Salmonella typhimurium Initiates Murine Infection by Penetrating and Destroying the Specialized Epithelial M Cells of the Peyer's Patches

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Summary

Salmonella species are known to initiate infection of mammalian hosts by penetrating the intestinal epithelium of the small bowel. These bacteria preferentially interact with Peyer's patches which are collections of lymphoid follicles making up the gut-associated lymphoid tissue. We infected murine ligated intestinal loops with invasive and noninvasive *Salmonella typhimurium* strains for 30, 60, 120, and 180 min and examined the infected tissue by transmission electron microscopy. Within 30 min, we found that invasive *S. typhimurium* exclusively entered M cells found within the follicle-associated epithelium (FAE) of the Peyer's patches. Initially, interactions between invasive bacteria and enterocytes adjacent to the M cells were not found. Invasion of M cells was associated with the ability of the bacteria to invade tissue culture cells. *S. typhimurium* mutants, which were noninvasive for tissue culture cells, could not be found in ligated loops associated with M cells or enterocytes after incubations of 30, 60, 120, or 180 min. At 60 min, internalized invasive *S. typhimurium* were cytotoxic for the M cells. Destruction of an M cell formed a gap in the FAE which allowed organisms to invade enterocytes adjacent to the dead cell. Later in the infection process (120 and 180 min), the presence of bacteria beneath the FAE correlated with changes in the cytoarchitecture of the lymphoid follicle. In addition, replicating *Salmonella* began to enter both the apical and basolateral surfaces of enterocytes adjacent to infected M cells.

Salmonella species cause infections ranging from asymptomatic carriage and localized gastroenteritis to systemic enteric fevers (1). Infection is initiated when invasive organisms pass through the epithelial surface of the small bowel. Efforts have been made to identify the components of the process that allows *Salmonella* to penetrate the membrane of a mammalian cell. In one study, the steps leading to *Salmonella typhimurium* entry of ileal absorptive cells were examined after oral inoculation of starved, opium-treated guinea pigs (2). Organisms in close contact with the epithelial cell surface induced degeneration of enterocyte microvilli. At later stages of the entry process, cytoplasmic projections from the host cells surrounded invading bacteria until they were contained within membrane-bound vacuoles. Over time, the apical surface of the invaded enterocyte regenerated and the cell apparently returned to normal. The *Salmonella* entry process has also been studied in vitro. These studies have confirmed the in vivo observations that invasive *Salmonella* strains induce membrane rearrangements as part of the internalization process (3–8). Tissue culture systems have also been used to demonstrate that *Salmonella*-induced membrane rearrangements are blocked by inhibitors of actin polymerization (9–11).

Infection models have been used to trace the *Salmonella* route of infection. Carter and Collins (12) experimentally demonstrated that the terminal ileum is the primary site of *Salmonella* invasion. Furthermore, they found that orally inoculated *Salmonella enteriditis* are almost exclusively associated with the Peyer's patch follicles of the ileum rather than the intestinal wall. Hohmann et al. (13), using the oral route of infection, also found that *S. typhimurium* associates quickly with Peyer's patch tissue. Bacteria were isolated from the gut-associated lymphoid tissue (GALT)[1] within 6 h of infection and the number of bacteria that were recovered increased logarithmically for 2 d. Furthermore, the organisms could not be removed from the Peyer's patch tissue with washing. In contrast, the majority of the organisms that were associated with the small intestine and cecum were dissociated from the tissue by rinsing with PBS. Recently, an effort to find a model system for the study of the human pathogen *Salmonella*

[1] *Abbreviations used in this paper:* FAE, follicle-associated epithelium; GALT, gut-associated lymphoid tissue.

typhi was published which examined the interactions of this species with murine Peyer's patch tissue (14). Microscopic examination revealed that invasive *S. typhi* are able to attach to and destroy the M cells of Peyer's patches, although the authors concluded that there was no evidence of bacterial internalization.

Since invasive *Salmonella* strains seem to specifically associate with Peyer's patch tissue, we have used a ligated loop model to examine the interactions between *S. typhimurium* strains and ileal lymphoid follicles. We were particularly interested in determining whether the bacteria could productively interact with M cells, which are the specialized cells found exclusively in the follicle-associated epithelium (FAE) (15, 16). These cells are believed to play an active role in sampling the antigenic content of the bowel. Our findings suggest that specific interactions between these cells and invasive *S. typhimurium* initiates infection of the host.

Materials and Methods

Bacterial Strains and Growth Conditions. *S. typhimurium* SL1344 is a well-characterized invasive mouse virulent strain (17). BJ66 is an SL1344 derivative containing a Tn5*lac* insertion in the 58–59-min region of the *Salmonella* chromosome which renders the strain noninvasive for tissue culture cells (Jones, B. D., and S. Falkow, manuscript in preparation). χ3643 is a noninvasive SL1344 derivative which contains an *inv*A mutation (18). All *S. typhimurium* strains were grown in a low oxygen environment, which induces the expression of the *S. typhimurium* invasive phenotype (19).

