

*Corresponding author

Published: 13 August 2002

Received: 14 May 2002

Accepted: 13 August 2002

This article is available from: http://www.biomedcentral.com/1471-2482/2/6

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**Keywords:** bacterial translocation, green fluorescent protein, gut, obstruction, ischemia, E. coli, intravital microscopy, rat

**Abstract**

**Background:** Existing animal models provide only indirect information about the pathogenesis of infections caused by indigenous gastrointestinal microflora and the kinetics of bacterial translocation. The aim of this study was to develop a novel animal model to assess bacterial translocation and intestinal barrier function in vivo.

**Methods:** In anaesthetized male Wistar rats, 0.5 ml of a suspension of green fluorescent protein-transfected E. coli was administered by intraluminal injection in a model of small bowel obstruction. Animals were randomly subjected to non-ischemic or ischemic bowel obstruction. Ischemia was induced by selective clamping of the terminal mesenteric vessels feeding the obstructed bowel loop. Time intervals necessary for translocation of E. coli into the submucosal stroma and the muscularis propria was assessed using intravital microscopy.

**Results:** Bacterial translocation into the submucosa and muscularis propria took a mean of 36 ± 8 min and 80 ± 10 min, respectively, in small bowel obstruction. Intestinal ischemia significantly accelerated bacterial translocation into the submucosa (11 ± 5 min, p < 0.0001) and muscularis (66 ± 7 min; p = 0.004). Green fluorescent protein-transfected E. coli were visible in frozen sections of small bowel, mesentery, liver and spleen taken two hours after E. coli administration.

**Conclusions:** Intravital microscopy of fluorescent bacteria is a novel approach to study bacterial translocation in vivo. We have applied this technique to define minimal bacterial transit time as a functional parameter of intestinal barrier function.

**Background**

Bacterial translocation has been defined as the passage of viable bacteria from the gut lumen to extraintestinal organs [1]. Major conditions contributing to bacterial translocation are a breakdown of the intestinal barrier, an impairment of host immune defense and a loss of the col-
onization resistance [2,3] with bacterial overgrowth in the intestinal tract [4]. Gut bacteria may penetrate the mucosa – that consists of a tight lining of enterocytes covering the villi and M-cells in the depth of the crypts [5] – by different mechanisms specific to each species [6]. However, when the mucosa is injured and the intestinal barrier is compromised [7] an unspecific and unlimited translocation of intestinal micro-organisms can occur [8]. Intestinal barrier disorders have been studied in a modified Ussing chamber in vitro [9]. Markers, such as inulin-fluorescein [10] or polyethylene-glycol 4000 [11], fluorescent microspheres [12] or plasmid-labelled bacteria [13] are used to study mucosal permeability in vivo. Gut obstruction and ischemia are severe and often fatal conditions in patients that can result in sepsis and multiple organ failure [14,15]. Obstruction [16,17] and ischemia [18,19] cause mucosal injury with a subsequent increase of mucosal permeability and thus bacterial translocation.

We describe a novel animal model to study bacterial translocation under conditions of intestinal obstruction and ischemia in vivo. Translocation of Escherichia coli (E. coli) transfected with green fluorescent protein (GFP) can be observed using intravital microscopy.

Methods

Animals and experimental design
Translocation of GFP-transfected E. coli from an obstructed segment of terminal ileum across the gut wall and into distant organs was investigated by intravital microscopy. Male Wistar rats (200 – 250 g body weight, 12–14 weeks old) were used for the experiments that were performed in accordance with German legislation on protection of animals and the NIH recommendations for the use of laboratory animals [20]. The animals had free access to water and standard pellet food prior to surgery, so that the integrity of the small bowel mucosa was maintained. Animals were randomly placed in groups that were either subjected to non-ischemic or ischemic bowel obstruction. Bowel obstruction was induced by intestinal ligation. Ischemia was induced by additional clamping of the mesenteric blood supply feeding the obstructed segment of ileum. Sham operated animals without obstruction served as controls for bacteriological and histopathological examinations. Each group consisted of eight animals.

Transfection and cultivation of E. coli
DH 5a-E-coli were heat-shock transfected with the commercial GFP-uv vector (BD Biosciences Clontech Inc., Palo Alto, USA) to produce a stable green fluorescence. E. coli were incubated with 250 ng DNA, stirred on ice and heat shocked at 42°C for 60 sec before SOC-transfection medium was added. The suspension was placed onto plates covered with Luria-Bertani Medium (LB-medium; Quiagen, Hilden, Germany). To obtain the necessary bacterial concentration before use in the experiments, we incubated E. coli in 100 ml LB-medium over night. Bacterial concentration (optical density at 600 nm) was checked and aliquots for the experiments were taken at an optic density of 1 (8 × 10^8 E. Coli/ml).

