CALL FOR PAPERS | Innovative and Emerging Technologies in GI Physiology and Disease

Ex vivo perfusion of the isolated rat small intestine as a novel model of Salmonella enteritis

Erin C. Boyle,1,2 Heike Dombrowsky,3 Jürgen Sarau,3 Janin Braun,4,5 Martin Aepfelbacher,1,* Ingmar Lautenschläger,3,6* and Guntram A. Grassl4,5,7*

1Institute for Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; 2Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany; 3Priority Area Asthma and Allergy, Research Center Borstel, Borstel, Germany; 4Priority Area Infections, Models of Inflammation, Research Center Borstel, Borstel, Germany; 5Institute for Experimental Medicine, Christian-Albrechts University Kiel, Kiel, Germany; 6Department of Anesthesiology and Intensive Care Medicine, University Medical Center Schleswig-Holstein, Kiel, Germany; and 7Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School and German Center for Infection Research (DZIF), Hannover, Germany

Submitted 12 December 2014; accepted in final form 11 November 2015

Boyle EC, Dombrowsky H, Sarau J, Braun J, Aepfelbacher M, Lautenschläger I, Grassl GA. Ex vivo perfusion of the isolated rat small intestine as a novel model of Salmonella enteritis. Am J Physiol Gastrointest Liver Physiol 310: G55–G63, 2016. First published November 12, 2015; doi:10.1152/ajpgi.00444.2014.—Using an ex vivo perfused rat small intestinal model, we examined pathological changes to the tissue, inflammation induction, as well as dynamic changes to smooth muscle activity, metabolic competence, and luminal fluid accumulation during short-term infection with the enteropathogenic bacteria Salmonella enterica serovar Typhimurium and Yersinia enterocolitica. Although few effects were seen upon Yersinia infection, this system accurately modeled key aspects associated with Salmonella enteritis. Our results confirmed the importance of the Salmonella Pathogenicity Island 1 (SPI1)-encoded type 3 secretion system (T3SS) in pathology, tissue invasion, inflammation induction, and fluid secretion. Novel physiological consequences of Salmonella infection of the small intestine were also identified, namely, SPI-1-dependent vacuolization and SPI-1-independent reduction in the digestive and absorptive functions of the epithelium. Importantly, this is the first small animal model that allows for the study of Salmonella-induced fluid secretion. Another major advantage of this model is that one can specifically determine the contribution of resident cell populations. Accordingly, we can conclude that recruited cell populations were not involved in the pathological damage, inflammation induction, fluid accumulation, nutrient absorption deficiency, and vasoconstriction observed. Although fluid loss induced by Salmonella infection is hypothesized to be due to damage caused by recruited neutrophils, our data suggest that bacterial invasion and inflammation induction in resident cell populations are sufficient for fluid loss into the lumen. In summary, this model is a novel and useful tool that allows for detailed examination of the early physiopathological effects of Salmonella infection on the small intestine.

ex vivo infection; Salmonella; Yersinia; intestinal inflammation

INFECTIOUS DIARRHEA CAUSES high morbidity and mortality worldwide, with the World Health Organization estimating there to be 1.7 billion cases every year (http://www.who.int/mediacentre/factsheets/fs330/en). Nontyphoidal Salmonella enterica serovars and enteropathogenic Yersinia spp. are major causes of diarrheal disease.

Enteropathogenic Yersinia spp. primarily colonize the distal ileum and cecum. Bacteria cross the intestinal epithelial barrier primarily via M cells, which are specialized intestinal epithelial cells that are naturally phagocytic. Using its chromosomally encoded adhesin called invasin, enteropathogenic Yersinia specifically binds to β1-integrins exposed on the apical surface of M cells located in the follicle-associated epithelium overlying Peyers’ patches and scattered throughout the villous epithelium (4, 18, 34). Much of Yersinia’s pathogenicity can be attributed to a 70-kb virulence plasmid that encodes a type 3 secretion system (T3SS) and its associated secreted effectors. Once across the epithelium, enteropathogenic Yersinia employ this T3SS to inject effectors into phagocytes to prevent phagocytosis, dampen inflammatory responses, and induce apoptosis (2). Subsequently, bacteria reside and replicate extracellularly within the lamina propria.