Mice. 7–8-wk-old BALB/c female mice were used for all experiments and were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained by the Stanford University Department of Laboratory Animal Medicine.

Ligated Loop Model. The interactions between *S. typhimurium* strains and murine intestinal tissue were examined using a ligated intestinal loop model (20). In preparation for each experiment, mice were starved for 24 h before experiments to clear the contents of the bowel. Mice were anesthetized before surgery by intraperitoneal injection of 1.5–2.0 mg per mouse of Nembutal (Abbott Laboratories, North Chicago, IL). When the mouse entered the surgical stage of anesthesia, a small incision was made through the abdominal wall and the small bowel was exposed. A loop was formed by ligating the intestine with silk thread at the ileocecal junction and at a site ~4–5 cm proximal to the cecum. Care was taken to determine that at least one Peyer's patch was present in the section of ligated bowel. Before closing the loop with silk thread, the bacterial inoculum (~200 μl of a 4 × 10^8 CFU/ml culture) was injected into the intestine through a 25-gauge needle. The bowel was then returned to the abdomen and the incision was stapled closed. The mice were kept alive, under anesthesia, for periods of time ranging from 30 to 180 min before they were killed by cervical dislocation, and the intestinal loops were removed for processing.

Preparation of Tissue for Transmission Electron Microscopy and Hematoxylin and Eosin Staining. After the appropriate incubation time, the intestines were gently pulled through the abdominal excision and the section of the small intestine containing the Peyer's patch was removed with a razor blade. The piece of tissue was cut longitudinally along the side of the intestine opposite the Peyer's patch. The tissue was pinned flat on dental wax and immediately fixed with an ice-cold fixation solution of 2.5% glutaraldehyde, 0.8% paraformaldehyde in a 0.1-M phosphate buffer, pH 7.4. After 15 min, the tissue sections were removed from the dental wax and placed in vials containing fresh cold fixative for an additional hour. The tissues were postfixed with 1% osmium tetroxide (Polysciences, Inc. Warrington, PA) in 0.1 M phosphate buffer for 1 h and stained with 0.25% aqueous uranyl acetate (Polysciences, Inc.) overnight (15 h). The following day the specimens were dehydrated through a graded series of ethanol washes and the ethanol was then replaced with propylene oxide. Each sample was infiltrated with embedding resin made according to the instructions of the manufacturer (Poly/Bed 812 EM; Polysciences, Inc.) and hardened in the resin by heating to 65°C overnight. Thick sections were cut and stained to locate the characteristic domes of the Peyer's patch. Thin sections were then cut and examined with a Philips model 201c transmission electron microscope.

When infected Peyer's patches were to be examined for tissue pathology, the tissue was fixed in ice-cold 10% formalin in PBS overnight. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results

Specialized M Cells within the Follicle Epithelium Can Be Distinguished from Epithelial Cells by Electron Microscopy. The GALT of murine small intestines can be identified visually as small white patches on the side of the intestine opposite to the mesenteric membrane attachment site. As reported by others (21), each patch is composed of several follicles that are populated with lymphocytes and macrophages. Approximately 10–20% of the cells in the murine FAE are specialized M cells and the remaining 80–90% of the cells are typical enterocytes (21). As seen in Fig. 1, an M cell was distinguished from surrounding enterocytes by shortened apical microvilli, an increased number of pinocytic vesicles, and a flexible cytoskeleton which allowed lymphoid cells to bulge into the cytoplasm of the cell and locate in close proximity to the luminal surface of the bowel.

Invasive S. typhimurium Preferentially Invade M Cells of the Terminal Ileum. Murine intestinal ligated loops were infected with invasive *S. typhimurium* SL1344 or noninvasive *S. typhimurium* BJ66 grown in a low oxygen environment. At 30 min, microscopic examination of tissue revealed that when invasive *S. typhimurium* were found within a section of tissue, the bacteria were exclusively associated with M cells (Fig. 2 *a*). Affected M cells contained one or more bacteria. In contrast, adjacent enterocytes contained no internalized bacteria and appeared completely unaffected by the presence of the invasive organisms. These experiments have been repeated five times and yielded similar results each time. Within 30 min of infection, significant numbers of invasive *S. typhimurium* preferentially bound to and passed through the apical surface of M cells, whereas no significant invasion of enterocytes was detected within the same period of time. In addition, we noted that M cell uptake of invasive *S. typhimurium* was associated with pronounced membrane ruffling (Fig. 2 *b*). In several instances, we also observed that several intracellular bacteria could be found within a single M cell (Fig. 2 *c*). Examination of cells containing bacteria revealed that *Salmonella* entry had a profound effect on the M cells. As seen in Fig. 2 *b*, the mitochondria of invaded M cells were swollen and dis-