Surgery
Animals were anaesthetized by i.m. administration of xylazine hydrochloride 100 mg/kg (Rompun, Bayer AB, Leverkusen, Germany) and ketamine 2.5–5 mg/kg (Hostaket, Parke-Davis, Freiburg, Germany). A silicone-catheter (27 G) was placed into the carotid artery to measure arterial blood pressure and monitoring blood gases. A second catheter was inserted into the contralateral jugular vein for continuous infusion of saline during experiments. An abdominal cavity chamber was implanted via an abdominal midline incision as previously described [21]. In this closed abdominal observation chamber temperature and humidity of the abdominal cavity and the exposed small bowel loop remain constant. Small bowel obstruction was simulated by proximal and distal ligation of a segment of terminal ileum two cm in length and two cm proximal to the cecum, thus forming a reservoir to hold the bacterial suspension. Perfusion of the obstructed segment of bowel was maintained through the feeding mesenteric vessels in one group [22]. In the other group ischemia was induced by selective clamping of the mesenteric vessels (Figure 1). The obstructed loop of terminal ileum was externalized onto a horizontal bar inside the working cylinder of the abdominal cavity chamber, thereby avoiding any tension on the mesentery. GFP-uv E. coli (4 × 10^8 suspended in 0.5 ml LB-medium) were injected into the obstructed bowel lumen using a 27G needle. Injection caused a moderate distension of the obstructed loop. We carefully avoided any contamination of the abdomen. In sham operated animals 0.5 ml of saline were injected into the terminal ileum. The abdominal cavity chamber was closed with an optical window leaving the serosal surface of the bowel exposed underneath. For intravital microscopy rats were placed on a heated operating table (K. Effenberger, Med. Tech. Geratebau, Pfaffing, Germany) in supine position. Systemic arterial blood pressure and heart rate were monitored continuously (Sirecust 404, Siemens, Erlangen, Germany).

Intravital microscopy
GFP-uv E. coli emit green light (peak at 509 nm, shoulder at 540 nm) when excited with blue light (at 395 nm maximum) [23]. This fluorescence at 540 nm was used to identify and monitor GFP-uv E. coli translocation through the gut wall by means of intravital microscopy using a modified Zeiss microscope (Axiotech, Carl Zeiss, Germany). Microscopy was performed in 5 minute-intervals to detect fluorescent bacteria. 10 high power fields (HPF) of tissue were observed for 10 to 15 sec each in two levels of...
focus (submucosa and muscularis propria) resulting in a total observation time of 200–300 sec per observation interval. This 5-minute interval provided a continuous microscopic observation of the chosen HPFs in both focus levels. The submucosa is characterized by the submucosal postcapillary venules, and the muscularis propria is characterized by its typical network of circular and longitudinal muscle capillaries. A 100 W mercury lamp was used for epiillumination (Carl Zeiss, Germany). A filter (Carl Zeiss, Germany) protected the tissue against UV illumination. GFP-uv 

**E. coli** were detected by their green fluorescence using a 200-fold magnification, and were identified by their typical shape using a 400-fold magnification. Microscopic images were recorded with a digital charge-coupled device video camera (KAPPA opto-electronics GmbH, Gleichen, Germany) and a video cassette recorder (AG 7350, Panasonic, USA) for off-line evaluation.

**Video analysis**

Minimal transit time was quantified on-line. Measurements were confirmed off-line by frame to frame computer-assisted image analysis (Capimage, Zeintl, Heidelberg, Germany). Minimal transit time was defined as the time period between GFP-uv 

**E. coli** administration into the obstructed small bowel segment and the detection of five or more fluorescent bacterial rods per HPF in ten HPFs.

**Bacteriological examination**

Samples of mesenteric lymph nodes and stool from the non-obstructed and the obstructed terminal ileum were homogenized in sterile isotonic saline using Tenbroeck tissue grinders (Wheaton, Millville, USA). Each sample was plated quantitatively on MacConkey and Columbia blood agar plates (Oxoid, Wesel, Germany) according to standard methods for culture of aerobic and microaerophilic bacteria. Smears of the abdominal cavity were cultured in the same manner. Anaerobic bacteria were not assessed. After 48 hours of incubation at 37°C, the colony-forming units (CFU) per gram of stool were determined. Bacterial species were identified by gram staining, by their characteristic biochemical pattern in a commercial system for biochemical identification of bacteria (API32E, bioMerieux, Nürtingen, Germany) and their characteristic susceptibility to antibiotics.

