

Role of M Cells in Initial Antigen Uptake and in Ulcer Formation in the Rabbit Intestinal Loop Model of Shigellosis

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Strains of *Shigella flexneri* with different invasive and pathogenic potentials were inoculated into the intestinal lumen of acutely ligated loops in nonimmune rabbits. After 90 min, tissues processed for ultrastructural as well as light microscopy showed that the bacilli were phagocytosed by M cells over lymphoid follicles of Peyer's patches and carried in vacuoles into the epithelium. Nonpathogenic as well as pathogenic strains were readily taken up regardless of the presence of the 140-megadalton virulence plasmid. More virulent than avirulent shigellae were found in M cells at 90 min, reflecting replication or preferential uptake of the virulent strains. Heat-killed shigellae of the virulent strain were taken up by M cells to the same degree as the avirulent strains. Incubation of the bacteria for 18 h resulted in surface ulceration which was limited to epithelium overlying lymphoid follicles (M cell areas) in acute loops exposed to the virulent shigellae. Villus epithelium adjacent to the ulcerated follicular domes was intact, although there was mucus depletion. In the present study, we found that pathogenic shigellae appear to replicate in the M cells, escape from the phagocytic vesicles, and thereby initiate the ulcerations in this experimental model of dysentery. While initial antigen processing in the gut for a mucosal immune response may require uptake of luminal microorganisms by M cells, this may pose a threat under some circumstances.

Shigellosis is an acute bacterial enteritis, which in humans involves principally the colon. Often both the ileum and the colon are affected with focal mucosal ulceration. The classical picture is abdominal cramps, colicky pains, and an early diarrheal phase (likely due to the action of *Shiga* toxin) characterized by the passage of voluminous watery stools, followed hours or days later by a dysentery phase. In the latter, *Shigella flexneri* bacilli invade the surface epithelium, multiply, migrate laterally from cell to cell, and enter the lamina propria (16). Finally, superficial ulceration and bleeding occur. As a result, patients with dysentery pass frequent small-volume stools containing blood, mucus, and pus in addition to the previously mentioned symptoms (8, 16, 23, 25).

Our laboratory has used a chronically isolated ileal loop model in rabbits to follow, in intestinal secretions, the secretory immunoglobulin A (IgA) response to immunization with *Shigella flexneri*. In those studies, either oral or intra-loop immunization with invasive or noninvasive *S. flexneri* consistently initiated a secretory IgA response to *Shigella* lipopolysaccharide (LPS) (9-11). Furthermore, invasive *Shigella* strains containing the 140-megadalton virulence plasmid were no more effective at achieving a strong secretory IgA response to *Shigella* LPS than were noninvasive strains. A noninvasive strain, *S. flexneri* M4243A₁, which lacks the 140-megadalton virulence plasmid, given orally was as effective at eliciting a mucosal memory response to *Shigella* LPS as was locally invasive *Shigella* strain X16 (containing the virulence plasmid) (12). In the present studies, we used an acute ileal loop model in rabbits to determine whether the ability of noninvasive strains to stimulate a mucosal immune response depends on their phagocytosis by specialized lymphoid follicle-associated epithelial cells (M cells) previously described to be responsible for the initial processing of luminal macromolecules and microorganisms (1, 5, 7, 17-20, 22, 27, 28). In addition to providing information by sampling

of intact shigellae, the present findings indicate that the initial ileal ulcerations in this model occur over intestinal lymphoid follicles.

MATERIALS AND METHODS

Bacterial strains used. Strains of *S. flexneri* used were originally provided from the laboratory of Samuel B. Formal at the Walter Reed Army Institute of Research. *S. flexneri* M4243 is a virulent strain which can invade the epithelium, proliferate, and cause superficial ulceration. It contains the 140-megadalton virulence plasmid and produces a positive Sereny test (2). *Shigella* strain X16 is a hybrid of *S. flexneri* and *Escherichia coli*. It also contains the 140-megadalton virulence plasmid and is able to invade the surface epithelium. However, it does not persist after invasion, does not replicate, causes no ulceration (3), and does not give a positive Sereny test. *S. flexneri* 2457-0 is a noninvasive strain, although it retains the 140-megadalton virulence plasmid. It neither causes ulcer formation in rabbit intestinal mucosa nor gives a positive Sereny test (4). *S. flexneri* M4243A₁ is a noninvasive strain which lacks the virulence plasmid (24). Predictably, this strain neither causes ulcer formation nor gives a positive Sereny test. To see the difference in uptake between live and killed bacteria, we studied the uptake of heat-killed M4243. The overnight bacterial culture was heated in boiling water for 10 min. The heat-killed M4243 culture did not grow when streaked on a MacConkey plate.

