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In Vivo Apoptosis in *Shigella flexneri* Infections

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***Shigella flexneri*, an etiological agent of bacillary dysentery, causes apoptosis in vitro. Here we show that it also induces apoptosis in vivo. We were able to quantify the number of apoptotic cells in rabbit Peyer's patches infected with *S. flexneri* by detecting cells with fragmented DNA. Infection with virulent *S. flexneri* results in massive numbers of apoptotic cells within the lymphoid follicles. In contrast, neither an avirulent strain nor an avirulent strain capable of colonizing Peyer's patches increases the background level of apoptotic cells. Macrophages, T cells, and B cells are shown to undergo apoptosis in vivo. These results indicate that apoptosis may play a crucial role in the pathogenesis of shigellosis.**

Shigellae cause bacillary dysentery, a severe form of bloody diarrhea, which is endemic in developing countries. Shigellosis outbreaks also occur in industrialized nations, especially where proper hygiene is difficult to maintain. Children younger than 5 years are the most susceptible victims, with over half a million deaths occurring annually (12).

The syndrome caused by shigellae consists of painful abdominal cramps, nausea, fever, tenesmus, and frequent emissions of bloody and mucopurulent stools (12). These symptoms reflect the invasion of the colonic mucosa by shigellae. Histopathological analysis of colonic biopsies from patients with shigellosis reveals destruction of the epithelium, mucosal erosion, and the typical signs of inflammation, namely, edema and infiltration of polymorphonuclear cells into the lamina propria (2, 11).

Shigella flexneri is incapable of invading epithelial cells through their apical membrane and can penetrate the mucosa only through M cells (13, 21). M cells are found in the follicle-associated epithelium that covers both lymphoid nodules and lymphoid aggregates (i.e., Peyer's patches), the sites where mucosal immunity is generated (10). Once inside the mucosa, shigellae encounter resident tissue macrophages.

In vitro, *S. flexneri* kills macrophages (6) by apoptosis (25). During apoptosis, interleukin-1β (IL-1β) is processed to its mature form and released from macrophages (22). Blocking IL-1 activity by using IL-1 receptor antagonist (IL-1ra) abrogates inflammation and decreases the number of bacteria in mucosal tissue of experimentally infected animals (16), suggesting that IL-1 initiates the inflammatory response. IL-1 recruits inflammatory polymorphonuclear cells that migrate through the epithelium into the lumen of the colon and destabilize the integrity of the epithelial barrier, allowing massive entry of bacteria into the mucosa. Further colonization of the epithelium aggravates the inflammation, and extensive tissue destruction ensues (24).

Since apoptosis appears to play an essential role in the pathogenesis of dysentery, we tested whether shigellae induce apoptosis in vivo. *S. flexneri* invasiveness and virulence are encoded in a 220-kb plasmid. Strains cured of this plasmid are completely noninvasive and avirulent (19). IpaB, an invasin encoded by the *Shigella* virulence plasmid, is necessary for the induction of apoptosis (23). We infected rabbit ligated ileal loops with three different *Shigella* strains: (i) a wild-type *S. flexneri* strain (M90T), (ii) a plasmid-cured, noninvasive, avirulent derivative (BS176), and (iii) BS15, which is BS176 transformed with a plasmid that codes for AFR1 (4), an adherence pilus of the rabbit-specific enteropathogenic *Escherichia coli* strain RDEC-1 (5). The AFR1 pilus mediates specific adherence to rabbit Peyer's patch M cells (9). In fact, we demonstrate that the expression of this rabbit-specific adhesin allows numerous bacteria to penetrate the follicle-associated epithelium through M cells. BS15, like the wild-type *Shigella* strain, infects subepithelial phagocytes but, in contrast, does not induce apoptosis or IL-1β release. Therefore, BS15 serves as a control for bacteria that colonize Peyer's patches but do not harbor the *Shigella* virulence plasmid and therefore lack *ipaB*, the *Shigella* gene necessary to induce apoptosis (23). Infection with the virulent strain M90T increased the number of cells undergoing apoptosis in the lymphoid follicles of Peyer's patches. Control animals infected with either BS176 or BS15 present background levels of apoptotic cells. Cells with apoptotic morphology were identified as macrophages and B and T cells. These results show that extensive apoptosis is induced by virulent shigellae and may represent a crucial step in the pathogenesis of dysentery.

MATERIALS AND METHODS

Bacteria. The wild-type, serotype 5, *S. flexneri* strain M90T and its plasmid-cured, avirulent derivative BS176 were described previously (25). BS15 is strain BS176 transformed with plasmid pRBK15, a pUC plasmid encoding the minimum number of cloned genes (*afr*) (4a) necessary for biogenesis of the AFR1 adherence pilus (5) of the *E. coli* strain RDEC-1 (4). Bacteria were grown at 37°C overnight in tryptic soy broth, subcultured, washed, and adjusted to the appropriate concentration prior to infection.

