Acute Inflammation Causes Epithelial Invasion and Mucosal Destruction in Experimental Shigellosis

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Summary

The gram-negative pathogen *Shigella flexneri* causes bacillary dysentery, an invasive disease of the human colonic mucosa. A major characteristic of the infectious process is the occurrence of an acute inflammatory reaction of mucosal tissues which is generally considered as a consequence of primary invasion and destruction of colonic epithelial cells by the pathogen. Confirming in vitro demonstration that *S. flexneri* is unable to invade the apical pole of colonic cells and that polymorphonuclear (PMN) cells may assist them in reaching the basal side of epithelial cells where they can invade, we have provided here in vivo evidence that *S. flexneri* enters the epithelial barrier essentially through the dome of lymphoid follicles at the early stage of infection and that subsequent invasion and destruction of the epithelium is primarily due to immigration of leukocytes, particularly PMN that destroy cohesion of the epithelial barrier. These conclusions are based on experiments carried out in infected rabbit ligated intestinal loops, with some animals treated by an anti-CD18 monoclonal antibody that blocked immigration of leukocytes into infected tissues.

Shigella flexneri, a gram-negative bacillus belonging to the family Enterobacteriaceae, is the major aetiological agent of the endemic form of bacillary dysentery, an invasive disease of the colon prevalent among young children of the developing world (1, 2).

It is currently considered that the key event in the pathogenesis of shigellosis is invasion of colonocytes (3). This is supported in vitro since *S. flexneri* invades mammalian epithelial cells (3–5). Bacteria enter these cells via directed phagocytosis (6), gain access to the cytoplasm after lysing the phagocytic vacuole (7), and then express a motility phenotype that allows intracellular movement and cell to cell spread (8). This movement is based on the capacity of bacteria to interact with the host–cell cytoskeleton since shigellae can slide along actin cables (9, 10), and organize F-actin on their surface as a polarized motor that pushes the bacterium forward and allows formation of protrusions that bring it into the cytoplasmic compartment of the next cell (11–14). These protrusions are then actively endocytosed by the recipient cell through a cadherin-mediated process at the level of intercellular junctions (15). This cycle of intracellular infection may allow colonization of large surfaces of the epithelium, the bacteria being protected from host immune surveillance. At an advanced stage, an inflammatory response may enhance tissue destruction. In this scheme, mucosal destruction is primarily due to invasion of the epithelial lining by *Shigella*, followed by mucosal inflammation and subsequent tissue destruction.

There are, however, inconsistencies in this scheme. First, in in vitro assay systems, we have demonstrated that, unlike *Salmonella* (16, 17), *S. flexneri* is unable to enter the apical pole of polarized intestinal epithelial Caco-2 or T-84 cells established on filters (18). Entry occurs only on the basal side of these cells. In vitro systems may be further sophisticated by combining two cell populations: intestinal epithelial cells grown on filters, and PMN (19). In a work using the T-84 human colonic cell line, addition of human PMN to the basal pole of the epithelial monolayer, and of shigellae to the apical pole, allowed rapid paracellular transmigration of PMN followed by bacterial invasion of epithelial cells (20). This process of PMN-assisted invasion depends on specific binding of PMN to T-84 cells since it is neutralized by an anti-CD18 monoclonal antibody which blocks the function of the Mac-1 receptor (21). Subsequent incubation (beyond 3 h) showed destruction of the monolayer (Perdomo, O. J. J., and P. J. Sansonetti, unpublished data). This indicates that the conflict between bacteria and PMN leads to the release of highly toxic substances from PMN that may kill enterocytes more efficiently than bacterial invasion per se. These experiments suggest that luminal bacteria themselves express or induce cells of the intestinal barrier to produce potent chemotactic signals that

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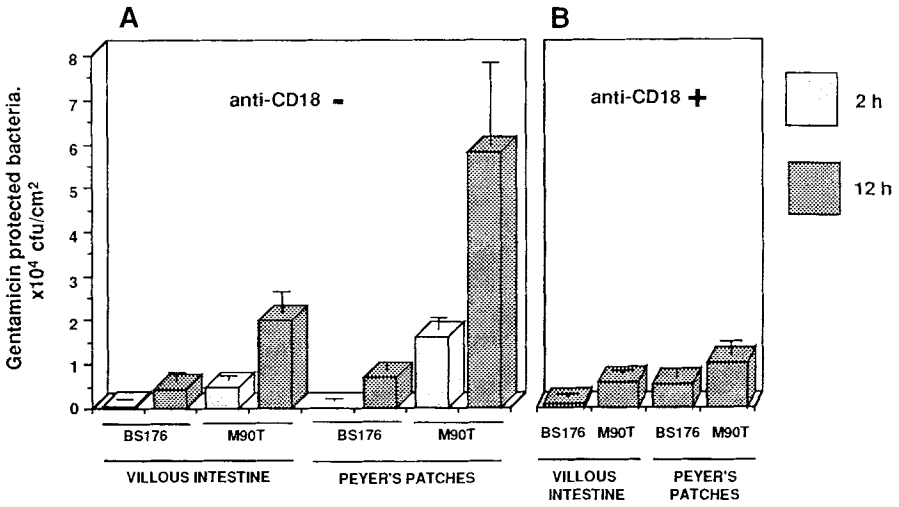


Figure 1. *S. flexneri* entry into rabbit intestinal mucosa. Tissue samples of villous intestine and Peyer's patches were processed in order to assess the number of gentamicin-protected bacteria inside samples of equivalent surface. (*A*) Rabbits infected with invasive (M90T) and noninvasive (BS176) strains for 2 and 12 h. (*B*) Rabbits pretreated with anti-CD18 monoclonal antibody and subsequently infected with M90T and BS176 for 12 h.

elicit an early inflammation initiating epithelial invasion and causing mucosal damage.

Examination of histopathological data obtained from experimental animal models and from clinical cases shows a complex pattern of invasion and emphasizes the importance of the inflammatory process elicited during shigellosis. Experiments involving intragastric infection of macaque monkeys, either with an invasive isolate of *S. flexneri*, or with its *icsA* mutant which has lost the capacity to spread from cell to cell, pointed to M cells as an early and major site of entry for bacteria (22). These results are supported by observations made in naturally infected humans (23) where small ulcers are observed, particularly in the epithelium that overlays lymphoid follicles. In addition, local blood capillaries subjacent to the epithelium demonstrate swollen endothelial cells and numerous marginating and transmigrating PMN. Ulcerated abscesses then extend from these areas of epithelial destruction, leaving a dense exudate that consists essentially in PMN.

In this work, using the rabbit ligated intestinal loop model of invasion by *S. flexneri*, we have tried to analyze how, from the initial site of bacterial entry, early inflammation may promote mucosal invasion as well as subsequent tissue destruction. To evaluate the role of the inflammatory reaction, we provoked a functional tissue leukopenia in a group of rabbits by injection of a monoclonal antibody directed against CD18, the β_2 subunit of several leukocyte adhesion molecules such as LFA-1 and Mac-1 (24), known to block migration of leukocytes, especially PMN, from blood vessels (21) to infectious foci.

