INFECTION AND IMMUNITY, July 1996, p. 2752–2764 Vol. 64, No. 7
0019-9567/96/$04.00+0

Infection of Rabbit Peyer's Patches by *Shigella flexneri*: Effect of Adhesive or Invasive Bacterial Phenotypes on Follicle-Associated Epithelium

PHILIPPE J. SANSONETTI,[1]* JOSETTE ARONDEL,[1] J. ROBERT CANTEY,[2] MARIE-CHRISTINE PRÉVOST,[3] AND MICHEL HUERRE[4]

Unité de Pathogénie Microbienne Moléculaire, U389, Institut National de la Santé et de la Recherche Médicale,[1] Station Centrale de Microscopie Electronique,[3] and Unité d'Histopathologie,[4] Institut Pasteur, 75724 Paris Cédex 15, France, and Infectious Disease Division, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425[2]

Received 4 December 1995/Returned for modification 8 January 1996/Accepted 1 April 1996

In order to invade the colonic mucosa, the bacterial pathogen *Shigella flexneri* must find a site of entry. Experiments with the rabbit ligated intestinal loop model described here confirm that M cells of the follicle-associated epithelium (FAE) that covers lymphoid structures of the Peyer's patches represent a major site of entry for invasive microorganisms. In addition, in an isogenic *Shigella* background, expression of an adhesive phenotype, or of an invasive phenotype, is required for bacteria to efficiently colonize the FAE. A nonadhesive, noninvasive mutant barely interacted with FAE. Adhesive and invasive strains induced dramatic but different alterations on FAE. Invasive strain M90T caused major inflammation-mediated tissue destruction after 8 h of infection. Adhesive strain BS15 caused limited inflammation, but major architectural changes, characterized by an increase in the size of M cells that became stretched over large pockets containing an increased number of mononuclear cells, were observed. M cells progressively occupied large surface areas of the FAE at the expense of enterocytes. This contributed to enterocytes losing contact with the lumen. These experiments demonstrate that various remodeling patterns may occur in Peyer's patches in response to bacterial pathogens, depending on the virulence phenotype expressed by the pathogenic strain.

In order to infect the intestinal mucosa, invasive bacterial pathogens must find a site of entry. Although major progress in understanding the cross-talk between enteroinvasive bacteria, such as *Salmonella*, *Shigella*, and *Yersinia* spp., and their host cells in in vitro assays has been made, questions about the initial route of entry they exploit and the consequences of this early interaction in further subversion of the barrier function of the mucosa remain (13, 30). Shigellosis, or bacillary dysentery, a model of bacterial invasion of the human colon, is currently analyzed at the tissue level. *Shigella flexneri*, a gram-negative bacillus, is the major etiological agent of the endemic form of this disease, which occurs in young children in developing areas of the world (5). The key step in the pathogenesis of shigellosis is invasion of colonic epithelial cells by the pathogen (16). Bacteria enter cells via a process involving rearrangement of the host cell cytoskeleton (1, 4), they escape into the cytoplasm by lysing the phagocytic vacuole (40), and then they express a motility phenotype caused by polar directional assembly of actin elicited on the bacterial surface by the protein IcsA (3). Cellular protrusions are then formed and endocytosed by adjacent cells in areas of intermediate junctions in polarized epithelia (39). Finally, bacteria that have reached adjacent cells lyse their two surrounding membranes via expression of the *icsB* gene (2) and reach the cytoplasm before repeating the same cycle.

In spite of the capacity of *S. flexneri* to invade epithelial cells in in vitro assays (35), current in vivo evidence indicates that this organism uses lymphoid structures associated with the intestinal mucosa as a primary site of entry. This is suggested by infection of macaques, which develop bacillary dysentery after intragastric inoculation. In such animals, administration of an *icsA* mutant which does not spread intracellularly and from cell to cell caused a markedly attenuated disease characterized by small colonic and rectal abscesses or ulcerations which colocalized with lymphoid follicles (37). In the intestinal epithelium, lymphoid follicles are covered by the follicle-associated epithelium (FAE), containing M cells which have the capacity to translocate macromolecules, particles, and also microorganisms (25). Follicular lymphoid structures are found as aggregates in the ileum (i.e., Peyer's patches) and as numerous solitary nodules in the colonic and rectal epithelia (12, 17, 26, 27). Therefore, there may be as many potential sites for microbial entry as FAE structures. It is now well established in intestinal invasion models that several pathogens, such as *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes*, penetrate through the FAE (6, 13, 20). Similar analysis of the invasion process carried out with rabbit ligated intestinal loops containing Peyer's patches indicated that entry of *S. flexneri* essentially occurs via M cells of the FAE (30, 42). Demonstration that FAE may represent the actual entry site of shigellae helped resolve the in vitro paradox of the inability of shigellae to invade cells via their apical pole (24).

Recent in vitro and in vivo experiments have allowed us to propose a scheme of the intestinal invasion process which starts with entry into M cells (30). Bacteria then reach resident macrophages of the dome, which are killed by a process of apoptosis (44, 45). Apoptotic killing is likely to facilitate bacterial survival, but it also seems to cause the release of important quantities of interleukin-1 (43). This major proinflammatory cytokine elicits early inflammation starting at the level of FAE and spreading laterally, attracting polymorphonuclear

* Corresponding author. Mailing address: Unité de Pathogénie Microbienne Moléculaire, U389 INSERM, Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris Cédex 15, France. Phone: 33 1 45688352. Fax: 33 1 45688953. Electronic mail address: psanson@pasteur.fr.

leukocytes (PMN) in subepithelial areas of the lamina propria. As modeled in vitro (31), this influx of PMN is also able, in vivo, to disrupt the epithelium, a process characterized by intense edema of the lamina propria and extensive sloughing of the epithelial layer. This process facilitates entry of bacteria at a distance of the FAE (30). The role of interleukin-1 in facilitating intestinal tissue invasion and destruction by *S. flexneri* has recently been shown (36).

As FAE appears to be essential in the early events of *Shigella* pathogenesis, we have studied the natural history of infection of Peyer's patches by *S. flexneri* in the rabbit ligated intestinal loop model. Attention has been given to the respective roles of adhesive and invasive phenotypes in penetration of the bacteria into these structures, as well as to the role played by inflammation caused by the invasive phenotype in destroying FAE and subjacent lymphoid tissues. In addition, changes in the cell populations of the FAE were monitored. A striking observation was the expansion of FAE surface areas occupied by M cells as infection proceeded.

MATERIALS AND METHODS

Bacterial strains and plasmids. Three *S. flexneri* isolates were used in this study. Strain M90T is an invasive isolate belonging to serotype 5 (38). BS176 is a noninvasive derivative of strain M90T which has been cured of its virulence plasmid (38). In order to obtain a variant expressing a phenotype of adhesion to the rabbit intestinal mucosa, plasmid pRKB15 was introduced by transformation into BS176. It contains an insert encoding the rabbit-specific adherence pilus AF/R1 of enteropathogenic *Escherichia coli* RDEC-1 (11). Bacteria were routinely grown in Trypticase soy broth or on Trypticase soy agar plates (Diagnostic Pasteur, Marnes la Coquette, France).

Infection of animals. New Zealand White rabbits, weighing 3 kg (CEGAVs.s.c., Les Hautes Noës, France), were used in this study. Results are based on histopathological analysis of intestinal tissues obtained from six rabbits. From each of these animals, ligated loops were prepared as described in previous publications (30, 36). Briefly, rabbits were anesthetized with 0.5 ml of sodium pentobarbital (SANOFI-Santé Animale, Libourne, France) per kg of body weight and laparotomized, and four to five intestinal loops of 10 cm, each containing a Peyer's patch in its center, were ligated, starting from the ileal-cecal junction, with care taken to preserve the mesenteric vasculature. Into each loop, a 0.5-ml bacterial culture, resuspended in saline to a concentration of 10^9 CFU/ml, was injected. A control loop was included for each rabbit by injecting 0.5 ml of saline.

Histopathological analysis of tissue samples. Following 2 or 8 h of infection, animals were sacrificed and relevant intestinal loops were dissected, longitudinally opened on their mesenteric side, and extensively washed with saline. Peyer's patches were punched off the tissue by using a skin biopsy punch 8 mm in diameter (Biopsy punch; Stiefel, Nanterre, France), fixed in 10% formalin, dehydrated, and embedded in historesin (Leica Instruments, Heidelberg, Germany) for histopathological preparation. Blocks were sectioned in 5-μm-thick slices and stained with hematoxylin-eosin for observation. For immunocytochemistry, tissues were fixed in 4% formalin; otherwise, preparation was similar to the procedure described above. Bacterial lipopolysaccharide (LPS) was labelled by using a primary mouse monoclonal antibody (immunoglobulin G3, kappa chain) directed against the *S. flexneri* serotype 5 somatic antigen (32). In order to label the mouse anti-LPS primary monoclonal antibody, we used the DAKO LSAB Kit, Peroxidase (Dako Corporation, Carpinteria, Calif.). In brief, the technique is based on the labelled streptavidin-biotin technique. Endogenous peroxidase activity is quenched by first incubating the samples in 3% H_2O_2 for 5 min. Nonspecific staining is eliminated by incubation with a blocking reagent for 5 min. Samples are then incubated with the primary antibody, and this is followed by sequential 10-min incubations with biotinylated-link antibody and peroxidase-labelled streptavidin. Staining is obtained by a 10-min incubation with the freshly prepared substrate-chromogen solution.

Histopathological sections from 12 different infected Peyer's patches were analyzed by optical microscopy. This was done for tissues infected by strains BS176 and BS15 but not for those infected by M90T, because of rapid destruction of Peyer's patch architecture by massive PMN infiltration. Three parameters were recorded: (i) the total number of superficial follicular epithelial pockets containing mononuclear cells, each one supposedly corresponding to at least one overlying M cell, (ii) the total number of mononuclear cells present in these pockets, and (iii) the total number of enterocyte nuclei (i.e., non-M cells and nonmononuclear cells) which were visible, aligned along the fibroblastic layer that delimits the basal side of the follicular epithelium. We then calculated the average number of pockets per enterocyte (P/E) and the average number of mononuclear cells per pocket (M/P). In toto, these calculations were based on counting 2,000 enterocyte nuclei in each of the three cases (i.e., BS176, BS15, and control), each section of FAE showing between 100 and 200 nuclei. Only clearly transverse sections were analyzed. Large pockets were counted as single pockets.

