INFECTION AND IMMUNITY, Mar. 1989, p. 673–678
0019-9567/89/030673-06$02.00/0

Vol. 57, No. 3

Immunohistochemical and Electron Microscopic Study of Interaction of *Yersinia enterocolitica* Serotype O8 with Intestinal Mucosa during Experimental Enteritis

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Received 24 August 1988/Accepted 15 November 1988

The experimental infection of mice with *Yersinia enterocolitica* serotype O8 was investigated in a quantitative and histological study. The course of bacterial penetration and spreading was precisely determined by immunohistochemical staining. After oral administration, the bacteria passed the epithelial barrier of the ileum and spread into the lamina propria. By preference they entered Peyer's patches, which were about 1,000 times more heavily colonized than the surrounding epithelium of a comparable surface area. The bacteria proliferated in the follicles, from which they spread into the lamina propria of the villi. At either site most of the bacteria multiplied extracellularly, with only a small percentage observed to be present within the phagocytes. The bacteria did not appear to be able to pass the intact basement membrane; hence, the integrity of the basement membrane is likely to play a role in determining the route of entry and limit of spread of *Y. enterocolitica* infection.

Pathogenic strains of *Yersinia enterocolitica* are known to cause ileitis, mesenteric lymphadenitis, and septicemia in humans (1); and a similar reaction has been observed in the established model of murine yersiniosis (2). Oral infection of mice causes a suppurative infection of Peyer's patches and mesenteric lymph nodes. With a high challenge dose (i.e., 5×10^8 organisms per mouse) the microorganisms can traverse the mesenteric lymph nodes, leading to systemic infection of the spleen, liver, and lungs. Although virulence has been shown to correlate with the presence of a 41- to 47-megadalton plasmid in the pathogenic strains (15), the marked tropism of the bacteria for lymphoid tissue, their remarkable resistance to the immune response of the host, and the manner in which they spread within the tissue have not yet been elucidated (for recent reviews, see references 4 and 15).

The invasion of cultured cells by *Y. enterocolitica* has been shown in vitro with HeLa and HEp-2 cells (7, 16, 18) and has been assumed to reflect the penetration of *Y. enterocolitica* into intestinal mucosa in vivo. However, antigens that are absent from a bacterial surface in vitro may appear within the intestinal lumen during proliferation (19). Furthermore, the surface membrane of epithelial cells in culture differs greatly from that of the intestinal mucosa. Therefore, the cellular interactions observed in vitro may not always reflect the in vivo situation.

Very few studies have been carried out on the interaction of *Y. enterocolitica* with intestinal cells in vivo. It has been shown that when the plasmid-bearing serotype O9 is introduced into the duodenum, it penetrates the epithelial lining of the rabbit ileum and is phagocytosed by mononuclear cells in the lamina propria (20). However, it has also been shown that the plasmid-bearing bacteria of serotype O3, which also penetrate the intestinal mucosa after intragastric administration, are not phagocytosed but proliferate extracellularly (11). This may reflect differences in the interaction of the two strains with intestinal tissue. The interaction of the highly invasive serotype O8 with the intestine at cellular and subcellular levels has not yet been studied.

The aim of the present study was to investigate in detail the infection route and the interaction of mouse intestinal cells with plasmid-bearing *Y. enterocolitica* serotype O8 by immunohistochemistry and electron microscopy under experimental conditions closely resembling the conditions in naturally acquired human infections.

MATERIALS AND METHODS

Bacteria. A plasmid-bearing *Y. enterocolitica* serotype O8 (strain NCTC 10938) was obtained from the National Collection of Type Cultures, Central Public Health Laboratory (London, England). The bacteria were grown overnight at 24°C in brain heart infusion broth (Oxoid Ltd., London, England) supplemented with 20 mM magnesium chloride and 20 mM sodium oxalate.

Infection of mice. Prior to application the bacteria were centrifuged, washed with saline, and diluted to approximately 8×10^6 organisms per ml. The exact number of bacteria in the suspension was determined by counting the CFU on Endo agar plates. Male CD-1 mice (age, 2 months; weight, about 40 g) were deprived of water for 24 h before they were infected. They were then allowed to drink 5 ml of a bacterial suspension containing 4×10^7 organisms. The mice drank the suspension within 30 min. To prevent reinfection by coprophagy, the animals were placed in separate cages with grid bottoms. For the duration of the experiment they were then given water and food ad libitum.