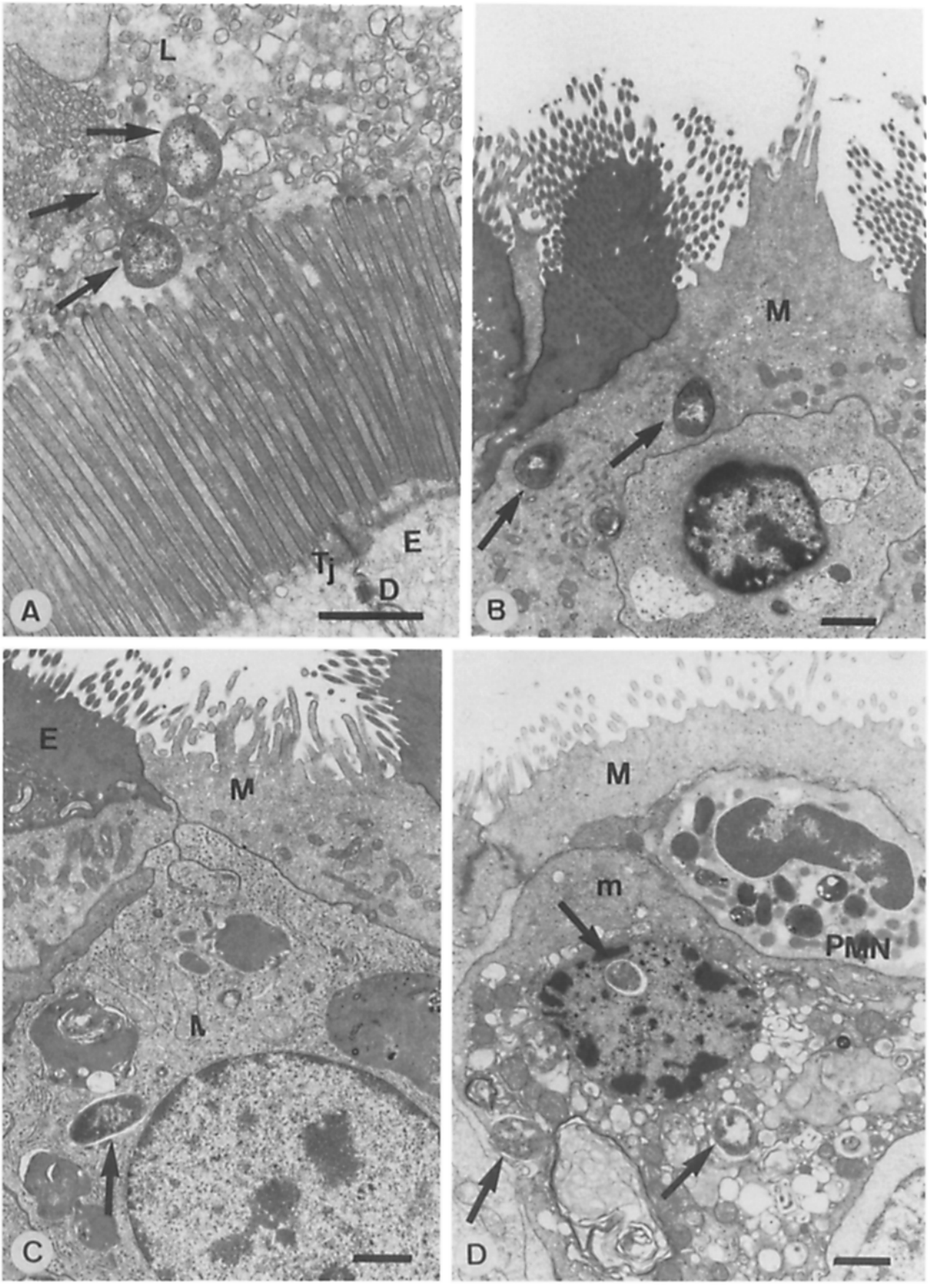
Materials and Methods

Bacterial Strains and Growth Media. Two *S. flexneri* strains were used in this study: M90T, an invasive isolate belonging to serotype 5, and BS176, an isogenic noninvasive derivative that has been cured of the virulence plasmid pWR100. Both strains were routinely grown on trypticase soy broth (TCS; Diagnostics Pasteur, Marnes la Coquette, France) at 37°C with aeration. For infection of ligated rabbit intestinal loops, an overnight culture on Congo red plates was diluted in sterile physiological serum to obtain 10^{10} bacteria/ml.

Rabbit Intestinal Loop Assay. We used 18 New Zealand White rabbits, weighing 2.0–2.5 Kg (Charles River Laboratories, St-Aubin, France) for a total of 216 intestinal loops. Animals were fastened 24 h before infection, and anesthesized intravenously with 6% sodium pentobarbital (0.5 ml/kg). 2% xylocaine was administered intradermally in the abdominal region where the laparotomy was to be performed. Intestinal segments of 10 cm length were ligated, some of which contained a Peyer's patch. The blood supply to the loops was carefully preserved, and bacterial strains were injected into the closed loops in a volume of 0.5 ml/loop. Control loops were obtained by injecting the same volume of saline. Loops were returned to the abdominal cavity for 2, 4, 8, and 12 h, after which animals were killed. The exudate was aspirated, measured, and replaced by the same volume of fixative when samples were to be processed for morphological analysis, or of a solution of gentamicin (50 μg/ml) in PBS, when bacterial counts were to be performed by measuring CFU. Specimens consisted of villus intestinal tissue, and of intestinal loops containing a Peyer's patch. In the latter, only Peyer's patches themselves were analyzed.

Bacterial Counts in Tissue Samples. Loops were treated with gentamicin (50 μg/ml in 0.1 M PBS), an aminoglycoside antibiotic that penetrates poorly into cells, to eliminate the extracellular or adherent population of bacteria present on the mucosal surface. This treatment was carried out twice; first, by replacing volume for volume the fluid accumulated inside the loop with the antibiotic solution (1 h), and second by soaking biopsy samples obtained from these loops in a fresh solution of the same antibiotic. Tissue samples of similar size were obtained by using an 8-mm diameter punching disk, on both intestinal and Peyer's patch tissues. Thorough washing was carried out with 0.1 M PBS to eliminate re-