Preparation of samples and observation by TEM. Loops containing a Peyer's patch were opened, and the major part of the overlying mucus was removed by direct contact with absorbing paper followed by several extensive washings with phosphate-buffered saline. Peyer's patches were then punched off the tissue sample as already described and fixed with 2.5% glutaraldehyde–1.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. Tissues were then minced into small pieces and fixed for one extra hour. They were rinsed in the same buffer and postfixed with reduced osmium (0.1% osmium tetroxide and 1% K_4FeCN_6 in cacodylate buffer) for 1 h, washed, dehydrated in a graded ethanol series, and treated with epoxy-1,2-propane before being embedded in Araldite. Thin sections were cut, stained, and observed by transmission electron microscopy (TEM) at 80 kV (CM-12 TEM; Philips, Eindhoven, Holland).

RESULTS

Invasion via FAE and comparison of *S. flexneri* colonization potentials depending on expression of invasive or adhesive phenotype. In Fig. 1A, immunostaining of *S. flexneri* serotype 5 LPS shows that, even after 8 h of infection, invasive bacteria (M90T) remained essentially associated with the FAE and subjacent domes of the lymphoid nodules in Peyer's patches. In comparison, bacteria were seen to be associated neither with the epithelial surface nor with the lamina propria in the villi adjacent to the FAE.

Figures 1B and 2 show that expression of an invasive or an adhesive phenotype had considerable influence on the capacity of bacteria to invade the FAE and colonize the domes of the lymphoid structures. Views shown in the figures correspond to 8 h of invasion; they are representative of observations made for five Peyer's patches for each of the three strains. Control Peyer's patches originating from loops injected with saline showed no background labelling (data not shown). Figure 1B shows intense invasion of the dome by bacteria. Most of the FAE is undergoing necrotic destruction by abscesses ulcerated into the lumen. Bacterial material, however, was present far beyond the FAE, being associated with many of the cells constituting the dome, which showed strong edema reflecting the intensity of the inflammatory response.

Infection by the noninvasive, nonadhesive strain BS176 is shown in Fig. 2A and B. Even after 8 h of infection, only a few bacteria appeared to be associated with the FAE, particularly with M cells and their subjacent pocket. A limited amount of bacterial material was seen within the dome, and edema remained very limited. No interaction with adjacent intestinal villi was observed.

Infection by the noninvasive but adhesive strain BS15, which expresses the rabbit-specific AF/R1 adherence pilus, is shown in Fig. 2C to E. Previous evidence that this adherence system directs bacteria essentially to Peyer's patches was confirmed (10). Panel C of Fig. 2 shows the massive amount of bacterial material that became associated with both the FAE and the dome of the lymphoid follicles. This amount was equivalent to that observed with invasive M90T. On the other hand, as shown in Fig. 3, the overall structure of the Peyer's patch was conserved, with intact FAE and limited edema in the dome.

These results confirmed that FAE was the major area of interaction between bacteria and the intestinal mucosa and showed that all microorganisms were not equal with regard to their capacity to be taken up by M cells.

Alterations of Peyer's patch FAE and the dome depending on the infecting strain and duration of infection. A combination of histopathological (Fig. 3, hematoxylin-eosin staining) and ultrastructural (Fig. 4 to 8, TEM) observations is reported; these techniques allowed us to analyze the alterations characterizing infection of Peyer's patches by the three strains, according to the duration of incubation. In addition to qualitative

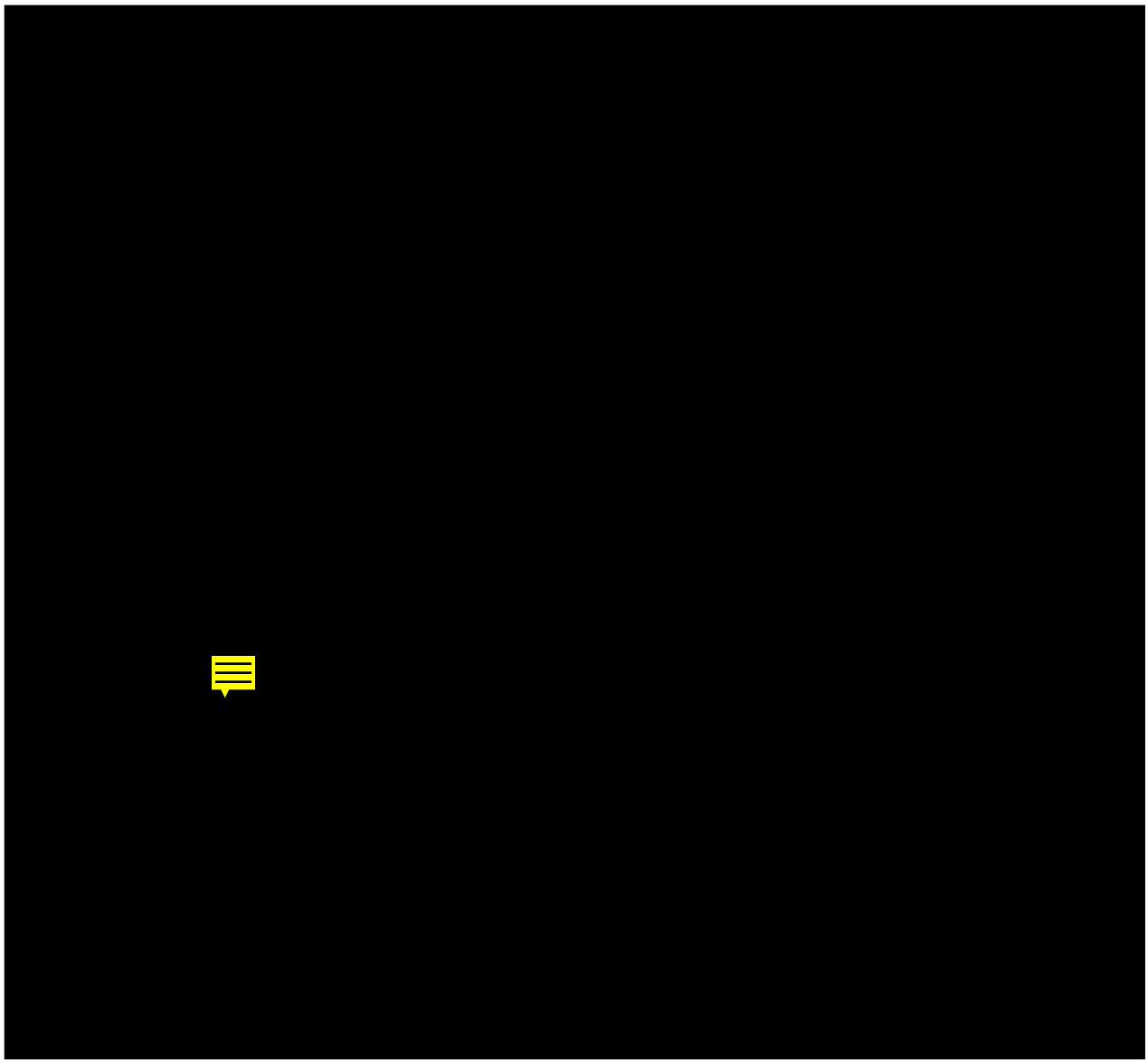


FIG. 1. Immunoperoxidase labelling of serotype 5 somatic antigen by anti-LPS monoclonal immunoglobulin G. Both panels show infection of rabbit Peyer's patch by invasive *S. flexneri* M90T. Bars = 10 μm.

analyses of differences among samples, a quantitative analysis was performed in order to monitor the evolution of the different cell populations during infection (i.e., enterocytes, M cells, and mononuclear and polymorphonuclear cells). Analytical methods have been detailed in Materials and Methods.

(i) Analysis of Peyer's patch tissue in control samples. The data from analysis of Peyer's patch tissue in control samples served as a reference for subsequent observation of infected tissue samples. Figure 3A shows the typical aspect of control samples after 8 h of infection. The overall architecture of both the FAE and the dome was maintained. M cells could be detected on the basis of the few mononuclear cells present inside their pocket (Fig. 3A, arrow). In summary, 8 h after ligation and saline injection, the P/E ratio was 0.32 and the M/P ratio was 2.1. These data were consistent with the ultrastructural views shown in Fig. 4. M cells, which are easily recognized by their lack of glycocalix, shorter and more flexible brush border, and associated mononuclear cells, were regularly alternating with enterocytes. Comparison of the two pictures (Fig. 4A and B) shows that the overall aspect of the FAE was not altered with time, in spite of loop ligation. However, the ratio of M cells/enterocytes was 0.5, a number higher than the P/E (i.e., 0.32). In our observation, the 0.5 ratio was actually a reflection of the situation prevailing in the periphery of the FAE and not necessarily at its center, thus explaining why the global P/E ratio was lower. Also noticeable were the roughly equal widths of enterocytes and M cells which appeared evenly positioned. No PMN was observed.

(ii) Invasion and inflammation-mediated destruction of FAE and dome of Peyer's patches caused by invasive strain M90T. Figures 3C and D show the typical aspect of Peyer's patches obtained from loops infected by M90T. After 2 h (Fig. 3C), an influx of PMN invading the dome and starting to dissociate the FAE could be observed. The FAE, even at this

