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Pretreatment of Mice with Streptomycin Provides a *Salmonella enterica* Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host

Manja Barthel,[1,2] Siegfried Hapfelmeier,[1,2] Leticia Quintanilla-Martínez,[3] Marcus Kremer,[3,4] Manfred Rohde,[5] Michael Hogardt,[2] Klaus Pfeffer,[6] Holger Rüssmann,[2] and Wolf-Dietrich Hardt[1]*

Institute of Microbiology, ETH Zürich, 8092 Zürich, Switzerland,[1] and Max von Pettenkofer-Institut, Ludwig Maximilians Universität, 80336 Munich,[2] Institute of Medical Microbiology, Immunology, and Hygiene[6] and Institute of Pathology,[4] Technical University of Munich, 81675 Munich, GSF-Research Center for Environment and Health, 85764 Neuherberg,[3] and GBF, 38124 Braunschweig,[5] Germany

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Salmonella enterica **subspecies 1 serovar Typhimurium is a principal cause of human enterocolitis. For unknown reasons, in mice serovar Typhimurium does not provoke intestinal inflammation but rather targets the gut-associated lymphatic tissues and causes a systemic typhoid-like infection. The lack of a suitable murine model has limited the analysis of the pathogenetic mechanisms of intestinal salmonellosis. We describe here how streptomycin-pretreated mice provide a mouse model for serovar Typhimurium colitis. Serovar Typhimurium colitis in streptomycin-pretreated mice resembles many aspects of the human infection, including epithelial ulceration, edema, induction of intercellular adhesion molecule 1, and massive infiltration of PMN/CD18$^+$ cells. This pathology is strongly dependent on protein translocation via the serovar Typhimurium SPI1 type III secretion system. Using a lymphotoxin β-receptor knockout mouse strain that lacks all lymph nodes and organized gut-associated lymphatic tissues, we demonstrate that Peyer's patches and mesenteric lymph nodes are dispensable for the initiation of murine serovar Typhimurium colitis. Our results demonstrate that streptomycin-pretreated mice offer a unique infection model that allows for the first time to use mutants of both the pathogen and the host to study the molecular mechanisms of enteric salmonellosis.**

Salmonella spp. are gram-negative enterobacteria that cause diseases ranging from a self-limiting enterocolitis to systemic infection (typhoid fever). *Salmonella enterica* serovar Typhimurium evokes a common form of nonsystemic enterocolitis in humans and cattle, whereas mice are intrinsically resistant to serovar Typhimurium enterocolitis (68, 81). Although resistant to intestinal salmonellosis, certain susceptible mouse strains that carry mutations in the NRAMP gene develop a disease similar to typhoid fever (30, 75).

After oral infection of susceptible mice, serovar Typhimurium does not replicate efficiently in the intestine but penetrates the epithelial barrier by invasion of M cells (12, 41, 64) or (less efficiently) by transport via CD18$^+$/dendritic cells (67, 80) and possibly by penetration of enterocytes (72). After penetration of the epithelial barrier, *Salmonella* spp. colonize Peyer's patches and mesenteric lymph nodes and then spread to the liver and spleen, and the mice finally succumb to systemic infection (10, 35, 75, 81). However, mice show few signs of the intestinal inflammation observed in cattle or humans.

Due to the lack of a versatile animal model, much less is known about the mechanisms of the enteric salmonellosis (21, 33, 62, 75, 81). To overcome these limitations, the pathogenesis of enteric salmonellosis has been studied by extrapolating data from tissue culture (review by Galan [26]) or from intestinal organ culture (1) or by infection of ligated murine and rabbit ileal loops (11, 12, 20, 41, 63, 64). However, it remains unclear how these results relate to enteric salmonellosis.

For this reason, bovine infection models with serovar Typhimurium (and serovar Dublin) have been established recently to study *Salmonella*-associated enterocolitis (reviewed previously [68, 75, 81]). Bovine models have allowed the identification of several *Salmonella*-associated factors required to evoke enterocolitis, including flagella (71), *aroAD* (42, 73), lipopolysaccharide (LPS) (73), and the SPI1 type III secretion system (75, 81, 86). This type III secretion system allows *Salmonella* spp. to inject (translocate) bacterial toxins (effector proteins) directly into the cytosol of host cells (13, 83) to manipulate host responses (23, 26) in order to invade the bovine intestinal epithelium and the Peyer's patches and induce inflammatory responses (28, 73, 74, 82). However, due to severe technical limitations, the bovine model has allowed only limited analysis of the contribution of the host in the complex interplay with serovar Typhimurium leading to enterocolitis. For example, it is still a matter of dispute whether intestinal inflammation is initiated by direct interaction with epithelial cells (reviewed in reference 26) or by colonization of the gut-associated lymphatic tissues (GALT; see reviews in references 79 and 81).

The gut-associated immune system is composed of diffusely distributed (dendritic, B, cytotoxic T, and NK) cells and organized lymphoid tissues (i.e., Peyer's patches and mesenteric lymph nodes) and coordinates appropriate responses to antigens derived from food, commensal bacteria, and pathogenic microorganisms (32, 58). Organogenesis of the gut-associated lymphoid tissues is determined by developmental programs,

* Corresponding author. Mailing address: Institute of Microbiology, ETH Zürich, Schmelzbergstr. 7, 8092 Zürich, Switzerland. Phone: 41-1-632-5143. Fax: 41-1-632-1129. E-mail: hardt@micro.biol.ethz.ch.

cytokines, and the exposure to antigens (46). Gene targeting has shown that the interleukin-7 receptor, the tumor necrosis factor (TNF), lymphotoxin (LT α_3, LT $\alpha_2\beta_1$, and LT $\alpha_1\beta_2$), and the TNF receptor (TNFR) superfamily are of fundamental importance in this process (24). However, only lymphotoxin β receptor (LTβR; a member of the TNFR superfamily) knockout mice (LTβR$^{-/-}$) are completely devoid of Peyer's patches, colon-associated lymphoid tissues, the cecum-associated lymphatic patch, and all lymph nodes (25). Therefore, LTβR$^{-/-}$ mice allow one to analyze the functional requirement of organized GALT in bacterial infection.

We have found that streptomycin-pretreated mice develop colitis upon infection with serovar Typhimurium. This inflammation is evoked by specific bacterial virulence factors. Furthermore, our data show that the organized GALT are dispensable for murine serovar Typhimurium colitis. Due to the availability of a broad variety of knockout mouse strains, the streptomycin-pretreated mouse model will be of great advantage compared to existing animal models: it allows study of the role of the host defense mechanisms in intestinal salmonellosis in great detail.

MATERIALS AND METHODS

Bacterial strains. The naturally streptomycin-resistant wild-type strain *S. enterica* serovar Typhimurium SL1344 (36) and the isogenic mutants SB161 (SL1344, Δ*invG* [43]), SB302 (SL1344, *invJ*::*aphT* [15]), and SB241 (SL1344, *sipD*::*aphT* [44]) were generously provided by J. E. Galan. The nonpathogenic *Lactobacillus* strain was a gift from A. Macpherson.

Serovar Typhimurium strains were grown for 12 h at 37°C in Luria-Bertani broth supplemented with 0.3 M NaCl, diluted 1:20 in fresh medium, and subcultured for 4 h under mild aeration. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS) and then suspended in cold PBS (2×10^3 or 10^8 CFU/50 μl).

Lactobacillus spp. were grown on MRS agar (Biolife, Milano, Italy) at 37°C under an anaerobic CO_2-enriched atmosphere (Anaerocult A; Merck, Darmstadt, Germany). Bacteria were scraped from the plates, washed with cold PBS, and resuspended in PBS to yield a final concentration of 10^8 CFU/50 μl.

Animal experiments. Specific-pathogen-free (SPF) female mice were from Harlan Winkelmann (C57BL/6; 6 to 8 weeks old; Borchen, Germany) or Charles River (LTβR$^{-/-}$ mice, C57BL/6 background; 6 to 8 weeks old; Sulzfeld, Germany [25]). Genotypes were verified by PCR typing with the primers 5′-CGG GTC TCC GAC CTA GAG ATC-3′ and 5′-GAG GTG GGT GGA TTG GAA AGA G-3′.

For the experiments, animals were housed individually or in groups of up to five animals under standard barrier conditions in individually ventilated cages (Tecniplast, Buguggiate, Italy) equipped with steel grid floors and autoclaved filter paper at the Max von Pettenkofer Institute (Munich, Germany) or BZL (Zurich, Switzerland).

Water and food were withdrawn 4 h before per os (p.o.) treatment with 20 mg of streptomycin (75 μl of sterile solution or 75 μl of sterile water [control]). Afterward, animals were supplied with water and food ad libitum. At 20 h after streptomycin treatment, water and food were withdrawn again for 4 h before the mice were infected with 10^8 CFU of serovar Typhimurium (50-μl suspension in PBS p.o.) or treated with sterile PBS (control). Thereafter, drinking water ad libitum was offered immediately and food 2 h postinfection (p.i.). At the indicated times p.i., mice were sacrificed by CO_2 asphyxiation, and tissue samples from the intestinal tracts, mesenteric lymph nodes, spleens, and livers were removed for analysis.

Animal experiments were approved by the German and Swiss authorities and performed according to the legal requirements.

Analysis of serovar Typhimurium loads in intestines, mesenteric lymph nodes, spleens, and livers. Two fresh fecal pellets were placed in 500 μl of 4°C cold PBS and suspended homogeneously on ice by vortexing and pipetting. Intestinal contents from the ileum, cecum, or colon were collected at the indicated times p.i. and weighed before resuspending them in 500 μl of 4°C cold PBS. The numbers of CFU were determined by plating appropriate dilutions on MacConkey agar plates (streptomycin at 50 μg/ml). The minimum detectable value was 5 CFU/fecal pellet and 10 CFU/sample (between 25 and 150 mg) of intestinal content.

Intestinal colonization by *Lactobacillus* spp. was determined by resuspending 50 mg of cecal content in 500 μl of ice-cold PBS, plating appropriate dilutions on MRS agar (Biolife, Milano, Italy) without delay, and incubation for 48 h at 37°C under an anaerobic CO_2-enriched atmosphere (Anaerocult A).

To analyze the colonization, mesenteric lymph nodes, spleens, and livers were removed aseptically and homogenized in 4°C cold PBS (0.5% Tergitol, 0.5% bovine serum albumin) by using a Potter homogenizer. The numbers of CFU were determined by plating appropriate dilutions on MacConkey agar plates (streptomycin at 50 μg/ml). The minimal detectable values were 20 CFU/organ in the spleen, 100 CFU/organ in the liver, and 10 CFU/organ in the mesenteric lymph nodes.

Histological procedures. Segments of the ileum, cecum, and colon were fixed and embedded in paraffin according to standard procedures. Alternatively, tissue samples were embedded in O.C.T. (Sakura, Torrance, Calif.), snap-frozen in liquid nitrogen, and stored at −80°C. Cryosections (5 or 30 μm) were mounted on glass slides, air dried for 2 h at room temperature, and stained with hematoxylin and eosin (H&E). Pathological evaluation was performed by two pathologists in a blinded manner.

Based on an earlier study (49), we developed a scoring scheme for quantitative pathological analysis of cecal inflammation. H&E-stained sections (5 μm) were scored independently by two pathologists in a blinded manner as follows.

(i) Submucosal edema. Submucosal edema was scored as follows: 0 = no pathological changes; 1 = mild edema (the submucosa is <0.20 mm wide and accounts for <50% of the diameter of the entire intestinal wall [tunica muscularis to epithelium]); 2 = moderate edema; the submucosa is 0.21 to 0.45 mm wide and accounts for 50 to 80% of the diameter of the entire intestinal wall; and 3 = profound edema (the submucosa is >0.46 mm wide and accounts for >80% of the diameter of the entire intestinal wall). The submucosa widths were determined by quantitative microscopy and represent the averages of 30 evenly spaced radial measurements of the distance between the tunica muscularis and the lamina mucosalis mucosae.

(ii) PMN infiltration into the lamina propria. Polymorphonuclear granulocytes (PMN) in the lamina propria were enumerated in 10 high-power fields (×400 magnification; field diameter of 420 μm), and the average number of PMN/high-power field was calculated. The scores were defined as follows: 0 = <5 PMN/high-power field; 1 = 5 to 20 PMN/high-power field; 2 = 21 to 60/high-power field; 3 = 61 to 100/high-power field; and 4 = >100/high-power field. Transmigration of PMN into the intestinal lumen was consistently observed when the number of PMN was >60 PMN/high-power field.

(iii) Goblet cells. The average number of goblet cells per high-power field (magnification, ×400) was determined from 10 different regions of the cecal epithelium. Scoring was as follows: 0 = >28 goblet cells/high-power field (magnification, ×400; in the cecum of the normal SPF mice we observed an average of 6.4 crypts/high-power field and the average crypt consisted of 35 to 42 epithelial cells, 25 to 35% of which were differentiated into goblet cells); 1 = 11 to 28 goblet cells/high-power field; 2 = 1 to 10 goblet cells/high-power field; and 3 = <1 goblet cell/high-power field.

(iv) Epithelial integrity. Epithelial integrity was scored as follows: 0 = no pathological changes detectable in 10 high-power fields (×400 magnification); 1 = epithelial desquamation; 2 = erosion of the epithelial surface (gaps of 1 to 10 epithelial cells/lesion); and 3 = epithelial ulceration (gaps of >10 epithelial cells/lesion; at this stage, there is generally granulation tissue below the epithelium).