Rabbit tissue sections. The ileum of anesthetized (6% sodium pentobarbital solution; 0.5 ml/kg of body weight) New Zealand White rabbits (CEGAV, St. Mars d'Egrenne, France) was removed from the abdomen and ligated at intervals of 10 cm with 2-cm spacers. The loops were then injected intraluminally with 10^9 CFU (18). Control injections with avirulent *Shigella* strains and saline solution were done in a different loop of the same animal. After different times of survival, the animals were sacrificed. The loops were examined, and fragments were fixed in 4% paraformaldehyde (14, 17). The specimens were processed

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subsequently by standard histopathological procedures, embedded in paraffin, and cut at 10 μm.

Electron microscopy. Infected tissues were processed as described by Perdomo et al. (13).

Peritoneal macrophages and infection. Male Swiss Webster mice (6 to 8 weeks old; Charles River, Wilmington, Mass.) were injected intraperitoneally with 1 ml of thioglycolate medium (GIBCO-BRL, Gaithersburg, Md.) 4 days before they were sacrificed. Cells were recovered by peritoneal lavage with cold phosphate-buffered saline (PBS), washed in cold RPMI 1640 medium (GIBCO-BRL), and resuspended, and 10^6 cells were allowed to adhere to plastic plates in culture medium (RPMI 1640 containing 10% fetal calf serum [GIBCO-BRL], 100 U of penicillin per ml, 100 mg of streptomycin per ml, 2 mM L-glutamine). After 120 min of incubation, the adherent cells were washed three times with culture medium and stimulated overnight with 1 μg of purified *E. coli* O111:B4 lipopolysaccharide (LPS; Sigma Chemical Co. St. Louis, Mo.). Then, macrophages were washed five times with serum-free medium and infected with the indicated bacteria, and the plates were centrifuged at 700 × *g* for 10 min. After 90 min of incubation, the supernatants of the infected macrophages were collected, filtered through a 0.2-μm-pore-size membrane, aliquoted, and frozen immediately. The multiplicity of infection was 100 bacteria per macrophage. Macrophages were incubated at 37°C, under 5% CO_2, at all times.

Immunoblot analysis. Macrophage cell lysates or culture supernatant equivalent to 10^6 cells was resolved by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE), transferred to nitrocellulose, and incubated with anti-murine IL-1β polyclonal antibodies (R & D Systems, Minneapolis, Minn.) at a 1:200 dilution. After the blots were washed, they were further incubated with alkaline phosphatase-labeled secondary antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), revealed with the Western blotting (immunoblotting) enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, Ill.) and exposed to X-Omat X-ray films.

TUNEL. Terminal D-transferase-mediated dUTP-biotin nick end labeling (TUNEL) was done essentially as described by Gavrieli et al. (8). In brief, deparaffinized tissue sections were treated sequentially with acetone and proteinase K (Boehringer Mannheim, Indianapolis, Ind.). Endogenous peroxide was inactivated with 3% H_2O_2. The sections were then blocked with fetal bovine serum, avidin, and biotin (Vector, Burlingame, Calif.) and incubated with terminal D-transferase (Promega, Madison, Wis.) and biotinylated dUTP (Boehringer Mannheim). Biotinylated dUTP was detected with an avidin-biotin complex (Vector) and visualized with nickel-intensified 3,3′-diaminobenzidine (DAB; Sigma). Methyl green was used as the counterstain.

Quantification of DNA fragmentation. The number of apoptotic cells in lymphoid follicles of Peyer's patches was determined by counting labeled cells with a computer-interfaced mapping microscope (1). Three independent follicles were counted for each loop.

Bacterial counts in tissue samples. Eight hours after infection, fluid in the loops was replaced with a gentamicin solution, and tissue samples were obtained by punching an 8-mm-diameter disk with a skin biopsy apparatus (Biopsy Punch; Stiefel, Nanterre, France). The biopsied tissue was immersed in a fresh gentamicin solution and then washed extensively in cold 0.1× PBS. Tissue samples were ground with an Ultra-Turrax apparatus (Janke & Kunkel GmbH, Hamburg, Germany), and an aliquot was incubated at 37°C for 30 min before serial dilutions were plated onto tryptic soy agar plates. Colonies were counted, and CFUs were calculated for an area of 1 cm^2 of intestinal mucosa (16). Results are the average of four experiments.

Immunofluorescence and confocal microscopy. Deparaffinized sections were permeabilized with acetone at −20°C for 5 min, blocked with 10% goat serum, and incubated in a humid chamber with the respective primary and secondary antibodies. The sections were also stained with the DNA-binding dye propidium iodide for 15 min at room temperature and mounted with Citifluor (UKC, Canterbury, United Kingdom). Three different antibodies were used: RAM-11 (Dako, Carpinteria, Calif.) to identify macrophages, anti-CD4 (Spring Valley Labs, Woodbine, Md.) to identify T cells, and the anti-immunoglobulin A (anti-IgA) antibody MG-2 (Spring Valley Labs) to identify B cells. Secondary antibodies were labeled with fluorescein isothiocyanate. Slides were scanned with a confocal microscope (Molecular Dynamics) equipped with an argon laser. With double band pass filters sets, fluorescein and propidium iodide were visualized simultaneously. The optical sections were generated in intervals of 0.39 μm, filtered, and reconstructed in three-dimensional projections.