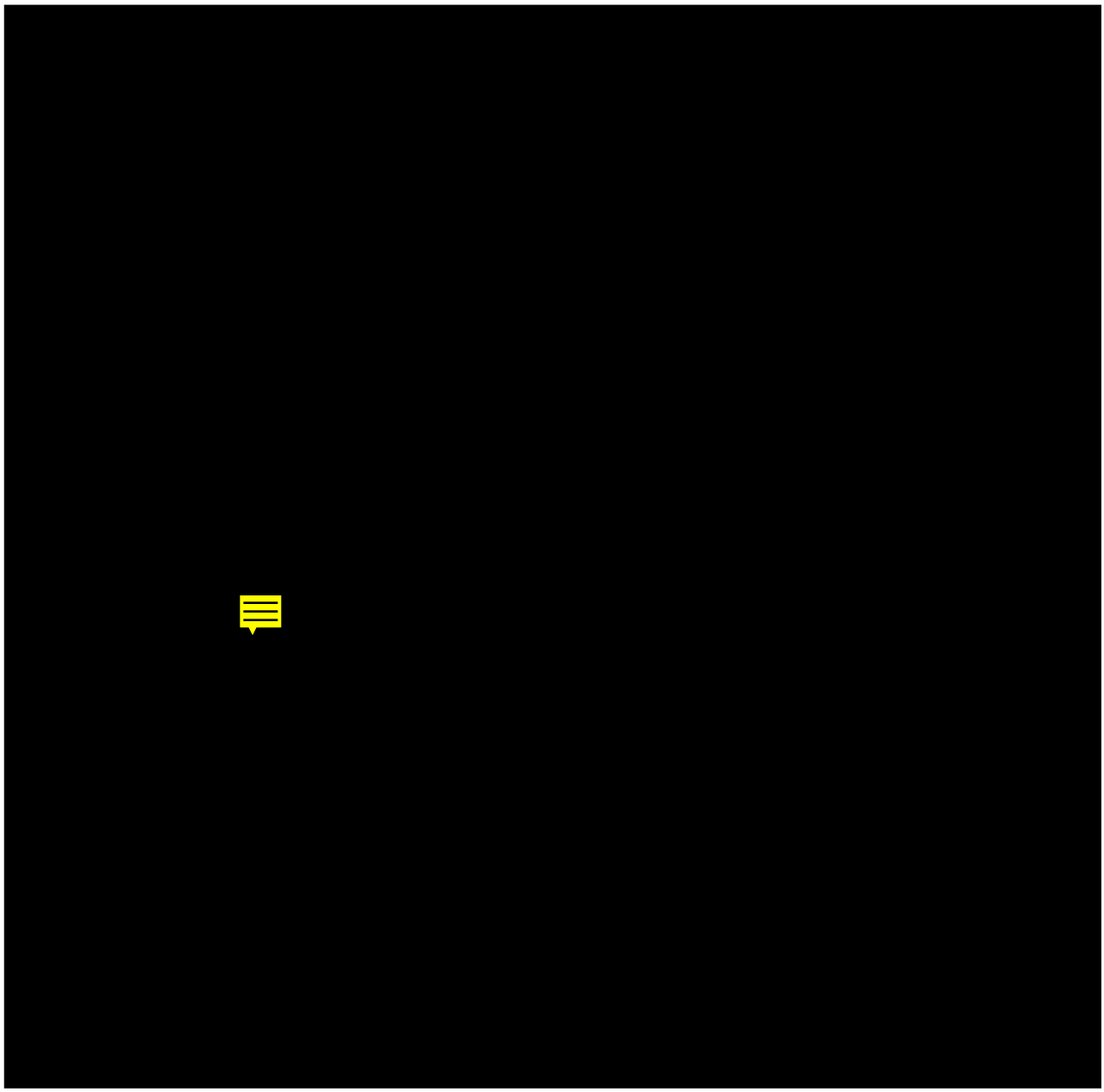


FIG. 2. Infection of rabbit Peyer's patches. Shown is Immunoperoxidase labelling of serotype 5 somatic antigen by anti-LPS monoclonal immunoglobulin G. (A and B) Infection by noninvasive strain BS176, (C, D, and E) infection by strain BS15, an adherent transformant of BS176 expressing the AF/R1 adherence pilus of *E. coli* RDEC-1. Bars = 10 μm.

early time of infection, showed ongoing ulcer formation with PMN accumulating in the pockets (Fig. 3C, arrow). After 8 h of infection (Fig. 3D), the FAE was disrupted by multiple abscesses (arrow), a massive influx of PMN being drained into the lumen through multiple openings. Total necrotic destruction of FAE was eventually observed to occur on some samples. PMN infiltration occurred in the dome and overlying FAE. This localization was consistent with immunostaining for bacterial LPS (Fig. 1B). The lymphoid nodules did not show such a strong inflammatory infiltrate (data not shown).

Figure 5 shows TEM observations suggesting how strain M90T entered Peyer's patches. At 2 h, numerous entry events could be seen occurring in M cells. As shown in Fig. 5A and B, major projections pushing the M-cell membrane away in the lumen were formed during entry of the microorganism. These striking modifications indicated that massive cytoskeletal rearrangement was induced by invasive shigellae, as previously observed in vitro in areas of contact with epithelial cells (1). Conversely, unlike what was previously observed to occur in epithelial cells grown in vitro (40), shigellae did not lyse their endocytic vacuole inside M cells. Among about 50 intracellular bacteria observed in different preparations, all clearly appeared in a membrane pocket. Having translocated throughout M cells, bacteria then resided in the intercellular space close to mononuclear cells present in the pocket, as shown in Fig. 5B to D. This demonstrated that once inside tissues, *S. flexneri* was

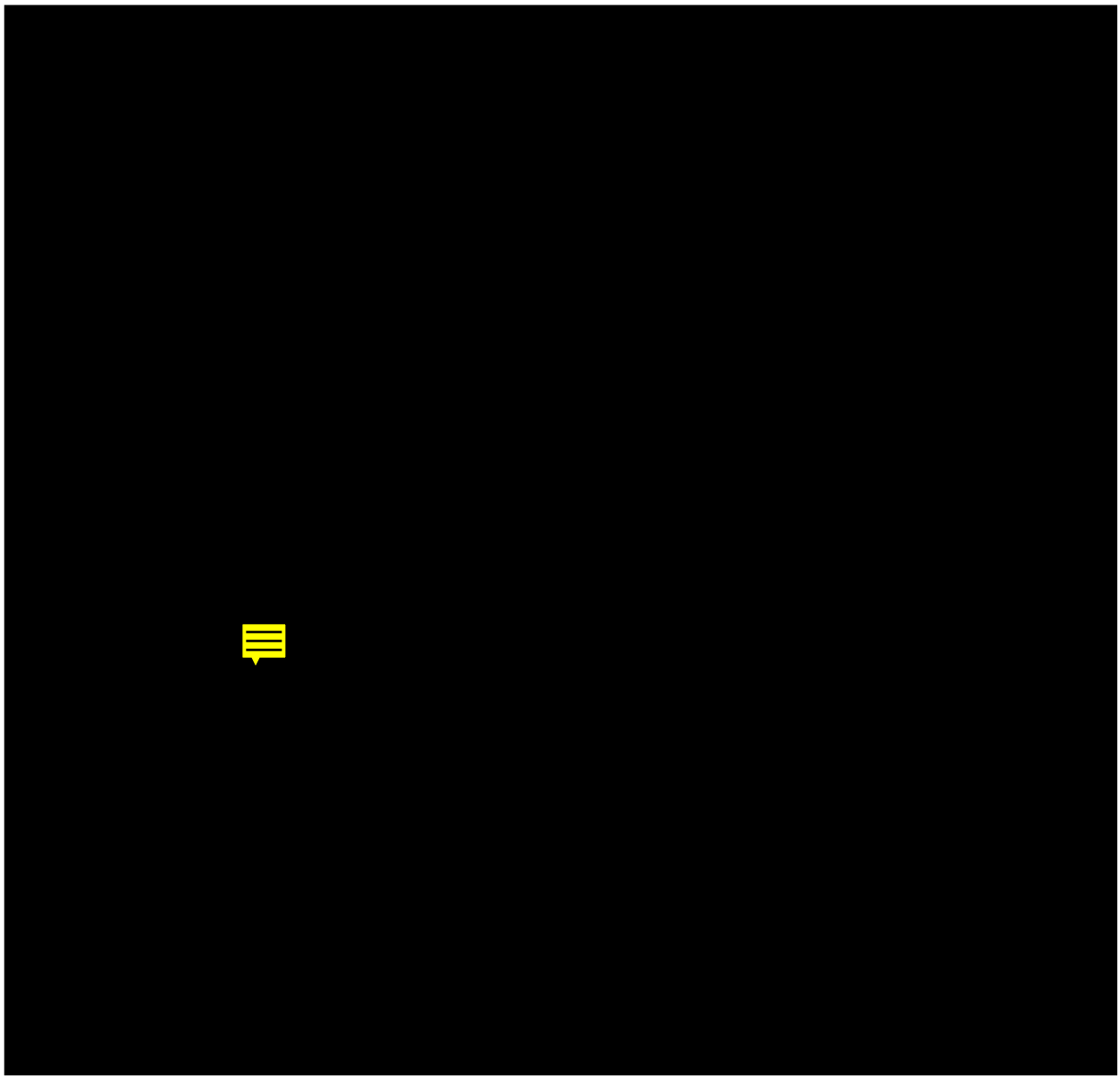


FIG. 3. Histopathological staining with hematoxylin-eosin on tissue sections of rabbit Peyer's patches. Views focused on the FAE have been selected. (A) Control (loop injected with saline), 8 h. (B) Infection with BS176, 8 h; the arrow points to an apical bump. (C and D) Infection with M90T, 2 and 8 h, respectively; the arrows point to a pocket filled with PMN (C) and to an abscess disrupting the FAE (D). (E and F) Infection with BS15 at 2 h (E) and 8 h (F). Arrows point to released spheroid structures from an enterocyte apex (E) and to an enlarged pocket filled with mononuclear cells (F). Bars = 10 μm.

not exclusively intracellular. At this early time, bacteria could also be seen inside mononuclear cells of the pockets, most likely corresponding to resident macrophages (Fig. 5C). M cells invaded by shigellae did not show significant cytotoxicity. If bacteria seen in the vicinity of or inside a mononuclear cell (Fig. 5C and D) originated from the adjacent M cell, the latter seemed to have fully recovered from the invasion-translocation process. Only after inflammation had occurred was significant cytotoxicity observed, and this concerned not only M cells, but also most of the Peyer's patch epithelial cells, as shown in Fig. 5.

Observations made after 8 h of infection are summarized in Fig. 6. As already described, at this stage infection of the Peyer's patch was characterized by strong inflammation due to influx of numerous PMN dissociating the structures of both the dome and the FAE. Of interest was the formation of "invasion units" (Fig. 6A and B) characterized by the presence of the following components: an M cell elongated and distended over an enlarged pocket containing cells such as a PMN, often infected by bacteria, and a mononuclear leukocyte, most probably a macrophage, characterized by the presence of giant lysosomes, some of which contained bacteria in the process of degradation, as well as several nuclei of apoptotic cells (Fig. 6C). Deeper in the dome, one or several macrophages, also containing giant lysosomes and apoptotic nuclei, were present, constituting a second line of defense (Fig. 6D). In the vicinity of these cells, transverse sections of capillaries, often containing PMN, were seen (data not shown). In many instances, the