Recovery of bacteria from infected mice. At specified times after infection, groups of three mice were killed by cervical dislocation. The distal part of the small intestine (15 cm in length) was removed and gently washed with 5 ml of saline.

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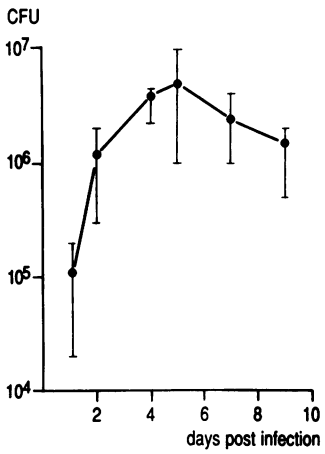


FIG. 1. Number of *Y. enterocolitica* organisms recovered from the contents of the small intestine at various times after infection with 4×10^7 bacteria per mouse. Each point represents mean values from three mice ± standard deviation.

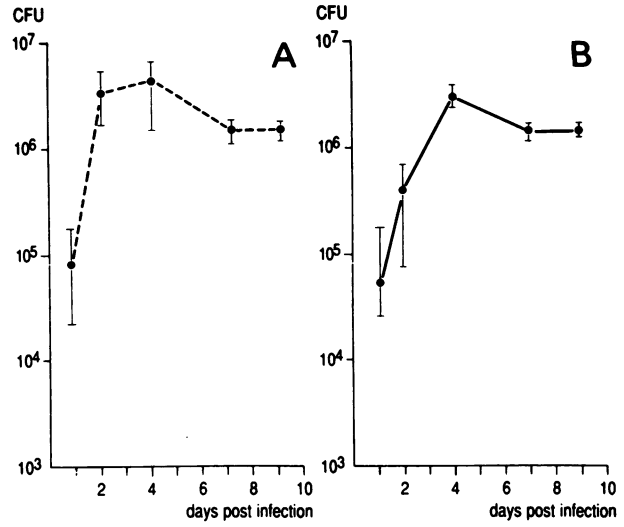


FIG. 2. (A) Colonization of a single isolated Peyer's patch. (B) Colonization of the distal 15 cm of small intestine without Peyer's patches. The infection dose was 4×10^7 bacteria per mouse. Each point represents mean values from three mice ± standard deviation.

This washing procedure removed 92 to 95% of the bacteria entrapped in the ileal contents and in the mucus. The Peyer's patches were excised, counted, suspended in saline, and homogenized in a Potter homogenizer. Some of the Peyer's patches were treated differently, as described below. The remainder of the ileum was also homogenized in a separate sterile homogenizer. Each homogenate was diluted in saline, and a suitable dilution was plated in duplicate onto Endo agar and incubated for 48 h at 24°C. The number of organisms recovered was expressed as CFU per a single Peyer's patch or in the remainder of the ileum. The colonies were routinely checked for their identity in an agglutination test with anti-*Yersinia* antiserum.

Determination of the approximate retention time of the ingested material in the mouse alimentary canal. Three mice, which were treated as described above for the mice used for infection, were given 5 ml of water colored with a foodstuff dye (Green No. SE 142; McCormick, Baltimore, Md.) which stained the excreted stool. The beginning and the end of excretion of the stained stool were monitored visually.

Preparation of antiserum. Bacteria were grown at 24°C to the early stationary phase. After centrifugation and two washes with saline, the bacteria were suspended in 3% formaldehyde and incubated for 1 h at 4°C. The fixed bacteria were washed twice in saline. About 10^9 bacteria in complete Freund adjuvant were injected in several loci in the back of a rabbit. Booster injections with 10^5 bacteria in incomplete Freund adjuvant were given after 2 and 4 weeks. The serum was collected 8 weeks after the first injection and was stored at −20°C.