RESULTS

BS15 penetrates Peyer's patches through M cells and infects macrophages in vivo. We determined whether BS15, an avirulent *Shigella* strain expressing the *E. coli* adhesin (4), is an appropriate control as a strain that can colonize the Peyer's patches in the absence of *Shigella*'s pathogenicity plasmid. We first determined whether BS15 traverses into the mucosa via M cells. Two hours after rabbit ileal loops were infected with BS15, the tissues were processed for electron microscopy. As shown in Fig. 1A, BS15 infects intestinal tissue through M cells. The M cells can be easily recognized by its very short brush border in comparison with the epithelial cells on either side. Two bacteria are visible traversing the M cell. There was no bacterial invasion of epithelial cells seen after an exhaustive screening of tissue sections.

Figure 1B shows a tissue section 8 h after infection with BS15. Bacteria were found in phagocytic cells that are most likely macrophages. These macrophages were localized to the dome of the lymphoid follicle, as demonstrated by the presence of the brush border of an M cell towards the lumen of the intestine. There are several bacteria in this field, and macrophages appear to be infected with more than one microorganism. The bacteria are contained within a vacuole, and the macrophages do not display an apoptotic morphology.

Taken together, these data show that BS15 infects the intestine through M cells and, once inside the mucosa, are phagocytosed by macrophages, mimicking the route of entry of wild-type shigellae (13, 21).

Quantification of bacteria in lymphoid follicles. Infections with BS15 or M90T result in comparable levels of bacterial invasion of lymphoid follicles. Two hours after infection, there were $(2.2 \pm 1.2) \times 10^4$ CFU/cm^2 (mean ± standard deviation) in Peyer's patches infected with BS15 and $(2.6 \pm 1.2) \times 10^4$ CFU/cm^2 in infections with M90T. Eight hours after infection, there were $(12.8 \pm 2.7) \times 10^5$ CFU/cm^2 in BS15 and $(13.2 \pm 6.8) \times 10^5$ CFU/cm^2 in M90T-infected Peyer's patches. The number of bacteria recovered from BS15 or M90T infections was not statistically significant in a Mann-Whitney test at either 2 h ($P = 0.51$) or 8 h ($P = 0.8$). There were only $(0.2 \pm 0.05) \times 10^5$ CFU/cm^2 in BS176 infections at 8 h after infection, which was significantly different from the results with both BS15 and M90T ($P = 0.06$; Mann-Whitney test).

Infection with BS15 does not lead to release of IL-1β. Induction of macrophage apoptosis leads to release of mature IL-1β (22). IL-1, in turn, seems to be the key inflammatory cytokine that initiates inflammation during shigellosis (16). Therefore, we tested whether in vitro infection of macrophages with BS15 leads to IL-1β release. The supernatants of macrophages infected with different *Shigella* strains were immunoblotted with anti-IL-1β antibodies. Lysates of peritoneal macrophages activated with LPS showed the expression of the IL-1β precursor exclusively (Fig. 2, lane 2), which is absent in nonactivated cells (Fig. 2, lane 1). The supernatant of activated macrophages infected with the wild-type *Shigella* strain M90T (lane 3) contained both the precursor and mature forms of IL-1β. In contrast, macrophages infected with either the avirulent strain BS176 (lane 4) or BS15 (lane 5) did not release IL-1β. These data show that release of IL-1β is specific to macrophages infected with the wild-type *Shigella* strain and directly associated with apoptosis. Cells infected with BS15 or BS176 remained viable after infection (data not shown).

In situ DNA fragmentation. To evaluate the extent of apoptosis induction in vivo, rabbits were injected intraluminally with either saline solution or one of the three different *Shigella* strains, i.e., the wild-type strain M90T, the avirulent derivatives BS176 and BS15.

Rabbits were infected as described in Materials and Methods and sacrificed at 2, 4, and 8 h postinfection. The tissue was processed immediately for histological analysis. The paraffin tissue sections were then labeled by the TUNEL procedure (8).