S. enterica serovar Typhimurium (S. Typhimurium) causes intestinal inflammation in both the small and large intestine. Critical for S. Typhimurium virulence is a T3SS encoded within the Salmonella pathogenicity island 1 (SPI1) (9, 26). SPI1 type 3-secreted effectors facilitate invasion of S. Typhimurium into epithelial cells. Once across the epithelium, S. Typhimurium resides and replicates intracellularly within phagocytic cells. As well as enabling invasion, SPI1 type 3-secreted effectors are known to induce early inflammatory events, polymorphonuclear neutrophil transmigration, fluid accumulation, chloride secretion, apoptosis, and tight junction disruption (35).

* M. Aepfelbacher, I. Lautenschläger, and G. A. Grassl contributed equally to this work.

Address for reprint requests and other correspondence: G. A. Grassl, Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School and German Center for Infection Research (DZIF), Carl-Neuberg-Straße 1, 30625, Hannover, Germany (e-mail: grassl.guntram@mh-hannover.de).

http://www.ajpgi.org 0193-1857/16 Copyright © 2016 the American Physiological Society G55
As well as mediating nutrient uptake and fluid balance, the intestinal epithelium essentially functions as a physical and innate immune barrier devoted to protecting the body against invasion and systemic dissemination of both commensal and pathogenic microorganisms. Despite this, many pathogens have evolved mechanisms that enable them to breach the epithelial cell barrier whereby they subvert host cell functions to survive, replicate, and disseminate. Various animal models have been established to investigate the physiological effects of S. Typhimurium and enteropathogenic Yersinia infection on the intestine. For enteropathogenic Yersinia spp., oral infection of mice or rabbits models intestinal colonization and inflammation seen in humans. Modeling human enterocolitis caused by nontyphoidal Salmonella serovars is more challenging. Infections of nonhuman primates (e.g., rhesus macaques) and calves causes robust intestinal inflammation and fluid secretion; however, these models are understandably restricted by cost and the limited possibility for genetic manipulation of the host. To induce intestinal inflammation in S. Typhimurium-infected mice, animals must either be pretreated with antibiotics (1) or have limited to no microbiota (7, 23, 36, 37). It is thought that by reducing and/or changing the composition of the intestinal microflora, antibiotic pretreatment allows S. Typhimurium to overcome “colonization resistance.”

We recently established an ex vivo perfused model of the rat small intestine that allows independent access to the vascular, lymphatic, and luminal fluids and enables direct measurements of vascular and luminal pressures (21). Under control conditions, functional and morphological integrity of the tissue is preserved for several hours. This system most closely resembles what happens in vivo yet has inherently less experimental variation than whole animal infections. We have adapted this model to study the early effects of S. Typhimurium and Y. enterocolitica infection on the physiology of the small intestine. Although few effects were seen upon infection, this system mimicked both important and novel cell populations to these effects. We have recently adapted this model to study the early effects of S. Typhimurium and Y. enterocolitica infection on the physiology of the small intestine. Although few effects were seen upon infection, this system mimicked both important and novel cell populations to these effects.

MATERIALS AND METHODS

Animals. Animals were housed in the Animal Care Facility at the Research Center Borstel. All experiments were conducted in accordance with the ethical requirements of the Animal Care Committee of the Ministry of Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Germany and in direct accordance with the German Animal Protection Law. All experiments were approved by the Ministry of Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Germany (Protocol: A56).

Perfusion model. Ex vivo perfusion of the isolated rat small intestine was performed as previously described (21) with slight modifications. In brief, the small intestines of anesthetized female Wistar rats weighing 200–275 g (Charles River Laboratories, Sulzfeld, Germany) were isolated and perfused both vasculally (7.5 ml/min) and luminally (0.05 ml/min) in a specialized perfusion chamber (Hugo-Sachs Elektronik, Harvard Apparatus, Holliston, MA). Vascular perfusate was collected via cannulation of the mesenteric vein and was recirculated 4.5 times during the course of the 4-h infection. Luminal effluent was collected via cannulation of the distal end of the intestine. Using pressure transducers connected to side ports of the cannulae, we recorded arterial, venous, and luminal pressures. Lymphatic fluid drained freely from the opened lymphatic vessels. Luminal effluent and lymph were aspirated separately by roller pumps and collected in preweighed tubes to allow for the calculation of fluid flows. The composition of the perfusates is described in Table 1. During the whole experiment, the intestinal preparation and all incoming fluids are maintained at 37°C. In all experiments, the luminal perfusate contained lactose and the subsequent appearance of galactose in the venous compartment was indicative of the digestive and absorptive functions of the intestine. FITC dextran (150 kDa, 1,700 mg/l) was included in the luminal buffer and fluorescence was analyzed in the venous, luminal, and lymphatic effluents. The main differences of the isolated perfused rat small intestine as a model of enteropathogen infection to the previously described model (21) are 1) recirculation of vascular perfusion buffer; 2) measurement of vascular perfusion; 2) application of FITC-dextran to the lumen to focus on changes to epithelial permeability and 3) reduction of the luminal perfusion rate from 0.15 ml/min to 0.05 ml/min to ensure sufficient infection of the pathogens with the intestinal tissue.