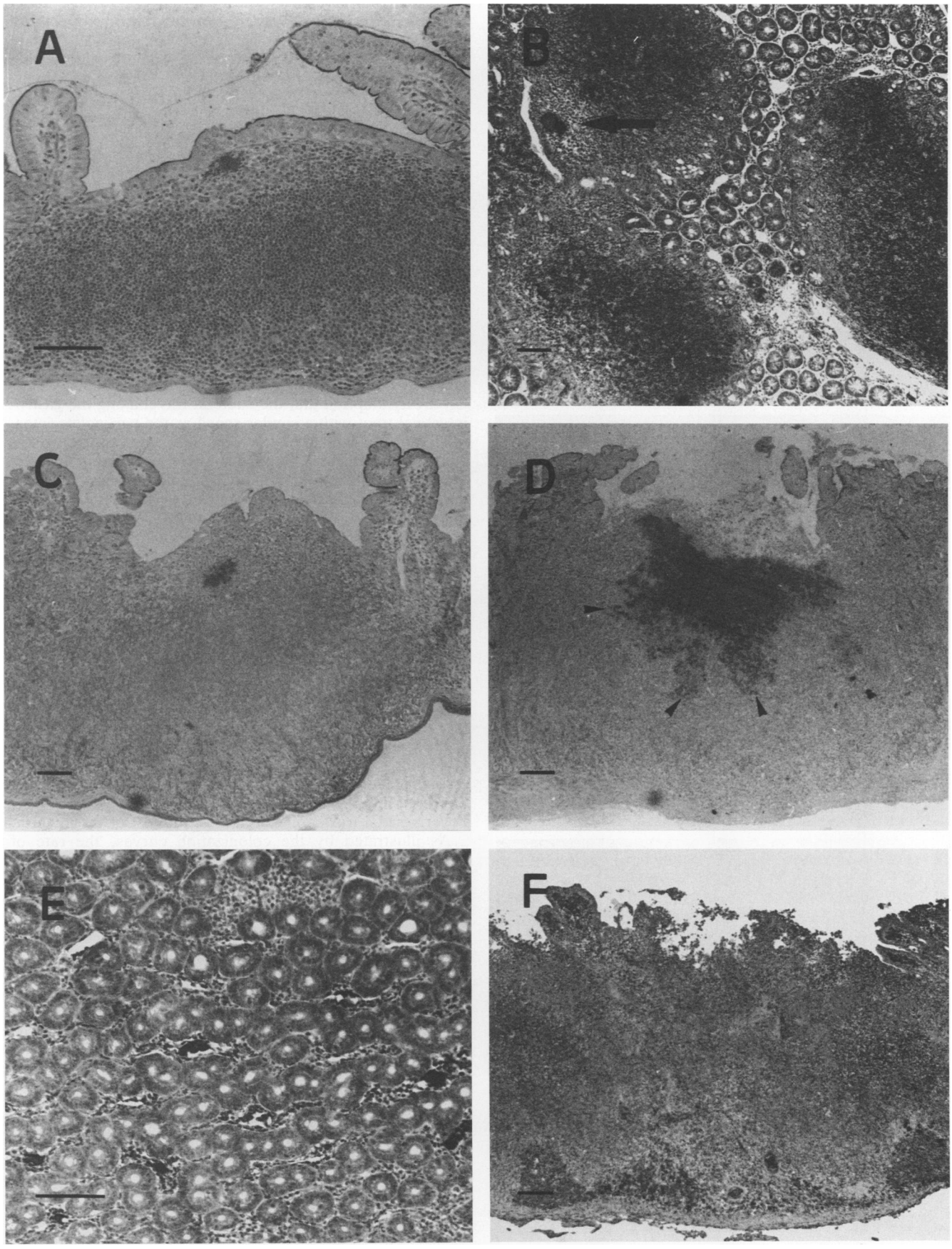
Immunohistochemical examination. Peyer's patches were fixed in 3% formaldehyde for 10 h, dehydrated in graded alcohol, and embedded in glycolmethacrylate (RWL-Histotechnologie, Bruckmühl, Federal Republic of Germany) or in paraffin. After this embedding procedure, 5-μm sections were cut and mounted onto glass slides. They were then incubated sequentially for 1 h with rabbit anti-*Yersinia* antiserum, mouse anti-rabbit immunoglobulin, and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (DiaNova, Hamburg, Federal Republic of Germany). As a substrate for alkaline phosphatase, AS-biphosphate (Sigma Chemical Co., Munich, Federal Republic of Germany) was used.