Figure 2. Initial site of entry for invasive *S. flexneri* (M90T). Transmission electron micrographs showing that the M cell is the initial site of entry of *S. flexneri*. (*A*) 8-h infection: bacteria (*arrows*) in the lumen (*L*) do not enter enterocytes (*E*) through their apical brush border. Tight junctions (*Tj*) and desmosomes (*D*) remain intact. (*B*) 4-h infection: bacteria (*arrows*) inside an M cell (*M*). (*C*) 8-h infection: bacteria (*arrow*) has translocated to subjacent monocyte–macrophage (*D*) 8-h infection: bacteria (*arrows*) inside a macrophage (*m*) showing perinuclear aggregation of chromatin and cytoplasmic vacuolization suggestive of apoptosis. PMN underlying the M cell (*M*). Bar, 1 μm.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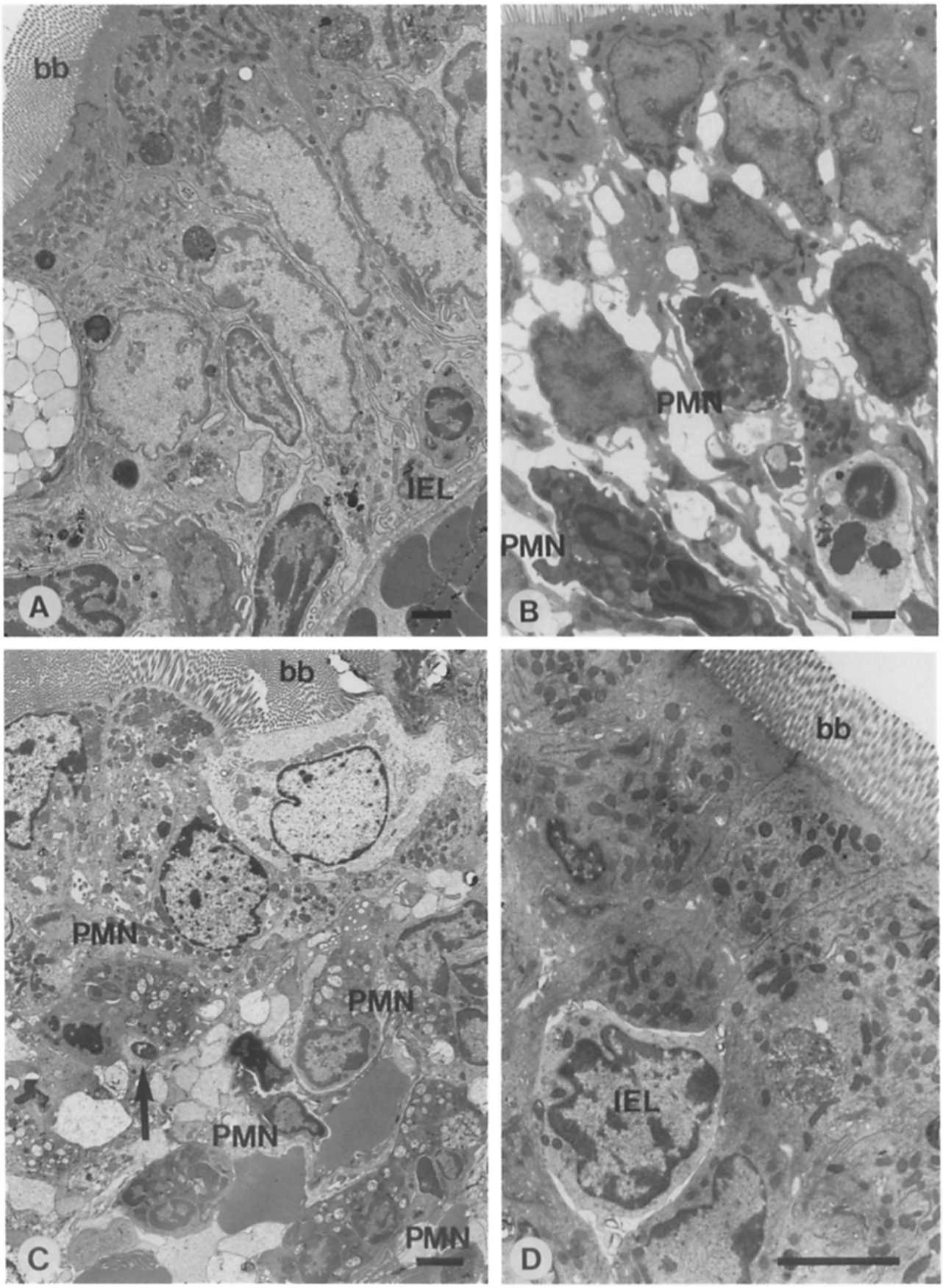
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sidual gentamicin, and ice-cold PBS was added to samples to stop bacterial proliferation. Tissue samples were then ground in ice-cold PBS. A 1/10 dilution was made in TCS and incubated for 30 min at 37°C with aeration. Serial dilutions of this culture were then plated on agar and incubated overnight at 37°C. CFUs were counted and the number of bacteria was calculated for an area of 1 cm^2 of intestinal tissue.

Tissue Sampling for Histopathological Analysis. All tissue samples obtained at necropsy were fixed in 10% paraformalin, dehydrated, and embedded in historesin (Leica Instruments, Heidelberg, Germany). Sequential sections were taken at different levels of the intestinal loops in order to study 10 sections/loop. Thin cuts of 1–2 μm were made and stained with hematoxylin-eosin for general observations, and with methyl blue or Giemsa to detect bacteria. We systematically analyzed 10 villi/section and 3 sections were read for every sample. Each villus was divided in two parts: epithelium and chorion. Observations were made in villi of the intestine, as well as over the dome of Peyer's patches. We looked for erosions, goblet cell depletion, edema, hemorrhage, and infiltration by monocytes and PMN. These histopathological sections were independently read and scored by three people.

Transmission Electron Microscopy (TEM)[1]. Tissues were minced to fragments of approximately 1 mm^3 and fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, containing 0.1 M sucrose, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. Samples were postfixed in 2% osmium tetroxide complemented with 1% $K_4[Fe(CN)_6]$. Progressive dehydration was followed by flat embedding in Epon. 1-μm thick sections were cut on an ultramicrotome (LKB Instruments Inc., Gaithersburg, MD), stained with toluidine blue, and examined. Areas presenting signs of inflammation were chosen in both villus and Peyer's patch samples. Ultrathin sections were then realized over these selected areas, stained with 1% uranyl acetate and 1% lead citrate, and observed on a transmission electron microscope operating at 80 kV (model CM-12; Philips Technologies, Cheshire, CT).

TNF Bioassay. Exudates recovered from the loops were frozen until the TNF bioassay was performed. These fluids were taken from villus and Peyer's patch loops. Essentially as described in (25), 3×10^4 L929 fibroblasts were cultured overnight in RPMI 1640, 10% FCS, in 69-well plates. Serial dilutions of culture supernatants were added in the presence of actinomycin D at a final concentration of 2 μg/ml. After 18 h of incubation, cells were stained with crystal violet. The microtiter plates were rinsed and the cells were solubilized in 1% SDS. Dye uptake was measured at 540 nm with a micro-ELISA automated reader. 1 U of TNF activity was defined as the amount required to lyse 50% of L929 target cells. In some cases, values were so high that several concentrations overpassed the detection levels (>3,000 U/ml). We considered >3,000 = 3,000 U/ml, and TNF titers were multiplied by the volume of the exudate found inside each of the loops assayed.