Biochemical assays. Arterial pH and oxygen supply as partial pressure of O2 were determined by use of an ABL700 blood gas analyzer (Radiometer). The concentration of FITC-dextran in the effluents was analyzed with a fluorometer (FL600FA, MWG Biotech, excitation 485 nm, emission 530 nm). Vascular galactose derived from luminal lactose was determined with a commercially available assay kit (Megazyme, Bray, Ireland).

Bacterial strains. Wild-type S. Typhimurium SL1344 and the isogenic invA mutant have previously been described (10, 17). Wild-type Y. enterocolitica serogroup O8 isolate WA-314 (WAP) and the virulence plasmid-cured derivative (WAC) have also been previously described (15).

Injection of the perfused intestine. S. Typhimurium was grown while being shaken for 16 h at 37°C in Luria-Bertani (LB) broth supplemented with streptomycin (100 µg/ml). Subsequently, cultures were diluted 1:33 into LB without antibiotics and grown for another 3 h to mid-log phase prior to infection. Y. enterocolitica was grown while being shaken for 16 h at 27°C in LB supplemented with 35 µg/ml nalidixic acid. Subsequently, cultures were diluted 1:20 and grown without antibiotics for another 4 h to mid-log phase prior to infection. Bacterial cultures were centrifuged at 8,000 rpm and resus-

<table>
<thead>
<tr>
<th>Table 1 Composition of vascular and luminal perfusates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
</tr>
<tr>
<td>Glucose, mM</td>
</tr>
<tr>
<td>Glutamine, mM</td>
</tr>
<tr>
<td>Lactate, mM</td>
</tr>
<tr>
<td>NaCl, mM</td>
</tr>
<tr>
<td>KCl, mM</td>
</tr>
<tr>
<td>KH2PO4, mM</td>
</tr>
<tr>
<td>MgSO4 · 7H2O, mM</td>
</tr>
<tr>
<td>CaCl2, mM</td>
</tr>
<tr>
<td>Lactobionic acid, mM</td>
</tr>
<tr>
<td>Mannitol, mM</td>
</tr>
<tr>
<td>HEPEPS, mM</td>
</tr>
<tr>
<td>NaHCO3, mM</td>
</tr>
<tr>
<td>BSA, g/l</td>
</tr>
<tr>
<td>Norepinephrine HCl, mM</td>
</tr>
<tr>
<td>pH (2 mol/l HCl/NaOH)</td>
</tr>
<tr>
<td>pH after equilibration with carbogen</td>
</tr>
<tr>
<td>Osmolarity, mosmol/l</td>
</tr>
</tbody>
</table>
pended in luminal perfusion buffer at a final concentration of $3 \times 10^8$ bacteria/ml. After an initial equilibration period of 30 min with luminal and vascular buffer alone, the intestinal lumen was perfused with the prepared inoculum. Data were recorded for another 240 min. At the end of the experiment, tissue sections were taken for histology, immunofluorescence, and quantitative RT-PCR.

**Histopathological analysis.** At 240 min after infection, tissue was removed and fixed in formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E). Images were obtained by using a Nikon Eclipse TS100 microscope connected to a Nikon D5000 digital camera. The fraction of fully intact, epithelialized villi was quantified from the mesenteric and antimesenteric sides of longitudinally and circularly cut sections.

**Immunofluorescence.** Five-micrometer-thick sections were deparaffinized and rehydrated. After antigen retrieval in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), samples were blocked in 1% BSA, 2% normal goat serum, 0.1% Triton X-100, 0.05% Tween 20 in PBS. Tissues were probed with the following primary antibodies: rabbit anti-*Y. enterocolitica* O:8 serum (Sifin, Berlin), rabbit anti-Salmonella O-antigen group B (factors 4, 5) (Difco), and mouse anti-E-cadherin (Abcam). Following incubation with primary antibodies, tissues were washed extensively with PBS and probed with anti-mouse-Alexa488- and anti-rabbit-Alexa568-conjugated antibodies (Molecular Probes). Tissues were mounted in ProLong Gold Antifade (Invitrogen) containing 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Images were obtained via an Olympus FV1000 confocal microscope. Confocal sections were projected by use of ImageJ v.1.45 and were imported into Adobe Photoshop CS5 (San Jose, CA).