Preparation of acute ileal loops. For each strain of *Shigella* studied (Table 1), three New Zealand White rabbits weighing about 3 kg each were anesthetized with Xylazine and Ketamine. A midline abdominal incision was made, and the ileum was identified. Then, 8- to 10-cm loops containing a grossly identifiable Peyer's patch were constructed by first tying off the proximal end with 4.0 silk, flushing the intestinal contents distally with sterile saline, and then tying off the distal end with 4.0 silk. The blood supply to the loop was kept intact.

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TABLE 1. Rabbits studied and main characteristics of each bacterial strain

Rabbit group	Bacterial strain	Virulence plasmid	Mucosal ulceration	Mucosal uptake	Sereny test
1	M4243	+	+	+	+
2	X-16	+	-	+	-
3	2457-0	+	-	+	-
4	M4243A ₁	-	-	+	-
5	Heat-killed M4243	+	-	+	-

An overnight culture (2 ml, 2×10^9 bacteria) in brain heart infusion broth (Becton Dickinson and Co., Cockeysville, Md.) was injected into the closed loops, which were returned to the abdominal cavity. Peyer's patches were arranged in a dependent position, and the loops were gently massaged every 10 to 15 min to ensure even exposure of the Peyer's patches to the shigellae. The loops were allowed to incubate for 30 or 90 min. For studies on pathogenicity of each strain, an 18-h incubation was used. After the specified incubation period, the loop was excised intact and opened along the mesenteric border, and the Peyer's patch with a surrounding rim of the intestinal wall was sampled. One piece of the sample was fixed in glutaraldehyde-formaldehyde for electron microscopy, and the rest was snap-frozen in liquid nitrogen for histologic assessment of bacterial uptake.

Electron microscopy. Tissues were minced to approximately 1 mm³ and fixed in 3% glutaraldehyde-formaldehyde in 0.1 M cacodylate buffer, pH 7.3 (Tousimis Corporation, Rockville, Md.). The samples were postfixated in 2% osmium tetroxide. After being stained en bloc with 2% uranyl acetate, tissues were dehydrated in alcohol and embedded in Epon. Sections (1- μ m thick) were cut, stained with toluidine blue, and examined. Areas of follicle-associated epithelium

(FAE) containing shigellae were chosen. Thin sections approximately 80 nm thick were then cut from the selected areas on a Porter-Blum MT-2 ultramicrotome. These sections were stained with lead citrate and examined with a Zeiss 109 transmission electron microscope. Photomicrographs were taken of the characteristic rod-shaped bacteria in the FAE.

Light microscopy. Frozen sections (6- μ m thick) were obtained along the longitudinal axis of the bowel through the Peyer's patch and adjacent intestine. The sections were immediately fixed in 100% ethanol and stained with Giemsa. These sections were examined with an oil immersion lens.

The surface epithelium was divided into two regions for this study. The dome area over the Peyer's patches, which is enriched with M cells, was the first region. Few goblet cells are found in this epithelium. Wandering lymphocytes and macrophages and connective tissues underlie the FAE (18). The second area examined consisted of villi outside the Peyer's patches. These structures contain primarily absorptive columnar cells and numerous goblet cells. An image analyzer (Bioquant, Biometrics, Nashville, Tenn.) with an IBM PC was used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of Peyer's patches. The mean of actual length of 100 areas for dome and villus areas from representative rabbits was calculated. The average length of dome epithelium was 1.26 ± 0.03 mm (standard error) and of villus epithelium was 1.36 ± 0.04 mm.

We examined five coded slides per rabbit and counted bacteria taken up over each dome area and over 10 randomly selected villi. These numbers were presented as bacteria per millimeter of surface epithelium of villi or domes. The mean and standard error of the mean were then calculated from