Inhibition of Leukocyte Transmigration in Tissues. To block transmigration of leukocytes, and particularly of PMN into infected intestinal loops, two rabbits received an intravenous injection of a mouse monoclonal antibody, R-15.7 (3 mg/kg), 4 h before surgery and loop-infections were carried out. This antibody (a kind gift from Dr. R. Rothleim, Boehringer Ingelheim Pharmaceuticals), is directed against CD18, the β_2 subunit of LFA-1 and Mac-1 integrins which has been shown to crossreact in the rabbit. This antibody is known to neutralize the integrin–receptor interaction between leukocytes and the vascular endothelium, preventing, in particular, PMN to extravasate (24) and transmigrate through an epithelial layer (26). Infection was then carried out in a total of 30 loops as described above. Induction of functional tissue-neutropenia was only performed in 12-h infections since this was a critical time point at which maximal PMN transmigration was observed before total tissue destruction occurred by 16 h of infection (data not shown). Results were compared with four untreated rabbits, on a total of 56 loops.

[1] *Abbreviation used in this paper:* TEM, transmission electron microscopy.

Results

Initial Site of Entry of S. flexneri

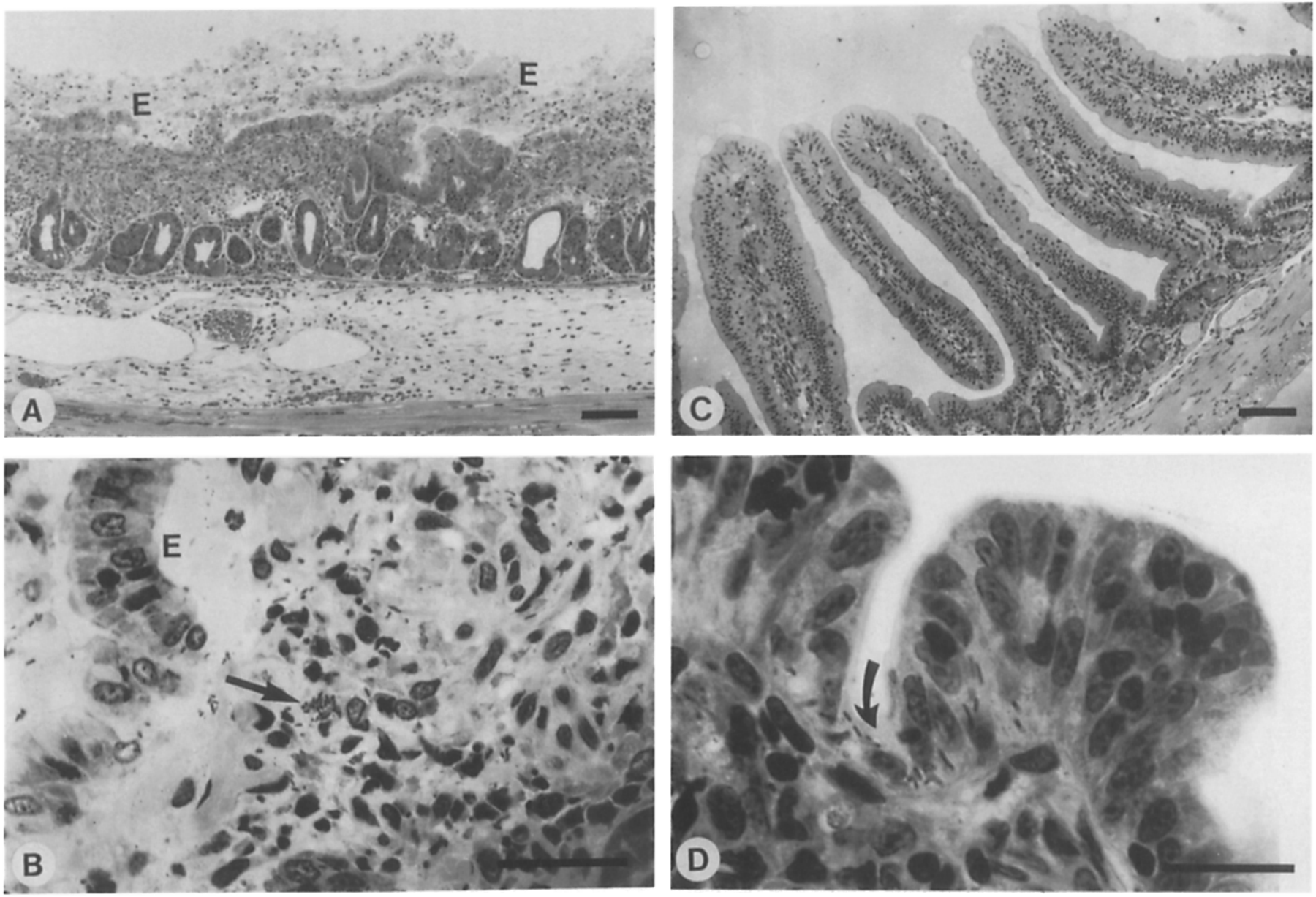
Data clearly point to Peyer's patches, and more specifically M cells, as the initial site of entry for shigellae. At 2 h after bacteria were inoculated inside the loops, Peyer's patches contained 3.2 times more bacteria if infected with M90T than the villus loops that did not contain lymphoid follicles (Fig. 1). There was also a large difference between initial uptake of the invasive strain ($1.62 \pm 0.29 \times 10^4$ CFU/cm^2) compared with the noninvasive strain ($0.02 \pm 0.01 \times 10^4$ CFU/cm^2) in follicular samples.

Closer analysis was performed by TEM in order to follow bacterial traffic in follicular structures. M90T was always associated with M cells of the dome areas, either inside vesicles or free in the cytoplasm of these cells (Fig. 2, *B* and *C*). Bacteria were also seen in the intercellular space between M cells and adjacent enterocytes, or in the process of being phagocytosed by subjacent macrophages or lymphocytes (Fig. 2 *D*). In contrast to these observations, and in support of the data obtained by enumerating bacteria in the tissues, BS176 was never observed inside any of the cells constituting the dome area.

Peyer's patches did not present obvious alteration in the presence of M90T during the first hours of infection. Nevertheless, after 8 h, severe inflammation began to take over the dome area. Bacteria were seen inside dead cells, or inside the phagolysosomes of live cells in areas that appeared highly disrupted by intense PMN accumulation.

At an advanced stage of infection (12 h), the number of invasive bacteria in Peyer's patches (Fig. 1) appeared nine times

Figure 3. Rabbit villous intestinal loop infected by *S. flexneri*. Electron micrographs showing different times of infection. (*A*) 8-h infection with noninvasive strain BS176: normal morphology of the epithelium. (*B*) 8-h infection with invasive strain M90T: PMN transmigration and swelling of intercellular spaces.(*C*) 12-h infection with M90T: overall architecture of the epithelium is disorganized with necrotic epithelial cells, infiltrating PMN, and swelling of intercellular spaces. *Arrow* points to a bacterium inside a PMN. (*D*) 12-h infection with M90T, animals pretreated with anti-CD18 monoclonal antibody: normal morphology of the epithelium. IEL, intraepithelial lymphocyte; bb, brush border. Bar, 2 μm.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greater than the number observed with the noninvasive strain ($5.90 \pm 1.86 \times 10^4$ compared with $0.69 \pm 0.16 \times 10^4$ CFU/cm^2, respectively). Uptake of M90T by the dome epithelium appeared more than twice as efficient as the villus intestine during this period.