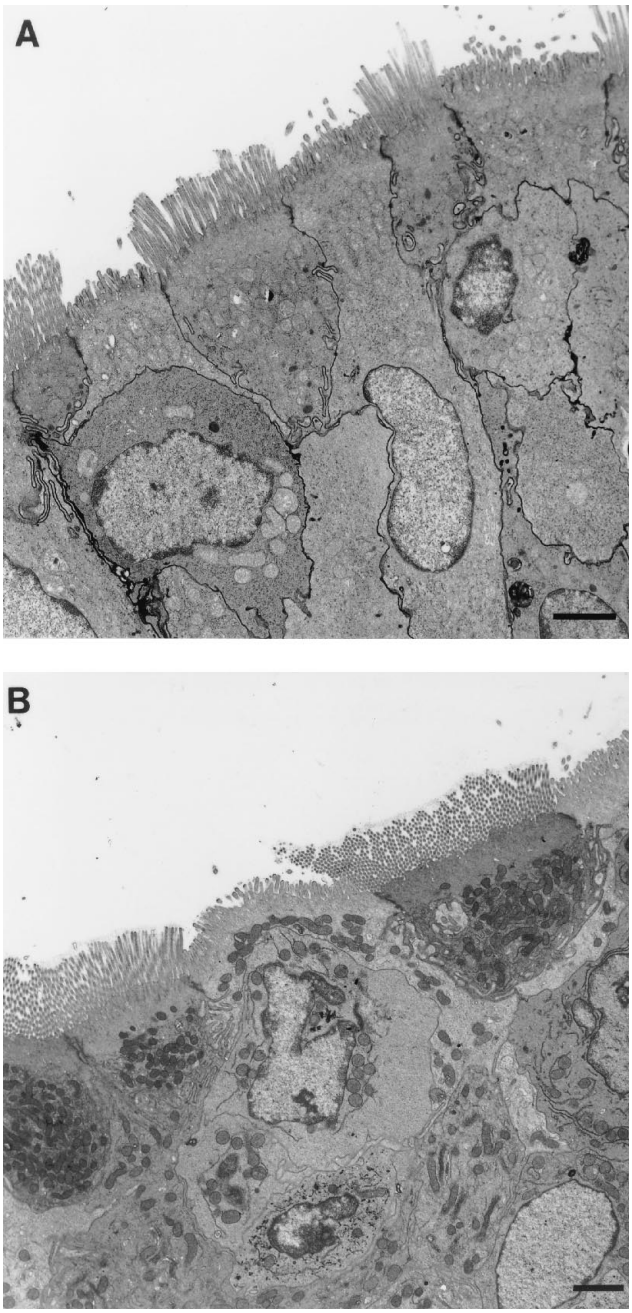


FIG. 4. TEM. Shown are rabbit Peyer's patches injected with saline at 2 h (A) and 8 h (B). Bars = 1 μm.

architectures of the FAE and of the dome were totally destroyed by the extensive inflammatory reaction which eventually caused tissue necrosis (data not shown).

(iii) Alterations in FAE architecture caused by strains BS176 and BS15. Figure 3B shows the limited alteration appearing after 8 h in Peyer's patches infected by the nonadhesive-noninvasive strain BS176. The overall architectures of both the FAE and the dome were preserved, and PMN were rarely seen. The only noticeable modifications were an increase in the thickness of the epithelium and an enlargement of the M-cell-associated pockets, which contained an increasing number of mononuclear cells. This was confirmed by calculations made for samples taken at 8 h, which showed a P/E ratio of 0.36 and an M/P ratio of 3.5, indicating that the number of pockets did not significantly increase but that more mononuclear cells migrated into them than into those of the noninfected controls. In addition, numerous bumps appeared at the apical surface of the FAE (Fig. 3B, arrow). They were particularly obvious in areas close to the pockets after 8 h of infection. The nature of these bumps will be considered in the next section.

Figures 3E and F show alterations appearing with time (panel E, 2 h; panel F, 8 h) in Peyer's patches infected by the adhesive strain BS15. The overall architecture of the FAE did not change, with the exception of an increase in thickness already obvious after 2 h. Inflammation remained minimal. On the other hand, alterations similar to those observed with BS176 were seen. They appeared earlier and with stronger intensity in this case. After 8 h of infection, the P/E ratio was 0.38, indicating that, compared with values for the uninfected controls and BS176-infected Peyer's patches, the number of pockets did not significantly increase. This suggested that the number of M cells remained constant. Conversely, they appeared strikingly enlarged, particularly after 8 h, with an increased number of immigrating mononuclear leukocytes inside (arrow in Fig. 3F). The M/P ratio of 5.7 indicated a threefold increase. In addition, numerous large bumps were observed, emerging from the apical side of the FAE, as soon as 2 h after infection had started (arrow in Fig. 3E). At 8 h (Fig. 3F), most of these bumps had disappeared.

(iv) TEM analysis of the alterations observed in FAE and the dome of Peyer's patches infected by strains BS176 and BS15. Histopathological observation suggested that the noninvasive strains BS176 and BS15 did not cause destruction of the FAE, since they did not elicit the strong inflammation characteristic of M90T, but they induced significant alteration of its architecture which could be analyzed by TEM. Few BS176 bacteria were observed to be associated with Peyer's patch tissue (data not shown), in agreement with the immunohistochemistry data in Fig. 2A and B. The presence of macrophages harboring large lysosomes, mostly located in the dome (data not shown), indicated, however, that the few bacteria internalized were rapidly cleared by the local reticuloendothelial system. Conversely, numerous BS15 bacteria were observed to be associated with the FAE and the dome. At an early stage of infection (i.e., 2 h [Fig. 7A]), bacteria could be detected in M cells and mononuclear phagocytes associated with M-cell pockets, always localized in vacuoles. No significant alteration of the M-cell surface was observed. At a later stage of infection (i.e., 8 h [Fig. 7B]), bacteria were observed to be undergoing lysis in the giant lysosomes of macrophages present in the dome. Figures 7C and D show examples of enormous lysosomal pockets in which bacteria underwent degradation.

However, besides the fate of the bacteria that—not surprisingly for noninvasive microorganisms—followed a classical degradation pathway, it was important to monitor by TEM evolution of the FAE and dome in the presence of microorganisms that did not cause their destruction. This analysis explained observations made by optical microscopy.

As shown in Fig. 8, two major features could be observed: the formation of bumps by protrusion of the enterocyte apex into the lumen (Fig. 8A to C) and the stretching process affecting M cells which occupied an increased surface area of the FAE (Fig. 8C and D).

The apex of enterocytes was squeezed between expanding M cells, and a bottleneck consequently formed, which constituted the bump (Fig. 8A). Tight intercellular junctions were maintained between cells of both types (Fig. 8B). In some cases, the

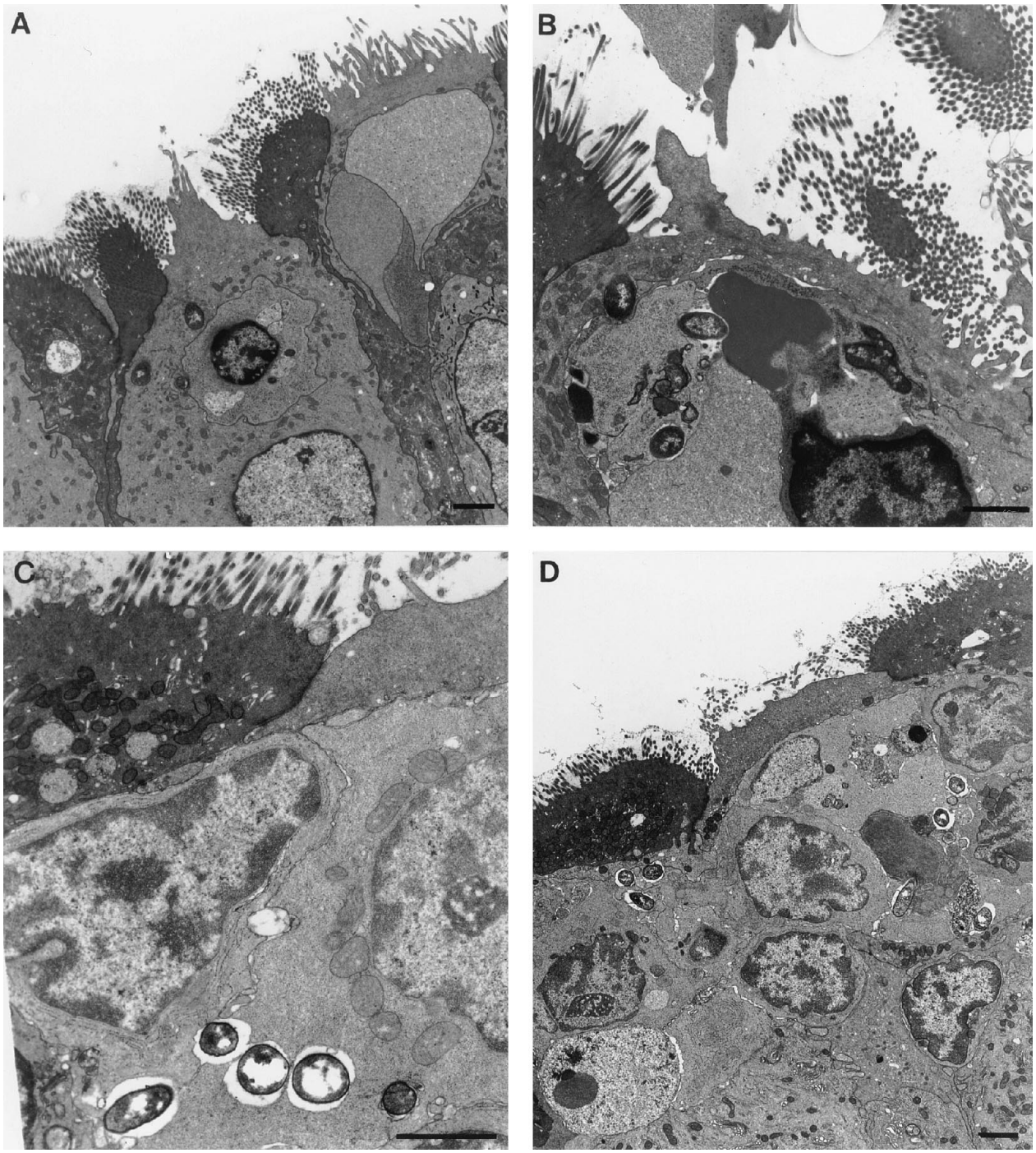


FIG. 5. TEM. Shown are rabbit Peyer's patches infected by M90T at 2 h. (A) Entry of bacteria via M cells; (B) presence of bacteria in the intercellular space located between the M cell itself and the mononuclear cells present in the M-cell pocket; (C and D) presence of bacteria in the intercellular space, but also intracellularly in a membrane-bound vacuole (C). Bars = 1 μm.

enterocyte apex appeared linked to a pedestal structure formed by the adjacent M cell (Fig. 8C). Serial sections showed that, in some circumstances, the enterocyte apex eventually closed itself and was released from the FAE. The basal portion of the enterocyte then seemed to retract in the depths of the FAE, leaving to M cells exclusive occupancy of the apex.