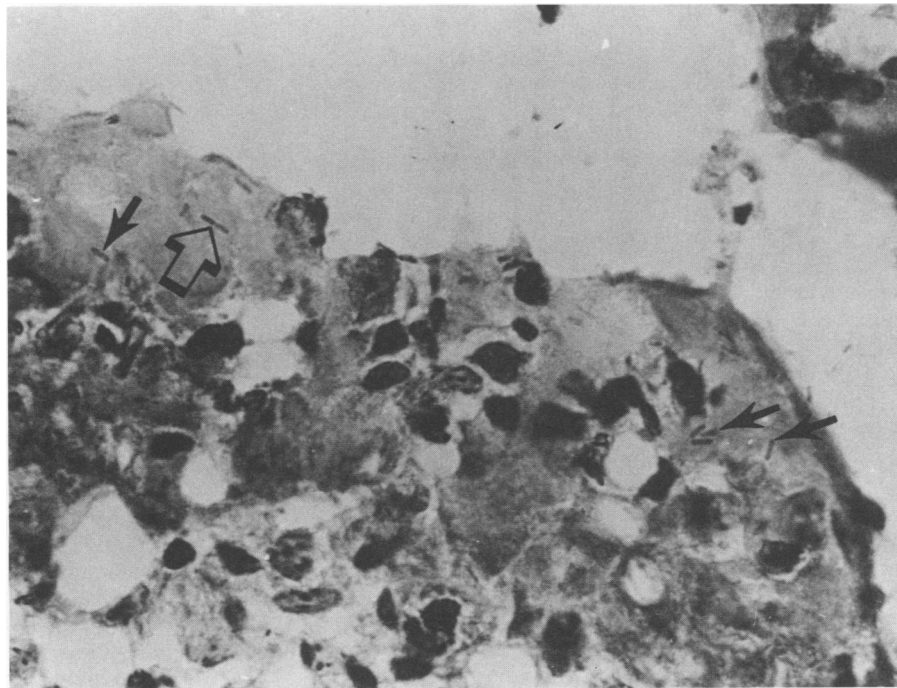


FIG. 1. Longitudinal section in Peyer's patch incubated for 90 min with M4243 and stained with Giemsa. Note the characteristic rod-shaped bacilli incubated for 90 min and taken up by epithelium over dome areas (arrows). The open arrow points to a bacterium undergoing binary fission. Bacteria may be seen in cross sections or oblique sections. Magnification, $\times 825$.



FIG. 2. Transmission electron micrograph of M cells containing shigellae packaged in vacuoles (arrows). Magnification, $\times 13,000$.

150 observations over villus epithelium or about 120 to 130 observations over FAE for each group of rabbits studied.

Statistical analysis. Results were compared by Student's *t* test on RS/1 software.

RESULTS

Uptake of shigellae at 30 min. At the 30-min time point, there was almost no demonstrable uptake of *S. flexneri* by either the FAE or the villus epithelium. No damage or evidence of inflammation was seen at this early time. A rare shigella was seen in the apical portion of the epithelium over the lymphoid follicles in the M4243 strain group.

Uptake of shigellae at 90 min. All four *Shigella* strains were readily detected in the dome epithelium by 90 min. We

required that the entire shigellae be within the cytoplasm to be counted (Fig. 1). Bacteria which were adherent to the surface epithelium but not clearly present within the cytoplasm were not counted. As shown by ultrastructural studies, the bacteria were taken up by the M cells in vesicles. All the bacteria seen at the 90-min point by ultrastructural studies were contained within membrane-lined vesicles (Fig. 2). The pathogenic strain *S. flexneri* M4243 had significantly greater numbers of bacteria within the dome epithelium than did the three nonpathogenic strains ($P < 0.01$) (Fig. 3). In several areas, the M4243 strain showed clustering of bacteria, some of which were undergoing binary fission (Fig. 1). The nonpathogenic strains were taken up with equal efficiency regardless of their invasive abilities or the presence of

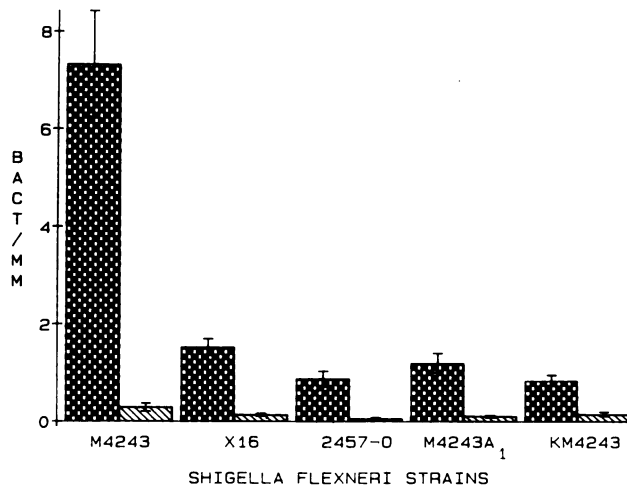


FIG. 3. Uptake of different *Shigella* strains by 1 mm of FAE (checked columns) versus 1 mm of villus epithelium (hatched columns) after 90 min of incubation. KM4243 is heat-killed M4243. Data are expressed as mean \pm SEM. BACT/MM, Bacteria per millimeter.

the 140-megadalton virulence plasmid (Fig. 3). Interestingly, heat-killed pathogenic M4243 were taken up by M cells as well as live avirulent bacteria (Fig. 3).