There were striking differences in the number of apoptotic cells in loops infected with the virulent or avirulent *Shigella* strain. Four hours after infection with M90T, there were numerous labeled cells in the lymphoid follicles of Peyer's patches (Fig. 3A). In contrast, there were very few labeled cells

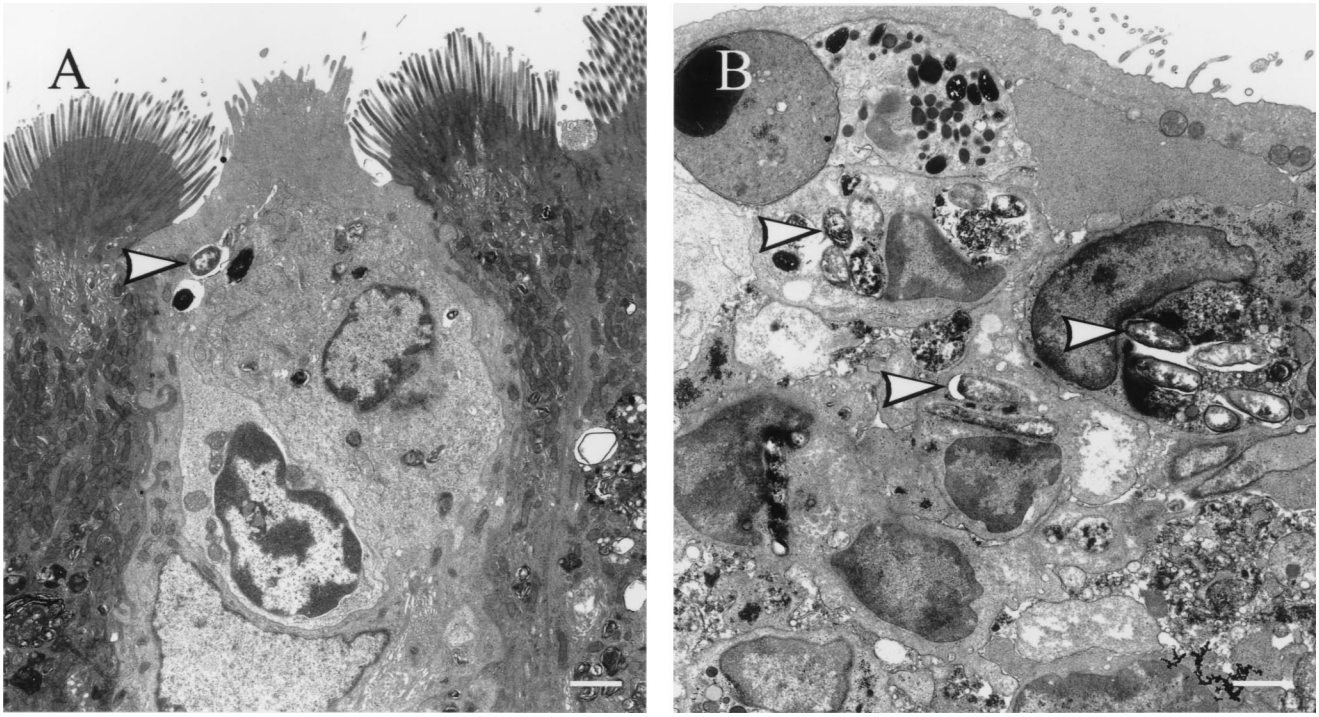

FIG. 1. BS15 penetrates Peyer's patches through M cells and infects phagocytes in lymphoid follicles. Rabbit ligated ileal loops were infected with the avirulent strain of *S. flexneri* transformed with a plasmid that codes for the AFR1 adherence pilus of the rabbit-specific enteropathogenic *E. coli* strain RDEC-1 (BS15). At 2 and 8 h postinfection, the tissues were fixed, processed, and sectioned for transmission electron microscopy. (A) An M cell, clearly distinguished by its short brush border, is infected with bacteria (arrowhead). (B) Bacteria are present in the cytoplasm (some identified with arrowheads) of several cells that appear to be macrophages. Bars, 1 μm.

in the lymphoid follicles of a loop infected with BS176 (Fig. 3B). The DNA fragmentation label was restricted to the nuclei. Similarly, 8 h after infection, there were large numbers of apoptotic cells in loops infected with the virulent strain (Fig. 3C), but there were only a few cells undergoing programmed cell death in loops infected with the avirulent strain (Fig. 3D). At both 4 and 8 h after infection, apoptotic cells appeared to be evenly distributed throughout the lymphoid follicle.

DNA fragmentation was restricted to the Peyer's patch after infection with shigellae, since very few labeled cells were observed in the villi of intestinal loops infected with either the BS176 (Fig. 3E) or M90T (Fig. 3F) strain.

Infection with BS15 results in massive bacterial invasion of the Peyer's patches. However, as shown in Fig. 3G, this strain did not induce cells to undergo apoptosis, demonstrating that programmed cell death activation is specific to *Shigella* pathogenesis and that the presence of a large number of gram-negative bacteria in the Peyer's patch does not by itself induce apoptosis. Finally, Fig. 3H shows a lymphoid follicle of a loop that was injected with saline solution and indicates the background level of apoptosis in this tissue. Control experiments for the TUNEL procedure, where terminal D-transferase, biotinylated nucleotides, or DAB was excluded, were negative.