**Quantitative real-time polymerase chain reaction.** Three-millimeter sections of the intestine were excised and placed into RNAlater (Qiagen). Samples were homogenized by using a Qiagen TissueLyser. Sections of the intestine were excised and placed into RNAlater (Qiagen). RNA was extracted with a High Pure RNA Tissue kit (Roche). Samples were homogenized by using a Qiagen TissueLyser. Tissues were assessed 240 min following infection. Tissues were collected, fixed, paraffin embedded, and processed for H&E staining or immunofluorescence (IF). H&E staining allowed general tissue architecture to be observed while E-cadherin staining specifically assessed the integrity of the intestinal epithelium. The extent of bacterial infection into tissue was also evaluated by IF. In agreement with previous findings, when perfused with only buffer, tissue structure was preserved after 270 min (Fig. 1A). Epithelial cell junctions remained intact with E-cadherin localizing to lateral membranes (Fig. 2).

Perfusion with wild-type *S. Typhimurium* caused massive disruption of the epithelium, with most villi being devoid of epithelial cells, while some crypts maintained their epithelium (Figs. 1B and 2). Significant submucosal edema was also observed (Fig. 1B). Perfusion with wild-type *S. Typhimurium* resulted in a robust infection as demonstrated by significant

**RESULTS**

**Bacterial- and virulence factor-dependent changes to the ultrastructure and integrity of the intestinal epithelium.** After an initial equilibration period of 30 min, ex vivo intestines were perfused with either buffer alone or buffer containing bacterial inocula. After 240 min, tissues were collected, fixed, paraffin embedded, and processed for H&E staining or immunofluorescence (IF). H&E staining allowed general tissue architecture to be observed while E-cadherin staining specifically assessed the integrity of the intestinal epithelium. The extent of bacterial infection into tissue was also evaluated by IF. In agreement with previous findings, when perfused with only buffer, tissue structure was preserved after 270 min (Fig. 1A). Epithelial cell junctions remained intact with E-cadherin localizing to lateral membranes (Fig. 2).

Perfusion with wild-type *S. Typhimurium* caused massive disruption of the epithelium, with most villi being devoid of epithelial cells, while some crypts maintained their epithelium (Figs. 1B and 2). Significant submucosal edema was also observed (Fig. 1B). Perfusion with wild-type *S. Typhimurium* resulted in a robust infection as demonstrated by significant

**Fig. 1.** Histopathological analysis of the intestinal epithelium upon ex vivo infection of the perfused rat small intestine. Tissues were assessed 240 min following mock or bacterial infection. Rats were mock infected (A; control) or infected with wild-type *Salmonella enterica* serovar Typhimurium (*B: S.t. SL1444, ΔinvA*; *S. Typhimurium* (*C: S.t. invA*), wild-type *Vesicular stomatitis virus* (*D; V.e. WAP*), or plasmid-cured *Y. enterocolitica* (*E; V.e. WAC*). Scale bars = 100 μm. F: scoring of the fraction of fully epithelialized villi. Five sections were analyzed per intestine, 4–6 independent experiments per condition. ***P < 0.001 by 1-way ANOVA with Bonferroni posttest.
invasion of bacteria into the tissue (Fig. 2). To test whether SPI-1-T3SS-delivered effectors played a role in the observed changes to the intestinal epithelium, intestines were also perfused with the \( \text{Y. enterocolitica} \) invA mutant. Despite a close proximity of \( \text{S. Typhimurium} \) to the intestinal epithelium, the intestinal epithelium structure remained intact and mucosal invasion was rarely observed (Figs. 1C and 2). Perfusion with either wild-type \( \text{Y. enterocolitica} \) (WAP) or the plasmid-cured \( \text{Y. enterocolitica} \) (WAC) did not alter the tissue or epithelial architecture as observed by H&E and IF staining over the course of a 240-min infection (Figs. 1, D and E, and 2). Both WAP and WAC demonstrated an ability to invade the intestinal epithelium, however, to a lesser extent than wild-type \( \text{S. Typhimurium} \) (Fig. 2). The fraction of fully intact, epithelialized villi was quantified from the mesenteric and antimesenteric sides of longitudinally and circular cut sections. Infection with wild-
type *S*. Typhimurium resulted in a significant reduction in fully epithelialized villi compared with uninfected controls (Fig. 1F, \( P < 0.001 \)) and in contrast to *S*. Typhimurium \( \Delta \text{invA} \) or *Yersinia* strains. Therefore, ultrastructural changes to the epithelium were both pathogen and virulence factor dependent and, because of the nature of the model (no circulating cells), these effects were independent of recruited cell populations.