All strains examined had relatively few shigellae within the villus epithelium (uptake by dome epithelium was at least 10-fold greater than that by villus epithelium for each strain). The uptake of pathogenic M4243 strain by villus epithelium was significantly greater ($P < 0.01$) than uptake of the nonpathogenic strains.

Incubation for 18 h. Mucoïd fluid accumulation was seen in the acute loops incubated with every *Shigella* strain studied. With the pathogenic *S. flexneri* M4243, the fluid was bloody, and the wall of the loop was noticeably edematous, very

friable, and sometimes coated with clotted blood. This was not the case with any of the other strains studied except the X16 strain, where there was some hemorrhage in the lumen and corresponding small focal ulcerations over some dome regions. Corresponding to the gross observations, microscopic examination revealed profound mucosal ulceration exclusively with the *S. flexneri* M4243 strain (Fig. 4). Many microorganisms were seen in the exudate over the ulcer and within the lymphoid follicle tissue. The surface was hemorrhagic, with marked acute inflammation throughout the lamina propria. Ulcerations were present almost exclusively in the dome regions over the Peyer's patches (Fig. 4), but occasionally extended to the bases of adjacent villi and caused their avulsion. These sloughed villi left flat hemorrhagic ulcers. Although there was mucosal damage in the attached or sloughed villi (edema, mucus depletion), the surface epithelium was in general intact (not ulcerated). In contrast, the nonpathogenic shigellae were not found within the surface epithelium at this time point. The three nonpathogenic strains showed no ulceration after the 18-h incubation.

DISCUSSION

Rabbit ileum was chosen as the site for these studies because it is enriched in Peyer's patches, which are a major site for uptake of luminal antigens by the intestine (1). In humans the ileum is the second most frequent site, after the colon, of mucosal ulceration in bacillary dysentery (25).

In the present study, we found that both pathogenic and nonpathogenic *Shigella* strains were taken up by the specialized M cells in the FAE which overlies dome areas in Peyer's patches and isolated lymphoid follicles in the gastrointestinal tract. M cells have been shown by other workers to be involved in pinocytosis of macromolecules as well as in sampling of luminal microorganisms (1, 5, 7, 17-20, 22, 27, 28). Consequently, the M cells are thought to play an important role in the initial uptake of antigens to stimulate the mucosal immune response.

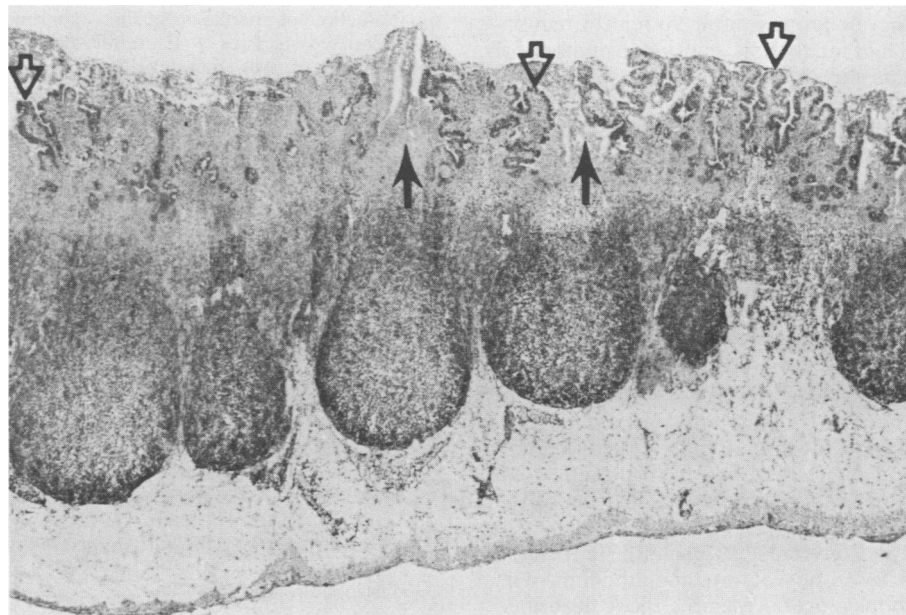


FIG. 4. Longitudinal section in rabbit Peyer's patch after incubation for 18 h with pathogenic M4243. Note the profound mucosal ulceration replacing the epithelium over the dome areas (solid arrows) and that although the villus epithelium is abnormal, it is intact (open arrows). Magnification, $\times 41.25$.