The ratio of M cells to enterocytes on the FAE surface increased. In some areas the M cells represented the exclusive population on extended surfaces. This might be consistent with an increase in the absolute number of these M cells. However, analysis of histopathological and TEM data suggested a different phenomenon. Stability of the P/E ratio indicated that the actual number of M cells did not significantly increase and that only their size was augmented. M cells became progressively stretched over giant pockets filled with numerous mononuclear cells. Figure 8D shows two adjacent M cells with an arrow

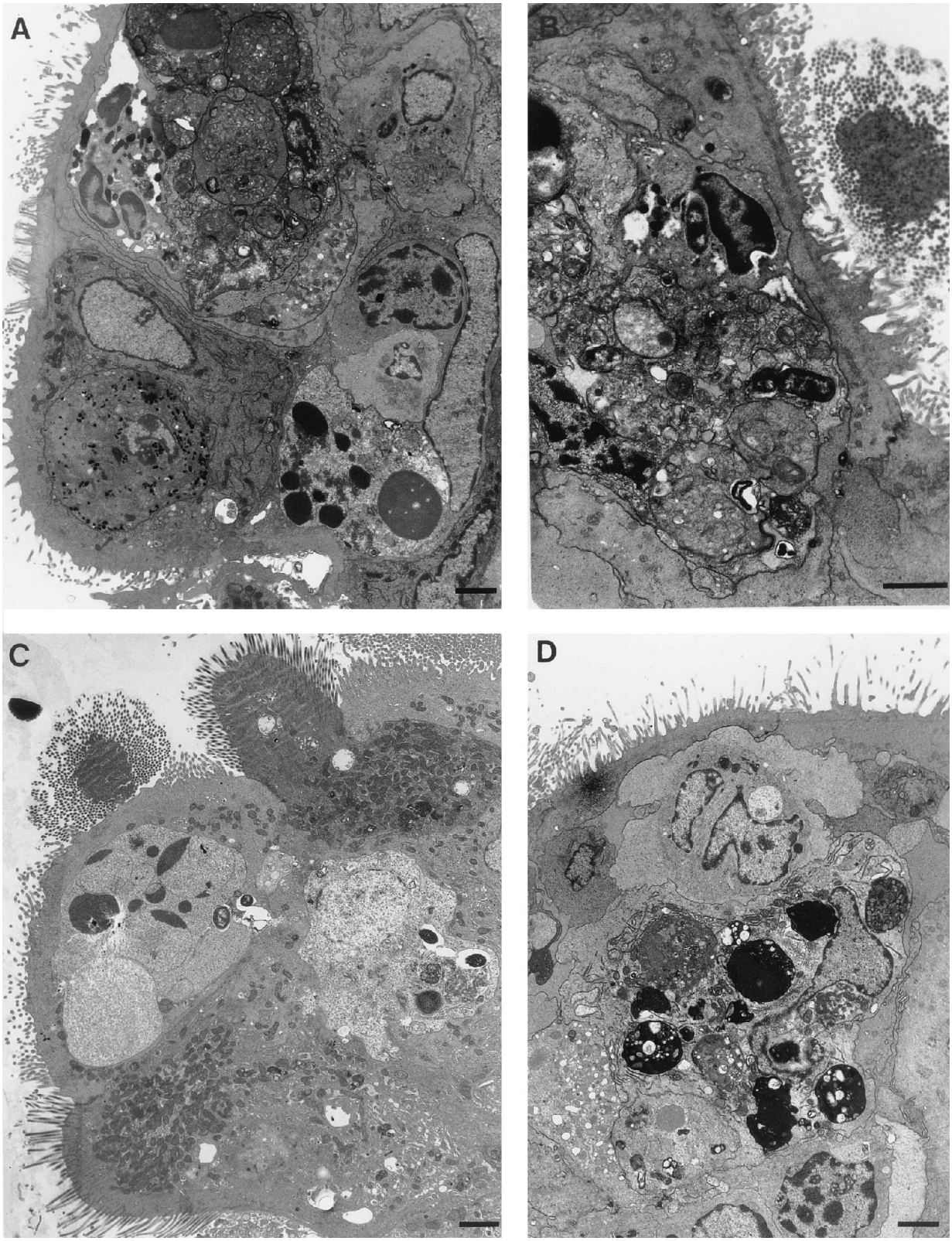


FIG. 6. TEM. Shown are rabbit Peyer's patches infected by M90T at 8 h. (A and B) Distension of M-cell pocket by numerous mononuclear cells and PMN. Bacteria can be seen inside PMN. (C) Numerous cells with apoptotic nuclei are located inside M-cell pockets with bacteria in the vicinity. (D) Presence of macrophages with giant lysosomes in, or in the vicinity of, M-cell pockets. Bars = 1 μm.

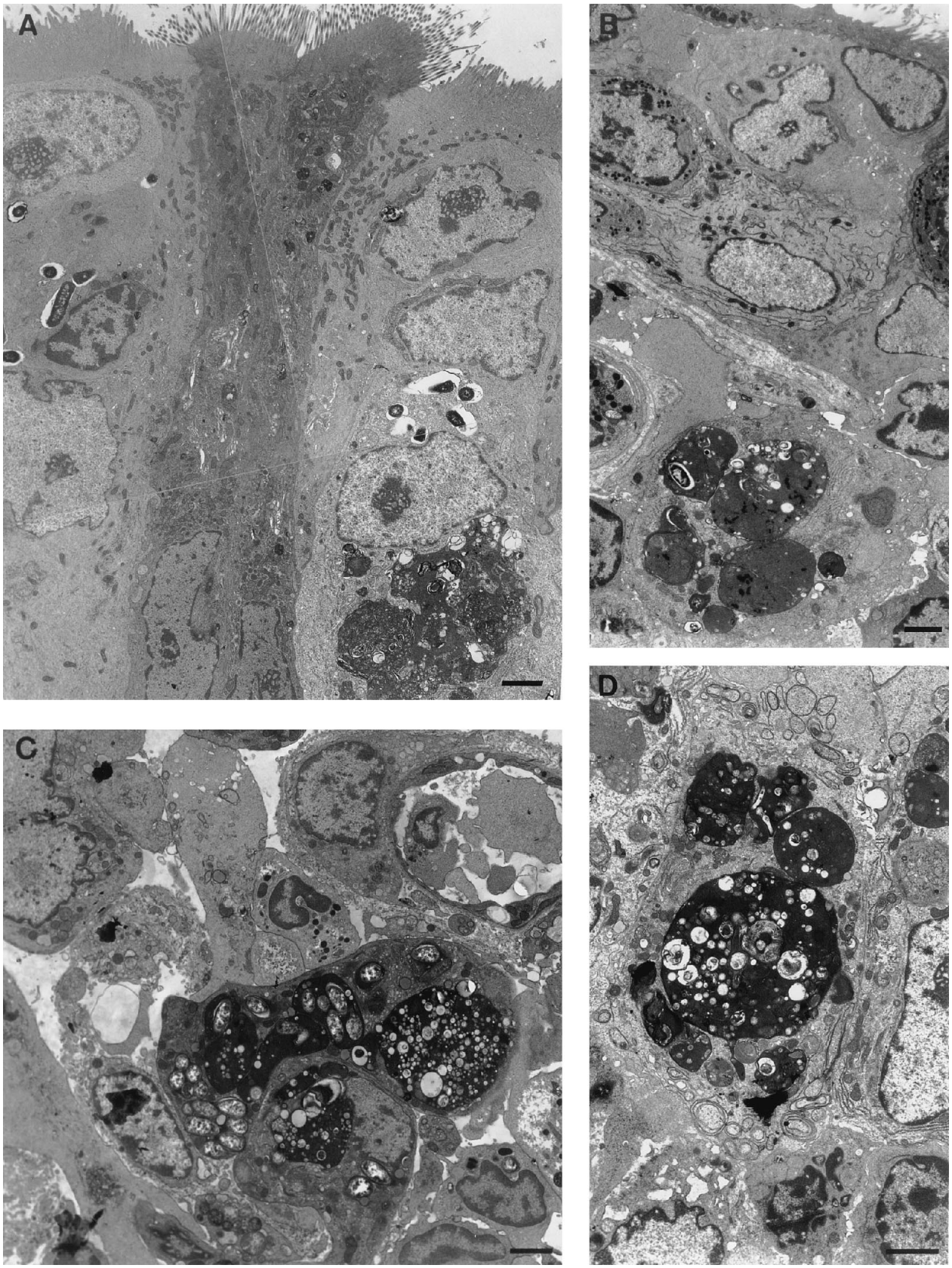


FIG. 7. TEM. Shown are rabbit Peyer's patches infected by BS15 at 2 h (A) and at 8 h (B to D). Note the presence of giant lysosomes with bacteria at various stages of degradation inside macrophages located below the fibroblast layer that delimits the FAE. Bars = 1 μm.