**Histopathology**

Specimens of the obstructed small bowel segment, mesentery, liver and spleen were taken two hours after GFP-uv 

**E. coli** administration for frozen sectioning and paraffin histology. Tissue samples were collected in tissue glue (Tissue Tek, Sakura, Torrance, USA) which was fixed in ice-cold isopentane (3-Methyl-1-Butan). Blocks were kept at -80°C until frozen sections of tissue samples were prepared in 10 µm slices on a Leica Cryostat (Leica, Wetzlar, Germany). Frozen sections were fixed in mounting medium and examined for translocation of GFP-uv 

**E. coli** using 400-fold and 630-fold magnification (Leica, Wetzlar, Germany). GFP-uv fluorescence allowed identification and a topographical localization of the 

**E. coli** within the examined tissues. Patterns of angioarchitecture were used as landmarks to correlate cryosections with corresponding hematoxylin-eosine (HE) crossections.

For HE histology 10 µm slices were deparaffinized with ethanol (99%, 96%, 80%), stained for 10 min with hematoxylin, washed and counterstained with 0.1% eosin before being fixed in ethanol. Slides were embedded with glycerol. Specimens of the obstructed small bowel were examined for ischemic injury after HE staining, as suggested by Meddah and co-workers [19]. Lesions were scored on a scale of 0–3: no or minimal lesions (grade 0), loss of epithelial cells from the villi (grade 1), sporadic necrosis of villi (grade 2), and complete mucosal necrosis (grade 3).

**Statistical analysis**

Minimal transit times of GFP-uv 

**E. coli**-translocation into the submucosal stroma and into the muscularis propria are expressed as means ± s.d. The significance of differenc-
es was evaluated using Student’s t-test. Differences were considered significant, if the p-value was <0.05. SAS 8.0® software (SAS, Cary, NC) was employed for statistical analysis.

**Results**
All animals survived the experimental period of two hours. Mean arterial blood pressures and heart rates remained constant and did not show marked differences between groups. GFP-uv E. coli were detected as single fluorescent rods or small clusters of bacteria in the submucosal stroma and, subsequently, in the muscularis propria of the small bowel wall using serosa sided intravital microscopy.

**Transit time of bacterial translocation**
During non-ischemic obstruction bacterial translocation into the submucosa and muscularis propria took a mean of 36 ± 8 min and 80 ± 10 min, respectively (Figure 2). Ischemia of the obstructed small bowel resulted in a significant acceleration of minimal transit time into the submucosa (11 ± 5 min, p < 0.0001) and muscularis propria (66 ± 7 min; p = 0.004).

**Small bowel microbiota and bacterial examination of tissue**
The variety of gram-positive and gram-negative bacteria per ml of stool from the small bowel proximal of the obstructed bowel segment and from the obstructed loop of terminal ileum did not show any quantitative nor qualitative differences in any groups. The most common bacteria found in the terminal ileum were fermentative and non-fermentative gram-negative rods, Streptococci and Staphylococcus aureus. GFP-uv E. coli predominated among these species in the stool from the obstructed loop (Table 1). The same species were found in homogenized mesenteric lymph nodes, liver and spleen. There were no differences between ischemic and non-ischemic animals regarding gram-positive and gram-negative intestinal microflora. Bacterial translocation to mesenteric lymph nodes, liver and spleen was not present in sham operated animals. Bacterial smears from the abdominal cavity were negative in all animals.

**Histopathology**

**Ischemic lesions**
No or minor mucosal lesions (grade 0–1) were found in HE stained specimens of obstructed terminal ileum in non-ischemic animals. Severe lesions (grade 3) were present in all animals subjected to ischemic small bowel obstruction.Specimens from unaffected terminal ileum proximal to the obstructed loop and from sham operated animals demonstrated no mucosal lesions in both groups of animals (grade 0).