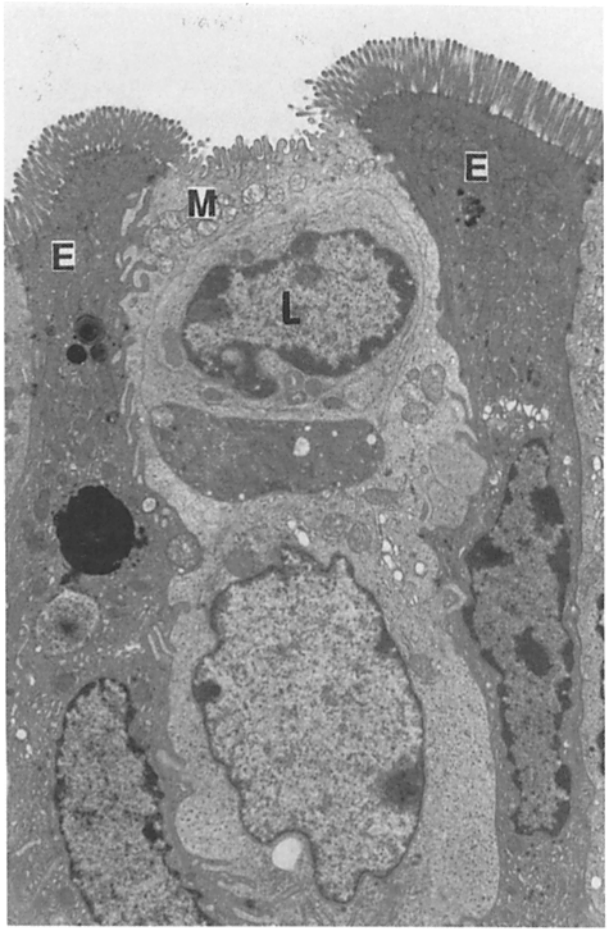


Figure 1. Transmission electron micrograph of uninfected mouse Peyer's patch tissue. The M cell (*M*) has short, irregular microvilli when compared to the microvilli of the neighboring enterocytes (*E*). A lymphocyte (*L*), which pushed into the cytoplasm of the M cell, can be seen immediately beneath the apical membrane of the cell. ×4,900.

rupted and the cytoplasmic content of the cell began to contract from the surrounding cells. We also examined the effect of inoculating ligated loops with the adherent, noninvasive *S. typhimurium* mutant BJ66. The LD50 of strain BJ66 is significantly higher than SL1344 when inoculated orally into mice but is equally as virulent as strain SL1344 when introduced by the intraperitoneal route of infection (our unpublished data). We were unable to find noninvasive *S. typhimurium* strain BJ66 attached to or within cells of the murine Peyer's patch after a 30-min infection, although this strain is as adherent for tissue culture cells as *S. typhimurium* SL1344.

M Cells Are Destroyed within 60 min after Infection of Murine Ligated Loops. The impact of invasive *S. typhimurium* on M cells, first observed within 30 min, was more apparent after the bacteria had been allowed to interact with the intestinal epithelium for 60 min. Observations of many different sections revealed that the apical membranes of M cells did not regenerate microvilli after invasion by *S. typhimurium* but were extruded into the lumen of the gut along with the cytoplasmic contents of the cell (Fig. 3 *a*). Destruction of these cells disrupted the integrity of the membrane and presumably caused depolarization of adjacent cells. We observed that in the presence of such tissue damage, invasive *S. typhimurium* were capable of entering enterocytes. Lymphoid cells which were beneath the damaged M cells appeared to be in the process of migrating into the lumen of the intestine (Fig. 3 *b*). Often, remnants of an M cell that contained intracellular bacteria were seen moving away from the basement membrane (basal lamina) of the epithelial layer. The tight junctions of adjacent enterocytes were disrupted and the cells were dying or were dead. Underlying cells were exposed to the intestinal lumen and pathogenic bacteria had free access to the basal lamina. In contrast, noninvasive *S. typhimurium* strain BJ66 was not detectable in the murine tissue after 60 min. Thus, the invasiveness of *S. typhimurium* was directly responsible for the M cell destruction that we observed.

Virulent S. typhimurium SL1344 Induces Damage of the Follicle Epithelium after it Destroys the M Cells. Major changes were observed in the structure of the Peyer's patch epithelium 120 min after the initial invasion of M cells. A gap in the follicle epithelium, formed by the destruction of an M cell, allowed bacteria to move to the basement membrane of the epithelium. In many sections, invading bacteria were seen following a path through a dead M cell to reach the basal lamina (Fig. 4). Bacteria that had passed through M cells usually encountered lymphoid cells. Invasive organisms were frequently found within the lymphoid cells that lay immediately beneath the M cells (Fig. 5). Pathogenic organisms which gained access to the basal lamina layer but did not enter a lymphoid cell, induced general damage to the epithelium. Bacteria, moving laterally from the site of initial penetration, had a deleterious effect on the enterocytes. In some instances it was possible to find enterocytes that had begun to slough away from the epithelium but that were still physically attached to the adjacent cells by the tight junctions. It was more common, however, to find clusters of dead and dying enterocytes that had lifted away from the intact epithelium and that were laying within the lumen of the gut (Fig. 6). At 180 min after infection, large stretches of the follicle epithelium were completely denuded of enterocytes and the basal lamina was completely exposed to organisms within the small intestine. The noninvasive strain BJ66, inoculated into ligated loops for 120 and 180 min, did not visibly damage the follicle epithelium when the tissue was examined by electron microscopy. However, a single example was found of a healthy M cell containing intracellular bacteria (Fig. 7). The microvilli of this cell appeared normal and the characteristic membrane rearrangements associated with *Salmonella*-induced invasion were absent. A second noninvasive *S. typhimurium* mutant χ3643 was also tested in our system. As with strain BJ66, it had no effect on murine Peyer's patches.