Immunofluorescence staining. Basement membranes in the small intestine and in the Peyer's patches were immunostained on 9-μm frozen tissue sections with affinity-purified antibodies against collagen type IV, which was obtained as described by Risteli et al. (17), or with antibodies against laminin (Collaborative Research, Inc., Bedford, Mass.). Nonimmune rabbit immunoglobulin G and goat serum were used as controls for nonspecific staining. Fluorescein isothiocyanate or rhodamine (TRITC) conjugates (second antibodies) were obtained from Dakopatts (Hamburg, Federal Republic of Germany).

Electron microscopy. Peyer's patches were fixed in ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 12 h, postfixed in 1% OsO_4 in 0.2 M cacodylate buffer (pH 7.4) for 1 h, washed in the cacodylate buffer, dehydrated in graded ethanol, and embedded in Epon 812 resin (Serva, Heidelberg, Federal Republic of Germany). Semithin sections were mounted onto glass slides and stained with toluidine blue for quick evaluation. Ultrathin sections (50 nm) were picked up on copper grids and stained with uranyl acetate and lead citrate. Evaluation was carried out with an electron microscope (Philips 410).

Determination of epithelial surface of murine ileum. Mor-

FIG. 3. Spreading of bacteria in Peyer's patches after defined time intervals after infection with 4×10^7 bacteria per mouse. Bacteria were visualized immunohistochemically in paraffin- or glycolmethacrylate-embedded tissue, with alkaline phosphatase used for detection. (A) Glycolmethacrylate section at 19 h after infection. Bacteria are proliferating beneath the virtually intact epithelium. (B) Paraffin section at 19 h after infection. Only a single colony (arrow) is visible. (C) Glycolmethacrylate section at 22 h after infection. Spreading of the colony is toward the germinal center of the follicle. (D) Glycolmethacrylate section at 28 h after infection. Small microcolonies (arrows) are detached from the main lesion. (E) Glycolmethacrylate section at 45 h after infection. Bacterial colonies in the lamina propria are in the vicinity of the dome. (F) Paraffin section at 63 h after infection. Complete colonization and destruction of the Peyer's patch. Bars, 0.1 mm.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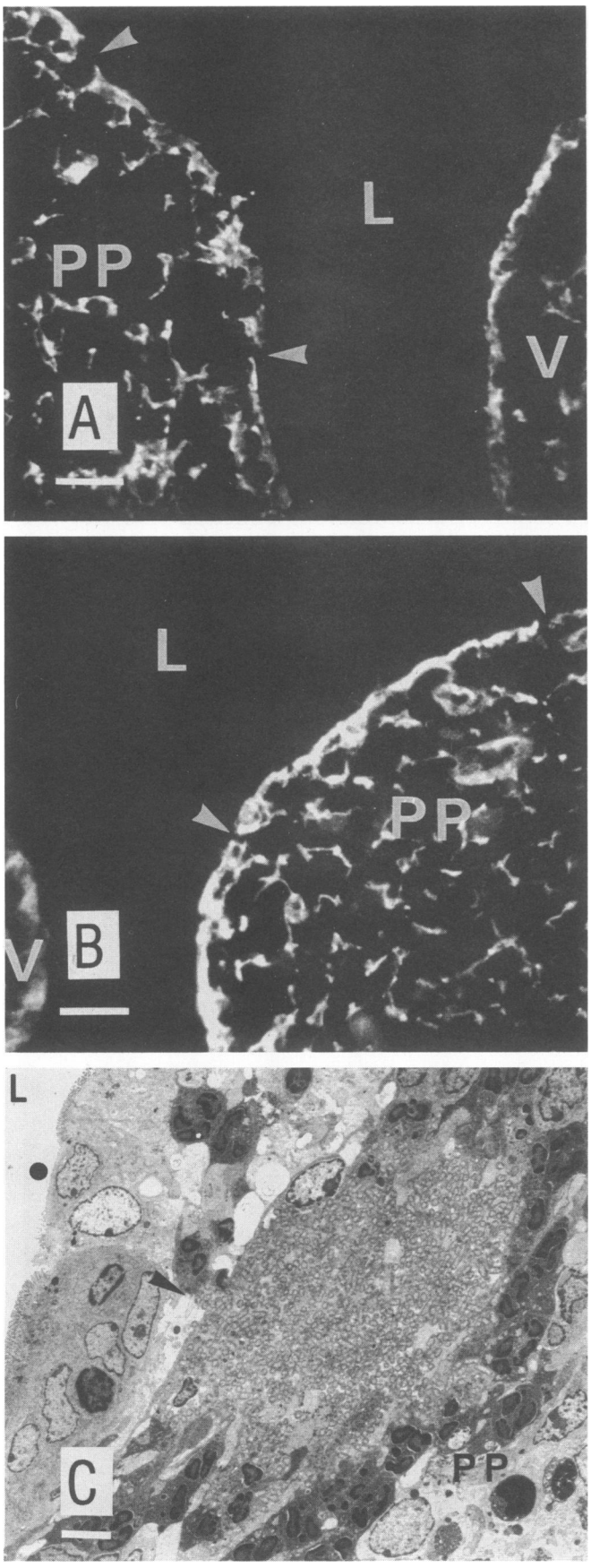