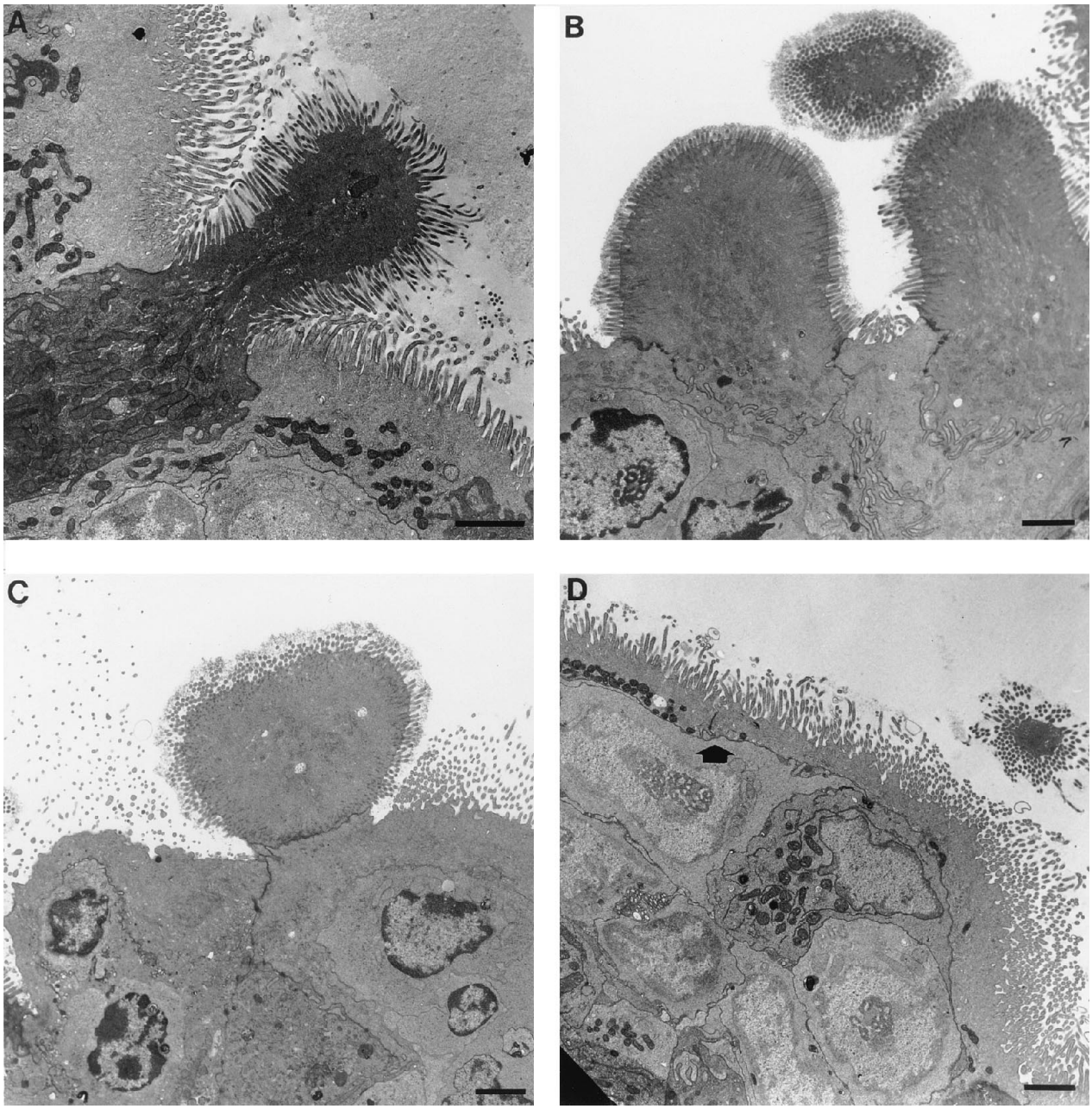


FIG. 8. TEM. Shown are rabbit Peyer's patches infected with BS15. (A and B) After 2 h of infection, note the squeezing and extrusion of the apical pole of enterocytes. (C) After 8 h of infection, note the ongoing elimination of one enterocyte apex with conservation of intercellular junctions and progressive spread of M cells. (D) After 8 h of infection, expansion of M cells over an enlarged pocket is seen. Intercellular junctions are maintained (arrow). This picture is characteristic of the modification affecting the FAE. Bars = 1 μm.

pointing to their conserved intercellular junction. Measurements indicated that they reached, on the apical side, a width about five times that of M cells in control samples, still maintaining intact junctions.

DISCUSSION

The intestinal epithelium is an impermeable barrier formed by tightly coupled epithelial cells which, among other properties, prevent entry of microorganisms into the host. Enteroinvasive bacteria such as *S. flexneri* therefore need to find their way through this barrier. In spite of its limited surface relative to that of the villous epithelium (i.e., 1/10,000), the FAE which covers the lymphoid structures associated with the intestinal mucosa appears to represent a major site of initial entry for bacterial pathogens, due to the presence of M cells interspersed among enterocytes. M cells can engulf viruses, bacteria, and even protozoa (8, 19, 29). They are present at a frequency varying according to the animal species (i.e., 10% of the FAE in humans and mice and about 50% in rabbits). In the digestive tract, these lymphoid structures and their overlying FAE can be found as aggregates in the ileum (i.e., Peyer's patches) or as solitary nodules in the colon and the rectum. There are differences in expression of glycoconjugates on the

epithelial surfaces of M cells in the mouse intestine, thus suggesting potential differential targeting of microbial pathogens through their adhesins (7). As a global system, these lymphoid structures form the inductive site of intestinal immunity (15). It is striking that many pathogens have taken advantage of its existence in order to invade their host.

The aim of this work has been to use the *Shigella* infection model of ligated intestinal loops in rabbits (30) to evaluate, in an isogenic background, the respective contributions of adhesive and invasive phenotypes to infection of FAE. To achieve this, the wild-type invasive *S. flexneri* M90T was used, as well as two of its derivatives: a noninvasive-nonadhesive mutant (BS176) which has lost the 220-kb virulence plasmid (38) and an adhesive transconjugant of BS176, BS15, which shows strong adherence to the rabbit intestinal mucosa because of its expression of AF/R1, which mediates attachment to M cells and induction of actin pedestals on their surface (10, 11).

The present work has confirmed previous evidence that *S. flexneri* used FAE as a primary site of entry into the intestinal barrier (30, 37, 42). The nonadhesive-noninvasive mutant BS176 showed a very limited capacity to infect the FAE, thus indicating that in order to be efficiently taken up by M cells, a microorganism must express an adhesive or an invasive phenotype. Random sampling of luminal bacteria is likely to occur, but large quantitative differences probably exist, depending on the degree of interaction that can be established. This observation has several important implications, among which the design of live bacterial vaccine candidates has to be considered. In *Shigella* vaccine development, it has been shown that a noninvasive mutant, such as strain T32 Israti, could achieve efficient but short-lasting protection, provided that multiple oral doses were administered (21). On the other hand, live attenuated, but still invasive, mutants, such as streptomycin-dependent strains, could elicit protective immunity with a limited number of oral doses (22). This practical observation suggests that in order to minimize the number of oral doses to be delivered to vaccinees, vaccine strains should express an efficient mechanism of interaction with the intestinal follicular epithelium (i.e., adherence or invasion). However, expression of an invasive phenotype by vaccine strains may cause significant side effects, due to its proinflammatory potential (43). Considering that the type-specific antigen of LPS is the major protective antigen of *S. flexneri* (33), a new approach which would make use of noninvasive *Shigella* mutants expressing LPS and an efficient, heterologous, human-specific adherence system can be suggested.

Having demonstrated the relative contributions of the adhesive and invasive phenotypes to infection of FAE, our second aim in this work was to describe the natural history of lymphoid follicle infection by the three isolates of *Shigella*. A major characteristic of Peyer's patches infected by the invasive strain M90T was the extent of inflammation-mediated destruction of the FAE. After 8 h, dislocation of the tissue architecture was observed, essentially caused by a major infiltration by PMN, which eventually led to necrosis and ulcer formation. We have already shown that pretreatment of animals with an antibody blocking migration of PMN into tissues abrogated inflammation-mediated ulcer formation (30). It is therefore possible that in infected humans, such ulcerations constitute the initial tissue lesion of shigellosis. A similar suggestion has recently been made concerning the small intestinal ulcerations that may cause bleeding and/or perforations in the course of typhoid fever (28).

Observations made for tissues infected for 2 h and for tissues that had not yet undergone major destruction after 8 h allowed us to reconstitute the sequence of events characteristic of wild-type *Shigella* invasion of FAE. Initial entry into M cells was seen to occur essentially as already described (30, 42). Major rearrangement of the apical membrane at the entry site was observed, very reminiscent of the membrane projections caused by massive cytoskeletal rearrangement occurring during *Shigella* entry into epithelial cells in vitro (1, 4). This suggests that the *Shigella* complex of invasins is actually used to penetrate M cells. A similar observation has been made with *S. typhimurium* invading murine M cells (13). This is not surprising since *Shigella* and *Salmonella* spp. have recently been shown to share homologous invasion-associated proteins (9, 14). However, the next steps appeared different. Instead of being cytotoxic to M cells and causing their destruction, as seen during *Salmonella* invasion, internalized shigellae appeared to be translocated to the intraepithelial and subepithelial areas, often remaining extracellular in spaces between M cells, mononuclear cells localized inside their pockets, and adjacent enterocytes. However, no invasion of enterocytes was observed at this point. We have shown that upon contact with epithelial cells in vitro, *S. flexneri* released its pool of cytoplasmic invasins via the activated Mxi-Spa secretory apparatus (23). We have also shown that, in spite of the presence of many intracellular bacteria, *S. flexneri* was not cytotoxic for its host epithelial cell before bacteria had massively grown inside the cytoplasmic compartment (18). It is therefore possible that, having exhausted their pool of invasins to enter M cells, shigellae find themselves passively translocated, unable to reinfect other cells until they have reconstituted this pool for the next round of invasion. Bacteria were subsequently found inside the mononuclear phagocytes located within enlarged pockets formed by M cells stretched around an increasing number of immigrating mononuclear cells. Whether bacteria entered resident macrophages or recently immigrated monocytes is not clear. We are currently investigating whether dendritic cells also participate in bacterial capture. On the basis of our data, there are two possible outcomes to this process. One possibility is that infection aborts, in which case numerous apoptotic nuclei are seen inside mononuclear phagocytes and bacteria are barely detectable inside giant lysosomes in an advanced stage of degradation. In this case, nonspecific local defenses could prevent bacterial growth and control inflammation, a deleterious effect which seems to be related to the capacity of shigellae to induce apoptotic death of macrophages, as demonstrated in vitro (43, 44) and recently in vivo (45). The second possibility is that infection proceeds, with numerous bacteria appearing intact inside macrophages, some of which show signs of ongoing apoptosis. In parallel, PMN rapidly appear, accumulating in the M-cell pocket, along with mononuclear cells, causing disruption of the FAE architecture.

In summary, when invasive shigellae are present it is difficult to describe the evolution of the cell populations that normally constitute the FAE, the process being rapidly dominated by an influx of PMN with deleterious consequences. In contrast, infection of Peyer's patches by noninvasive BS176 and BS15 strains allowed better observation of the modifications undergone by the FAE, since only limited inflammation occurred. In spite of quantitative differences due to poor colonization of Peyer's patches by the nonadhesive strain BS176, the alterations observed with the two strains were qualitatively similar. We have therefore considered here data obtained with the adhesive strain BS15.

At early time points, bacteria appeared to follow a route quite similar to that of M90T, although a major change in the apical surface of M cells was seen. Translocation was observed, and intercellular migration occurred, even beyond the layer of fibroblasts which delimits the lower part of the FAE. Bacteria

were then sequestered and destroyed inside giant lysosomes present in macrophages in this area of the dome.

The effect of BS15 on the cell population constituting the FAE was characterized, after 8 h of infection, by three essential features: (i) the occurrence of major morphological changes in enterocytes of the FAE, some of which seemed to lose their apical domain; (ii) the occupation of large surfaces of this FAE by M cells; and (iii) the migration of numerous mononuclear cells inside M-cell pockets which increased in size. We do not believe that this effect is shigella specific. It has already been shown that after infection by *Streptococcus pneumoniae* of rabbit ligated intestinal loops containing a Peyer's patch, marked changes in the FAE were observed (34). These included immigration of mononuclear cells, particularly lymphocytes, into M-cell pockets, as well as transmigration of some of these lymphocytes through M cells into the lumen. Some degree of bump formation by the apical domain of the FAE-associated enterocytes could be observed. *Shigella* spp. and *S. pneumoniae* may therefore express factors that directly or indirectly account for this process. These factors, as well as the putative host molecules—possibly cytokines produced by mononuclear leukocytes present in the dome—which relay signals to lymphocytes, enterocytes, and M cells, now need to be identified. The fact that a gram-positive organism and a gram-negative microorganism can elicit similar changes suggests that bacterial LPS is not a major factor. One could argue that elimination of the enterocyte apex is a direct response of these cells to a bacterial factor and that M cells are only filling the gap. However, we have never observed *Shigella* spp. to cause this phenomenon, either in vitro on polarized monolayers of human intestinal cells or in vivo on the villous intestine of rabbit ligated intestinal loops. The actual physiological status of spreading M cells is currently unknown. If their endocytic capacity is maintained, the actual surface of efficient interaction with pathogens should increase, leading to more invasion as infection proceeds. *S. typhimurium* has been shown to induce M-cell formation in germ-free mouse Peyer's patch tissue (41). One day after oral administration of 5×10^9 *aroA* mutants of *S. typhimurium*, the number of M cells detected via their alkaline phosphatase-negative phenotype showed a two- to threefold increase. It was then hypothesized that *S. typhimurium* induced formation of M cells by changing the local subepithelial environment of the follicles. Within the limits of our experimental design, we have not seen significant increase in M-cell numbers with *Shigella* infection, but rather we have seen considerable enlargement of their surface at the expense of enterocytes. We cannot explain this difference at the moment. Host factors, bacterial factors, or the infection procedure may account for it. Future models reconstituting the FAE in vitro will hopefully help to define the molecules involved in its profound remodeling in the presence of infectious agents.