We averaged the two independent scores for submucosal edema, PMN infiltration, goblet cells, and epithelial integrity for each tissue sample. The combined pathological score for each tissue sample was determined as the sum of these averaged scores. It ranges between 0 and 13 arbitrary units and covers the following levels of inflammation: 0 = intestine intact without any signs of inflammation; 1 to 2 = minimal signs of inflammation (this was frequently found in the ceca of SPF mice; this level of inflammation is generally not considered as a sign of disease); 3 to 4 = slight inflammation; 5 to 8 = moderate inflammation; and 9 to 13 = profound inflammation.

Statistical analysis. Statistical analysis of the cecum weight, the individual pathological scores for submucosal edema, PMN infiltration, goblet cells, and epithelial integrity and for the combined pathological score was performed by using the exact Mann-Whitney U test and SPSS version 11.0 software. *P* values of <0.05 were considered statistically significant. This procedure was adopted from that of Madsen et al. (49).

To allow the statistical analysis of the bacterial loads, values for animals yielding "no CFU" were set to the minimal detectable value (fecal pellet = 5 CFU; spleen = 20 CFU; liver = 100 CFU; mesenteric lymph nodes = 10 CFU;

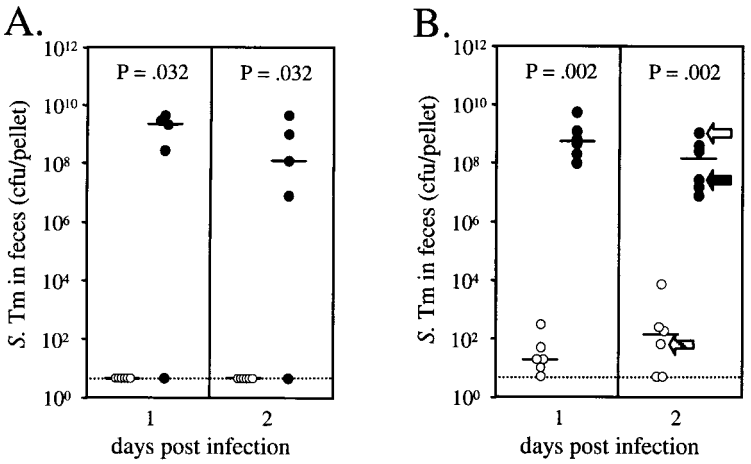


FIG. 1. Streptomycin pretreatment allows efficient colonization of the murine intestine by serovar Typhimurium. (A) Infection with an inoculum of 2×10^3 serovar Typhimurium. Five mice (C57BL/6) were treated with 20 mg of streptomycin (●) or water (○) p.o. After 24 h, they were infected with 2×10^3 CFU of serovar Typhimurium SL1344. (B) Infection with an inoculum of 10^8 CFU of serovar Typhimurium. Six mice (C57BL/6) were treated with 20 mg of streptomycin (●) or water (○) p.o. After 24 h, they were infected with 10^8 CFU of serovar Typhimurium SL1344. We monitored the excretion of serovar Typhimurium (CFU per fecal pellet) at days 1 and 2 p.i. (see Materials and Methods). The dashed line indicates the limit of detection; bars indicate the median; and "P" refers to the *P* value (Mann-Whitney U test; see Materials and Methods). Arrows indicate mice selected for histopathological analysis (see Fig. 2).

intestinal content = between 67 and 400 CFU [see above]). Afterward, the median values were calculated by using Microsoft Excel XP, and statistical analysis was performed by using the exact Mann-Whitney U test and the SPSS version 11.0 software. *P* values of <0.05 were considered statistically significant.

Immunohistological procedures. Cryosections (5 μm) of OCT-embedded tissue samples from the ileum, cecum, and colon of each animal were mounted on glass slides (Sigma), air dried at room temperature for 2 h, fixed (PBS, 3.7% formaldehyde; 1 h at room temperature), washed in PBS, permeabilized with Triton X-100 (0.1% in PBS, 10 min at room temperature), washed in PBS, and blocked with goat serum (20% in PBS, overnight at 4°C). Monoclonal rat αCD18 (1:100), hamster α-intercellular adhesion molecule-1 (ICAM-1; 1:100), and polyclonal rabbit α-*Salmonella* O antigen group B (factors 1, 4, 5, and 12) antiserum (1:300) were from Becton Dickinson (San Diego, Calif.). Fluorescein isothiocyanate (FITC)-conjugated goat α-rabbit (1:200), FITC-conjugated goat α-rat (1:100), and Cy3-conjugated goat α-hamster (1:200) polyclonal antibodies were from Dianova (Hamburg, Germany). Controls with appropriate species- and isotype-matched monoclonal antibodies (Becton Dickinson) were performed to ensure specific detection of the antigens. DAPI (4′,6′-diamidino-2-phenylindole; 0.5 μg/ml; Sigma) was used for nucleic acid staining. Staining was performed with PBS (20% goat serum). Sections were washed with PBS and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, Calif.). Images (see Fig. 11E and F and 14) were obtained with a Leica DM epifluorescence microscope and a Visitron spot charge-coupled device camera system. Other images (see Fig. 9I to P and 11G and H) were recorded by using a Perkin-Elmer Ultraview confocal imaging system and a Zeiss Axiovert 200 microscope: red and green fluorescence was recorded confocally, and the blue fluorescence was determined by epifluorescence microscopy. Afterwards, the red, green, and blue images were superimposed by using Adobe Photoshop software, ensuring that all panels from each figure were processed in the same way.

RESULTS

Serovar Typhimurium colonizes the lower intestine of streptomycin-pretreated mice and causes colitis. Murine salmonellosis is generally not associated with efficient colonization of the intestine and overt pathological changes of the intestinal mucosa. However, it has long been known that oral treatment with streptomycin reduces the oral 50% infectious dose of serovar Enteritidis or serovar Typhimurium by 10^5- to 10^6-fold and greatly improves intestinal colonization (2, 3, 9, 53, 54, 57, 66). This effect has been attributed to the elimination of commensal intestinal bacteria (4, 5, 55, 65). However, to our knowledge it has never been determined whether streptomycin-pretreated mice colonized with serovar Typhimurium develop enterocolitis.

In a first experiment, five streptomycin-pretreated (20 mg p.o.) and five water-pretreated C57BL/6 mice were placed in separate cages. At 24 h after the pretreatment the mice were infected p.o. with a dose of 2×10^3 CFU of the virulent serovar Typhimurium strain SL1344. In agreement with earlier reports (2, 3, 9, 53, 54, 57, 66), we found that the streptomycin-pretreated mice were excreting significantly larger quantities (10^7 to 10^{10} CFU/fecal pellet) of serovar Typhimurium than the water-pretreated control mice at days 1 and 2 p.i. (Fig. 1A; $P = 0.032$). However, one of the five streptomycin-pretreated mice was not excreting detectable quantities (<5 CFU/fecal pellet) of serovar Typhimurium (Fig. 1A). Therefore, we repeated the experiment with six animals per group with a higher inoculum (10^8 CFU of serovar Typhimurium p.o.; Fig. 1B). Again, we found that the streptomycin-pretreated mice were excreting significantly higher amounts (10^7 to 10^{10} CFU/fecal pellet; $P = 0.002$) of serovar Typhimurium than the water-pretreated control mice at days 1 and 2 p.i. (Fig. 1B). In this experiment we detected large quantities of serovar Typhimurium in the feces of all six streptomycin-pretreated animals. Therefore, we have used an inoculum of 10^8 CFU p.o. throughout the rest of the present study.

To analyze serovar Typhimurium-induced intestinal pathology, two of the streptomycin-pretreated mice (Fig. 1B) and one mouse from the control group (Fig. 1B) were sacrificed 2 days p.i., and the internal organs were fixed and embedded in paraffin (see Materials and Methods). Histopathological analysis of 5-μm thin sections revealed pronounced inflammation of the cecum (Fig. 2A and C) of the streptomycin-pretreated mice colonized with serovar Typhimurium. In the cecum of both mice, we observed pronounced edema in the submucosa, edematous changes in the lamina propria, crypt elongation, disruption of the crypt architecture, reduced numbers of goblet

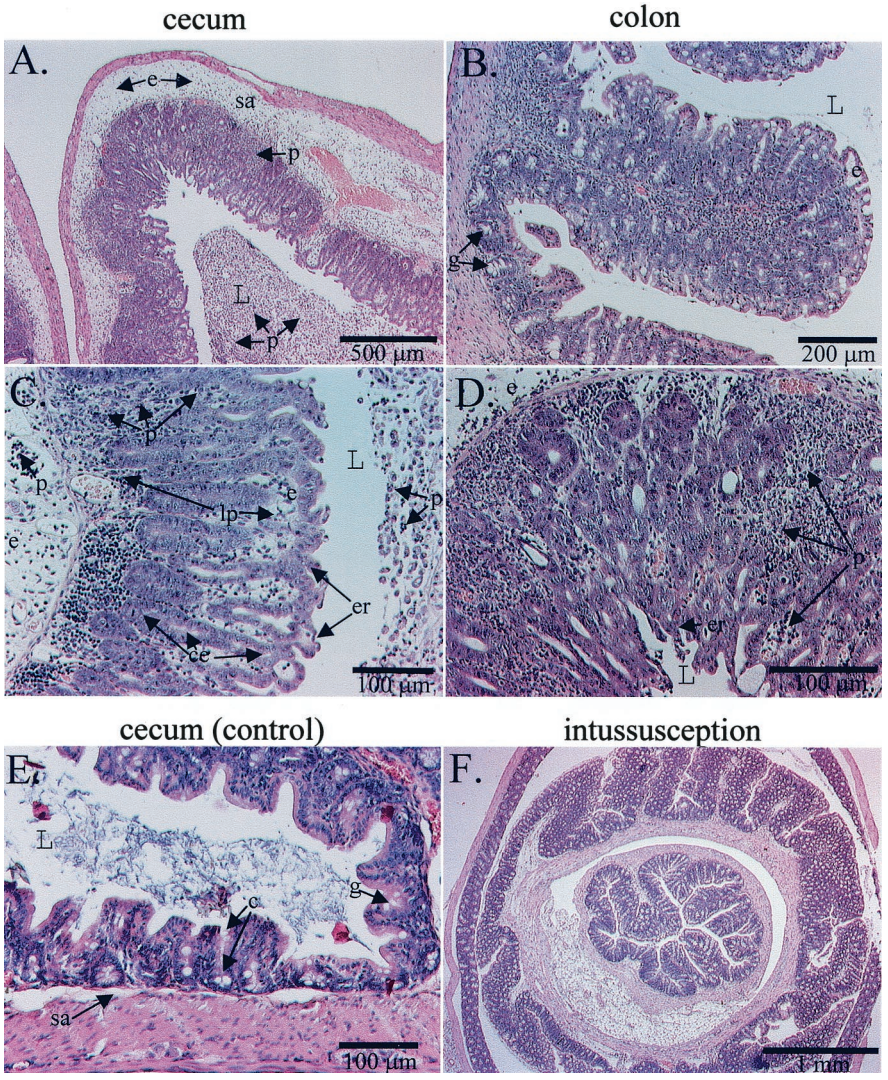


FIG. 2. Histopathological analysis reveals colitis in streptomycin-pretreated mice infected with serovar Typhimurium. Intestines of three mice from the experiment shown in Fig. 1B were fixed, embedded in paraffin, and 5-μm thin sections were stained with H&E. (A and C) Inflammation of the cecum of the streptomycin-pretreated serovar Typhimurium-infected mouse at 2 days p.i. (marked by an open arrow in Fig. 1B). (B and D) Inflamed colon from the same mouse as in panels A and C; identical inflammatory responses were observed in the mouse marked by the black arrow in Fig. 1B (data not shown). (E) No signs of severe inflammation were observable in the cecum (and colon; data not shown) of the water-pretreated serovar Typhimurium-infected control mouse (striped arrow; see Fig. 1B). (F) Intussusception was observed in the large intestine. The tissue was from the streptomycin-pretreated serovar Typhimurium-infected mouse marked with the black arrow in Fig. 1B. L, intestinal lumen; e, edema; p, PMN; lp, lamina propria; er, erosion of the epithelial layer; c, crypt; ce, crypt elongation; g, goblet cell; sa, submucosa. Magnifications are indicated by the black bars.

cells, epithelial erosion and/or ulceration and pronounced PMN infiltration of the submucosa, the lamina propria, and the epithelial layer, as well as transmigration of PMN into the intestinal lumen (Fig. 2A and C; compare to Fig. 2E). Though less severe, the colons of these mice were also inflamed, as judged by the edema and PMN infiltration in the lamina propria and epithelial erosion and/or ulceration (Fig. 2B and D). In contrast, no signs of pronounced inflammation were observed in the ilea of these mice (data not shown). These data suggested that serovar Typhimurium causes colitis in streptomycin-pretreated mice.

Enterocolitis is frequently associated with increased motility of the smooth muscles of the intestine. In severe cases this can lead to an intussusception, a serious complication that results from folding of one segment of the intestinal tube forward into an adjacent intestinal segment. This was observed in one of the streptomycin-pretreated mice colonized with serovar Typhimurium (Fig. 2F). During the entire course of our study, intussusceptions occurred in the lower intestines of ca. 3% of all streptomycin-pretreated mice colonized with wild-type serovar Typhimurium (data not shown).