phological measurements were carried out on the small intestines from three mice of the same age and strain as those that were used for infections, and the average values were calculated. The average length of the whole small intestine was 45 cm, of which the distal third, the ileum, was investigated. The circumference of the ileum was measured on a longitudinally slit and spread intestine. For determination of the surface area of the Peyer's patches, the spread tissue was covered with a transparent copy of graph paper. The surface area was determined under a stereomicroscope by counting the number of 1-mm^2 fields overlying the Peyer's patch.

RESULTS

Colonization of ileal mucosa and Peyer's patches after oral challenge. The approximate retention time of ingested bacteria in the alimentary canal was estimated by using a foodstuff dye, which irreversibly stained the particulate material of the intestinal contents. Under the experimental conditions, the mice excreted the dye in the stool between 4 and 7 h after they were given access to the drinking water. On the assumption that the bacteria travel along the gastrointestinal tract with a similar speed as the particulate intestinal contents, most of the originally ingested organisms would enter the ileum within 3 h and leave it within 6 h after the start of the experiment. However, as shown in Fig. 1, the number of bacteria in the lumen of the small intestine increased for 4 days after infection, indicating that their proliferation was faster than their excretion within the feces. This continuous challenge of the intestinal wall with a high number of bacteria present in the lumen resulted in a very uneven colonization of different areas of the ileum.

During the whole period of infection the number of bacteria in a single Peyer's patch was comparable to that in the rest of the ileal mucosa (Fig. 2A and B). The surface area of a murine Peyer's patch varied between 5 and 8 mm^2, while that of the small intestinal mucosa was about 1,200 mm^2 (150 by 8 mm). The epithelial surface is enlarged by the surface of the villi by a factor of about 5 (5). Therefore, the effective epithelial surface of the investigated mouse ileum is about 6,000 mm^2; i.e., it is 750 to 1,200 times greater than the epithelial surface of a single Peyer's patch. Notwithstanding this great difference in surface area, the colonization of one Peyer's patch was similar to that of the rest of the mucosa.

As illustrated by the colonization curves, the rate of bacterial multiplication in both areas was roughly the same but the initial entrance into Peyer's patches was about 1,000 times greater than that into the remaining mucosa with a comparable surface area. This identifies the Peyer's patches as the main route of infection by *Y. enterocolitica*.

Spread of the bacteria within Peyer's patches. Nineteen hours after oral challenge, sections treated with the antibody raised to *Y. enterocolitica* disclosed small bacterial colonies beneath the intact epithelium of the Peyer's patches (Fig. 3A and B). At this time bacteria were only rarely seen in the

FIG. 4. Influence of basement membrane structure on bacterial spreading. Gaps (arrowheads) in the basement membrane under the intact epithelium of the dome of a Peyer's patch with anti-laminin staining (A) and anti-collagen type IV staining (B). Abbreviations: L, intestinal lumen; V, villus; PP, Peyer's patch. Bars, 30 μm. (C) Passage of bacteria through a gap in the basement membrane (arrowhead) underlying the dome epithelium. Bar, 5 μm. The epithelial cell marked with a dot and overlying the gap has the morphology of the M cell.

lamina propria of the villus base. They proliferated mainly within the Peyer's patches and migrated into the germinal center and then into the surrounding lamina propria (Fig. 3C, D, and E). By day 3 the whole Peyer's patch was colonized and its normal architecture was completely destroyed (Fig. 3F). At higher doses of bacteria (5×10^9 bacteria per mouse) the same histopathological changes occurred at a faster pace, followed usually by the death of the mouse within 7 days.