ACKNOWLEDGMENTS

We thank Colette Jacquemin for secretarial assistance and Armelle Phalipon and Wandy Beatty for careful reading of the manuscript.

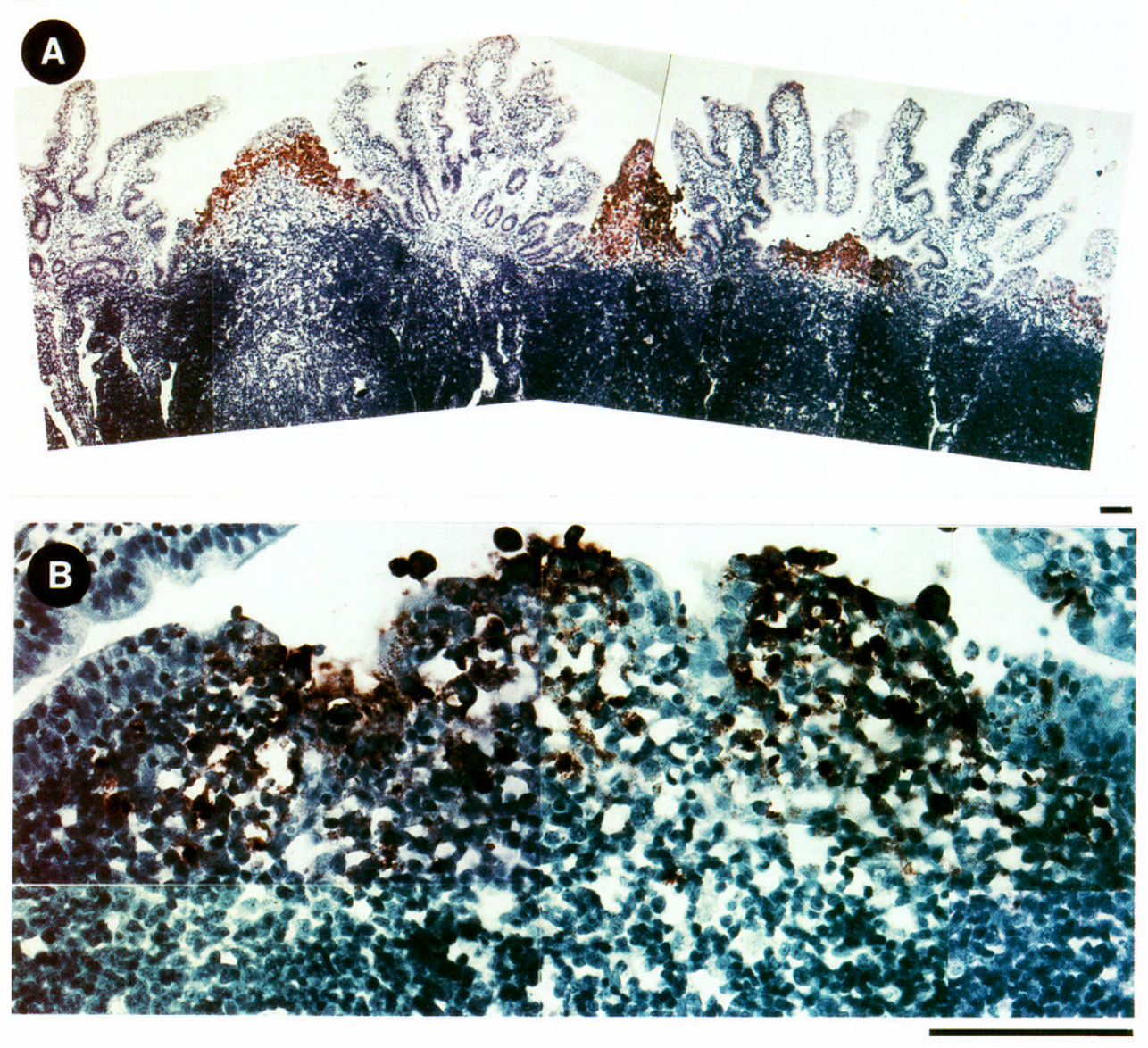
This work was funded by a Direction des Recherches et Techniques (DRET) grant, 94 092.