In the villus loops, very few invasive bacteria were counted in the early times (2 h) of infection (Fig. 1). By 4 h of infection with M90T, the intestine did not show significant alteration, although scanning electron microscopy demonstrated an early phase of inflammation with leucocytes and erythrocytes appearing in the lumen, in areas where bacteria interacted with the surface of the villi (data not shown). After 8 h, histopathological examination confirmed that the inflammatory reaction had increased with broad areas of PMN accumulation in the case of M90T infection (data not shown). In addition, compared with BS176, TEM demonstrated enlargement of intercellular spaces in the epithelial layer, even though tight junctions remained unaltered (Fig. 3, *A* and *B*). PMN were seen transmigrating in these areas.

Bacterial counts in villus loops showed that after 12 h of infection, M90T presented $3.86 \pm 0.94 \times 10^4$ CFU/cm^2, whereas BS176 gave a fivefold lower value, $0.85 \pm 0.34 \times 10^4$ CFU/cm^2 (Fig. 1). After tissue lysis, bacterial counts should reflect the number of intracellular bacteria, not excluding the possibility that a few extracellular bacteria may be protected from the antibiotic simply because they are embedded in a shield of matrix proteins. Also, it is most likely that the noninvasive mutant represents a mixed population of bacteria, some being extracellular, others being trapped inside phagocytes.

Analysis and Role of the Inflammatory Reaction during S. flexneri Infection

Histopathological Evaluation of Tissues. Examination of intestinal tissue revealed profound mucosal alterations that were exclusively observed with the invasive strain M90T. The height of all villi was markedly decreased, due to oedema of the lamina propria and erosion of the epithelium, goblet cell depletion was severe, and hemorrhagic foci were seen in the chorion. Great numbers of PMN were seen emigrating from submucosal blood vessels and villi capillaries into the lamina propria. As seen in Fig. 4 (*A* and *B*), complete detachment of the epithelial layer could be seen in many places, leaving behind vast areas of the chorion infiltrated with PMN, where bacteria could be observed. In other areas, villi were totally sloughed off, leaving flat hemorrhagic ulcers infiltrated by numerous PMN.

Peyer's patch samples infected with M90T showed abcesses in 9 out of 10 domes or villi. Focal ulcerations were seen over all dome areas (Fig. 5, *A* and *B*). Many microorganisms were found in the exudate over the ulcers and within follicular tissues. Acute inflammation was observed throughout the chorion and follicular epithelium, PMN being the major inflammatory cell. Bacteria entered epithelial cells through their basolateral side that became exposed in destroyed areas. At this stage, microorganisms could easily reach the lamina propria and continue invading enterocytes throughout their basolateral pole in nondisrupted areas.

Fluid Accumulation in the Loops. By 12 h, fluid accumulation was observed in the loops as shown in Fig. 6. This luminal exudate contained mucus and PMN. With the invasive strain M90T, the fluid was hemorrhagic. M90T elicited a mean volume of 4.17 ± 0.13 ml/loop, which is six times higher than the volume elicited by the noninvasive strain BS176 (0.66 ± 0.38 ml/loop).

PMN and Monocyte Counts. To assess the intensity of mucosal inflammation, monocytes and PMN were counted in the epithelium and in the chorion of villi. A total of 10 villi/section and 3 different sections/sample were examined. The mean value is expressed as the number of cells counted/villus, as shown on Fig. 7. Monocyte counts did not show any significant difference with either of the two strains, however tissues infected by M90T presented 2.6 times more PMN/villus than those infected by BS176. It is likely that we underestimated the number of PMN in the M90T-infected loops, due to severe destruction of the villi by this strain.

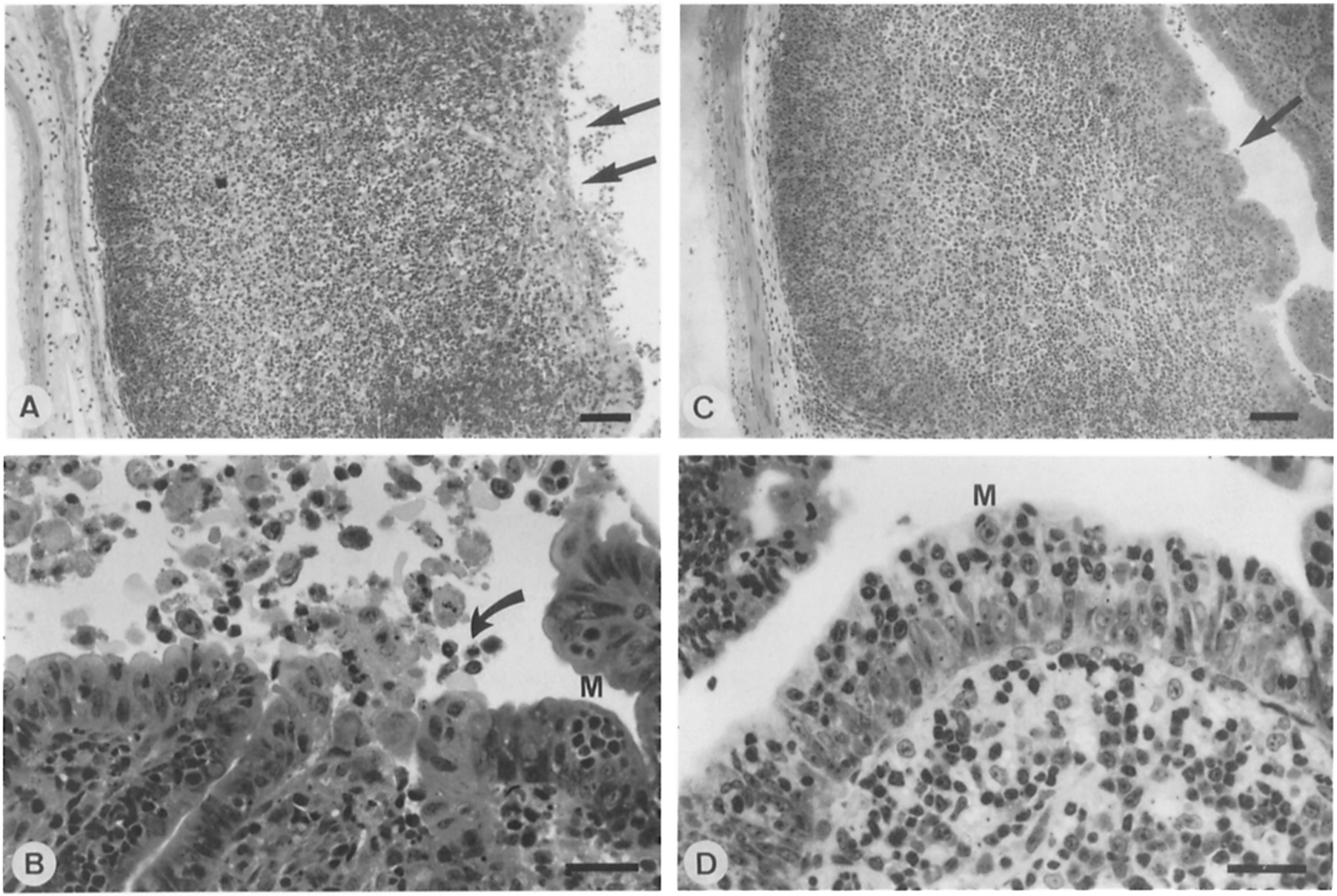
PMN and monocyte counts in Peyer's patches were carried out on the dome areas of lymphoid follicles. Areas infiltrated by PMN always presented more than 100 cells/villus which is more than twice the amount found in the intestinal villi, and nine times greater than the amount observed with the noninvasive strain. These results show a strong correlation between PMN transmigration and tissue invasion by M90T in Peyer's patches.