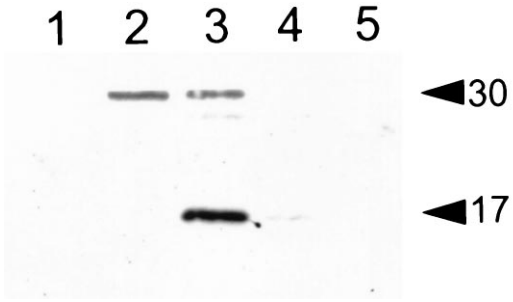

FIG. 2. Immunoblot of macrophage cell lysates or culture supernatants with anti-murine IL-1β antibodies. LPS-stimulated peritoneal macrophages infected with either the wild-type *S. flexneri* strain M90T (lane 3), an avirulent derivative BS176 (lane 4), or the avirulent strain that expresses the adherence pilus of the rabbit-specific enteropathogenic *E. coli*, BS15 (lane 5). The culture supernatants were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-IL-1β antibody. Lysates of LPS-stimulated (lane 2) and nonstimulated (lane 1) peritoneal macrophages are shown as controls. The 30-kDa immature form of IL-1β is present in the macrophage lysate. Macrophages infected with M90T release both the mature (17-kDa) and immature forms of the cytokine. IL-1 was undetectable in supernatants infected with either BS176 or BS15.

Quantification of cells undergoing apoptosis. The number of cells undergoing programmed cell death were counted with a computer-interfaced mapping microscope (1). Figure 4 shows a lymphoid follicle at 8 h after infection with M90T, the virulent strain, or BS176, the avirulent strain. The maps of the same follicles in strains M90T and BS176 are shown in Fig. 4C and D, respectively. This approach allowed us to count the number of apoptotic cells.

There were 30-fold more cells with fragmented DNA in the Peyer's patches infected with the virulent strain than that in loops injected with saline solution 8 h after infection (Fig. 5). The numbers of apoptotic cells in loops infected with either BS176 or BS15 were not significantly different compared with that of follicles in loops injected with saline solution. Four hours after infection, there were 1,204 ± 640 apoptotic cells per mm^2 in loops infected with the virulent strain and only 360 ± 96 cells per mm^2 in loops infected with the avirulent strain BS176. These data are similar to those shown for 8 h