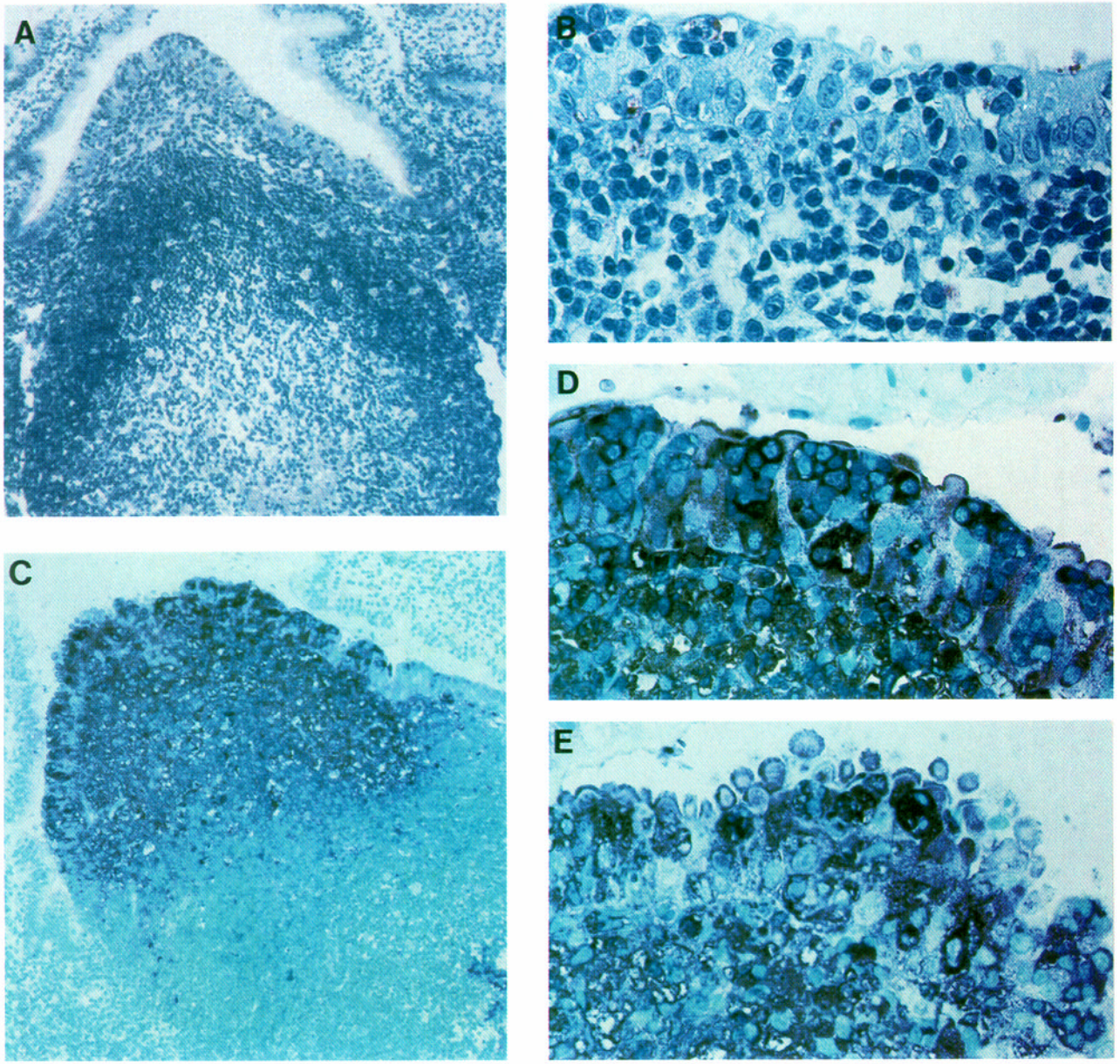
REFERENCES

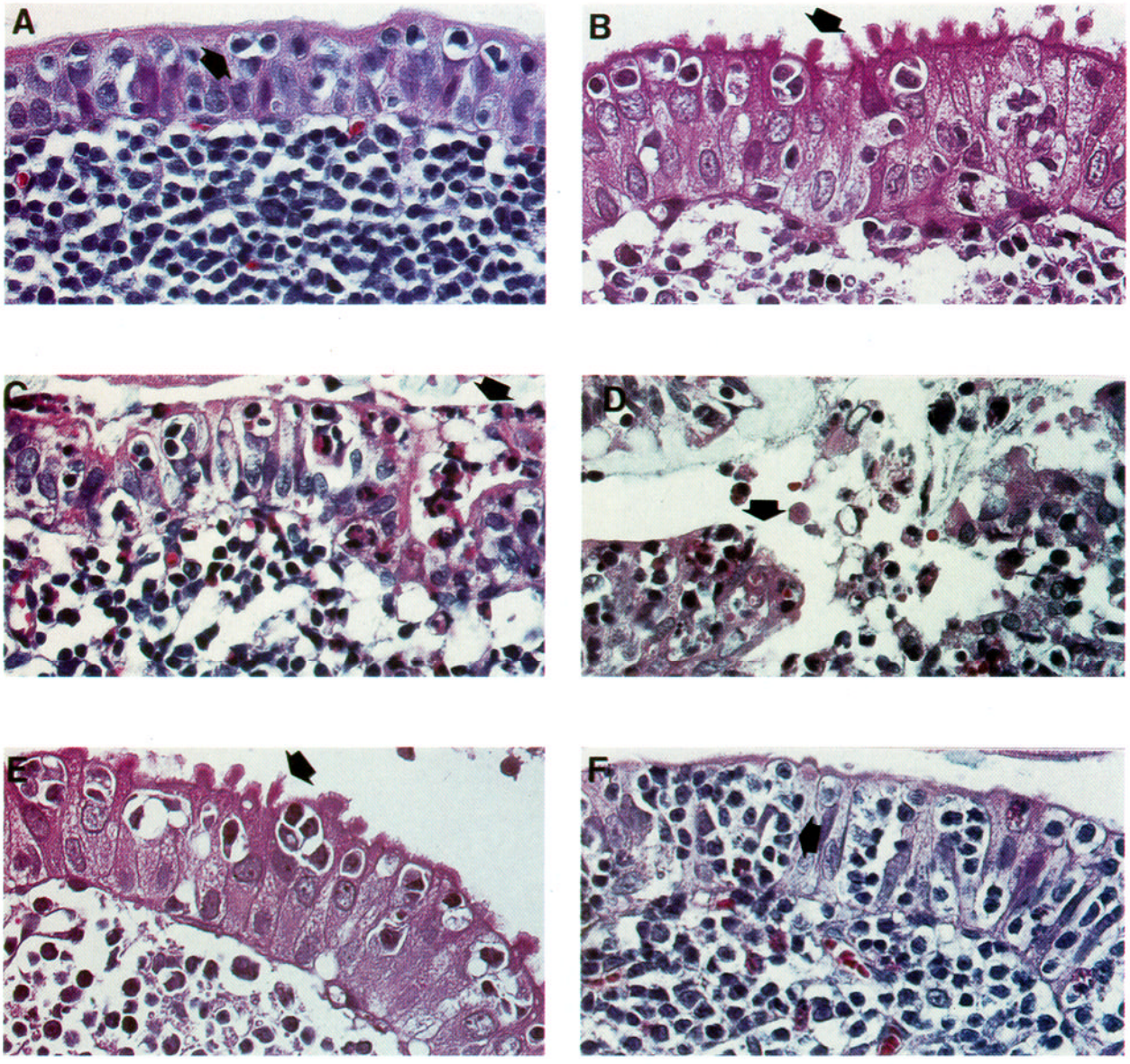
1. **Adam, T., M. Arpin, M. C. Prevost, P. Gounon, and P. J. Sansonetti.** 1995. Cytoskeletal rearrangements and the functional role of T-plastin during entry of *Shigella flexneri* into HeLa cells. J. Cell Biol. **129:**367–381.
2. **Allaoui, A., J. Mounier, M. C. Prevost, P. J. Sansonetti, and C. Parsot.** 1992. *ics*B: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intercellular spread. Mol. Microbiol. **6:**1605–1616.
3. **Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. Identification of *ics*A, a plasmid locus of *Shigella flexneri* which governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86:**3867–3871.
4. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55:**2681–2688.
5. **Formal, S. B., P. Gemski, Jr., R. A. Giannella, and S. Austin.** 1972. Mechanisms of *Shigella* pathogenesis. Am. J. Clin. Nutr. **25:**1427–1432.
6. **Fujimora, Y., T. Kihara, and H. Mine.** 1992. Membranous cells as a portal of *Yersinia pseudotuberculosis* entry into rabbit ileum. J. Clin. Electron Microsc. **25:**35–44.
7. **Giannasca, P. J., K. T. Giannasca, P. Falk, J. I. Gordon, and M. R. Neutra.** 1994. Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am. J. Physiol. **267:**1108–1121.
8. **Giannesca, P. J., and M. R. Neutra.** 1993. Interactions of microorganisms with intestinal M cells: mucosal invasion and induction of secretory immunity. Infect. Agents Dis. **2:**242–248.
9. **Hermant, D., R. Ménard, N. Arricau, C. Parsot, and M. Y. Popoff.** 1995. Functional conservation of the Salmonella and Shigella effectors of entry into epithelial cells. Mol. Microbiol. **17:**781–789.
10. **Inman, L. R., and J. R. Cantey.** 1983. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. J. Clin. Invest. **71:**1–8.
11. **Inman, L. R., and J. R. Cantey.** 1984. Peyer's patch lymphoid follicle epithelial adherence of a rabbit enteropathogenic *Escherichia coli* (strain RDEC-1). Role of plasmid-mediated pili in initial adherence. J. Clin. Invest. **74:**90–95.
12. **Jacob, E., S. J. Backer, and S. P. Swaminathan.** 1987. M cells in the follicle-associated epithelium of the human colon. Histopathology **11:**941–952.
13. **Jones, B. D., N. Ghori, and S. Falkow.** 1994. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. **180:**15–23.
14. **Kaniga, K., S. Tucker, D. Trollinger, and J. E. Galan.** 1995. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. J. Bacteriol. **177:**3965–3971.
15. **Kraehenbühl, J. P., and M. R. Neutra.** 1992. Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Rev. **72:**853–879.
16. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in pathogenesis of bacillary dysentery. J. Bacteriol. **88:**1503–1518.
17. **Langmann, J. M., and R. Rowmand.** 1986. The number and distribution of lymphoid follicles in the human large intestine. J. Anat. **194:**189–194.
18. **Mantis, N., M.-C. Prévost, and P. J. Sansonetti.** 1996. Analysis of epithelial cell stress response during infection by *Shigella flexneri*. Infect. Immun. **64:**2474–2482.
19. **Marcial, M. A., and J. L. Madara.** 1986. Cryptosporidium: cellular localization, structural analysis of absorbtive cell-parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. Gastroenterology **90:**583–594.
20. **Marco, A. J., M. Domingo, M. Prats, V. Briones, M. Pumarola, and L. Dominguez.** 1991. Pathogenesis of lymphoid lesions in murine experimental listeriosis. J. Comp. Pathol. **105:**1–15.
21. **Meitert, T., E. Pencu, L. Ciudin, and M. Tonciu.** 1984. Vaccine strain *Shigella flexneri* T32-Istrati. Studies in animals and volunteers. Anti-dysentery immunoprophylaxis and immunotherapy by live vaccine Vadizen. Arch. Roum. Pathol. Exp. Microbiol. **43:**261–278.
22. **Mel, D. M., A. L. Terzin, and L. Vuksic.** 1965. Studies on vaccination against bacillary dysentery: III. Effective oral immunization against *Shigella flexneri* 2a in a field trial. Bull. W.H.O. **32:**647–677.
23. **Ménard, R., P. J. Sansonetti, and C. Parsot.** 1994. The secretion of the *Shigella flexneri* Ipa invasins is induced by the epithelial cell and controlled by IpaB and IpaD. EMBO J. **13:**5293–5302.
24. **Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P. J. Sansonetti.** 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through their basolateral pole. Infect. Immun. **60:**237–248.
25. **Neutra, M. R., and J. P. Kraehenbühl.** 1992. Transepithelial transport and mucosal defence.I. The role of M cells. Trends Cell Biol. **2:**134–138.
26. **O'Leary, A. D., and E. C. Sweeney.** 1986. Lymphoglandular complexes of the colon: structure and distribution. Histopathology **10:**267–283.
27. **Owen, R. J., A. J. Piazza, and T. H. Ermark.** 1991. The number and distribution of lymphoid follicles in the human large intestine. Am. J. Anat. **190:**10–18.
28. **Owen, R. L.** 1994. M cells—entryways of opportunity for enteropathogens. J. Exp. Med. **180:**7–9.
29. **Owen, R. L., C. L. Allen, and D. P. Stevens.** 1981. Phagocytosis of *Giardia muris* by macrophages in Peyer's patch epithelium in mice. Infect. Immun. **33:**591–601.
30. **Perdomo, J. J., J. M. Cavaillon, M. Huerre, H. Ohayon, P. Gounon, and P. J. Sansonetti.** 1994. Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. J. Exp. Med. **180:**1307–1319.
31. **Perdomo, J. J., P. Gounon, and P. J. Sansonetti.** 1994. Polymorphonuclear leukocyte transmigration promotes invasion of colonic epithelial monolayer by *Shigella flexneri*. J. Clin. Invest. **93:**633–643.

32. **Phalipon, A., S. Barzu, J. Arondel, C. Fitting, J. M. Cavaillon, and P. J. Sansonetti.** Role of systemic antibodies directed against *Shigella flexneri* lipopolysaccharide, IpaB and IpaC antigens in protection against experimental shigellosis. Unpublished data.
33. **Phalipon, A., M. Kaufmann, P. Michetti, J. M. Cavaillon, M. Huerre, P. J. Sansonetti, and J. P. Kraehenbühl.** 1995. Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of *Shigella flexneri* lipopolysaccharide protects against murine experimental shigellosis. J. Exp. Med. **182:**769–778.
34. **Regoli, M., C. Borghesi, E. Bertelli, and C. Nicoletti.** 1994. A morphological study of the lymphocyte traffic in Peyer's patches after an in vivo antigenic stimulation. Anat. Rec. **239:**47–54.
35. **Sansonetti, P. J.** 1991. Genetic and molecular basis of epithelial cell invasion by *Shigella* species. Rev. Infect. Dis. **13:**S285–S292.
36. **Sansonetti, P. J., J. Arondel, J. M. Cavaillon, and M. Huerre.** 1995. Role of interleukin-1 in the pathogenesis of experimental shigellosis. J. Clin. Invest. **96:**884–892.
37. **Sansonetti, P. J., J. Arondel, A. Fontaine, H. d'Hauteville, and M. L. Bernardini.** 1991. OmpB (osmo-regulation) and *ics*A (cell to cell spread) mutants of *Shigella flexneri*. Evaluation as vaccine candidates. Probes to study the pathogenesis of shigellosis. Vaccine **9:**416–422.
38. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a large plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35:**852–860.
39. **Sansonetti, P. J., J. Mounier, M. C. Prevost, and R. M. Mege.** 1994. Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. Cell **76:**829–839.
40. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51:**461–469.
41. **Savidge, T. C., M. W. Smith, P. S. James, and P. Aldred.** 1991. *Salmonella*-induced M cell formation in germ-free mouse Peyer's patch tissue. Am. J. Pathol. **139:**177–184.
42. **Wassef, J., D. F. Keren, and J. L. Mailloux.** 1989. Role of M cells in initial bacterial uptake and in ulcer formation in the rabbit intestinal loop model in shigellosis Infect. Immun. **57:**858–863.
43. **Zychlinsky, A., C. Fitting, J. M. Cavaillon, and P. J. Sansonetti.** 1994. Interleukin 1 is released by macrophages during apoptosis induced by *Shigella flexneri*. J. Clin. Invest. **94:**1328–1332.
44. **Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti.** 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature (London) **358:**167–169.
45. **Zychlinsky, A., K. Thirumalai, J. Arondel, R. Cantey, A. Aliprantis, and P. J. Sansonetti.** *In vivo* apoptosis in *Shigella flexneri* infection. Submitted for publication.

Editor: J. R. McGhee


A
B


A
B
C
D
E


A
B
C
D
E
F