TNF Measurements in the Accumulated Fluid. To evaluate the degree of inflammation, TNF was measured in fluid accumulated inside the loops after 2 and 12 h of infection with both strains.

After a 2-h infection period with either strain, TNF activity was undetectable in the limited amounts of fluid (50–100 μl) present inside the loops. By 12 h, concentrations raised dramatically in the case of M90T invasion since the amounts of TNF accumulated in exudates inside villous and Peyer's patch loops reached $9,571 \pm 2,020$ U/ml and $8,893 \pm 2,527$ U/ml respectively (Fig. 8).

Ultrastructural Observations. By TEM, we confirmed that infection with M90T caused major tissue alteration and destruction associated with PMN transmigration (Fig. 3 *C*). Bacteria were always present in areas of epithelial desquamation and microorganisms entered epithelial cells through their basolateral pole where they were either found in vacuoles,

Figure 4. Villous intestinal loop infected with invasive *S. flexneri* (M90T) in untreated rabbits and rabbits treated with anti-CD18 monoclonal antibody. Histopathological analysis after 12 h of infection. (*A*) Disappearance of villi architecture and intense tissue destruction with massive immigration of PMN in the mucosa and detachment of the epithelium (*E*). Bar, 30 μm. (*B*) Intense infiltration by PMN, detachment of epithelial layer, bacterial invasion of lamina propria (*arrow*). Bar, 20 μm. (*C*) Anti-CD18 mAb-treated rabbits showing normal intestinal architecture. Bar, 30 μm. (*D*) Same as *C*. *Arrow* points to a focal invasion site in the epithelium. Bar, 10 μm.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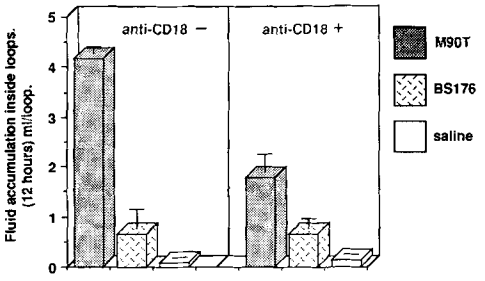

Figure 6. Fluid accumulation in intestinal loops after a 12-h infection period with *S. flexneri*. Infection of untreated and anti-CD18 mAb-treated animals with invasive M90T and noninvasive BS176 strains.

or free in the cytoplasm. Inside PMN, bacteria were found intact or in a degradation process. Some were also found in dead cells in the epithelium or fragments of epithelium.

Effect of Anti-CD18 Monoclonal Antibody Treatment in Rabbits. Two rabbits were treated with an anti-CD18 monoclonal antibody 4 h before infection, in order to inhibit leukocytes and particularly PMN adhesion to vascular endothelium, and subsequent epithelium transmigration. All the parameters described above were again analyzed in 24 different ileal loops, 12 h after infection with either of the two strains.

Histopathological analysis of M90T-infected loops in animals treated with the anti-CD18 monoclonal antibody showed striking differences compared with control animals. Intestinal villi appeared of normal height, and no tissue destruction was observed in spite of few foci of invasion. In these specific sites, bacteria remained intracellular, unable to cross the basal membrane and to reach the lamina propria. In addition, although a significant number of intracellular microorganisms could be seen, no epithelial cell death was observed (Fig. 4, *C* and *D*). Tissues of loops infected with the noninvasive strain BS176, appeared intact, even the slight edema of the lamina propria seen in untreated rabbits had disappeared. The use of anti-CD18 monoclonal antibody in rabbits before loops were infected by M90T diminished tissue invasion by a factor of 3.4, as compared with untreated rabbits (Fig. 1). This value almost reached the background level seen with BS176, although slightly higher due to the presence of scattered invasive foci.

In Peyer's patches, inhibition of tissue infiltration by PMN caused return to a normal morphology. No sign of tissue alteration or destruction was observed with the invasive strain M90T. Few inflammatory cells were found in the lumen and no significant difference was observed in the number of bacteria taken-up either by the villus loop tissue or the dome epithelium of Peyer's patches (Fig. 5, *C* and *D*).

This group of rabbits showed that fluid accumulation diminished by >50% with the invasive strain, whereas it remained constant with BS176 (Fig. 6). No blood was seen.

When PMN counts were performed in treated rabbits, only 5% PMN/dome were counted when compared with the amount seen in a normal infection with M90T (Fig. 7). No significant difference was observed in the number of PMN or monocytes with or without the antibody in the presence of the noninvasive strain.

The amount of TNF accumulated inside infected loops also presented a great difference between treated and untreated animals (Fig. 8). In villus intestinal loop exudates, values were 16 times lower (595 ± 310 U/ml), and in Peyer's patch loop exudates, 15 times lower (607 ± 349 U/ml), when anti-CD18 monoclonal antibody was administrated.