Quantitative analysis of serovar Typhimurium colitis in streptomycin-pretreated mice. To explore whether streptomycin-pretreated mice might provide a useful model for studying serovar Typhimurium colitis, we performed a more detailed analysis. A total of 24 mice were divided into four groups of 6

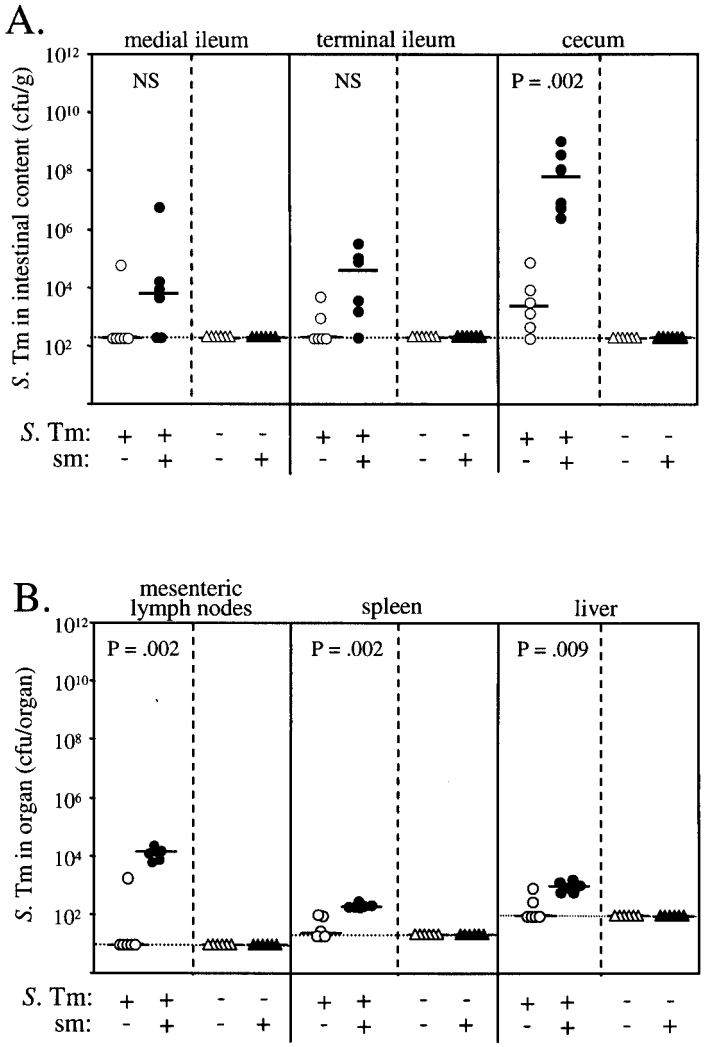


FIG. 3. Effect of the streptomycin pretreatment on the colonization of the intestine and internal organs by serovar Typhimurium. Four groups of six mice (C57BL/6) were pretreated with streptomycin (solid symbols) or sterile water (open symbols) and infected with 10^8 CFU of serovar Typhimurium SL1344 (circles) or mock infected with sterile PBS (triangles; see Materials and Methods). The mice were sacrificed 2 days p.i., and we analyzed the bacterial colonization. (A) Bacterial loads in the intestinal contents of the medial ileum (left panel), the terminal ileum (middle panel), and the cecum (right panel). (B) Bacterial loads in the mesenteric lymph nodes (left panel), the spleen (middle panel), and the liver (right panel; see Materials and Methods). The dashed line indicates the limit of detection; the bars indicate the median bacterial load; and "P" indicates the *P* value (Mann-Whitney U test; differences between the water and streptomycin-pretreated mice infected with serovar Typhimurium). NS, not statistically significant. Symbols: △, water-pretreated mice mock infected with PBS; ○, water-pretreated mice infected with serovar Typhimurium; ▲, streptomycin-pretreated mice mock infected with PBS; ●, streptomycin-pretreated mice infected with serovar Typhimurium.

mice. The first group was pretreated with streptomycin 24 h prior to p.o. infection with 10^8 CFU of serovar Typhimurium (Fig. 3). One control group was pretreated with water 24 h prior to infection p.o. with 10^8 CFU of serovar Typhimurium. The two other control groups were pretreated either with streptomycin or with water 24 h prior to mock infection by oral treatment with sterile PBS. The mice were killed 2 days p.i., and we analyzed the bacterial colonization (Fig. 3) and pathological changes in the ceca (Fig. 4, 5, and 6).

As expected, serovar Typhimurium was only detectable in the intestine and organs of the mice that had been infected (Fig. 3). We detected no serovar Typhimurium in the contents of the medial and terminal ilea of most of the water-pretreated mice infected with serovar Typhimurium. Low bacterial loads ($\leq 10^5$ CFU/g) were detected in the medial ileum of one, in the terminal ilea of two, and in the ceca of five of the six water-pretreated mice (Fig. 3A).

In streptomycin-pretreated mice we observed a slight but not significant ($P = 0.24$; $P = 0.065$) increase in the bacterial loads in the medial ($P = 0.24$) and terminal ($P = 0.065$) ilea and significantly increased (ca. 10^5-fold) bacterial loads in the cecum contents ($P = 0.002$; Fig. 3).

We observed significantly increased loads of serovar Typhimurium in the mesenteric lymph nodes of streptomycin-pretreated mice ($P = 0.002$; Fig. 3B). Slightly increased colonization was also detected in the spleens ($P = 0.002$) and livers ($P = 0.009$) of streptomycin-pretreated mice. However, even in the streptomycin-pretreated mice bacterial loads in the spleens and livers were only ca. 10-fold above the detection limit. Overall, these data are in line with earlier observations (57)

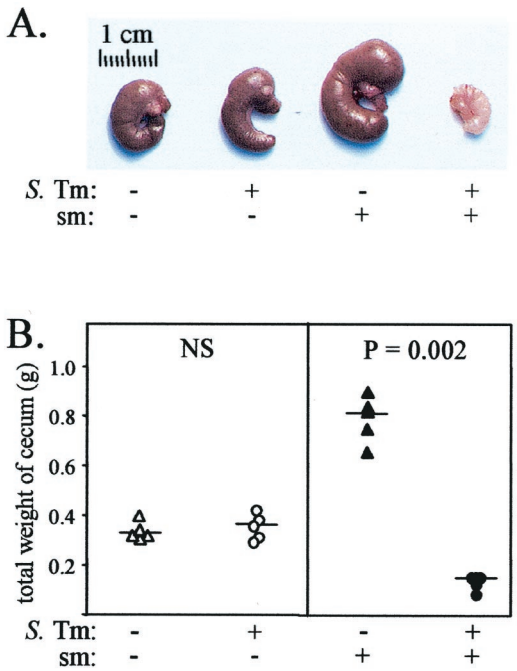


FIG. 4. Serovar Typhimurium induces macroscopic changes in the cecum of streptomycin-pretreated mice at 2 days p.i. We removed the ceca of mice from the experiment shown in Fig. 3 for macroscopic analysis. (A) Cecum morphology. The cecum of one mouse from each group was taken for photographic documentation. The photograph is representative of all six animals from each group. The centimeter scale indicates the magnification. (B) Total weight of the cecum. We determined the weight of the ceca of the remaining five mice from each group. P, *P* value (Mann-Whitney U test; differences between animals which were infected with serovar Typhimurium or mock infected with sterile PBS); NS, not statistically significant. "*S.* Tm + or −" and "sm + or −" indicate whether the mice were pretreated with streptomycin (sm) or water and whether the animals were infected with serovar Typhimurium (*S.* Tm). Bars indicate the median weight of the cecum. Symbols: △, water-pretreated mice mock infected with PBS; ○, water-pretreated mice infected with serovar Typhimurium; ▲, streptomycin-pretreated mice mock infected with PBS; ●, streptomycin-pretreated mice infected with serovar Typhimurium.

and suggest that even in streptomycin-pretreated mice the systemic infection is still at a very early stage at 48 h p.i. Therefore, the systemic infection in these mice is unlikely to affect the pathological changes observed in the lower intestine.

During the dissection we did not observe macroscopic signs of inflammation of the ilea, ceca, and colons of the water-pretreated mice that were infected with serovar Typhimurium or mock infected with sterile PBS. In accordance with earlier results (69), streptomycin-pretreated mice that were mock infected with sterile PBS had enlarged ceca. In contrast to the three control groups, fecal pellet formation was impaired in the proximal colons of all six streptomycin-pretreated mice infected with serovar Typhimurium, and the tissues of the proximal colons appeared pale and swollen in five of the six animals (data not shown). Furthermore, the ceca of all six streptomycin-pretreated serovar Typhimurium-infected mice were shriveled to a small size, pale, and filled with purulent exudate (Fig. 4A, right side). To document these macroscopic differences, we removed the cecum from one mouse of each group for photographic documentation (Fig. 4A) and determined the weight of the cecum, including the contents of the remaining five animals from each group (Fig. 4B). In water-pretreated mice, infection with serovar Typhimurium did not significantly affect the total weight of the cecum (Fig. 4B; $P = 0.458$). However, in streptomycin-pretreated mice we observed a significant reduction of the cecal weights in mice infected with serovar Typhimurium (Fig. 4B, right panel; $P = 0.002$).

For histopathologic analysis, intestinal tissue samples were cryoembedded, and 5-μm thin sections were stained with H&E (see Materials and Methods). The intestines of all three control groups appeared histologically normal (Fig. 5A to C and E to G and data not shown). However, in line with our initial observations (Fig. 2), pronounced inflammation was detected in the ceca and, to a lesser extent, also in the colons of all streptomycin-pretreated mice colonized with serovar Typhimurium (Fig. 5D and H; data not shown). This included pronounced edema in the submucosa and the lamina propria, crypt elongation, disruption of the crypt architecture, reduced numbers of goblet cells, epithelial erosion, and pronounced PMN infiltration of the submucosa, the lamina propria, and the epithelial layer, as well as transmigration of PMN into the intestinal lumen. No signs of severe inflammation were observed in the ilea of these mice (data not shown).

To improve comparison of the inflammatory responses between the different groups of mice, we devised a histopathologic scoring scheme for H&E-stained cecal tissue sections (see Materials and Methods). This scheme considers the extent of the submucosal edema (0 to 3 [arbitrary units]), PMN infiltration (0 to 4), loss of goblet cells (0 to 3), and the epithelial integrity (0 to 3) and yields a total pathological score of 0 to 13 U (Materials and Methods). Using this scoring scheme, we have found no significant differences between the water-pretreated mice that had been infected with serovar Typhimurium or mock infected with sterile PBS (Fig. 6, left panel). The minimal signs of cecal inflammation observed in these mice and in the streptomycin-pretreated mice that had been mock infected with sterile PBS are frequently found in untreated SPF mice and are generally not considered as a sign of disease. In contrast, the streptomycin-pretreated mice infected with serovar Typhimurium displayed profound inflammation that was significantly stronger than in the animals of the control group (Fig. 6, right panel; $P = 0.008$). These data are in line with the macroscopic observations (Fig. 4) and demonstrate that streptomycin-pretreated mice offer a robust model for studying serovar Typhimurium colitis.

***Lactobacillus* spp. do not induce colitis in streptomycin-pretreated mice.** Streptomycin treatment is known to diminish the intestinal flora and to render mice susceptible to intestinal colonization by various microorganisms. It was conceivable that any dramatic change in the composition of the intestinal microflora might lead to inflammation and that the colitis induced by serovar Typhimurium might not be attributable to specific virulence factors. To test this hypothesis, we pretreated two groups of mice with 20 mg of streptomycin given p.o. 24 h prior to oral infection with 10^8 CFU of nonpathogenic *Lactobacillus* spp. or serovar Typhimurium SL1344. The mice were sacrificed 2 days p.i., and we verified efficient colonization by both bacterial species by plating samples of the cecum contents on suitable agar plates (see Materials and Methods; $\gg 10^4$

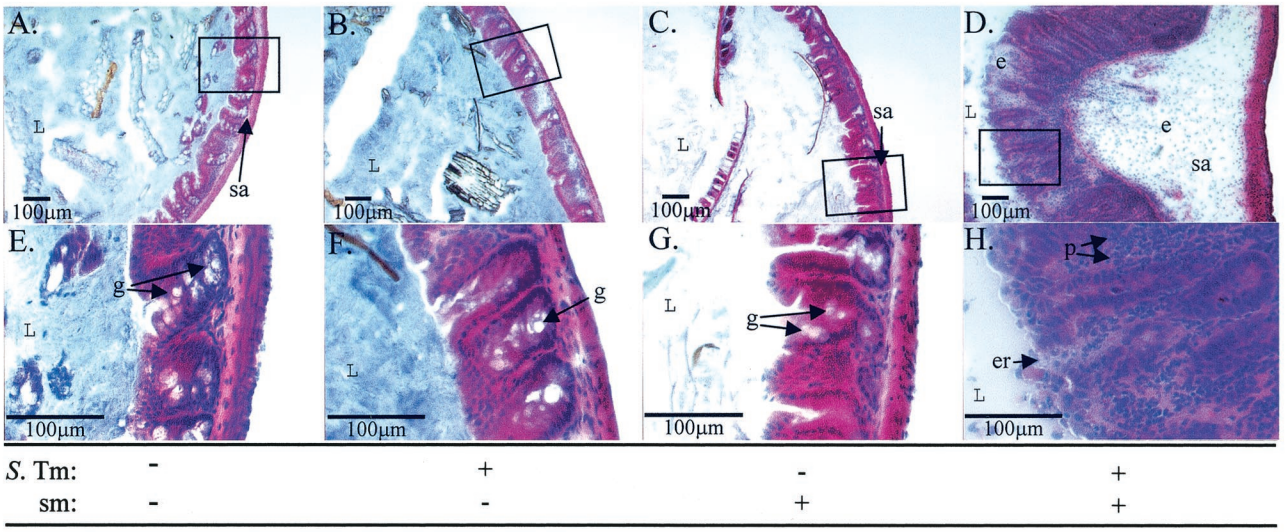
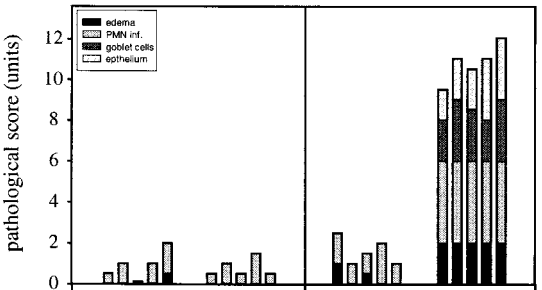


FIG. 5. Cecal inflammation at 2 days p.i. The ceca of five of the six animals from each group of the experiment shown in Fig. 3 were cryoembedded, and 5-μm thin sections were stained with H&E for histopathological analysis. (A and E) Cecum of a water-pretreated mouse mock infected with PBS; (B and F) cecum of a water-pretreated mouse infected with serovar Typhimurium; (C and G) cecum of a streptomycin-pretreated mouse mock infected with PBS; (D and H) streptomycin-pretreated mouse infected with serovar Typhimurium. The images are representative for all animals from each group. Boxes in panels A, B, C, and D indicate the area shown at the higher magnification. L, intestinal lumen; e, edema; p, PMN; er, erosion of the epithelial layer; g, goblet cell; sa, submucosa. Magnifications are indicated by the black bars.