Pathology of Murine Tissue Infected with Invasive and Noninvasive S. typhimurium Strains. The gross effects of invasive and noninvasive *S. typhimurium* on the Peyer's patch tissue over the 3-h time course of the ligated loop experiments were also assessed by examining infected murine tissue stained with hematoxylin and eosin. Tissue infected with the noninvasive

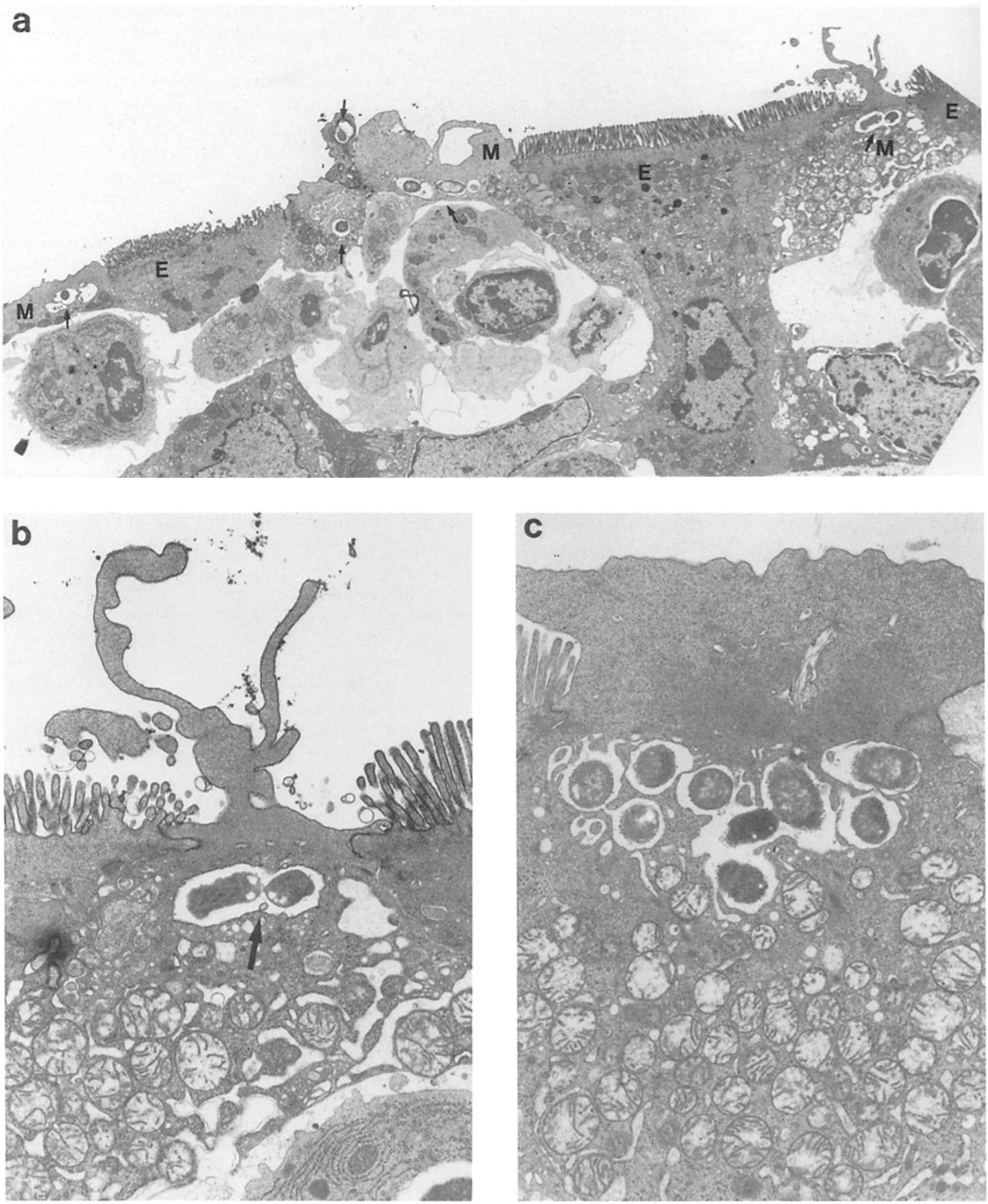


Figure 2. Invasion of M cells 30 min after infection of murine ligated loops with invasive *S. typhimurium* SL1344. (*a*) A contiguous region of FAE containing three M cells (*M*) is shown. Each of the M cells contains intracellular bacteria (*arrows*). Intervening enterocytes (*E*) are unaffected by the presence of the bacteria. Changes in the underlying tissue are obvious. ×3,800. (*b*) The M cell from the far right in *a* is shown at higher magnification. Two internalized bacteria (*arrow*) are seen within the M cell after inducing a dramatic rearrangement of the membrane. ×12,000. (*c*) Several bacteria have been internalized into a single M cell. ×12,600.

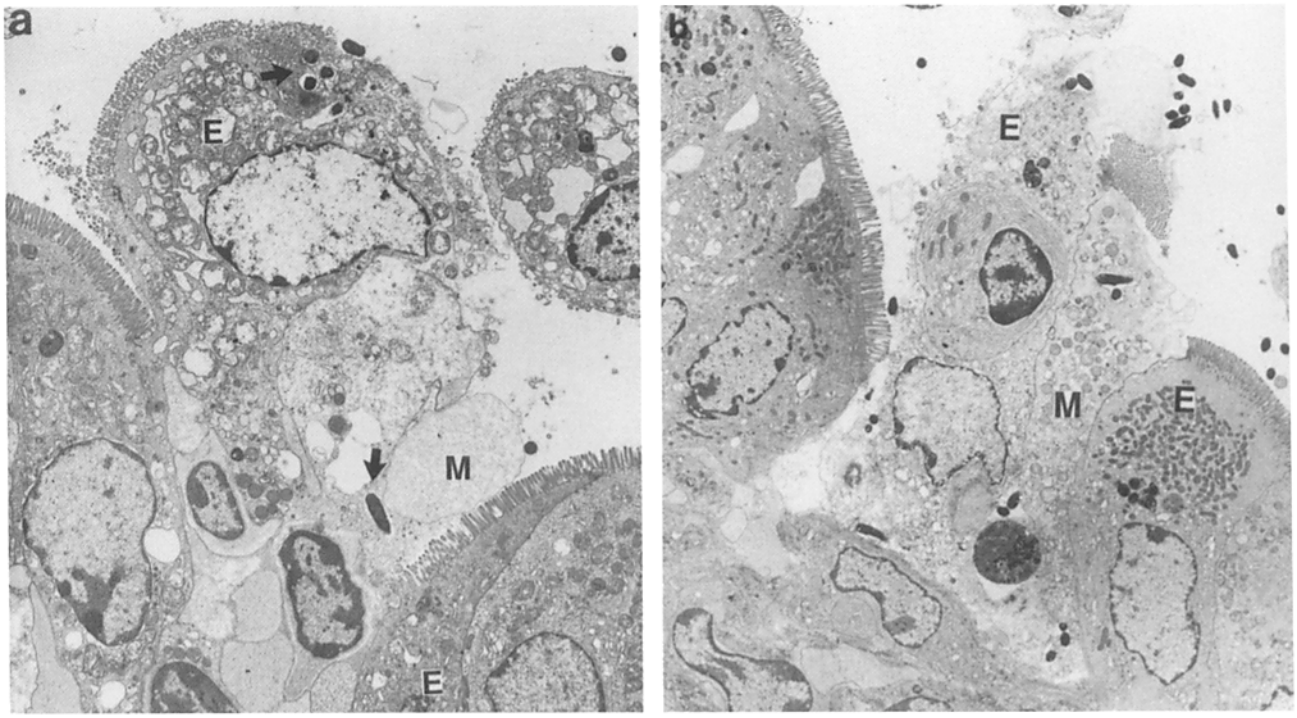
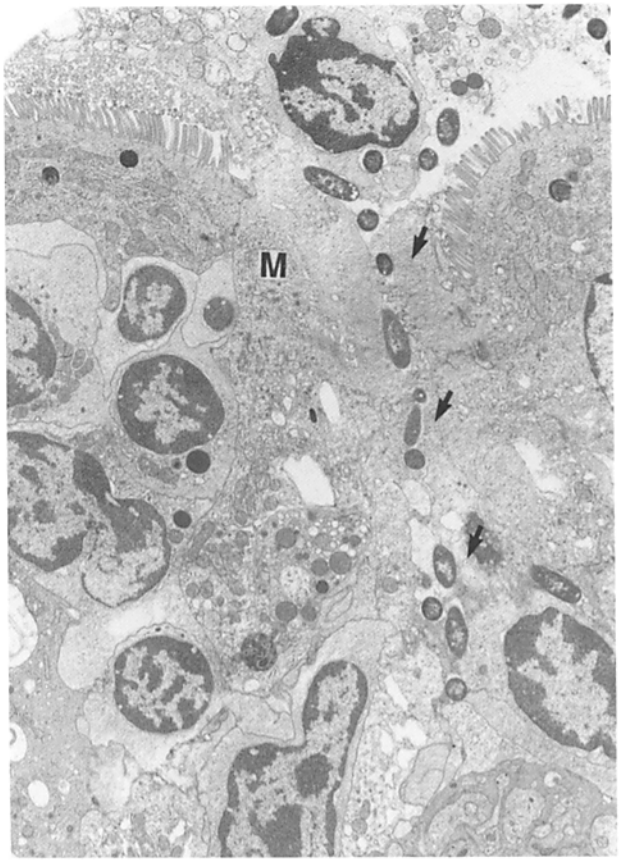