Whereas the four live strains and one heat-killed strain of *S. flexneri* studied have dramatically different invasive and virulence potentials, all five preparations have been found to elicit significant mucosal immune responses in our previous studies, where direct intestinal stimulation was given in chronically isolated ileal (Thiry-Vella) loops (9–12). When the three nonpathogenic strains were administered orally, all three could prime the rabbits for a memory mucosal response regardless of their ability to invade the surface epithelium or of the presence of the 140-megadalton virulence plasmid (11, 13). We predicted that all three should be sampled with equal efficiency by the surface epithelial M cells. The findings in the present study are consistent with this hypothesis. The killed preparation, however, was not able to elicit a memory mucosal immune response even when large doses of antigen were given orally (13). Since Owen et al. had shown previously that live but not killed *Vibrio cholerae* were taken up by M cells (20), we had hypothesized that the killed shigellae did not elicit a mucosal memory response due to ineffective sampling by the M cells. The present studies clearly demonstrate that this earlier hypothesis was wrong. The killed shigellae were taken up to the same extent by M cells as were the avirulent strains which can elicit a vigorous mucosal memory response. The reason that killed shigellae were unable to elicit a memory mucosal immune response is not known at present. Perhaps the process of bacterial division is necessary to prolong the existence of these antigens in the lumen; alternatively, memory cells may be stimulated by epitopes which were destroyed by the heat treatment.

Although there was no significant difference in the number of shigellae within the epithelium for the live, avirulent strains or the heat-killed M4243 strain, there was a significant increase in the number of bacteria found in dome epithelium by 90 min with the pathogenic *S. flexneri* M4243. This may reflect either preferential uptake of the virulent strain by the FAE or intracellular proliferation after the initial uptake. We believe that the latter mechanism is best supported by the results of the present study. First, some bacteria were undergoing binary fission in addition to being present in scattered foci or groups (after 90 min of incubation). Second, after 18 h of incubation, enormous numbers of shigellae were in the tissues and the follicular surface epithelium was completely destroyed only by the pathogenic strain. Third, the four- to eightfold difference between the numbers of bacteria in loops incubated with virulent versus avirulent strains corresponds well to the intracellular rate of growth of shigellae in HeLa cell cultures (15). On the other hand, avirulent shigellae appeared as scattered, rare, and individual bacteria, not in clusters.

It is notable that when the pathogenic bacteria were allowed to incubate for 18 h in the acute loops, ulcerations preferentially occurred over the dome regions of the Peyer's patches. While the entire epithelium was damaged, only the areas over the dome regions of the Peyer's patches were completely denuded (Fig. 4). Ulcerative lesions extended to the bases of neighboring villi and crypts. Many villi appeared, solely or as a cluster, detached from the intestinal wall and free in the lumen. Microscopic examination of the ulcer revealed fibrinopurulent exudate matted together with sloughed epithelium and myriads of bacteria. Ulcerated areas were separated by zones of intact epithelium. The lesions in these ileal loops closely resemble those described for shigellosis in humans (21, 25). This suggests that in addition to being a site for antigen sampling and stimulation of the mucosal immune response, M cells may serve as the

preferential portal of entry for pathogenic microorganisms such as *S. flexneri*. Indeed, others have proposed the M cell as a portal of entry for intestinal pathogens (5, 7, 14, 17, 20, 27) and even the human immunodeficiency virus (26). The presence of abundant M cells in the FAE is the main characteristic that differentiates this specific epithelium from that found throughout the gastrointestinal tract. These findings support our hypothesis that FAE is both the site of antigen sampling and the site of disease propagation and ulcer initiation in shigellosis.

It will be important to determine whether the uptake of virulent shigellae by the epithelium overlying lymphoid follicles can be prevented by the production of antigen-specific secretory IgA. Mucosal immunity has been shown to prevent the pathologic effect of *V. cholera* and cholera toxin (20). Since nonpathogenic strains are effective immunogens for developing a secretory IgA response to *S. flexneri* LPS, future studies with live nonpathogenic mucosal vaccines may be useful in the prevention of enteropathogenic diseases by interference with uptake of the causative microorganisms.

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