CFU/g of *Lactobacillus* spp. or *Salmonella* spp.). Intestinal tissue samples were cryoembedded, and we analyzed 5-μm thin H&E-stained sections for signs of inflammation. In the *Lactobacillus*-infected mice we did not observe significant inflammation in the small or large intestine (Fig. 7A, C, and E; data not shown), whereas serovar Typhimurium-infected mice showed severe inflammation of the cecum and the proximal colon (Fig. 7B, D, and E). This indicates that colitis in streptomycin-pretreated mice is a specific response to colonization by serovar Typhimurium.

Time course of serovar Typhimurium colitis in streptomycin-pretreated mice. To analyze the early stages of serovar Typhimurium colitis, we investigated the time course of the infection. Groups of five mice were pretreated with streptomycin, infected with 10^8 CFU of serovar Typhimurium SL1344 (see Materials and Methods), and sacrificed at 2, 8, 20, and 48 h p.i. We analyzed pathological changes, as well as bacterial loads in the liver, mesenteric lymph nodes, and the intestinal content.

At 2 h p.i. the highest bacterial loads were present in the small intestine, and only low loads ($<10^4$ CFU/g) were present in the cecal contents (Fig. 8A and B). In accordance with earlier results (66), bacterial loads in the cecum increased significantly between 2 h and 8 h p.i. ($P = 0.008$). Even higher bacterial loads were detected in the cecum at 20 h p.i. ($P = 0.008$; median = 4.4×10^7 CFU/g) (Fig. 8B; Table 1). Between 20 h and 48 h p.i. we did not observe any further increase in the serovar Typhimurium loads in the cecum ($P = 0.841$; Table 1), which indicated that colonization of the ceca of streptomycin-pretreated mice by serovar Typhimurium had reached a steady-state level within 20 h after oral infection. We did not observe a specific enrichment of serovar Typhimurium at the intestinal epithelium at any time between 8 h and 48 h p.i. and



statistics:	stat. significance	stat. significance
combined score:	NS	P = .008
edema:	NS	P = .008
PMN infiltration:	NS	P = .008
goblet cells:	NS	P = .008
epithelium:	NS	P = .008

FIG. 6. Scoring of inflammatory changes at 2 days p.i. In order to quantify pathological changes, we scored H&E-stained sections of the ceca from five of the six mice from each group of the experiment shown in Fig. 3 as described in Materials and Methods. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately and plotted as stacked vertical bars. The combined score equals the sum of the separate scores. "*S.* Tm + or −" and "sm + or −" indicate whether the mice were pretreated with streptomycin (sm) or water and whether the animals were infected with serovar Typhimurium (*S.* Tm). Statistical analyses are shown for the separate scores and for the combined score. P, *P* value (Mann-Whitney U test; see Materials and Methods; difference between mice infected with serovar Typhimurium and mice mock infected with sterile PBS); NS, not statistically significant.

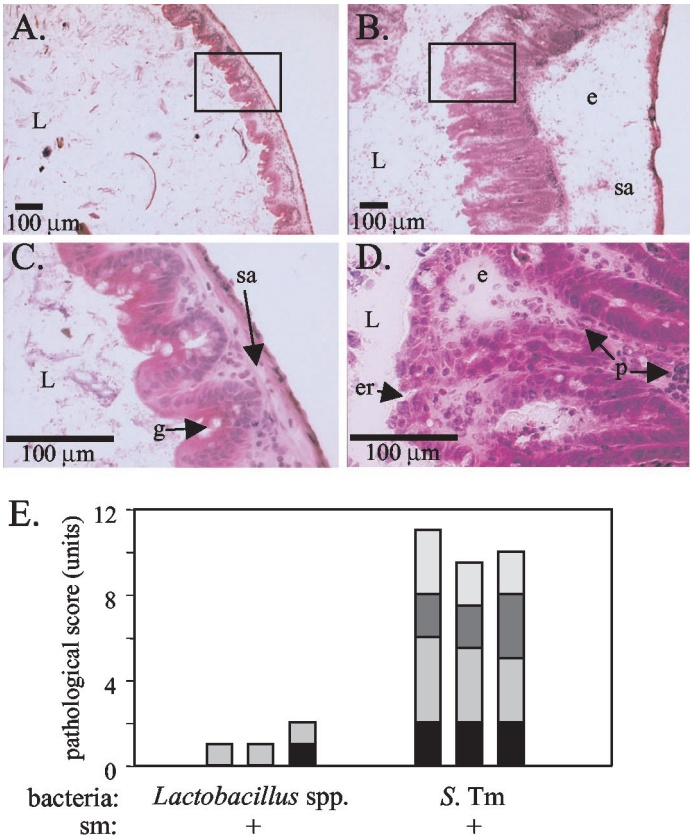


FIG. 7. Infection of streptomycin-pretreated mice with *Lactobacillus* spp. does not lead to colitis. Groups of three streptomycin-pretreated mice (C57BL/6) were infected with 10^8 CFU of *Lactobacillus* spp. or 10^8 CFU of serovar Typhimurium. At 48 h p.i. the animals were sacrificed and 5-μm cryosections of the cecum were stained with H&E. (A and C) Cecum of streptomycin-pretreated mouse infected with *Lactobacillus* spp.; (B and D) cecum of streptomycin-pretreated mouse infected with serovar Typhimurium. The images are representative for all three animals from each group. The boxes in panels A and B indicate the areas shown at the higher magnification in panels C and D. L, intestinal lumen; e, edema; p, PMN; er, erosion of the epithelial layer; g, goblet cell; sa, submucosa. Magnifications are indicated by black bars. (E) Scoring of inflammatory changes as described in Materials and Methods. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately and are plotted as stacked vertical bars. The combined score equals the sum of the separate scores. *S.* Tm, mice infected with serovar Typhimurium; sm, mice were pretreated with streptomycin.

observed that only a small minority of the bacteria ($\ll$5%) were located in crypts (Fig. 9J, K, and L).

At 2 h p.i. macroscopic inspection did not reveal any signs of inflammation in the small and the large intestine (median weight of the cecum = 0.59 g; Fig. 8D; data not shown). This was confirmed by histopathological analysis (Fig. 8F and 9A and E). At 8 h p.i. two of the mice appeared to be normal, and histopathological analysis did not reveal signs of inflammatory disease (Fig. 8F). Interestingly, these two mice also carried the lowest loads of serovar Typhimurium in their cecal contents (Fig. 8C), suggesting that a slower bacterial colonization might result in a delayed onset of the inflammatory response. The ceca of the three other animals sacrificed 8 h p.i. were pale, shriveled to a small size, and filled with purulent exudate (Fig. 8E). Histopathological analysis confirmed that the ceca of these mice were inflamed, as judged by submucosal edema, PMN infiltration into the lamina propria, epithelial erosion or ulceration, and a reduced number of goblet cells (Fig. 8F and 9B and F). Substantial infiltration of PMN ($CD18^+$, segmented nuclei) into the lamina propria was confirmed by immunofluorescence microscopy (Fig. 9N). In addition, we observed increased expression of ICAM-1 (compare Fig. 9M and N), a key transmembrane receptor that binds CD18–β_2-integrin dimers and is involved in the extravasation of leukocytes from capillary blood vessels into tissues.

At 20 h p.i. the ceca of all five mice showed characteristic signs of inflammation, including a significantly reduced cecum size (Fig. 8E and Table 1) and histopathologic changes such as submucosal edema, a reduced number of goblet cells, and epithelial erosion or ulceration (Fig. 8F and 9C and G). In contrast to the three mice that showed intestinal inflammation already at 8 h p.i., PMN were present in high numbers not only in the lamina propria but also in the submucosa and in the intestinal lumen (Fig. 9G and O), and we observed a concomitant increase in ICAM-1 expression (compare Fig. 9O and N).

In accordance with our first observations, the ceca of all five mice sacrificed at 48 h p.i. showed signs of profound inflammation (Fig. 8E and F and 9D, H, and P). Compared to the findings at 20 h p.i., we observed a further progression of the disruption of the villus architecture (compare Fig. 9C and G with 9D and H), increased ICAM-1 expression, and a significant exacerbation of the PMN infiltration ($P = 0.032$) and the

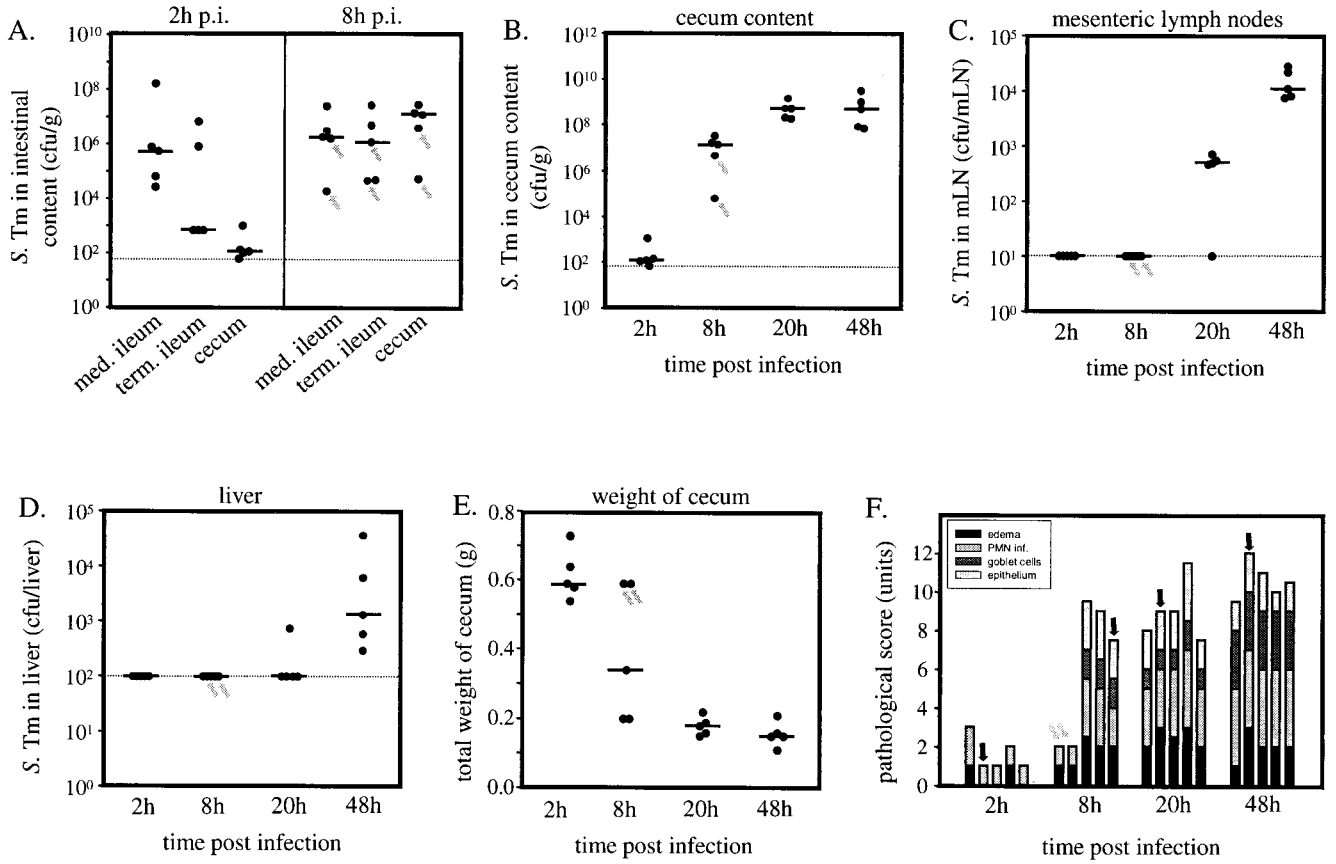


FIG. 8. Time course of serovar Typhimurium colitis in streptomycin-pretreated mice. Groups of five streptomycin-pretreated mice were infected for 2, 8, 20, or 48 h with 10^8 CFU of serovar Typhimurium SL1344 p.o. We determined bacterial colonization and pathological changes as described in Materials and Methods. (A) Bacterial loads in different regions of the intestine at 2 h p.i. (left panel) and 8 h p.i. (right panel). (B) Time course of the bacterial loads in the cecum content. (C) Time course of bacterial loads in the mesenteric lymph nodes. (D) Time course of bacterial loads in the liver. (E) Time course of the total weight of the cecum (determined before the removal of samples for bacteriological or histopathologic analysis). (F) Time course of the histopathologic changes. Samples of the cecal tissue were cryoembedded and 5-μm thin sections were stained with H&E. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately (see Materials and Methods) and are plotted as stacked vertical bars. The combined score equals the sum of the separate scores. For the statistical analysis, see Table 1. *S.* Tm, serovar Typhimurium; dashed line, limit of detection; bars, median bacterial load; gray arrows, values determined for the two mice without cecal inflammation at 8 h p.i. The black arrows indicate the pathological scores of the tissue sections shown in Fig. 9.

loss of goblet cells ($P = 0.008$) (Fig. 8F; Table 1; see also Fig. 9L and P).