Figure 3. *S. typhimurium*–induced cytoplasmic extrusion of M cells. (*a*) The cytoplasm of an M cell (*M*) appears to have erupted into the lumen of the small intestine after internalization of *S. typhimurium* SL1344 (*arrow*). One of the adjacent enterocytes (*E*) contains intracellular bacteria (*arrow*) and is detaching from the epithelium whereas the enterocyte on the other side is still intact. ×3,200. (*b*) *Salmonella*-induced destruction of an M cell and a neighboring enterocyte has created a hole in the epithelium. Lymphoid cells are moving outward and bacteria have free access to the lamina propria. ×2,400.



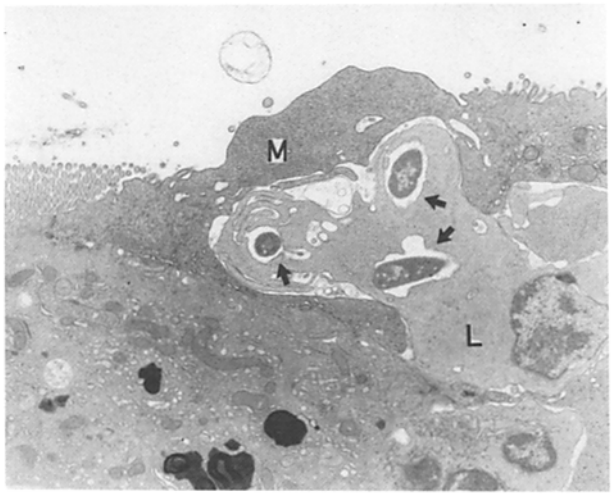


Figure 5. Invasive *S. typhimurium* SL1344 enters underlying lymphoid cells. 120 min after infection of a mouse intestinal loop three bacteria were found within a lymphoid cell (*L*) immediately below an M cell (*M*). ×5,500.

Figure 4. Penetration of pathogenic *S. typhimurium* through an M cell 120 min after infection of a murine ligated loop. Numerous bacteria (*arrows*) are penetrating the epithelium through the remnants of an M cell (*M*). ×4,500.

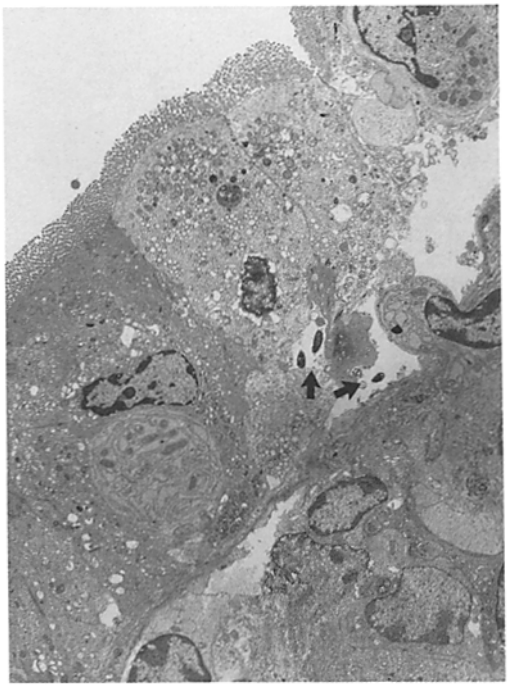


Figure 6. Sloughing of enterocytes from the dome epithelium. Organisms (*arrows*) which reached the basal lamina membrane caused general destruction of the FAE. Large groups of enterocytes around the site of invasion are lifting off. ×2,100.