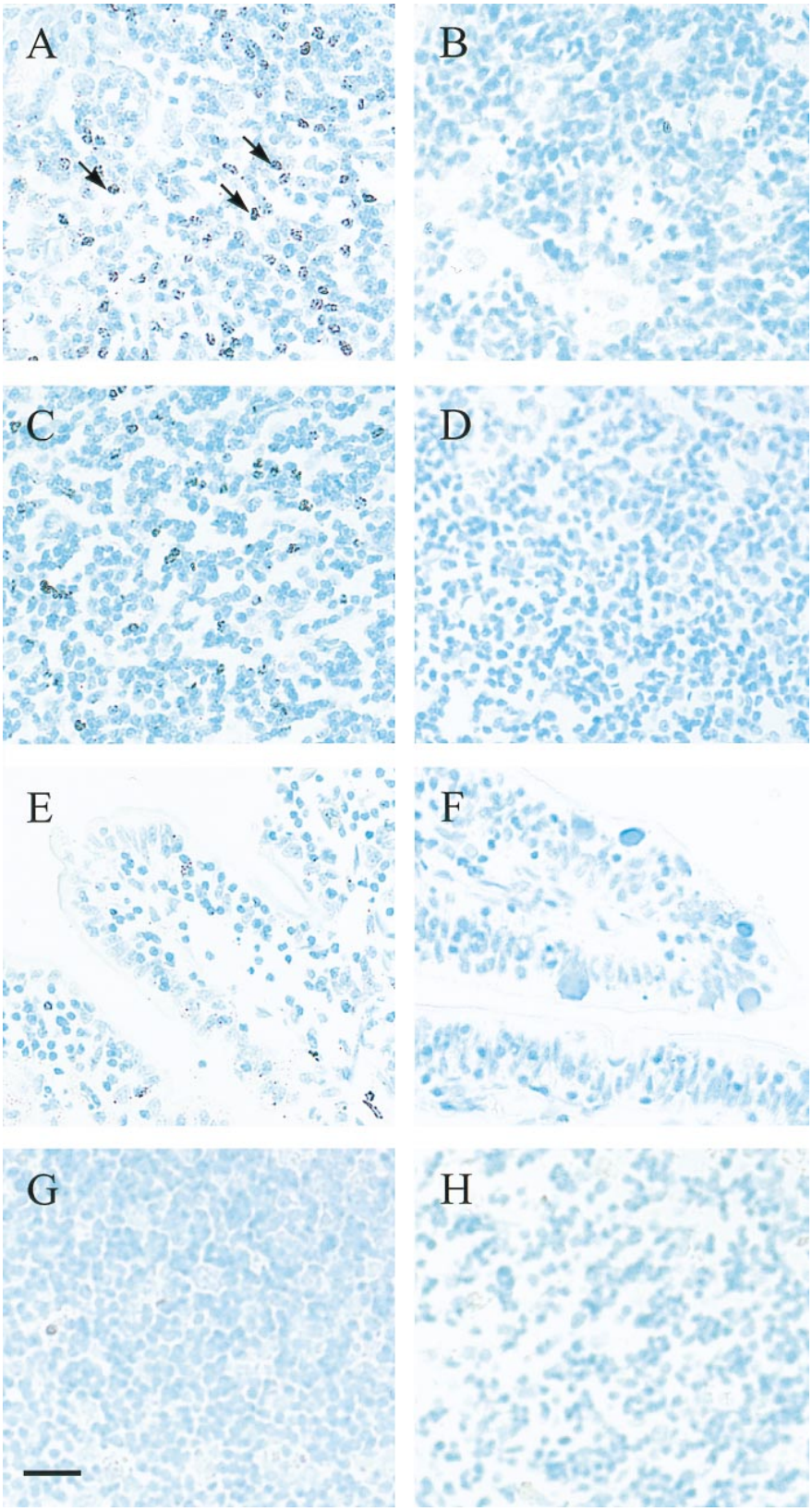

FIG. 3. In situ DNA fragmentation in *Shigella*-infected Peyer's patches. Rabbit ligated ileal loops were infected with either the avirulent (BS176) or the virulent (M90T) strain of *S. flexneri*. At different times postinfection, the tissues were fixed, embedded in paraffin, sectioned, and labeled for in situ DNA fragmentation. Cells undergoing apoptosis are stained black by DAB. The tissue sections were counterstained with methyl green. (A) View of a lymphoid follicle infected with the virulent strain M90T for 4 h. The localization of the DNA fragmentation label on the nuclei is clearly visible; three labeled nuclei are indicated by arrows. (B) Lymphoid follicle infected with the avirulent strain BS176 for 4 h. (C) Lymphoid follicle of a rabbit infected for 8 h with the virulent strain M90T. As in panel A, many cells appear distributed throughout the follicle. (D) Lymphoid follicle of a rabbit infected for 8 h with the avirulent strain BS176. (E) Villi of a rabbit infected for 8 h with the virulent strain. (F) Villi of a rabbit infected for 4 h with the avirulent strain. Panels E and F show a low abundance of labeled cells. (G) Lymphoid follicle of a loop infected for 8 h with strain BS15 showing few apoptotic cells. (H) Lymphoid follicle of a rabbit 8 h after injection with the saline solution, containing a low abundance of apoptotic cells. Bar, 20 μm.