To assess a possible correlation between intestinal inflammation and the onset of systemic infection, we monitored the time course of colonization of mesenteric lymph nodes and liver. Serovar Typhimurium could not be detected in the mesenteric lymph nodes and liver of any mice up to 8 h p.i. (Fig. 8C and D). However, at 8 h p.i. three of the five mice already had an inflamed cecum (see above). This suggests that colonization of the mesenteric lymph nodes or spread to internal organs is not a prerequisite for intestinal inflammation. At 20 h p.i. we detected a significant increase in the median serovar Typhimurium load in the mesenteric lymph nodes ($P = 0.032$) and a further increase at 48 h p.i. ($P = 0.008$; Fig. 8C; Table 1). In the livers we detected serovar Typhimurium in only one of five animals at 20 h p.i. ($P = 0.690$; Fig. 8D; Table 1). However, bacterial loads in the liver were significantly increased at 48 h p.i. (Fig. 8D and Table 1). In conclusion, colonization of the

TABLE 1. Statistical analysis of disease parameters in the time course of serovar Typhimurium colitis in streptomycin-pretreated mice[a]

Comparison	*P* at time (h) p.i.				
	2 vs 8	2 vs 20	2 vs 48	8 vs 20	20 vs 48
Cecum CFU	0.008	0.008	0.008	0.008	NS
mLN CFU	NS	0.032	0.008	0.032	0.008
Liver CFU	NS	NS	0.008	NS	0.032
Cecum wt	NS	0.008	0.008	0.032	NS
Combined score	NS	0.008	0.008	NS	NS
Edema	0.032	0.008	0.008	NS	NS
PMN infiltration	NS	0.008	0.008	NS	0.032
Goblet cells	NS	0.008	0.008	NS	0.008
Epithelium	NS	0.008	0.008	NS	NS

[a] The data shown in Fig. 8 were analyzed by using the Mann-Whitney U test (see Materials and Methods). NS, not significant; mLN, mesenteric lymph nodes.

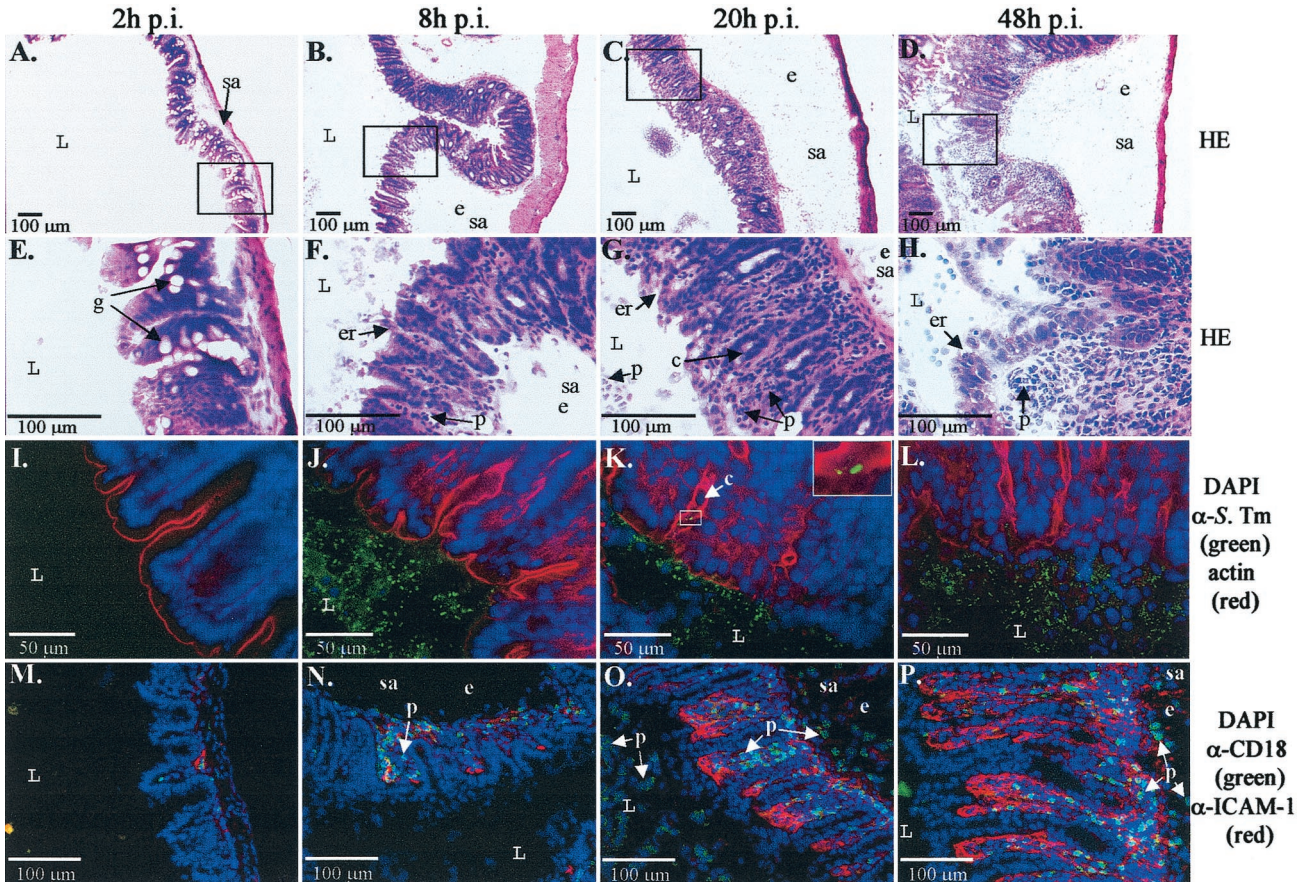


FIG. 9. Analysis of the time course of murine serovar Typhimurium colitis by histology and immunofluorescence microscopy. Cecal tissues of the mice from the experiment described in Fig. 8 (sacrificed at 2, 8, 20, or 48 h p.i.) were cryoembedded, sectioned, and stained as described in Materials and Methods. (A to H) Histopathology of thin sections (5 μm) of the cecum of mice infected for 2 h (A and E), 8 h (B and F), 20 h (C and G), or 48 h (D and H; marked by black arrows in Fig. 8F). (I to L) Localization of the bacteria. Thin sections (30 μm) of the cecum at 2 h (I), 8 h (J), 20 h (K), or 48 h (L) p.i. (marked by black arrows in Fig. 8F) were stained with DAPI, TRITC (tetramethyl rhodamine isothiocyanate)-phalloidin, a rabbit α-*Salmonella* LPS antiserum, and a secondary α-rabbit-FITC conjugate (DNA = blue; actin = red; serovar Typhimurium = green fluorescence). (M to P) Expression of ICAM-1 and infiltration of $CD18^+$ cells. Thin sections (5 μm) of the cecum at 2 h (M), 8 h (N), 20 h (O), or 48 h (P) p.i. (marked by black arrows in Fig. 8F) were stained with DAPI, rat α-mouse CD18, hamster α-mouse ICAM-1, and polyclonal preadsorbed α-rat IgG-FITC and α-hamster IgG-Cy3 antibodies (DNA = blue; ICAM-1 = red, CD18 = green fluorescence; see Materials and Methods). The boxes in panels A, B, C, and D indicate areas shown at a higher magnification in panels E, F, G, and H. The inset in panel K is a higher magnification of the area marked by the white box in the upper right corner of the panel. L, intestinal lumen; e, edema; p, PMN; er, erosion of the epithelial layer; g, goblet cell; sa, submucosa; c, crypt. Magnifications are indicated by bars.

mesenteric lymph nodes and liver does not seem to be required for the initiation of cecal inflammation. However, we cannot rule out that colonization of the mesenteric lymph nodes or the early systemic infection might play a role in the exacerbation of the inflammatory response observed between 8 h and 48 h p.i.

Overall, the observed pathological changes correlated well with the kinetics of intestinal colonization of streptomycin-pretreated mice by serovar Typhimurium. In some animals we observed cecal inflammation as early as 8 h p.i. These animals carried bacterial loads of ca. 10^7 CFU/g in the cecal contents. At 20 h p.i. the cecum was fully colonized (median = 4×10^8 CFU/g; Fig. 8B), and the maximum levels of cecal inflammation were reached by between 20 and 48 h p.i. These observed symptoms are indicative of inflammation and a rapid regeneration of the cecal epithelium in response to colonization by serovar Typhimurium and resemble many aspects of human (nontyphoid) salmonellosis and bovine and rabbit models of *Salmonella* enterocolitis. Therefore, streptomycin-pretreated mice might offer a versatile alternative model for studying the pathogenesis of gastrointestinal serovar Typhimurium infections.

Role of SPI1 type III secretion in murine serovar Typhimurium colitis. The SPI1 type III secretion system plays a key role in the early, gut-associated stages of the infection in susceptible animals (75, 81). In calves, the SPI1 type III secretion system plays a key role in the induction of inflammatory diarrhea. However, it has remained unclear whether the SPI1 type III secretion system can play a similar role in mice. To explore this question, we used SB161 (SL1344, Δ*invG*), an isogenic mutant that lacks an essential subunit of the SPI1 type III apparatus and is incapable of secreting and translocating any proteins via this route (14, 43). Eight streptomycin-pretreated C57BL/6 mice were infected with wild-type serovar Typhimurium SL1344, and eight mice were infected with SB161 (10^8

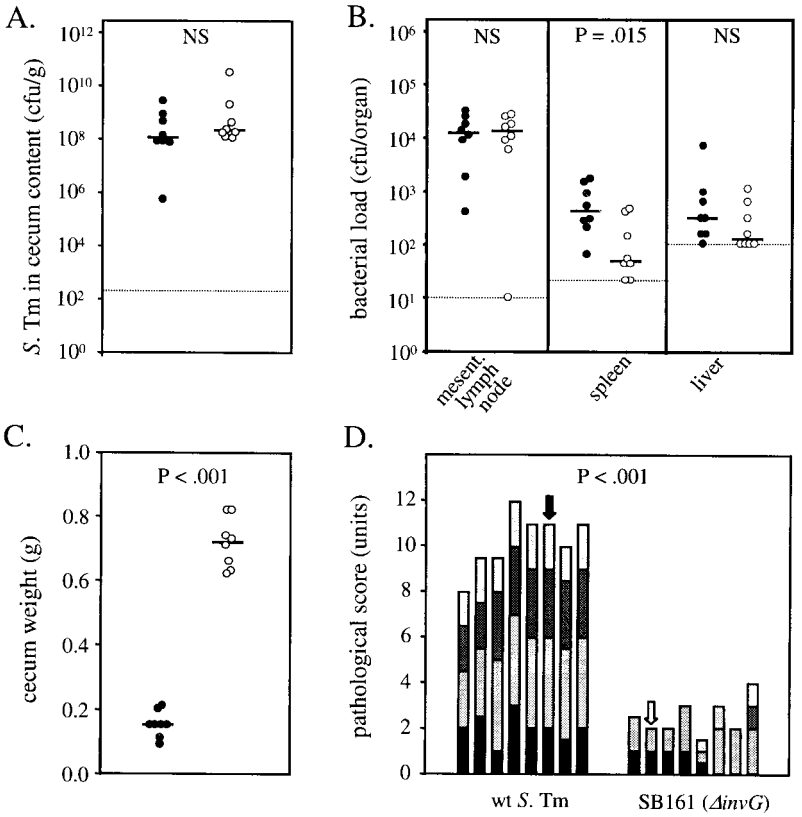


FIG. 10. Role of the SPI1 type III secretion system in serovar Typhimurium colitis. Eight streptomycin-pretreated C57BL/6 mice were infected p.o. with 10^8 CFU of wild-type serovar Typhimurium SL1344 (black circles in panels A, B, and C; left side of panel D) or with SB161 (SL1344, Δ*invG*; open circles in panels A, B, and C; right side of panel D) and analyzed at 2 days p.i. (A) Loads of serovar Typhimurium present in the cecum content. (B) Colonization of mesenteric lymph nodes (left panel), spleen (middle panel), and liver (right panel). (C) Total weight of the cecum, including the cecal contents (determined before the removal of samples for bacteriological or histopathologic analysis). (D) Histopathological analysis. Samples of the cecal tissue were cryoembedded, and 5-μm thin sections were stained with H&E. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately (see Materials and Methods) and plotted as stacked vertical bars. The combined score equals the sum of the separate scores. Black and white arrows indicate the pathological scores of the tissue sections from the wild-type- and SB161-infected mice, respectively, shown in Fig. 11. For the statistical analysis, see Table 2. P, *P* value (Mann-Whitney U test; see Materials and Methods). NS, not statistically significant; *S*. Tm, serovar Typhimurium; dashed line, limit of detection; bars, median bacterial load or weight.