**Bacterial- and virulence factor-dependent induction of cytokines in resident cell populations.** Next, we wanted to know whether in this model, *S*. Typhimurium and *Y*. enterocolitica could induce an early proinflammatory immune response. Because of the absence of circulating cells, only resident cell populations would be responsible for any proinflammatory cytokines produced. qRT-PCR was used to assess TNF-\( \alpha \), IL-17, and MCP-1 gene expression in intestinal tissue homogenates 240 min postinfection. In response to wild-type *S*. Typhimurium infection, MCP-1 (\( P < 0.05 \)), TNF-\( \alpha \) (\( P < 0.01 \)), and IL-17 (\( P < 0.001 \)) were significantly upregulated in resident cell populations compared with uninfected controls (Fig. 3). In contrast, infection by *S*. Typhimurium \( \Delta \text{invA} \) or *Y*. enterocolitica WAP or WAC did not significantly increase expression of these cytokines compared with uninfected controls. Therefore, in this model, *S*. Typhimurium induced expression of MCP-1, TNF-\( \alpha \), and IL-17 in resident cell populations in a SPI-1-T3SS-dependent manner.

*S*. Typhimurium increases vascular resistance in the small intestine in a SPI-1-T3SS-dependent manner. Arterial pressure was recorded in real time during the perfusion period. Beginning at 90 min postperfusion (60 min postinfection), there was a significant increase in arterial pressure compared with control conditions upon infection with wild-type *S*. Typhimurium (Fig. 4). Conversely, there were no significant changes in arterial pressure upon infection with *S*. Typhimurium \( \Delta \text{invA} \) or *Y*. enterocolitica WAP or WAC strains. Therefore, *S*. Typhimurium induces transient vasoconstriction in the small intestine that is dependent on SPI-1-T3SS-delivered effectors, but independent of recruited cell populations.

*S*. Typhimurium compromises the digestive and absorptive functions of the small intestine. In our ex vivo perfusion model, the transfer of galactose (from the digestion of luminal lactose) to the venous compartment was monitored, thereby providing further critical information on vital organ function during control conditions and over the course of the bacterial infection. Due to recirculation of the vascular buffer, a steady increase in venous galactose was expected over time when the metabolic competence of the intestinal tissue was maintained. Indeed, this was the case under control conditions (Fig. 5A). After a 240-min infection by wild-type *S*. Typhimurium, significantly less galactose was present in the vascular effluent compared with uninfected control conditions (Fig. 5B). Although impaired nutrient absorption and digestion might be expected due to the significant destruction of the intestinal epithelium upon wild-type *S*. Typhimurium (Figs. 1 and 2), interestingly, we also observed a significant reduction in venous galactose upon infection with *S*. Typhimurium \( \Delta \text{invA} \) (Fig. 5B). Vascular galactose was also reduced upon infection with *Y*. enterocolitica WAP and WAC strains; however, this decrease was not statistically different than control conditions during the 240-min infection period (Fig. 5B).

**Bacterial invasion and inflammation induction in resident cell populations are sufficient for fluid accumulation in the lumen upon *S*. Typhimurium infection.** Our previous studies demonstrated that this ex vivo model system is receptive to infection with *S*. Typhimurium. Interestingly, we also observed a significant reduction in venous galactose upon infection with *S*. Typhimurium \( \Delta \text{invA} \) (Fig. 5B). Vascular galactose was also reduced upon infection with *Y*. enterocolitica WAP and WAC strains; however, this decrease was not statistically different than control conditions during the 240-min infection period (Fig. 5B).