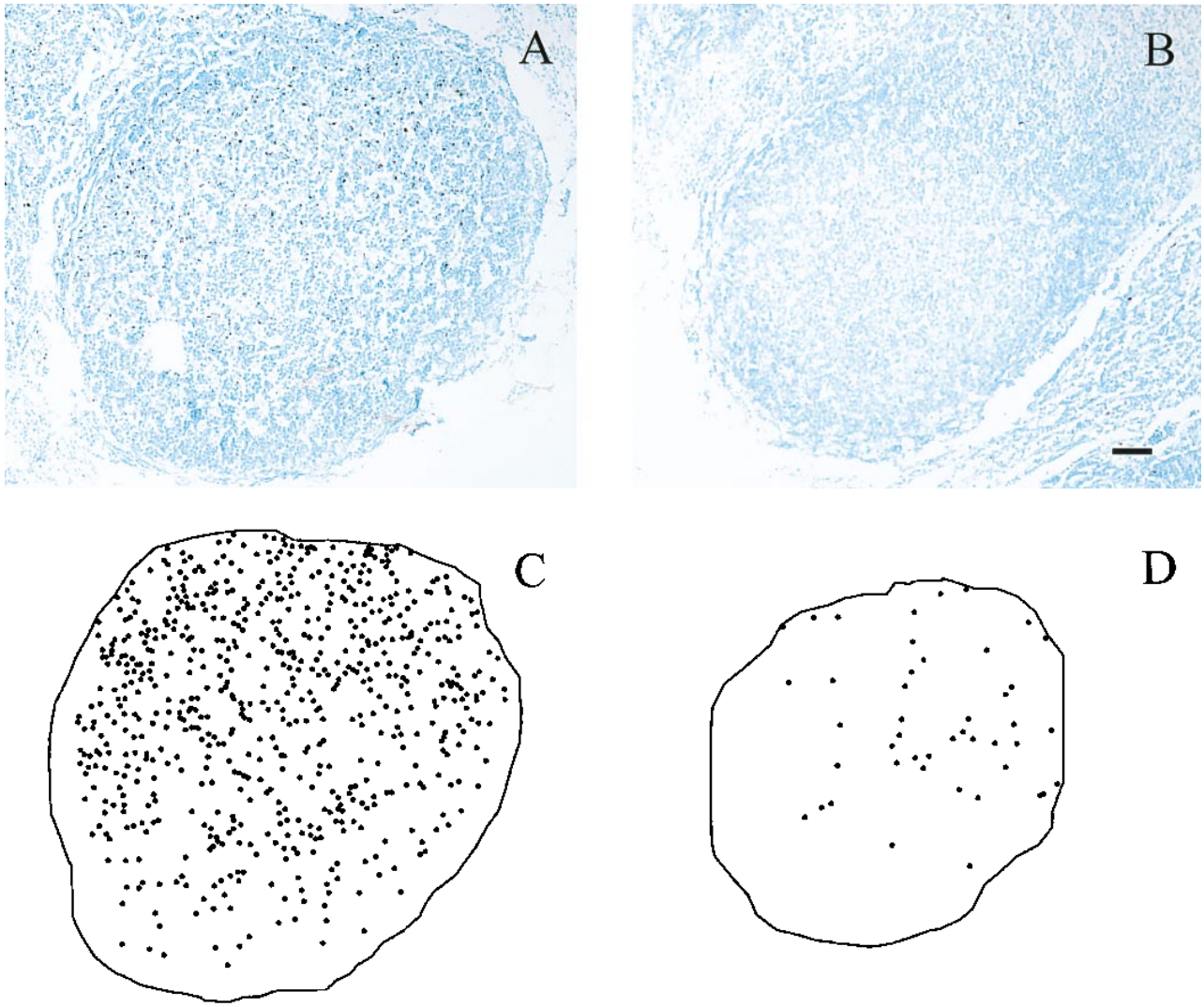

FIG. 4. Quantification of cells undergoing DNA fragmentation. (A and B) View of a complete lymphoid follicle of a Peyer's patch infected with either the virulent (M90T) (A) or avirulent (BS176) (B) *Shigella* strain. Labeled cells were mapped with a computer-interfaced mapping microscope. (C and D) Maps of the follicle infected with M90T shown in panel A (C) and of the follicle infected with BS176 shown in panel B (D). Bar, 50 μm.

after infection. Between 200 and 300 apoptotic cells per mm^2 were observed in Peyer's patches of animals sacrificed 2 h after infection regardless of whether the loops were infected with the virulent or avirulent *Shigella* strain. The number of apoptotic cells in M90T-infected animals correlates with the number of bacteria in the tissue as described above. There were significantly more bacteria and apoptotic cells at 8 h than at 2 h postinfection.

To assess the statistical significance of the number of labeled cells in infections with the different strains, we counted three lymphoid follicles in seven independently M90T-infected loops (total, 21 follicles) and three lymphoid follicles in five BS176-infected loops (total, 15 follicles) 8 h after infection. The mean number of apoptotic cells in loops infected with M90T was $847/mm^2$, whereas the mean number of labeled cells in follicles infected with BS176 was $189/mm^2$. The difference between these numbers was statistically significant ($P = 0.0027$) by the Mann-Whitney nonparametric test.

Identification of apoptotic cells. In vitro, *Shigella*-induced apoptosis was first identified in macrophages (25). In view of the large amount of apoptotic cells in M90T infections, we decided to identify the type of cells that undergo apoptosis by double labeling tissue sections with rabbit-specific cell markers and propidium iodide. Propidium iodide is a DNA-binding dye that permits the visualization of the typical apoptotic nuclear morphology, consisting of shrinkage, chromatin condensation, and marginalization of the DNA (3, 7).

Because *Shigella*-induced apoptosis was first identified in macrophages (25), we labeled the tissue sections with the rabbit macrophage-specific antibody RAM-11 (20). Figure 6A shows cells labeled with RAM-11 presenting normal nuclear morphology in Peyer's patches infected with the avirulent strain BS176. In follicles of loops infected with the virulent strain M90T, there were many RAM-11-positive cells that contained multiple apoptotic nuclei (Fig. 6B). These cells are likely to be macrophages that have phagocytized other apoptotic cells. In other fields, there were RAM-11-positive cells that contained only one apoptotic nucleus. These cells are likely to be macrophages undergoing apoptosis (Fig. 6C). In the same section, nuclei with apoptotic morphology were not labeled by

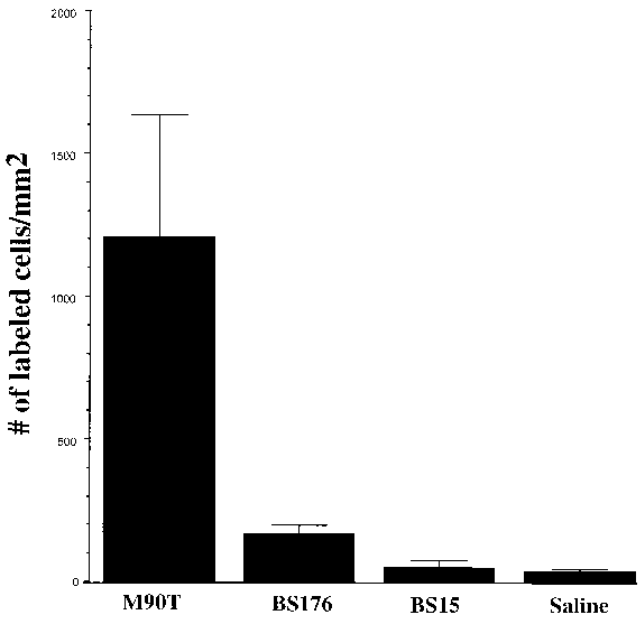

FIG. 5. Quantification of cells undergoing DNA fragmentation in *Shigella*-infected Peyer's patches. Tissue sections of rabbit ligated ileal loops were infected with the indicated strains and processed for DNA fragmentation as described in the legend to Fig. 3. The number of labeled cells was counted with a computer-interfaced mapping microscope as for Fig. 4. The means of counts of three independent lymphoid follicles from the same rabbit are shown.