CFU p.o.). At 48 h p.i., the mice were sacrificed and analyzed. Bacterial loads in the cecum contents, the mesenteric lymph nodes, and in the livers did not differ significantly between mice infected with wild-type serovar Typhimurium and those infected with SB161 (Fig. 10A and B). However, significantly lower loads of SB161 than of wild-type serovar Typhimurium were detected in the spleens ($P = 0.015$; Fig. 10B, middle panel). These observations are in line with earlier findings (27, 57) and indicate that the SPI1 type III secretion system plays a role in establishing systemic infection in streptomycin-pretreated mice. However, in spite of similar interaction patterns with the intestinal wall during the early stages of colonization (Fig. 11G and H) and similar *Salmonella* loads in the cecal contents (Fig. 10A), only mild symptoms of colitis were detected in the ceca (and proximal colons) of all eight mice colonized with SB161. This included significantly higher cecal weights ($P < 0.001$; Fig. 10C) and reduced histopathological signs of inflammation (combined score = $P < 0.001$; Fig. 10D, compare Fig. 11A and C with 11B and D; Table 2). Infiltration and transmigration of $CD18^+$ cells/PMN and expression of ICAM-1 were also alleviated in the ceca of mice infected with the serovar Typhimurium mutant SB161 (compare Fig. 11E and F). This demonstrates that serovar Typhimurium requires a functional SPI1 type III secretion apparatus in order to elicit profound intestinal inflammation.

Tissue culture experiments have suggested that mere secretion (and not direct injection into the host cell) of some effector proteins (i.e., SipA) might be sufficient to elicit proinflammatory responses in polarized epithelial monolayers (47). In order to assess whether protein secretion or direct injection (translocation) of bacterial effector proteins into host cells is required for the induction of colitis, we analyzed two serovar Typhimurium mutants that are incapable of injecting effector proteins into host cells but still retain the capacity to secrete some or all proteins across the bacterial cell envelope. In contrast to SB161, strain SB302 (SL1344, *invJ*::*aphT*) can secrete a few proteins (InvJ and SpaO [14]) and strain SB241 (SL1344, *sipD*::*aphT*) is even more efficient than wild-type serovar Typhimurium at secreting all known effector proteins into bacterial culture supernatants (44).

Groups of five C57BL/6 mice were infected with the isogenic mutants SB161, SB302 (SL1344, *invJ*::*aphT*), or SB241

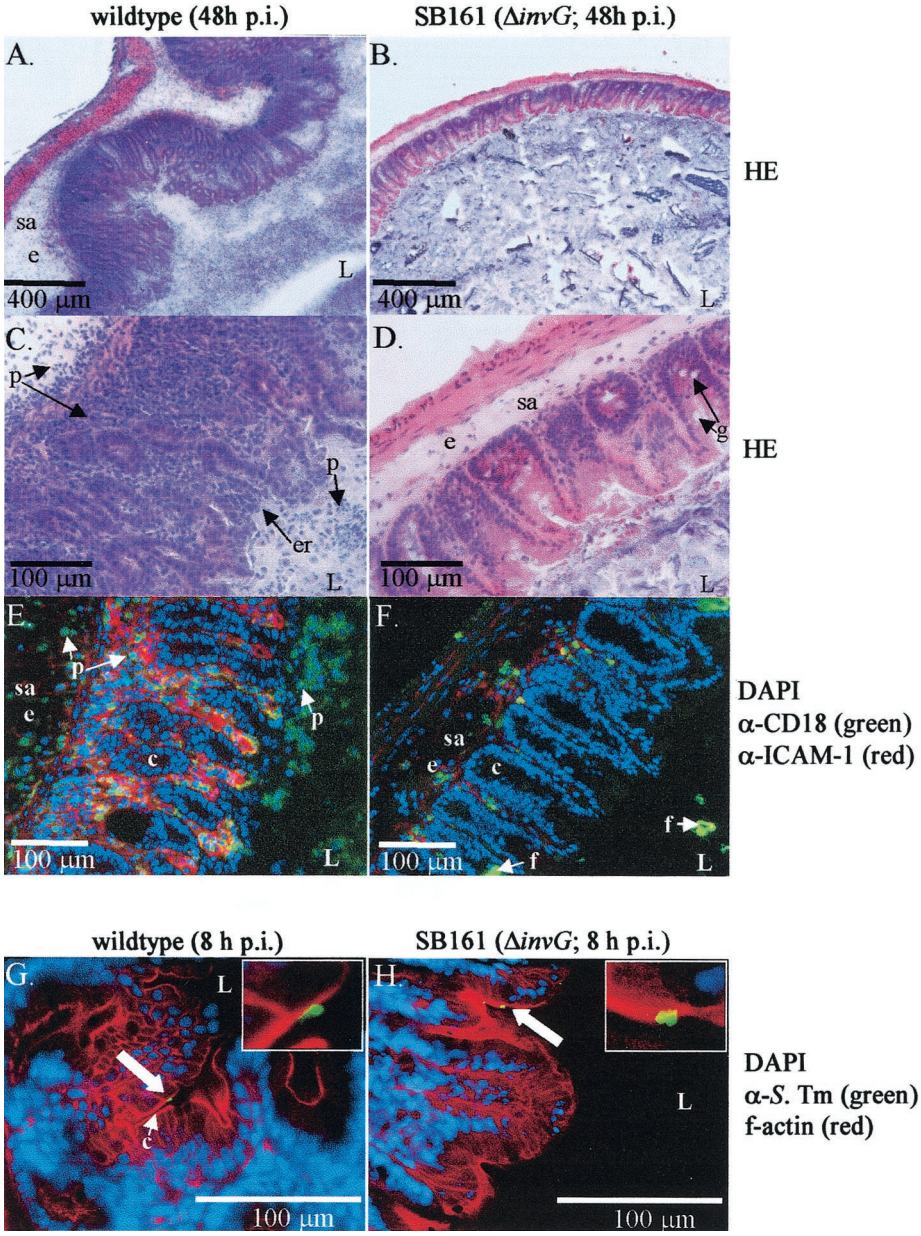


FIG. 11. Role of the SPI1 type III secretion system in cecal inflammation. (A to F) The ceca of all 16 mice from the experiment shown in Fig. 10 were cryoembedded and sectioned for histopathological evaluation and analysis by immunofluorescence microscopy. (A to D) SB161 causes a less-pronounced cecal inflammation. Thin sections (5 μm) were stained with H&E. (A and C) Representative image of mice infected for 2 days with wild-type serovar Typhimurium SL1344 (marked by white arrow in Fig. 10D); (B and D) representative image of mice infected for 2 days with the SPI1 mutant SB161 (marked by black arrow in Fig. 10D); (E and F) induction of ICAM-1 and infiltration of $CD18^+$ cells. Thin sections (5 μm) of the ceca of mice infected for 48 h with wild-type serovar Typhimurium SL1344 (E) or SB161 (F) were stained with DAPI, rat α-mouse CD18, hamster α-mouse ICAM-1, and polyclonal preadsorbed α-rat IgG-FITC and α-hamster IgG-TRITC antibodies (DNA = blue; ICAM-1 = red, CD18 = green fluorescence; see Materials and Methods). (G and H) Early interaction of wild-type serovar Typhimurium and SB161 with the intestinal epithelium. Groups of two streptomycin-pretreated mice were infected with 10^8 CFU of wild-type serovar Typhimurium (G) or SB161 (H) and sacrificed at 8 h p.i. Macroscopic inspection and histopathologic scoring confirmed that the ceca of all mice were not inflamed (data not shown). Thin sections (30 μm) of the ceca were stained with DAPI, TRITC-phalloidin, a rabbit α-*Salmonella* LPS antiserum, and a secondary α-rabbit-FITC conjugate (DNA = blue, actin = red; serovar Typhimurium = green fluorescence). Insets show higher-magnification views of the areas marked by the white arrows. L, intestinal lumen; e, edema; p, PMN; er, erosion or ulceration of the epithelial layer; g, goblet cell; sa, submucosa; c, crypt; f, autofluorescence of food particles. Magnifications are indicated by bars.

(SL1344, *sipD*::*aphT*) or with wild-type serovar Typhimurium (10^8 CFU p.o.). At 48 h p.i. the mice were sacrificed for analysis of bacterial loads in internal organs, intestinal colonization, and pathological symptoms. The capacity of SB302 and SB241 to colonize the cecum, the lymph nodes, and the liver and to cause inflammatory changes in the cecal tissue did not differ significantly from that of the secretion- and translocation-deficient serovar Typhimurium mutant SB161 (Fig. 12A

TABLE 2. Comparison of the infection with the secretion-deficient mutant SB161 and wild-type serovar Typhimurium: statistical analysis[a]

Comparison	*P*
Cecum CFU	NS
mLN CFU	NS
Spleen CFU	0.015
Liver CFU	NS
Cecum wt	<0.001
Combined score	<0.001
Edema	<0.001
PMN infiltration	<0.001
Goblet cells	<0.001
Epithelium	<0.001

[a] The data shown in Fig. 10 were analyzed by using the Mann-Whitney U test (see Materials and Methods). NS, not significant; mLN, mesenteric lymph nodes.

to E and Table 3). Altogether, these data are in accordance with observations from bovine models (75, 81, 86) and demonstrate that the SPI1 type III secretion system is dispensable for establishing colonization of the murine intestine and spread to the mesenteric lymph nodes. However, efficient dissemination to systemic sites and the elicitation of colitis are dependent on a functional SPI1 type III secretion apparatus. Furthermore, the results obtained with SB241 indicate that in vivo mere secretion is insufficient but that the SPI1 effector proteins inducing the inflammatory response must be translocated into host cells in order to exert their biological function.

Role of GALT in murine serovar Typhimurium colitis. It is still a matter of dispute how inflammation is primed in enteric salmonellosis. Data from tissue culture experiments suggest that the direct interaction of serovar Typhimurium with epithelial cells can trigger inflammatory responses, chloride secretion, and PMN chemotaxis (19, 34, 47, 50, 51, 61; for a review, see reference 26). Alternatively, it has been argued that the inflammation is primed within the GALT (79) that are colonized early on during infection and that are of general importance in the initiation of innate and acquired immune responses (58, 60). However, due to the lack of a suitable animal model, this issue could not be addressed directly.

We used LTβR$^{-/-}$ knockout mice, which lack Peyer's patches, colon-associated lymphoid tissues, and all lymph nodes (25), to analyze the role of the gut-associated organized lymphatic tissues in the induction of serovar Typhimurium colitis. Eight streptomycin-pretreated LTβR$^{-/-}$ mice and eight wild-type C57BL/6 mice were infected p.o. with 10^8 CFU of serovar Typhimurium SL1344. In addition, seven water-pretreated LTβR$^{-/-}$ mice and seven wild-type C57BL/6 mice were infected p.o. with 10^8 CFU of serovar Typhimurium SL1344. Furthermore, two streptomycin-pretreated LTβR$^{-/-}$ mice and two wild-type C57BL/6 mice were mock infected with sterile PBS. The mice were sacrificed at 48 h p.i. and analyzed with respect to bacterial colonization and intestinal inflammation (see Materials and Methods).

In streptomycin-pretreated C57BL/6 and LTβR$^{-/-}$ mice infected with serovar Typhimurium we did not detect significant differences of the bacterial loads in the feces, spleens, and livers (left panels of Fig. 13A to C). In all eight streptomycin-pretreated C57BL/6 and LTβR$^{-/-}$ mice infected with serovar Typhimurium the cecum was pale, shriveled to a small size, and showed signs of inflammation. No significant differences were detected between the cecal weights and the pathological scores of both mouse strains (left panels of Fig. 13D and E; Table 4). In both strains we observed edema, epithelial erosion or ulceration, disruption of the villus architecture, loss of goblet cells (Fig. 13 and 14A and C), migration of large numbers of PMN/CD18$^+$ cells into the lamina propria and the intestinal lumen (Fig. 14E and G), and increased ICAM-1 expression (Fig. 14I and K). Only the distribution of the B220^{high} B cells differed between C57BL/6 and LTβR$^{-/-}$ mice. In the cecal submucosae of uninfected C57BL/6 control mice we detected small clusters of B220^{high} B cells that were absent in LTβR$^{-/-}$ mice (compare Fig. 14P and N). At 48 h p.i. with serovar Typhimurium these small B-cell clusters had disappeared in the C57BL/6 mice (Fig. 14O). We have repeatedly observed significant numbers of B220^{high} B cells in the exudates of C57BL/6 mice colonized with wild-type serovar Typhimurium (data not shown), suggesting that transmigration into the intestinal lumen might play a role in the disappearance of the cecal B-cell clusters. In LTβR$^{-/-}$ mice, however, we observed massive infiltration of B220^{high} B cells into the lamina propria (Fig. 14M), whereas no B220^{high} B cells were present in the intestinal lumina of these mice. Therefore, LTβR signaling might play a role not only in the formation of Peyer's patches and mesenteric lymph nodes, but it might also affect B-cell (but not PMN) recruitment and/or function during acute inflammatory responses. Nonetheless, our data clearly demonstrate that organized GALT are dispensable for the induction of murine serovar Typhimurium colitis.

In the control experiments we analyzed the effect of the LTβR$^{-/-}$ mutation on the serovar Typhimurium infection in water-pretreated mice. As expected, intestinal colonization was low, and we did not detect signs of inflammatory disease in all seven LTβR$^{-/-}$ and wild-type C57BL/6 mice (Fig. 13A, D, and E). Interestingly, in spite of the lack of Peyer's patches, and all lymph nodes in the LTβR$^{-/-}$ animals (25), the bacterial loads in the livers and spleens did not differ significantly between the two mouse strains (Fig. 13B and C; Table 4). However, it should be noted that the variance of the bacterial loads in the spleens and livers was higher in this experiment than in the experiment described in Fig. 3. Furthermore, the median bacterial load in the water-pretreated mice was somewhat higher than in the experiment shown in Fig. 3B (compare results for wild-type C57BL/6 mice). The reason for this result is unclear. Nevertheless, our data indicate that colonization of the Peyer's patches and mesenteric lymph nodes is dispensable for the initiation of systemic infection.