*S*. Typhimurium compromises the digestive and absorptive functions of the small intestine. In our ex vivo perfusion model, the transfer of galactose (from the digestion of luminal lactose) to the venous compartment was monitored, thereby providing further critical information on vital organ function during control conditions and over the course of the bacterial infection. Due to recirculation of the vascular buffer, a steady increase in venous galactose was expected over time when the metabolic competence of the intestinal tissue was maintained. Indeed, this was the case under control conditions (Fig. 5A). After a 240-min infection by wild-type *S*. Typhimurium, significantly less galactose was present in the vascular effluent compared with uninfected control conditions (Fig. 5B). Although impaired nutrient absorption and digestion might be expected due to the significant destruction of the intestinal epithelium upon wild-type *S*. Typhimurium (Figs. 1 and 2), interestingly, we also observed a significant reduction in venous galactose upon infection with *S*. Typhimurium \( \Delta \text{invA} \) (Fig. 5B). Vascular galactose was also reduced upon infection with *Y*. enterocolitica WAP and WAC strains; however, this decrease was not statistically different than control conditions during the 240-min infection period (Fig. 5B).

**Bacterial invasion and inflammation induction in resident cell populations are sufficient for fluid accumulation in the lumen upon *S*. Typhimurium infection.** Our previous studies demonstrated that this ex vivo model system is receptive to...

---

Fig. 3. Induction of cytokine expression upon ex vivo infection of the perfused rat small intestine as determined by qPCR. Gene expression was assessed 240 min following mock or bacterial infection. Data were normalized to GAPDH levels and are relative to uninfected controls. Values are expressed as means \( \pm \) SE of 4 – 6 independent experiments, each performed with 2 tissue samples per animal. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) compared with control conditions by 1-way ANOVA with Bonferroni posttest. Y.e., *Y*. enterocolitica; S.t., *S*. Typhimurium.

Fig. 4. Changes in arterial pressure (A\( P \) arterial) during infection of isolated perfused rat small intestines. Data are displayed as the change in pressure over the indicated time period and represent the means \( \pm \) SE of 4 – 6 independent experiments. *\( P < 0.05 \), ***\( P < 0.001 \) compared with control conditions by 1-way ANOVA with Bonferroni posttest. Y.e., *Y*. enterocolitica; S.t., *S*. Typhimurium.
physiologically relevant stimuli and is suitable for studying fluid dynamics in the small intestine (21, 22). Accordingly, next, we wanted to test whether this model could be used to investigate the pathophysiological mechanisms leading to fluid loss during infectious diarrhea. The intestinal lumen was perfused with 0.05 ml of perfusate (± bacteria) per minute and the volume of luminal effluent was measured every 30 min. FITC-dextran (1,700 μg/ml) was included in the luminal perfusate and its concentration in the luminal effluent was monitored every 30 min. In the absence of absorption or secretion one would expect to collect 1.5 ml of luminal effluent (Fig. 6A, dotted line) containing 1,700 μg/ml of FITC-dextran (Fig. 6B, dotted line) every 30 min. Under control conditions, net absorption of fluid was observed as indicated by slightly less luminal effluent collected at the distal end of the intestine (Fig. 6A) and an increase in FITC dextran in the lumen over time (Fig. 6B). Beginning at 150–180 min, wild-type S. Typhimurium induced a sustained significant increase in luminal effluent volume (Fig. 6A). The S. Typhimurium ΔinvA mutant or the Y. enterocolitica WAP and WAC strains did not significantly alter the luminal effluent during the course of the experiment. As a result of the increase in luminal fluid upon wild-type S. Typhimurium infection, beginning at 150–180 min there was a concomitant significant decrease in the concentration of luminal FITC-dextran compared with control conditions due to dilution from fluids entering the lumen (Fig. 6, B and C, P < 0.01). On the other hand, similar to control conditions, S. Typhimurium ΔinvA as well as Y. enterocolitica WAP and WAC strains all demonstrated an increase in the concentration of FITC dextran in the lumen over time (Fig. 6, B and C). Therefore, in this model, SPI-1-T3SS-dependent S. Typhimurium invasion and inflammation induction in resident cell populations are sufficient for fluid accumulation in the lumen.

DISCUSSION

In this paper we describe the adaptation of an ex vivo perfused rat small intestinal model to study physiological changes to the intestine upon infection with invasive enteropathogenic bacteria. A major advantage of this system over whole mouse infections is that bacteria can synchronously infect the gut, minimizing experimental variation seen in whole mouse infections and allowing for a detailed analysis of early bacterial-host interactions in a time-dependent manner. Specifically, we monitored infection, inflammation induction, vasoactivity, nutrient absorption, and luminal fluid accumulation over the course of a 240-min infection with S. Typhimurium or Y. enterocolitica and the contribution of major virulence factors was assessed.