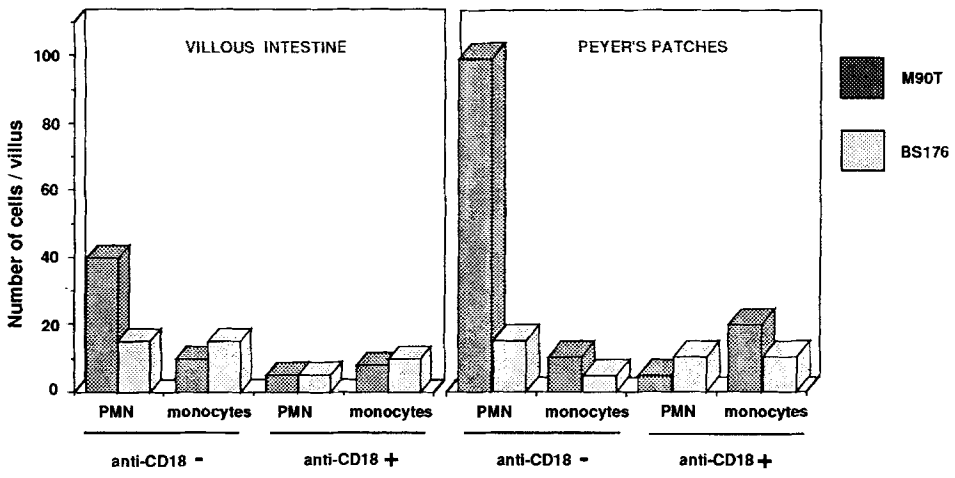

Figure 7. Histopathological grading of inflammation in villous intestine and Peyer's patch tissue samples. Columns show PMN and monocyte counts in normal and anti-CD18 mAb-treated rabbits infected with invasive M90T and noninvasive BS176 *S. flexneri* strains for 12 h.

Figure 5. Peyer's patches infected with invasive *S. flexneri* (M90T). Histopathological evaluation of the dome epithelium after 12 h of infection. (*A*) Lymphoid follicle showing destruction of dome epithelium and ulceration (*arrows*). Bar, 30 μm. (*B*) Massive PMN immigration to the lumen, disrupting the dome epithelium (*arrow*). Bar, 10 μm. (*C*) Intact dome epithelium (*arrow*) over lymphoid follicle of rabbits treated with anti-CD18 mAb. Bar, 30 μm. (*D*) Same as *C*. Integrity of the dome epithelium. Bar, 10 μm.

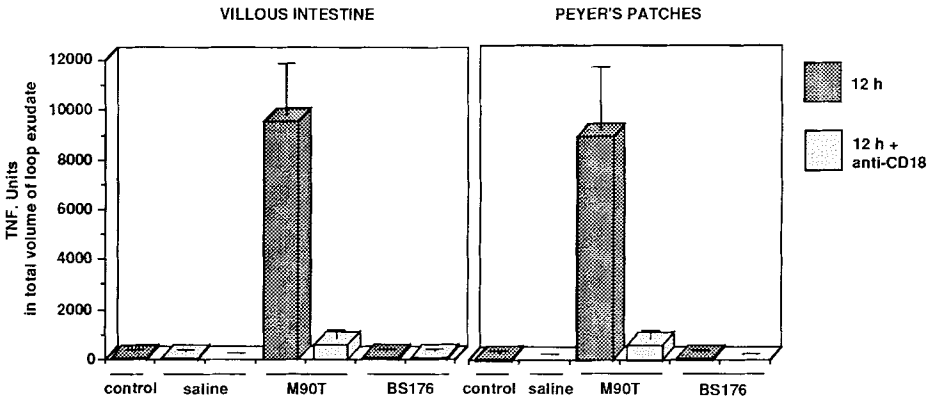

Figure 8. TNF present in total volume of luminal exudate in villous intestine and Peyer's patch-containing loops of anti-CD18 mAb-treated and untreated rabbits. Animals were infected for 12 h with invasive M90T and noninvasive BS176 *S. flexneri* strains.

Discussion

In shigellosis, which is characterized by bacterial invasion of the epithelium and massive PMN infiltration of colonic tissues, the rabbit ligated intestinal loop model provides a fair reflection of the infectious process. The present work has attempted to dissect this complex interplay between epithelial invasion and the inflammatory reaction in vivo. In this model, early entry occurred essentially via M cells in Peyer's patches, the bacteria being subsequently found primarily inside resident macrophages. The fact that shigellae could enter the epithelium via M cells confirms previous findings in rabbits (27, 28) and in macaque monkeys (22). We also found that the invasive strain M90T was present in higher numbers than the noninvasive mutant BS176 within Peyer's patches. Since the latter is not invasive and not toxic for macrophages (29), it is likely that it remains essentially extracellular, thus being killed by gentamicin treatment, and that the bacteria that are phagocytosed by macrophages are rapidly destroyed. However, ultrastructural observations did not show significant association of this mutant with M cells of the dome epithelium and subjacent macrophages. It is likely that M cells do not equally absorb luminal bacteria, and that expression of an adhesive or invasive phenotype allows more efficient apical uptake. This is consistent with data showing that expression by *S. flexneri* of the *Escherichia coli* RDEC-I pili that mediate adherence to rabbit intestinal epithelium, increased colonization of Peyer's patches (27).

After entry, invasive shigellae could be seen in an intercellular situation, a position enabling them to invade enterocytes basolaterally. This situation is quite reminiscent of reoviruses that also cross the intestinal barrier through M cells before infecting other intestinal epithelial cells via their basolateral surface (30, 31). However, invasive shigellae were also rapidly translocated to phagocytic cells associated with the follicular structures of Peyer's patches. Infected macrophages were killed by the pathogens. This was consistent with previous demonstration that *S. flexneri* causes rapid killing of macrophages in vitro (29). Moreover, the morphology of dying cells in our in vivo model was often characterized by large cytoplasmic vacuoles and perinuclear condensation of chromatin, confirming recent in vitro demonstration that invasive shigellae induce programmed cell death (apoptosis) in macrophages (29). Based on observation of multiple tissue sections, two events may account for the spread of the bacterium to adjacent cells after macrophage infection. First, fragments of dying infected cells, particularly apoptotic macrophages, can be phagocytosed by adjacent phagocytes and remain inside these cells as large phagolysosomes from which live bacteria may escape (data not shown). This is characteristic of the way apoptotic cells, which express specific receptors, are cleared from tissues by phagocytes (32). Alternatively, cell to cell spread via the actin-mediated *Ics* phenotype may also occur. In vivo evidence of actin polymerization inside phagocytes has also been observed (data not shown). Progressive infection of a large number of phagocytic cells by shigellae in these areas is therefore likely to cause production of high amounts of inflammatory cytokines accounting for early development of strong local inflammation. We have recently shown that after macrophages were infected by invasive *S. flexneri*, they secreted high quantities of IL-1 during the 2 h that preceded their apoptotic death (33).

At late stages of infection (12 h), M90T-infected Peyer's patches appeared edematous, hemorrhagic, with their surface becoming necrotic and ulcerated. This was not observed for BS176. However, the most striking feature was the enormous infiltrate of PMN which not only dissected lymphoid follicles, but also invaded adjacent intestinal villi. Bacteria could be seen in these areas. It is not clear whether infection can occur at the level of the villus intestinal mucosa, in the absence of lymphoid follicles, in humans or macaque monkeys. It is likely that in the villus intestine, invasion remains limited both by the presence of mucus and glycocalyx and by the structure of the brush border which keeps invasive bacteria such as *S. flexneri* from invading the epithelium. It is still possible, however, that transmigration of PMN, especially in the crypts, may open the way for microorganisms, once signaling has started from a neighboring follicle. The rabbit ligated loop model is likely to exaggerate the importance of invasion outside follicular zones, due to the closure of the loop and the high inoculum used.