DISCUSSION

It has long been noted that different *S. enterica* serotypes often have different host preferences and that the type of disease (i.e., inflammatory diarrhea or systemic infection) depends on the *S. enterica* serotype, as well as on the host species (75, 78). This concept has often been illustrated by the observation that oral infection of humans and calves with serovar Typhimurium results in enterocolitis, whereas susceptible mice succumb to a systemic typhoid-like disease without overt intestinal inflammation. The latter phenomenon has prohibited detailed analysis of intestinal salmonellosis. Here, we demon-

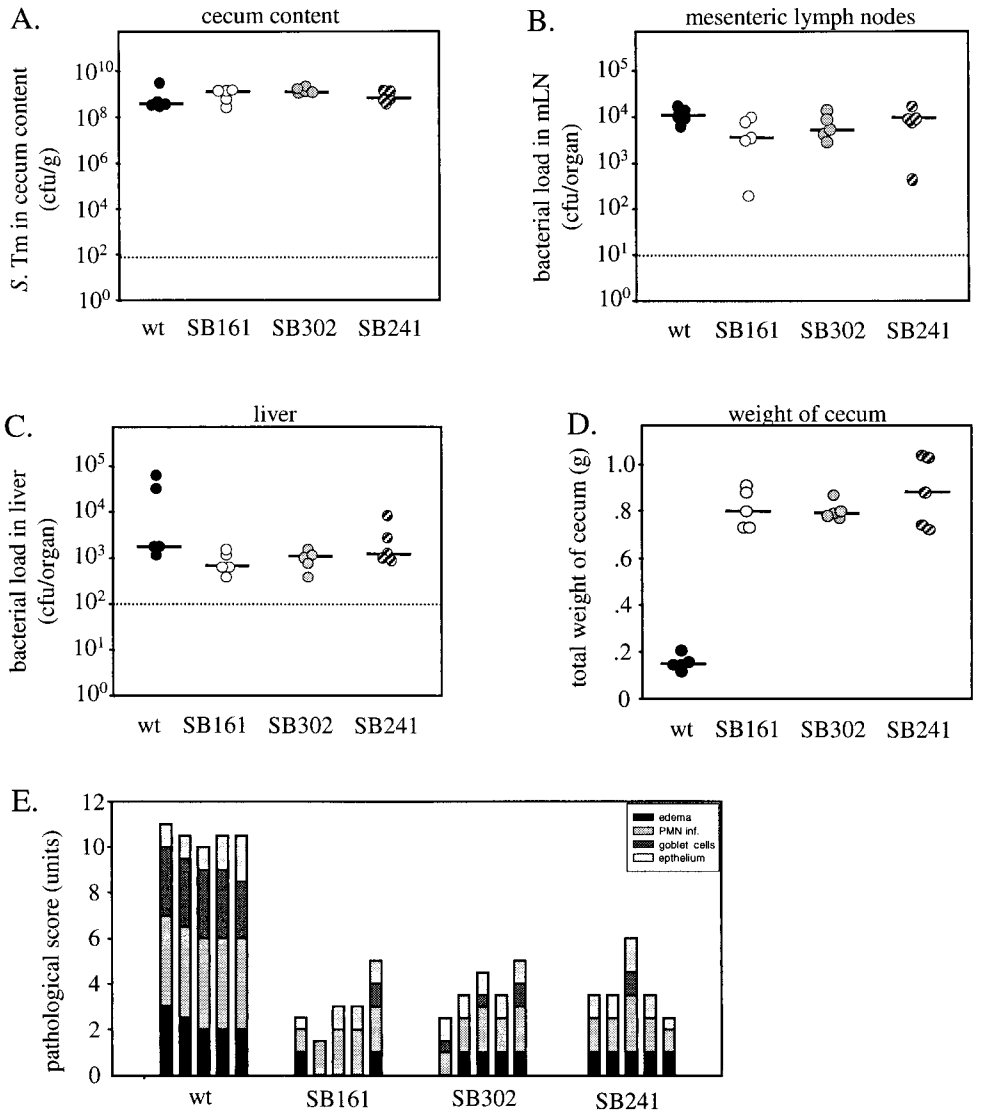


FIG. 12. Effect of different mutations compromising effector protein translocation on murine serovar Typhimurium colitis. Groups of five streptomycin-pretreated C57BL/6 mice were infected p.o. with 10^8 CFU of wild-type serovar Typhimurium SL1344 (black circles), SB161 (SL1344, Δ*invG*; white circles), SB302 (SL1344, *invJ*::*aphT*; gray circles), or SB241 (SL1344, *sipD*::*aphT*; striped circles), sacrificed 48 h p.i. and analyzed as described in Materials and Methods. (A) Bacterial load in the cecal content; (B) bacterial load in the mesenteric lymph nodes (mLN); (C) bacterial load in the liver; (D) total weight of the cecum, including the cecal contents (determined before the removal of samples for bacteriological or histopathologic analysis); (E) histopathological analysis. Samples of the cecal tissue were cryoembedded, and 5-μm thin sections were stained with H&E. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately (see Materials and Methods) and plotted as stacked vertical bars. The combined score equals the sum of the separate scores. *S*. Tm, serovar Typhimurium. For the statistical analysis, see Table 3. Dashed line, limit of detection; bars, median bacterial load.

strate that streptomycin-pretreated mice offer a versatile animal model for studying serovar Typhimurium colitis.

Earlier reports had demonstrated that oral treatment with streptomycin renders mice highly susceptible to oral infection with the *S. enterica* serovars Typhimurium and Enteritidis (2, 3, 9, 53, 54, 57, 66). This effect has been attributed to the elimination of commensal intestinal bacteria (4, 5, 55, 65). Indeed, gnotobiotic mice are also highly susceptible to infection with various bacteria, including *Salmonella* spp. (16, 22, 38, 59). Many of the early studies have not used well-defined inbred mouse strains, and the animals were housed under conventional hygiene conditions. Nevertheless, our results with inbred SPF C57BL/6 mice housed under barrier conditions are in perfect agreement with the earlier reports. The former studies had focused on the effect of streptomycin treatment on colonization by *Salmonella* spp. Our work extends these studies by demonstrating that streptomycin-pretreated C57BL/6 mice develop colitis upon oral infection with serovar Typhimurium.

The pathology of the murine serovar Typhimurium colitis is strikingly similar to that observed in bovine models, infections of rabbit ileal loops, rhesus monkeys, and humans (20, 42, 68, 73, 82, 84). This includes edema in the submucosa and lamina

TABLE 3. Statistical analysis of disease parameters in streptomycin-pretreated mice infected with different translocation-deficient serovar Typhimurium mutants[a]

Comparison	*P*				
	wt vs SB161	wt vs SB302	wt vs SB241	SB161 vs SB302	SB161 vs SB241
Cecum CFU	NS	NS	NS	NS	NS
mLN CFU	NS	NS	NS	NS	NS
Liver CFU	0.016	0.016	NS	NS	NS
Cecum wt.	0.008	0.008	0.008	NS	NS
Combined score	0.008	0.008	0.008	NS	NS
Edema	0.008	0.008	0.008	NS	NS
PMN infiltration	0.008	0.008	0.008	NS	NS
Goblet cells	0.008	0.008	0.008	NS	NS
Epithelium	NS	NS	NS	NS	NS

[a] The data shown in Fig. 12 were analyzed by using the Mann-Whitney U test (see Materials and Methods). NS, not significant ($P \geq 0.05$); wt, wild type.

propria, typical symptoms associated with fast regeneration of the intestinal epithelium (i.e., crypt elongation and loss of goblet cells), ulceration of the epithelial layer, upregulation of ICAM-1 expression (37), and pronounced PMN infiltration of the submucosa, lamina propria, and the epithelial layer, as well as inflammatory exudates in the intestinal lumen.

However, it should be noted that some differences from the bovine, rabbit, and human infections may also exist. In rabbits, calves, and primates, the infection is often associated with massive luminal fluid secretion (75, 81). In contrast, streptomycin-pretreated mice infected with serovar Typhimurium have a rather mild secretory response restricted to impaired formation of fecal pellets in the colon. Furthermore, it should be mentioned that the intestinal compartments most affected by the serovar Typhimurium infection often differ between different hosts. In cattle, both the ileum and the colon are affected (76), whereas streptomycin-pretreated mice develop colitis. Even though systematic analyses are lacking, anecdotal reports of the human disease have also identified the most severe pathological changes in the large intestine (8, 17, 52). In summary, our data show that streptomycin-pretreated mice provide an accurate model for many aspects of enteric salmonellosis.

Which serovar Typhimurium virulence factors induce intestinal inflammation? We found that serovar Typhimurium mutants with a defective SPI1 type III secretion system are still able to colonize the lower intestinal tract of streptomycin-pretreated mice but that the mutants cause much milder colitis than the isogenic wild-type strain. Similar observations have been made in bovine models and rabbit ileal loops, in which the SPI1 type III secretion system is required for intestinal inflammation and the induction of massive fluid secretion (20, 28, 73, 82). No evidence is available for the human infection, but work on human tissue culture models suggests that the serovar Typhimurium SPI1 type III secretion system can trigger proinflammatory responses such as cytokine expression and PMN transmigration (19, 34, 47, 51). Therefore, the attenuation of serovar Typhimurium mutants with a defective SPI1 type III secretion system further supports that streptomycin-pretreated mice are useful "surrogate" hosts to study the pathogenetic mechanisms of enteric salmonellosis.

The SPI1 type III secretion system allows serovar Typhimurium to translocate effector proteins directly into the cytosol of host cells. A large body of evidence suggests that the effector proteins exert their biological function inside the host cells. However, serovar Typhimurium can also secrete effector proteins into the culture supernatant via the SPI1 type III secretion system (39, 43–45, 83). Recently, it has been reported that addition of the purified effector protein SipA to the culture media is sufficient to induce PMN transmigration across polarized human intestinal epithelial cell monolayers (47). However, in streptomycin-pretreated C57BL/6 mice the translocation-deficient serovar Typhimurium mutant SB241 (SL1344, *sipD*::*aphT*), which secretes effector proteins such as SipA even more efficiently than the wild-type strain (44), is attenuated to the same extent as SB161 (SL1344, Δ*invG*), a mutant incapable of secreting and translocating effector proteins (43). In bovine infections a *sipD* mutant is also highly attenuated (74). These data cannot completely rule out that some SPI1 effector proteins can also exert functions from the outside. However, they strongly suggest that the effector proteins involved in murine colitis must be translocated directly into host cells.

Recognition of pathogen-associated molecular patterns by receptors of the innate immune system is thought to play an important role during the early responses to bacterial infection (40, 77). Indeed, serovar Typhimurium is known to express and release large amounts of LPS and flagellar subunits, and these bacterial factors have been shown to elicit "defensive" responses in a variety of in vitro and in vivo assays (29, 31, 85). Do these mechanisms contribute to serovar Typhimurium colitis in streptomycin-pretreated C57BL/6 mice? Serovar Typhimurium mutants with a defective type III secretion system induce only mild inflammation after 2 days of infection (Fig. 10D and 12E). However, these mutants release or express identical amounts of LPS and flagellar components (44; unpublished observations), and they colonize the murine intestine as efficiently as the isogenic wild-type strain (Fig. 10 and 12; Tables 2 and 3). These data are in line with results from bovine and rabbit ileal loop models (20, 28, 73, 82) and suggest that responses of the innate immune system to *Salmonella* pathogen-associated molecular patterns are insufficient to cause pronounced intestinal inflammation, at least in the absence of a functional SPI1 type III secretion system. However, serovar Typhimurium mutants with a defective SPI1 type III secretion system seem to retain a residual capacity to cause inflammation in streptomycin-pretreated C57BL/6 mice (compare Fig. 6 and 13E with Fig. 10D and 12E). Future experiments will have to determine whether innate immune responses might contribute to the SPI1-dependent or residual SPI1-independent inflammation.