Interaction with cells of the lamina propria and with the basement membrane. The immunohistochemical findings were corroborated and extended by ultrastructural analysis. The bacteria passed the dome epithelium without destroying it (Fig. 3A and 4C). Although the colonies were observed beneath the M cells (Fig. 4C), no organisms could be identified within these cells. The extensive proliferation of the bacteria within the Peyer's patch led to the gradual destruction of its epithelium (Fig. 5A and B), while the epithelium of the villi remained intact throughout the infection period (data not shown). The proliferating *Y. enterocolitica* were restricted by the intact basement membrane (Fig. 5A) but migrated freely through any gaps in this structure (Fig. 4C and 5B). These gaps, with a diameter of up to 15 μm, could be also visualized under the epithelium of intact, noninfected Peyer's patches with anti-collagen type IV and anti-laminin antibody (Fig. 4A and B). In the lamina propria the colonized area was surrounded with polymorphonuclear leukocytes. However, most of the leukocytes did not exhibit phagocytic activity toward *Y. enterocolitica* (Fig. 4C and 5C).

DISCUSSION

In the present study infection with *Y. enterocolitica* was investigated by determining the number of bacteria in different areas of the small intestine. At the same time the spreading of bacteria and their interaction with intestinal cells were visualized by immunohistochemistry and electron microscopy. Oral infection of mice with plasmid-bearing *Y. enterocolitica* serotype O8 resulted primarily in the colonization of Peyer's patches, from which the bacteria spread to surrounding tissue. Indeed, each Peyer's patch was more highly colonized (by a factor of about 1,000) than the remaining ileal mucosa of comparable surface area, probably because of the facilitated passage through the dome epithelium. As reported by Hohmann et al. (8), colonization of the murine intestine by *Salmonella typhimurium* exhibited a similar distribution of the organisms between Peyer's patches and the remaining mucosa. It has not been further investigated whether colonization of the rest of the mucosa is diffuse or limited only to the solitary intestinal lymph follicles along the ileum, which are known to have similar properties as Peyer's patches (14). The immunohistochemical visualization of the bacteria in tissue sections with subsequent histological staining permitted analysis of the time course of the infection, as well as of the manner in which the bacteria spread within the tissue. The results