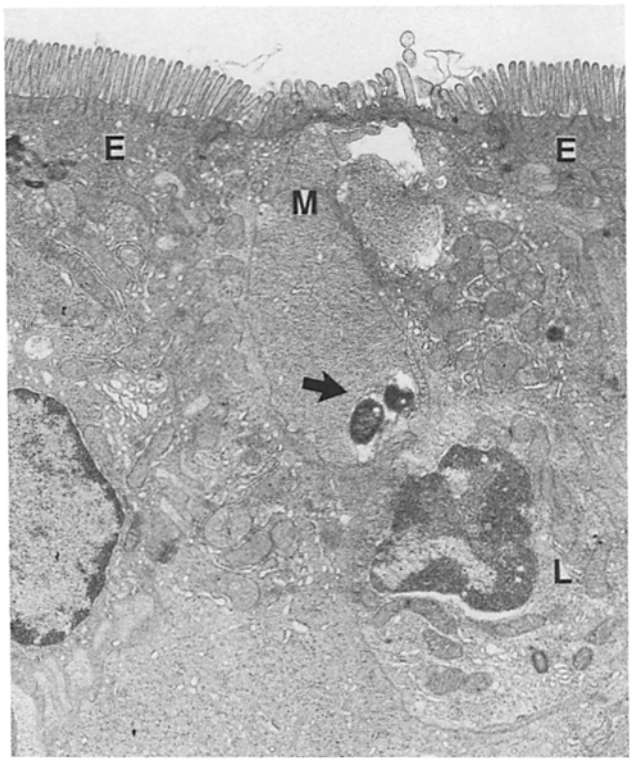


Figure 7. Noninvasive *S. typhimurium* strain BJ66 within a healthy M cell. Two intracellular organisms (*arrow*) are visible near the bottom of an M cell (*M*). The M cell, neighboring enterocytes (*E*), and underlying lymphoid cell (*L*) appear completely normal. ×7,900.

S. typhimurium BJ66 appeared healthy and normal. In contrast, Peyer's patches exposed to *S. typhimurium* SL1344 exhibited epithelial cell damage and activation of a substantial number of lymphoid cells (enlarged cytoplasms) (data not shown). In addition, there was some tissue necrosis and cellular debris present in the follicle dome (data not shown).

Discussion

We have examined the interactions between invasive *S. typhimurium* and murine ileal Peyer's patch tissue in an intestinal ligated loop model. The sequence of events leading to infection is diagrammed in Fig. 8. These observations are based on a high number of bacteria interacting with defined region of the ileum. Whereas the general epithelial damage that was observed at later time points may have been a consequence of the high numbers of bacteria present in the tissue, it was clear that invasive *S. typhimurium* selectively entered M cells within the FAE by induction of membrane ruffling (Fig. 8 *a*). Bacterial entry was followed closely by extrusion and death of the M cell (Fig. 8 *b*). After elimination of the M cell, organisms either moved laterally along the basal lamina or deeper into the dome of the follicle (Fig. 8 *c*).

M cells are specialized epithelial cells that are found exclusively in lymphoid FAE (15, 16). These cells form tight junctions with adjacent enterocytes, possess microvilli, and line up along the basement membrane (22). They possess a flexible cytoskeletal structure that allows lymphoid cells migrating toward the epithelial cell to deform their cytoplasm. M cells have the capacity to actively take up particles that range in size from small proteins to bacteria and protozoa (23–30). It is likely that pinocytosized material from the lumen of the intestine is transported from the apical to the basolateral surface of the cell where it is delivered to underlying lymphoid cells. This constant antigenic stimulation is believed to keep the intestinal immune system primed for a quick response to microbial insult.

Efforts have been made previously to understand how invasive *Salmonella* species cross the epithelial barrier of the intestine. Takeuchi (2), using an opium-treated guinea pig model, demonstrated that invasive *S. typhimurium* could invade and pass through enterocytes of the ileum 12 h after infection. He concluded that invasive *S. typhimurium* initially penetrates enterocytes of the small bowel. Others (12–14) have postulated that the lymphoid follicles of the small intestines are the host portal of entry since pathogenic *Salmonella* species initially localize to the Peyer's patches after oral inoculation. We have found that invasive *S. typhimurium* preferentially associates with and invades M cells within 30 min of infection. Furthermore, this entry is followed closely by the death of the cell. Within the same time period we were unable to detect interactions between invasive *S. typhimurium* and enterocytes. These results support the conclusion that *Salmonella* infection of a host is initiated by colonization of the GALT.

Other enteric pathogens are known to take advantage of the mucosal antigen sampling system. Reovirus type I promotes its own uptake by binding via specific receptors to the