**Bacterial translocation to distant organs**
Bacterial translocation to distant organs was confirmed by fluorescence microscopy on crossections of frozen tissue taken from the obstructed small bowel segment, liver and spleen two hours after injection of GFP-uv E. coli. GFP-uv E. coli were found adherent to the mucosa, penetrating the mucosa and translocating into the submucosal stroma (Figure 3), the muscularis propria and the attached mesentery in tissue of the obstructed small bowel loop. Single GFP-uv E. coli or small clusters of bacteria were also found in specimens taken from liver and spleen. Translocation of GFP-uv E. coli was observed in all animals subjected to injection of GFP-uv E. coli on intravital microscopy. On frozen sections too, GFP-uv E. coli were detected in all of these animals without any differences between non-ischemic and ischemic obstruction. No fluorescent bacteria were found in sham operated animals (Table 2).

**Discussion**
Translocation of intestinal bacteria has been observed as a spontaneous event in humans [24] leading some authors to question its clinical significance [25]. Others, however, consider bacterial translocation to be a crucial factor in the pathogenesis of sepsis and multiple organ failure in surgical and intensive care patients [15,25,26]. Intestinal obstruction [17] and ischemia are among the conditions most likely to be associated with bacterial translocation and sepsis. A failure of the intestinal barrier function, re-
sulting in an increase of mucosal permeability, has been hypothesized to be a major promoter of bacterial translocation [8].

In order to understand its pathophysiological relevance, bacterial translocation has been studied in animals subjected to various pathological conditions such as hemorrhagic shock [27], endotoxin-challenge [28], thermal body surface trauma [29], acute pancreatitis [30] and intestinal obstruction [31] and ischemia [19]. Animal models, developed to study mucosal permeability, involved the use of specific markers to follow the bacterial passage across the mucosa. Inulin-fluorescein was used to study permeability of the mucosa in inflamed small and large bowel. Permeability was determined by analyzing plasma-levels of the marker [10]. Others measured plasma levels of polyethylene glycol 4000 [11] or used fluorescence-activated cell sorting of fluorescent microspheres [12] that had been administered into the small bowel, to show an altered mucosal permeability during hemorrhagic shock and acute pancreatitis respectively. The most detailed proof of a gut-origin of endogenous infections was provided by the detection of plasmid-labelled \textit{E. coli} in pancreatic tissue of pancreatitic dogs via plasmid DNA analysis, seven days after colonizing them with a strain of \textit{E. coli} bearing the plasmid pUC4K [13].

In contrast to these more indirect approaches to demonstrate and investigate bacterial translocation, we developed a rat model allowing us to observe the passage of viable fluorescent \textit{E. coli}, that had been injected into an obstructed small bowel segment, across the mucosa and into the muscularis propria, and their further translocation to extraintestinal organs such as the liver and spleen.

| Species        | Sham operated | non-ischemic obstruction | ischemic obstruction | non-ischemic obstruction | ischemic obstruction |
|----------------|---------------|--------------------------|----------------------|--------------------------|----------------------|
| GFP-\textit{E. coli} | -             | -                        | -                    | +                        | +                    |
| \textit{E. coli}     | +             | +                        | +                    | +                        | +                    |
| \textit{S. aureus}   | +             | +                        | +                    | +                        | +                    |
| \textit{Strept. spp.} | +             | +                        | +                    | +                        | +                    |
| \textit{Past. spp.}  | +             | +                        | +                    | +                        | +                    |
| \textit{Bac. spp.}   | +             | +                        | +                    | +                        | +                    |

Table 1: \textit{Indigenous small bowel microflora in rats} Bacterial species found in the terminal ileum of sham operated animals and ischemic and non-ischemic rats after intraluminal injection of GFP-uv \textit{E. coli} (\textit{E. coli} = \textit{Escherichia coli}; \textit{Past.} = \textit{Pasteurella}; \textit{Strept.} = \textit{Streptococcus}; \textit{S.} = \textit{Staphylococcus}; \textit{Bac.} = \textit{Bacillus}).

| Group                | Organ | number of GFP-uv \textit{E. coli} per HPF (mean ± s.d.) |
|----------------------|-------|---------------------------------------------------------|
| sham operated        | MLN   | none                                                    |
|                      | liver | none                                                    |
|                      | spleen| none                                                    |
| non-ischemic obstruction | MLN | 1.8 ± 0.7                                               |
|                      | liver | 2.9 ± 1.2                                               |
|                      | spleen| 2.0 ± 0.9                                               |
| ischemic obstruction  | MLN   | 2.0 ± 0.9                                               |
|                      | liver | 2.8 ± 1.0                                               |
|                      | spleen| 1.8 ± 0.7                                               |

MLN = mesenteric lymph nodes; HPF = high power field

Table 2: Incidence of GFP-uv \textit{E. coli} translocation to distant organs
Online intravital microscopy allowed the assessment of the minimal time period necessary for GFP-uv E. coli to translocate into the submucosal stroma and muscularis propria (≥ 5 E. coli per HPF in 10 HPFs). We defined minimal transit time as a novel parameter characterizing intestinal barrier function in this model of small bowel obstruction.