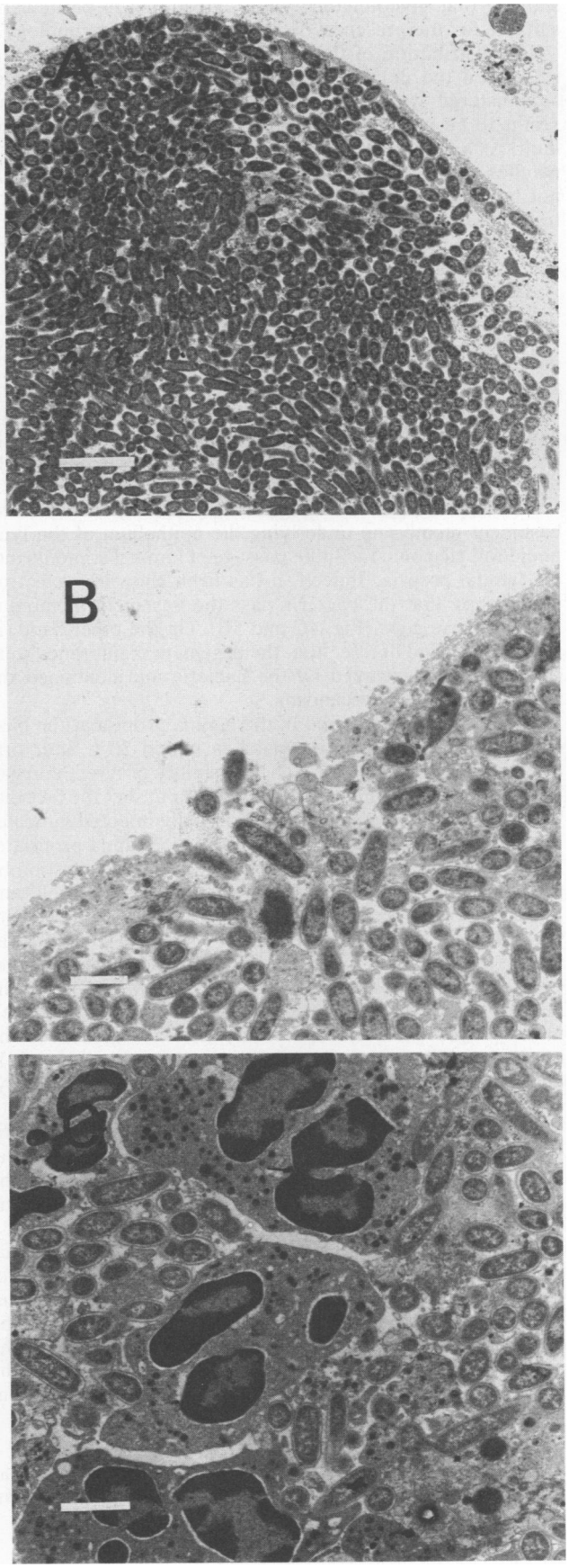

FIG. 5. Interaction of *Y. enterocolitica* with intestinal cells and basement membrane. (A) Extensive proliferation of bacteria in the apical part of the dome without rupturing the basement membrane. Bar, 5 μm. (B) Passage of bacteria through a gap in the basement membrane of the dome. Bar, 2 μm. (C) Resistance to phagocytosis by polymorphonuclear leukocytes in the lamina propria of a Peyer's patch. Bar, 2 μm.

showed that glycolmethacrylate embedding is particularly suitable for the preservation of intact follicle morphology.

The distribution of the bacteria within the tissue closely resembled the uptake by the intestine of carbon particles administered with the drinking water (9). Bacterial entry seems to be facilitated at certain areas of the dome epithelium. As a result the initial bacterial infection of the Peyer's patches was limited to sparse foci (Fig. 3A and B). It could not be determined whether M cells are responsible for the transfer of organisms into the lamina propria. The bacterial colonies were observed beneath the M cells (Fig. 4C), but no bacteria were found within M cells.

Another determinant of bacterial entrance into the Peyer's patches appeared to be the structure of the basement membrane underlying the dome epithelium. As shown by immunofluorescence with anti-laminin and anti-collagen type IV antibodies (Fig. 4A and B), the intact basement membrane exhibited gaps with a diameter of more than 10 μm through which the bacteria could freely pass into the lamina propria. By contrast, the basement membrane of the villi appeared to be thicker and did not exhibit any gaps.

As suggested by McClugage et al. (13), the porosity of the basement membrane underlying the epithelium of the lymphoid follicle could facilitate passage of luminal contents into the lamina propria. Indeed, it has been shown by electron microscopy that the bacteria pass the basement membrane through these gaps (Fig. 4C and 5B). On the other hand, in the initial period of infection, the basement membranes were generally not destroyed by the bacteria and contained the spread of the microorganisms.

The bacteria were found in the lamina propria at the base of the villi adjacent to the patches within 20 h after oral infection (data not shown). The possibility of direct entry of bacteria into villi cannot be totally ruled out, but the fact that the villus base and not the tip was initially infected indicates that the organisms migrated there via the lamina propria of the Peyer's patches. This could be the result of ingestion by macrophages, which then migrated to neighboring villi and mesenteric lymph nodes (10). However, in agreement with previous reports (6, 12), the invasive strain of *Yersinia* was found to be resistant to phagocytosis. The number of phagocytosed bacteria was negligible in comparison with the number of bacteria that were present extracellularly; i.e., macrophages could transport only a small fraction of the organisms. The other possibility is the migration of *Y. enterocolitica* via lymphatic channels associated with Peyer's patches.

The fast spreading of bacteria from the follicle into the surrounding lamina propria is consistent with the observation of Carter and Collins (3) that the dye injected into the Peyer's patches almost immediately spreads into lymphatic capillaries in the surrounding intestinal tissue.

Although the number of available bacteria in the intestinal lumen was high throughout the infection, the number of organisms in the Peyer's patches did not increase continuously. The immune response, however, did not prevent the destruction of Peyer's patches and the eventual death of the infected mice. The nature of bacterial resistance to the immune response of the host requires further investigation.

ACKNOWLEDGMENTS

This work was supported by grant An 152/1-1 from the Deutsche Forschungsgemeinschaft and by the Maria Sonnenfeld-Gedächtnisstiftung.

We thank A. J. Kenny for critical reading of the manuscript and many valuable suggestions.

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