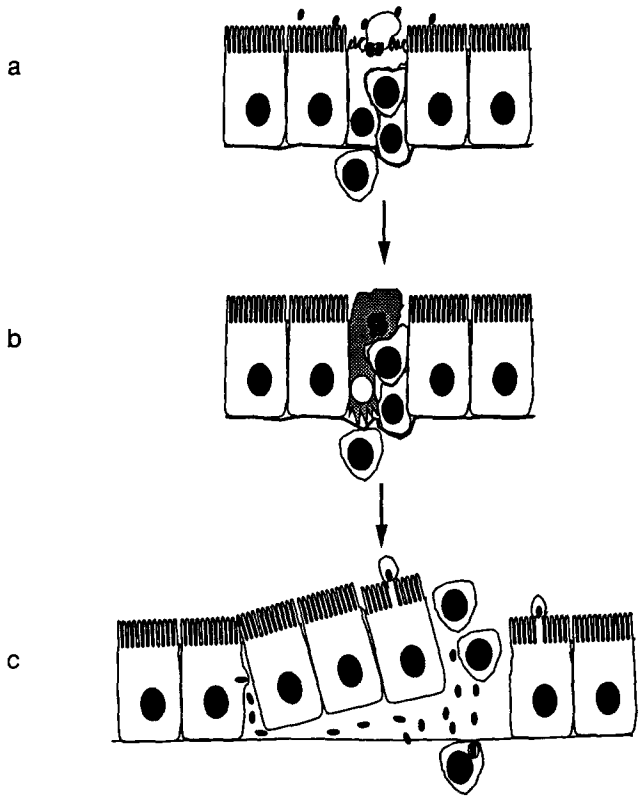


Figure 8. A diagram illustrating the events leading to establishment of *S. typhimurium* infection of a murine Peyer's patch. (*a*) Invasive bacteria initiate membrane ruffling at the apical surface of an M cell which leads to uptake of the bacteria (30 min). (*b*) M cells containing bacteria die, forming a gap in the FAE (30–60 min). (*c*) Destruction of M cells allows pathogenic bacteria to move unhindered to the basement membrane of the epithelium. Underlying lymphoid cells move upward into the lumen of the intestine. Bacteria beneath the epithelial surface induce sloughing of enterocytes and begin to invade lymphoid cells within the follicle dome.

M cell apical surface (23). *Escherichia coli* strain RDEC-1 specifically binds to M cells of the rabbit Peyer's patches (31). In contrast to *Salmonella*, this attachment appears to prevent M cell uptake of RDEC-1, allowing these organisms to colonize the host intestinal tract. Others have shown that *Mycobacterium bovis* BCG, *Shigella flexneri*, and *Yersinia* spp. are phagocytized and transported through rabbit M cells (28, 32, 33). *Listeria monocytogenes* is also known to associate with Peyer's patch tissue during infection (34). However, it has not been demonstrated that passage through M cells is necessary for *Listeria* virulence. Collectively, these data suggest that a variety of pathogenic microorganisms take advantage of the normal M cell sampling mechanism to move through the intestinal epithelium and establish infection of the host. However, our data indicate that a pathogenic organism actively induces its own uptake into M cells.

Our Stanford University laboratory, as well as others (3–6, 8) has demonstrated that *Salmonella* invasion of tissue culture cells is accompanied by actin polymerization and host cell membrane rearrangement. These cytoskeletal rearrangements are essential to entry as inhibitors of actin polymerization also block entry (9–11). In this study, *S. typhimurium* SL1344 invasion of murine M cells was always accompanied by membrane ruffling. Apparently, invasive *S. typhimurium* initiates a signal at the surface of M cells which induces membrane ruffling and uptake of the bacteria. However, unlike tissue culture cells or the invaded murine enterocytes described by Takeuchi (2), M cells that have been induced to ruffle by invasive *S. typhimurium* do not appear to return to an unactivated state. In fact, we found that bacterial internalization caused death of M cells. These results indicate that the murine ligated loop model is a relevant in vivo invasion assay. It is one more tool that can now be used to measure the importance of *Salmonella* virulence genes. The noninvasive *S. typhimurium* mutants that we tested were unable to induce apical membrane ruffling of M cells and had no pathological effect on the intestinal tissue. These results strongly suggest that membrane ruffling is an integral component of the in vivo *S. typhimurium* invasion mechanism.

Our laboratory recently published work (5) showing that noninvasive bacterial strains or even latex beads can passively enter tissue culture cells which have been induced to ruffle by invasive *Salmonella*. During the course of our observations, we noted that several bacteria were often present within a single M cell (Fig. 2 *b*). Although it is possible that these bacteria entered the cell by independent invasion events, it appears that ruffling induced by a single invasive organism may allow several bacteria to be passively internalized. We postulate that such a phenomenon would allow numerous bacteria to pass through the intestinal epithelium and initiate infection of a host from a single productive interaction between an invasive bacterium and an M cell.

Host-adapted invasive *Salmonella* species cause systemic disease whereas nonhost-adapted species seem to be restricted to localized infection of the intestinal epithelium (1). Our work and the work by Kohbata et al. (14) indicate that both the mouse-adapted *S. typhimurium* strains and the human-adapted *S. typhi* strains have the ability to destroy the M cells of murine Peyer's patches. Apparently, M cell destruction is a common first step in the establishment of either localized or systemic infection. It seems likely that, after passing through the M cells, an infection is limited to gastroenteritis (invasion and destruction of enterocytes) if a particular *Salmonella* strain is unable to survive within the environment of the host lymphatic system. Passage through M cells allows host-adapted strains, which are able to multiply within the lymphatic system, to invade circulating lymphoid cells within the Peyer's patch. Regardless of the type of infection that a particular strain of *Salmonella* establishes in a host, it appears that invasion of M cells is a critical first step.

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