Only in germ-free (23, 36, 37), low-complexity flora (7), or antibiotic-pretreated mice does S. Typhimurium infection elicit significant intestinal inflammation in a rodent model (1). The absence or reduction of intestinal microflora enables S. Typhimurium to overcome the colonization resistance presented by the natural microbiota and thereby efficiently colonize the intestine. In our ex vivo model, we observe significant pathology and induction of inflammatory cytokines upon infection with wild-type S. Typhimurium (Figs. 1 and 3). Despite a moderate perfusion rate of 0.05 ml/min, the microbiota is likely drastically reduced in our ex vivo model through physical,
rather than chemical means. Consequently, colonization resistance is overcome through physical disruption of the microbiota, allowing for examination of the pathophysiological effects of *S. Typhimurium* infection on the small intestine.

Robust infection of wild-type *S. Typhimurium* into the intestinal tissue was observed, leading to significant pathological damage to the epithelial layer (Fig. 1 and 2). Submucosal edema and epithelial erosion have similarly been observed in other in vivo models of *S. Typhimurium* enteritis including in rhesus macaques (19, 31, 32), cattle (33, 38, 41), and streptomycin-pretreated mice (1). Similar to *S. Typhimurium* infection of rhesus macaques and cattle, in our ex vivo model we observe infection and pathological changes in the small intestine within a short time period. In streptomycin-pretreated mice, pathology also occurs rapidly (as early as 8 h after oral infection) but is restricted to the large intestine (1). Wild-type *S. Typhimurium* also caused a significant increase in the expression of inflammatory cytokines. Both the pathological damage and the increase in cytokine expression were dependent on effectors secreted by the SPI1 T3SS, since infection with an isogenic mutant defective in SPI1 type 3 secretion (*S. Typhimurium invA*) did not result in any observable changes to the intestinal tissue or cytokine expression levels (Figs. 1–3). This dependence on the SPI1 T3SS agrees with findings from other in vivo models of *Salmonella*-induced enteritis, including cattle (38), rhesus macaques (32), and streptomycin-pretreated mice (1). Epithelial tight junction disruption by *S. Typhimurium* has been shown in vitro and is also dependent on SPI1 T3SS-delivered effectors (3, 30). The ability of SPI1 T3SS-delivered effectors to stimulate Rho GTPases, thereby disrupting tight junctions (3), likely contributes to the damage to the epithelium we observe. Therefore, our ex vivo model closely models aspects of early *S. Typhimurium* infection in the small intestine using a small animal model.

Despite invasion of wild-type *Y. enterocolitica* into the submucosa, no changes to the integrity of the epithelial layer could be observed by histological or immunofluorescent analysis (Figs. 1 and 2). This demonstrates that the pathophysiological effects of wild-type *S. Typhimurium* were specific to this pathogen and not a more generalized result of luminal treatment with bacteria. *Y. enterocolitica* were grown at 27°C prior to infection to maximize their invasion potential (25). Nevertheless, *Y. enterocolitica* likely has different requirements for a robust infection that were not met in this model system, whether that be the length of the infection period or the location of the intestine utilized. Histopathological changes and inflammation induction in the ileum of mice is often not seen until 24 h after oral infection with *Y. enterocolitica* (13, 14, 28). We chose to focus on the early interaction between pathogens and the intestine. For physiological reasons, the system is limited to studying the jejunum and proximal half of the ileum. In several animal models of intestinal infection (e.g., rodent or pig), *Y. enterocolitica* primarily infects the terminal ileum and cecum. Perhaps the distal segment of the ileum, where M cell-rich Peyer’s patches are more abundant, would have been required to observe a more pronounced infection of
both *Yersinia* and *Salmonella ΔinvA*. It is interesting to note that, despite invasion of *Y. enterocolitica* into the mucosa, we did not detect inflammatory cytokine induction (Fig. 3). The virulence plasmid of wild-type *Y. enterocolitica* encodes a T3SS and associated secreted effectors that are well known to dampen inflammatory responses during infection (2). Therefore, one could speculate that injection of these effectors may be responsible for the lack of inflammatory responses observed with the wild-type strain at these early time points.

Our ex vivo model enables the dynamics of localized vascular smooth muscle activity to be monitored in real time through online vascular pressure measurements. For the first time, we have been able to demonstrate that wild-type *S*. Typhimurium induces localized vasoconstriction in small intestine (Fig. 4). We have previously observed vasoconstriction with this model upon treatment with the proinflammatory mediator platelet-activating factor (PAF) (21). Interestingly, *S*. Typhimurium is reported to induce the release of PAF from human intestinal tissue and cell lines (6). Vasoconstriction was dependent on the presence of the SPI1 T3SS. Several SPI1 type 3 secreted effectors are known to activate Rho family GTPases (35). Vasoconstriction is highly dependent on Rho GTPase activation (24), and, therefore, future use of this ex vivo model will determine specifically which virulence factors are required for intestinal vasoconstriction and the signaling mechanisms involved.