Intestinal obstruction in our model was associated with translocation of GFP-uv E. coli within less than an hour after intraluminal administration of the bacteria. When we examined specimens of small bowel on crossections, we did not find severe mucosal damage in animals subjected to intestinal obstruction. Deitch and co-workers also found no mucosal damage during bowel obstruction and suggested that changes in the intestinal microflora and infectious mechanisms may promote bacterial translocation [16]. In our study, however, bacteriological examination did not reveal any differences in the variety of intestinal microorganisms in the non-obstructed or obstructed terminal ileum. We assume that even the moderate distension of the bowel by intraluminal administration of the E. coli suspension promoted bacterial translocation. A possible explanation for bacterial translocation resulting from bowel distension may be a loss of mucosal tight junction integrity.

The specific effect of segmental ischemia of the obstructed small bowel, in our study, was a marked acceleration of bacterial translocation. Ischemic mucosal injury, as detected by histopathological examination probably correlates with a breakdown of the intestinal barrier function. The concept that intestinal hypoperfusion and ischemia promote bacterial translocation is well established in literature [16,31–33]. By using our novel animal model, we can provide further functional and histopathological evidence supporting the hypothesis of a gut origin of microorganisms that translocate to extraintestinal organs under pathological conditions. This animal model was used to study bacterial translocation in ischemic and non-ischemic small bowel obstruction. Bacterial translocation observed in this model was initiated by inflammatory stress due to obstruction and distension of the gut. Gut ischemia serves as an additional promoter of bacterial translocation in this model. We were able to study the minimal transit time necessary for bacterial translocation across the intestinal barrier by means of intravital microscopy. Thus we have introduced an additional experimental tool to investigate specific pathological effects on intestinal barrier function. However, the quantification of bacterial translocation across the intestinal barrier into distant organs is difficult to accomplish in this model and may require a longer period of observation. GFP-uv E. coli are administered into the stool contained in the obstructed small bowel, which probably results in an uneven distribution along the mucosa. The number of translocated GFP-uv E. coli found in mesenteric lymph nodes, liver and spleen varied, but did not show significant differences between animals subjected to ischemic and non-ischemic bowel obstruction. Transit time did not correlate to the number of GFP-uv E. coli / HPF found in mesenteric lymph nodes, liver and spleen two hours after GFP-uv E. coli administration. Further elaboration of the model is needed to provide quantitative measures of bacterial translocation.

**Conclusions**

The presented animal model allows an online assessment of bacterial translocation by means of intravital microscopy. In addition to previous animal models of bacterial translocation, our novel approach defines minimal bacterial transit time as a function of the intestinal barrier and demonstrates a significant influence of ischemic injury of the gut on bacterial translocation. This novel parameter of intestinal barrier function can contribute to the understanding of bacterial translocation. The model allows studies of bacterial translocation under various pathological conditions, e.g. acute pancreatitis or increased intraabdominal pressure. Furthermore the impact of pharmacological agents on intestinal barrier function can be examined in this model. However, further studies are needed to determine the clinical significance of this novel kinetic parameter.

**Competing interests**

None declared.
Author's contributions
SS designed the animal model, performed animal experiments, microscopy and statistical analysis of data and drafted the manuscript. KM performed animal experiments and microscopy and co-drafted the manuscript. KM and LS performed animal experiments and microscopy. GN participated in design and co-ordination of the experimental work. HM performed GFP-transfection of E. coli. SJ had the initial idea to develop the presented model and participated in the design of the study. PS supervised and advocated the experimental work and reviewed the final draft of the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank our medical technicians, Mrs. B. Heidrich and Mrs. C. Jost for preparing the histological specimens. Bacteriological examinations were kindly performed by PD Dr. T. Nchterlein and microscopic photographs were taken by Dr. H. Hein, both from the Department of Medical Microbiology and Hygiene at our institution. Special thanks to Dr. E.A. Schober, who has reviewed language and style of the manuscript.

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2482/2/6/prepub