What is the role of organized lymphatic tissues in systemic disease? In the murine model several different mechanisms have been identified that allow serovar Typhimurium to breach the intestinal barrier and cause systemic disease. In particular, penetration of M cells, with subsequent colonization of Peyer's patches and mesenteric lymph nodes (10–12, 35, 41, 64) and active sampling of luminal serovar Typhimurium by $CD18^+$ phagocytes/dendritic cells (67, 80), has been discussed recently. However, their relative contributions have remained unclear. We did not find significant differences in the efficiency of systemic infection between wild-type C57BL/6 mice and $LT\beta R^{-/-}$ mice (Fig. 13C; Table 4). Since $LT\beta R^{-/-}$ mice lack Peyer's

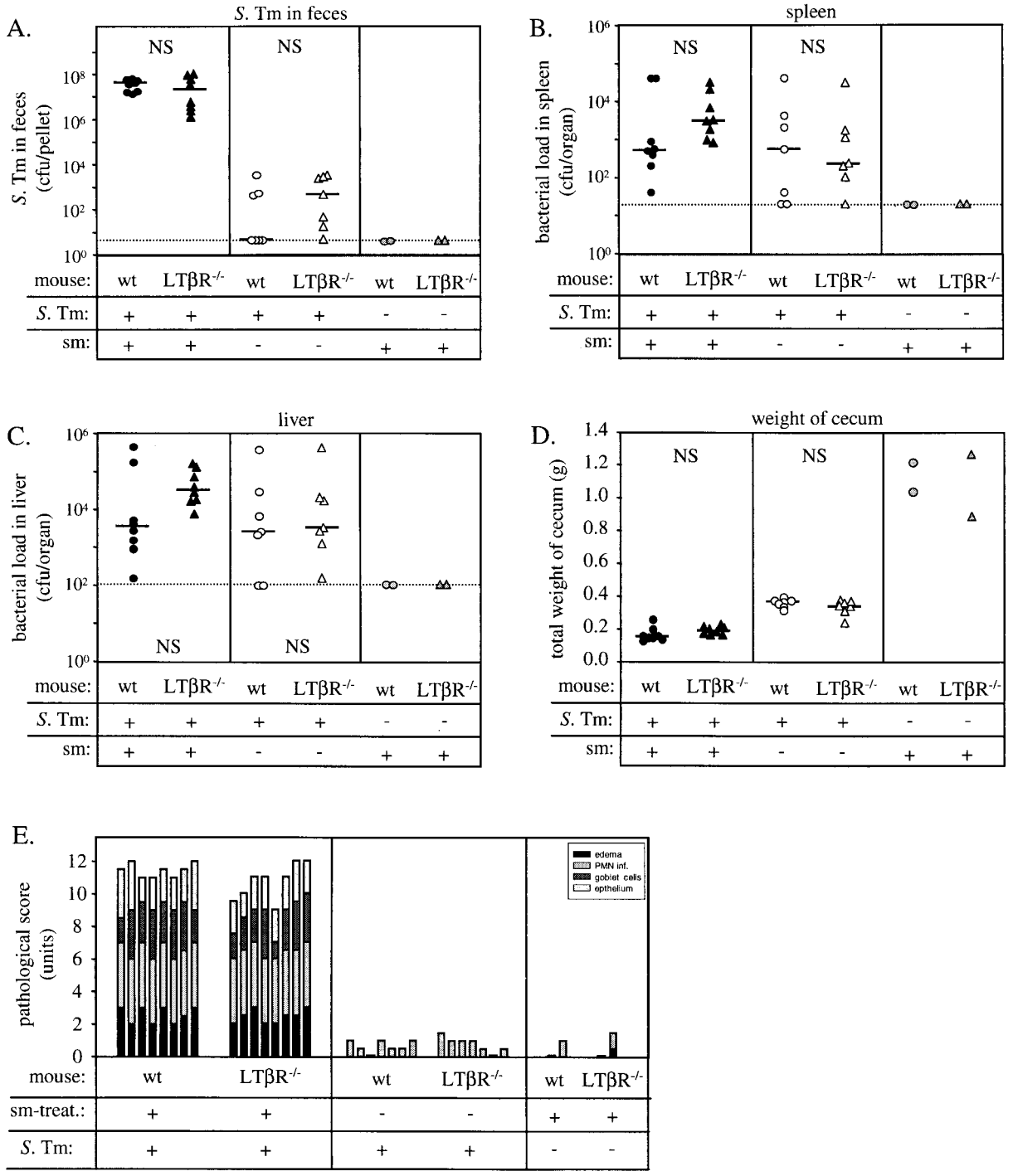


FIG. 13. Serovar Typhimurium colitis in streptomycin-pretreated LTβR$^{-/-}$ mice. (A to D) On the left side of each panel, eight wild-type C57BL/6 and eight LTβR$^{-/-}$ mice (genetic C57BL/6 background) were pretreated with streptomycin and infected with 10^8 CFU of serovar Typhimurium SL1344 p.o. (black circles and black triangles). In the middle of each panel, seven C57BL/6 and seven LTβR$^{-/-}$ mice were pretreated with water and infected with 10^8 CFU of serovar Typhimurium SL1344 p.o. (open circles and open triangles). On the right side of each panel, two C57BL/6 and two LTβR$^{-/-}$ mice were pretreated with streptomycin and mock infected with sterile PBS (gray circles and gray triangles). The mice were sacrificed 48 h p.i. and then analyzed as described in Materials and Methods. (A) Bacterial loads in the feces; (B) bacterial loads in the spleens; (C) bacterial loads in the livers; (D) total weight of the cecum (determined before the removal of samples for bacteriological or histopathologic analysis); (E) histopathological analysis. Samples of the cecal tissue were cryoembedded, and 5-μm thin sections were stained with H&E. Edema in the submucosa (black), PMN infiltration (medium gray), reduction of the number of goblet cells (dark gray), and erosion or ulceration of the epithelial layer (light gray) were scored separately (see Materials and Methods) and plotted as stacked vertical bars. The combined score equals the sum of the separate scores. For the statistical analysis, see Table 4. NS, not statistically significant; dashed line, limit of detection; bars, median bacterial loads.

TABLE 4. Statistical analysis of disease parameters in wild-type C57BL/6 and LTβR$^{-/-}$ mice infected for 2 days with wild-type serovar Typhimurium SL1344[a]

Comparison	*P* in C57BL/6 vs LTβR$^{-/-}$ mice[b]	
	+	−
Feces CFU	NS	NS
Spleen CFU	NS	NS
Liver CFU	NS	NS
Cecum wt	NS	NS
Combined score	NS	NS
Edema	NS	NS
PMN infiltration	NS	NS
Goblet cells	NS	NS
Epithelium	NS	NS

[a] The data shown in Fig. 13 were analyzed by using the Mann-Whitney U test (see Materials and Methods). NS, not significant ($P \geq 0.05$).
[b] +, streptomycin pretreated; −, not streptomycin pretreated.

patches and mesenteric lymph nodes, colonization of these GALT seems to be dispensable for the initiation of systemic infection. It is a bit more complicated to judge the involvement of M cells: due to the lack of M-cell markers, it has been difficult to formally prove the absence of all M cells in LTβR$^{-/-}$ mice. In addition, a rapid increase in the number of M cells might occur shortly after bacterial infection (6, 7, 70). However, LTβR$^{-/-}$ mice presumably have at least significantly reduced numbers of M cells (18, 25), which suggests that alternative pathways such as CD18^{+} phagocyte/dendritic cell transport (67, 80) might represent the major route for breaching the intestinal barrier in the initiation of systemic disease.

What is the role of organized lymphatic tissues in serovar Typhimurium colitis? A multitude of defense mechanisms, including the mucous layer, bactericidal peptides, innate immune responses, and the gut-associated immune system, ensure that potentially pathogenic intestinal microorganisms are detected and eliminated (48, 58, 60). As discussed above, it has been a matter of dispute whether serovar Typhimurium colitis is in-

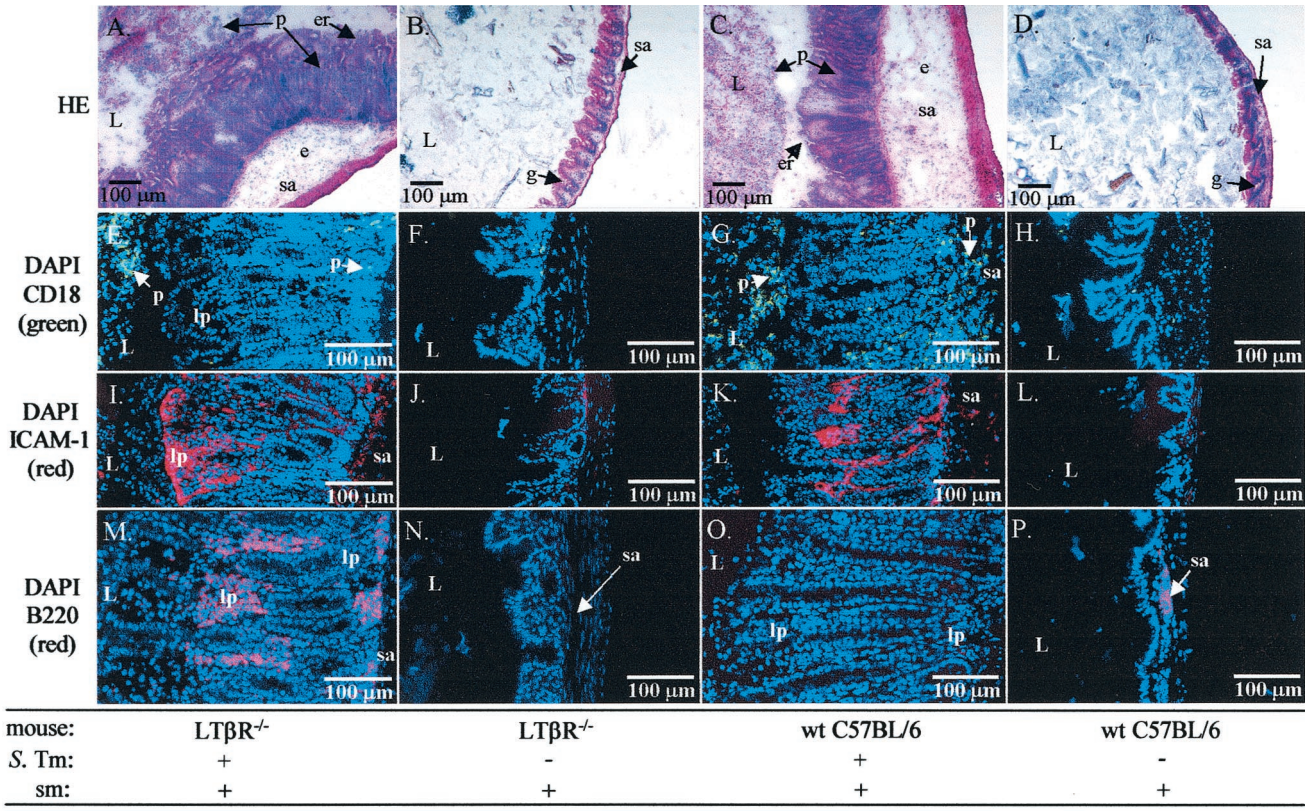


FIG. 14. Cecal inflammation in serovar Typhimurium-infected LTβR$^{-/-}$ and wild-type C57BL/6 mice. Representative images of the ceca of the LTβR$^{-/-}$ and wild-type C57BL/6 mice from the experiment in Fig. 13 are shown. Each cecum was cryoembedded, and 5-μm thin sections were stained with H&E (A to D) or processed for immunofluorescence microscopy (E to P). (A, E, I, and M) Streptomycin-pretreated LTβR$^{-/-}$ mice infected with serovar Typhimurium; (B, F, J, and N) streptomycin-pretreated LTβR$^{-/-}$ mice mock infected with PBS; (C, G, K, and O) streptomycin-pretreated wild-type C57BL/6 mice infected with serovar Typhimurium; (D, H, L, and P) streptomycin-pretreated wild-type C57BL/6 mice mock infected with sterile PBS. In panels A to D, the cecum sections of LTβR$^{-/-}$ and wild-type C57BL/6 mice were stained with H&E. In panels E to H, infiltration and transmigration of CD18^{+} cells is shown. Cecum sections were stained with DAPI, rat α-mouse CD18, and α-rat IgG-FITC antibodies (DNA = blue; CD18 = green). In panels I to L, induction of ICAM-1 expression is shown. Cecum sections were stained with DAPI, hamster α-mouse ICAM-1, and α-hamster IgG-TRITC antibodies (DNA = blue, ICAM-1 = red). In panels M to P, the distribution of B220^{high} B cells is shown. Cecum sections were stained with DAPI, rat α-mouseB220, and α-rat IgG-TRITC antibodies (DNA = blue, B220 = red). L, intestinal lumen; e, edema; p, PMN; er, erosion or ulceration of the epithelial layer; g, goblet cell; sa, submucosa; lp, lamina propria; c, crypt. Magnifications are indicated by bars. "*S.* Tm + or −" indicates whether the mice were infected with serovar Typhimurium SL1344 or mock infected with sterile PBS; "Sm + or −" indicates whether the mice were pretreated with streptomycin or with water.

duced by direct interaction of the bacteria with intestinal epithelial cells or as a consequence of colonization of Peyer's patches and mesenteric lymph nodes (79). We found that cecal inflammation actually precedes colonization of the mesenteric lymph nodes (Fig. 8). Furthermore, we found that serovar Typhimurium colitis in LTβR$^{-/-}$ mice, which lack all organized GALT, is just as strong as in wild-type C57BL/6 mice (Fig. 13 and Table 4). This suggests that Peyer's patches and mesenteric lymph nodes are not essential for the initiation of serovar Typhimurium colitis.

In conclusion, our results provide the first direct evidence that neither colonization of Peyer's patches and mesenteric lymph nodes nor host responses dependent upon these lymphoid structures are required for intestinal inflammation or spread of serovar Typhimurium to internal organs. This is further supported by preliminary results obtained with immunodeficient mice (SCID, BALB/c genetic background; unpublished data). However, the presence of bacteria (and presentation of their antigenic determinants) in the GALT is required later for the efficient development of protective immune responses (56, 60). The effects of such a response are not expected to affect our short-term infection experiments because adaptive immune responses generally take longer to come into effect.

In summary, pretreatment of SPF mice with streptomycin renders them susceptible to serovar Typhimurium colitis that closely resembles the inflammatory responses observed in the human colon and animal models for intestinal salmonellosis. The virulence defects that we have observed with serovar Typhimurium SPI1 mutants lend further support to this notion. In contrast to the other models that have been used so far to study the pathogenesis of enteric salmonellosis, streptomycin-pretreated mice offer several important advantages. (i) There is a wide variety of tools for immunohistologic analyses of inflammatory responses. (ii) A multitude of knockout mouse strains lacking specific organs, cell types, or proteins are available. (iii) Innate and adaptive immune responses have been studied in great detail in mice. This allows for the first time detailed analysis of the role of host responses in enteric salmonellosis. Our data demonstrate that the possibility to combine manipulation of both serovar Typhimurium and the murine host provides a very powerful system for unraveling the molecular pathogenesis of serovar Typhimurium colitis.

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