Villous loops presented no change upon gross examination during the first 4 h of infection by M90T. However, by 8 h, significant alteration in epithelial cell integrity was

observed, in addition to enlargement of intercellular spaces without alteration of junctions. This has also been observed in previous in vitro studies using polarized T-84 monolayers (20). After 2 h of incubation with apical bacteria, cells began to show cytotoxic symptoms in spite of the lack of significant invasion, and intercellular spaces also became enlarged. Moreover, tight and intercellular junctions showed no alteration, neither morphologically in TEM, nor functionally, since apical peroxidase did not reach these spaces. Likewise, in vivo, no intracellular bacteria were detected in either case. These observations suggest a transcellular effect in response to a bacterial component that has not yet been identified. This component may account for the early jejunal secretory diarrhea observed in humans and monkeys at the onset of shigellosis (34) and could therefore correspond to an enterotoxin stricto sensu. However, transendothelial and transepithelial migration of PMN has already started at this time of infection, and activated PMN are known to release compounds that can elicit Cl^- secretion from the crypts such as hydrogen peroxide (35), and adenosine (36). In the case of *Salmonella* infection, the inflammatory response may also account for electrolyte secretion (37). This is largely confirmed in our case since significant decrease in fluid production was observed in the anti-CD18–treated rabbits in which ligated loops were infected with M90T. The low level of fluid production induced by BS176 remained constant in anti-CD18–treated animals, thus indicating that fluid production is essentially due to the early inflammatory response induced by the invasive pathogen but also that remaining fluid production may reflect the presence of a chromosomally encoded enterotoxin (34).

In animals killed after 12 h of infection by M90T, the intensity of the PMN infiltrate was striking. These data confirmed clinical observations showing a great number of PMN within stools in the course of shigellosis (23). The enormous levels of TNF found in the exudates followed the high number of PMN observed in fluids and tissues since PMN are a significant source of this cytokine when activated (38). This and other cytokines have highly toxic effects when produced in excess. There is strong evidence, for instance, that the clinical manifestations of septic shock are due to such overproduction of cytokines, particularly IL-1 and TNF (39, 40).

A spectrum of lesions could be observed between detachment of the epithelial layer to complete destruction of the epithelial lining and lamina propria. Few bacteria could be observed inside epithelial cells, although many invaded the exposed lamina propria after epithelial detachment or necrosis had occurred. Microorganisms were also seen in areas of PMN accumulation and transmigration. In these sites, bacteria were able to make their way from the lumen to the basal side of the epithelial layer. However, the presence of large pieces of uninfected epithelium detached in the lumen as well as the fact that antibody-treated rabbits presented scattered sites of invasion, without tissue destruction, suggest that PMN and not bacterial invasion per se account for the major tissue destruction observed. The release of proteolytic enzymes from PMN (41), may promote destruction of the extracellular matrix that connects cells to the lamina propria. In addition, defensins and oxygen radicals are released by activated PMN in the presence of bacterial products (42) as well as TNF-α which induces tissue necrosis (40). Epithelium detachment would allow access of luminal bacteria both to the basal side of the epithelium where they are able to invade, and to fibroblasts, mononuclear–macrophages and vascular structures of the lamina propria. The fact that a majority of enterocytes may be killed as innocent bystanders is strongly supported by histological observations showing very few infected cells among the target epithelium.

The number of intracellular bacteria protected from gentamicin, which for the most part correspond to intracellular microorganisms, was significantly decreased when functional tissue leukopenia was established by treating rabbits with the anti-CD18 monoclonal antibody before infection was performed. Such experiments cannot, unequivocally, establish that only PMN are responsible for destabilization of the intestinal barrier in the presence of *S. flexneri*. Other CD18-bearing cells, such as monocytes and lymphocytes, are likely to migrate into tissues, even at early stages of infection. However, the overwhelming quantity of PMN infiltrating tissues as well as our recent in vitro demonstration that these cells are able to subvert an epithelial monolayer and cause its invasion by *S. flexneri* (20), indicates that they play a major role. A more specific monoclonal antibody such as RB6-8C5 which has been used in mice to study the role of PMN in hepatocyte invasion by *Listeria monocytogenes* (43) would be necessary. Unfortunately no such neutralizing antibody exists for rabbits. We therefore conclude that leukocytes, essentially PMN, play a major role in opening the epithelial barrier to invasive microorganisms. This may occur by at least three mechanisms, which are likely to cooperate. (*a*) PMN that migrate through the epithelial lining to its luminal side open up intercellular junctions and allow bacteria to gain access to the basal side where they can penetrate epithelial cells. This hypothesis is supported by our recent in vitro model (20) and confirmed here by some of the TEM pictures. (*b*) PMN may shuttle back and forth through the epithelium, according to local changes in chemotactic gradients or concentration of cytokines such as IFN-γ (44), thereby vehiculating phagocytosed bacteria to the basal side of the epithelium. (*c*) Detachment of the epithelial lining via destruction of the basal membrane by immigrating PMN that secrete high amounts of tissue-toxic components. It was striking to notice that when functional tissue-neutropenia was established, in the very few areas where M90T could invade the epithelium, infection proceeded as in in vitro models.

This series of experiments indicates that shigellosis is an acute inflammatory bowel disease triggered by an invasive microorganism. In acute noninfectious inflammatory colitis such as ulcerative colitis or severe forms of Crohn's disease, similar pathways are probably involved since tissue infiltration by PMN is a constant feature. We suggest that in shigellosis only a few microorganisms are sufficient to initiate this process. These few bacteria are taken up by M cells in the dome epithelium over lymphoid follicles at early stages of infection. Shigellae are rapidly translocated to macrophages subjacent to M cells. Phagocytosis of these invasive micro-

organisms induces cytokine release, IL-1 in particular, representing a strong signaling process which induces a cascade leading to recruitment of an enormous number of PMN to the initial site of bacterial entry. The inflammatory reaction then spreads to adjacent villi. PMN that emigrate at the edge of this inflammatory process not only facilitate entry of microorganisms into epithelial cells but also induce detachment of large pieces of the epithelial lining, causing the destruction of these cells. This reaction will then spread much further from the initial site of entry to areas devoid of lymphoid structures.

The rabbit bacterial meningitis model is another example in which over response of the host accounts, in a large part, for the observed lesions and for the poor outcome (45). In mice, if phagocytic cells are prevented from emigrating into foci of hepatocyte infection by *L. monocytogenes* (43), *Francisella tularensis*, and *Salmonella typhimurium*, no lysis of hepatocytes occurs, infection goes unrestricted within these cells (46), and infectious doses that would be sublethal in the presence of phagocytes become lethal (43). This indicates that in certain models of infection, a well adapted and early occurring inflammatory reaction is not only an important defense strategy, but also a major cause of tissue damage. Its absence enables clear identification of the primary cellular targets of the pathogen.

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