Impaired digestive and absorptive functions of the small intestine have been observed during periods of inflammation (29). Upon ex vivo infection in the rat small intestine with either wild-type *S*. Typhimurium or the ΔinvA mutant, we observed a significant reduction in the metabolic competence of the small intestine compared with control conditions. Although the decrease in galactose uptake upon ΔinvA infection is less pronounced than with wild-type infection, this difference is not statistically significant (Fig. 5B, NS). However, we speculate that mild inflammation triggered by the ΔinvA mutant (Fig. 3, TNF-α and IL-17) is sufficient to reduce nutrient uptake and digestion during ex vivo perfusion.

In streptomycin-pretreated mice, *S*. Typhimurium infection leads to only a very mild secretory response that is limited to a slight impairment in the formation of fecal pellets in the colon (1). Therefore this model is unsuitable to probe the mechanisms leading to diarrhea. Fluid secretion in response to *S*. Typhimurium infection has been observed in ileal loops of rabbits (8, 11, 12, 39, 40), rhesus macaques (20, 31, 32), and calves (5). Data from the calf model in particular has provided significant insight into the virulence factors required for fluid accumulation in the lumen. A functioning SPI1 T3SS is necessary for fluid secretion in calves and, more specifically, it is the SPI1-secreted effectors SipA, SopA, SopB, SopD, and SopE2 that are required (41). During ex vivo perfusion of the rat small intestine, we observed significant fluid secretion into the lumen upon infection with wild-type *S*. Typhimurium. This effect was dependent on SPI1 type 3-secreted effectors, since infection with an ΔinvA mutant did not lead to an increase in luminal fluid. The ability to study *Salmonella*-induced intestinal fluid dynamics in a small animal model is a major advantage of this model.

Mice and rabbits are known to experience fluid loss upon *Y. enterocolitica* infection. Limited studies have investigated the mechanisms of *Y. enterocolitica*-induced fluid loss in vivo. Intestinal epithelial barrier disruption by *Y. enterocolitica* has been observed in vitro (16), which could also occur in vivo, thereby contributing to fluid loss. However, during the 240-min ex vivo infection, we did not observe fluid secretion with either the wild-type or plasmid cured strain. As previously mentioned, this is likely because the infection time was too short or the intestinal location was not optimal for efficient infection.

One caveat of the ex vivo perfused small intestinal model is that it does not allow for the examination of recruited cell populations (e.g., neutrophils). On the other hand, it gives one the unique possibility to specifically determine the contribution of resident cells to pathological processes. In the case of *S*. Typhimurium infection, we can conclude that although the recruitment of neutrophils likely does contribute to pathology, inflammation, and fluid loss in vivo, our results clearly show they are not absolutely necessary for these effects. *S*. Typhimurium is thought to induce exudative diarrhea. In *S*. Typhimurium-infected rhesus macaques and cattle, neutrophilic influx into the intestinal mucosa correlates with the extent of fluid accumulation (27, 32, 41). Therefore, fluid loss has been hypothesized to be due to damage specifically caused by recruited neutrophils. The data presented here suggests that bacterial invasion and inflammation induction in resident cell populations are sufficient for fluid loss into the lumen.

In summary, this ex vivo infection system models inflammation induction and fluid accumulation and identifies novel aspects of the intestinal pathology associated with *S*. Typhimurium enteritis. This model can also now be used to probe the host and pathogen-mediated mechanisms involved in fluid secretion, nutrient absorption deficiency, and vasoconstriction upon *S*. Typhimurium infection. Transgenic rats, although more limited than mouse strains, offer the possibility to investigate host requirements for the pathophysiological effects on bacterial infection on the small intestine. Current work in our group is focused on the development of a similar system in the mouse small intestine.

ACKNOWLEDGMENTS

The authors thank Tobias Spielmann for use of his confocal microscope. Present address for E. C. Boyle: Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany.

GRANTS

E. C. Boyle was supported by a postdoctoral research fellowship from the Alexander von Humboldt Foundation. G. A. Grassl was supported by the Cluster of Excellence “Inflammation at Interfaces” (Borstel-Kiel-Lübeck-Plön; EXC306GTP4).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

G63 Type III secretion system.