this antibody, indicating that other cell types are also susceptible to *Shigella* cytotoxicity (Fig. 6D).

To identify the other cell types undergoing apoptosis in *Shigella*-infected tissues, we used either anti-rabbit CD4 to label helper T cells or anti-rabbit IgA to label B cells. Cells identified as helper T cells (Fig. 6E) or B cells (Fig. 6G) had a healthy appearance in follicles of loops infected with BS176. Apoptotic nuclei in cells labeled with anti-CD4 (Fig. 6F) or anti-IgA (Fig. 6H) antibodies were identified in follicles infected with M90T.

The apoptotic morphology of cells in Peyer's patches was further confirmed by transmission electron microscopy. Figure 7A shows a micrograph of a cell in a follicle infected with M90T containing multiple apoptotic nuclei similar to the cells shown in Fig. 6B. On the basis of our immunofluorescence results, this cell is likely to be a macrophage. The nuclei inside the cell present the chromatin condensation and segmented morphology typical of apoptosis. Figure 7B demonstrates the presence of bacteria in cells with clear apoptotic morphology. Similar images were not found in extensive searches of follicles infected with either BS176 or BS15.

DISCUSSION

The initial interaction between shigellae and cells in the gut-associated lymphoid tissue is crucial in the pathogenesis of dysentery. Shigellae invade the intestinal tissue through M cells, which deliver the bacteria directly into lymphoid follicles, where bacteria encounter tissue macrophages and other cells (13, 21). On the basis of the in vitro results, we proposed that *Shigella*-induced macrophage apoptosis, and the concomitant release of mature IL-1β, is a pivotal step in the initiation of the acute inflammatory response during shigellosis (22, 24). To be able to determine whether apoptosis induction was a relevant phenomenon in vivo, we used the rabbit ileal loop model to test the cytotoxicity of shigellae.

We engineered the BS15 strain as an avirulent control that expresses an *E. coli* adhesin, AFR1, and that can mimic the ability of shigellae to colonize the Peyer's patches. BS15 traverses the epithelial barrier through M cells and is then phagocytosed by macrophages (Fig. 1). Thus, this strain provides a control for bacteria that are capable of colonizing lymphoid follicles but do not contain the pathogenic determinants of shigellae. Furthermore, infection of peritoneal macrophages in vitro with BS15 does not result in apoptosis or release of IL-1β (Fig. 2). Taken together, these data show that while strain BS15 uses the same route of infection as the wild-type strain, infections with BS15 do not lead to cytokine release.

By use of the TUNEL technique to label cells undergoing DNA fragmentation, a unique feature of programmed cell death, we determined the number of apoptotic cells in follicles infected with different *Shigella* strains. At 4 and 8 h after infection, we observed a significant increase in the number of cells undergoing apoptosis in loops infected with the virulent strain M90T compared with loops infected with the avirulent strains (Fig. 3 and 4). It is interesting to note that 2 h after infection, there was no significant induction of apoptosis. Considering that bacteria will take some time to reach the lamina propria in in vivo infections, the appearance of apoptotic cells 4 h after infection of the rabbit ileal loop correlates with the kinetics of in vitro studies (25), where *Shigella*-induced cell death is apparent after 2 h of infection.

The plasmid-cured nonpathogenic strain BS176 is inefficient in invading the lamina propria in the rabbit ileal loop model. BS15 also did not induce apoptosis in lymphoid follicles (Fig. 3 and 4), demonstrating that programmed cell death activation is specific to *Shigella* pathogenesis and that the presence of a large number of gram-negative bacteria (BS15) in the Peyer's patch does not by itself induce apoptosis.

LPS, used as a bacterial marker, localized to the dome of the lymphoid follicles in rabbit ileal loops infected with *S. flexneri* (15). Interestingly, the distributions of bacteria and of apoptotic cells in these follicles are different. It is possible that the bacterial load beyond the follicular dome is low and undetectable by conventional immunohistochemistry. Alternatively, if bacteria are localized exclusively to the dome, the presence of apoptotic cells throughout the follicle could be explained by two different models. The first one would be that *Shigella*-infected cells migrate out of the dome to die. A second possibility is that after an initial bacterium-induced apoptosis in the dome, there is a cytokine-dependent apoptosis in the rest of the follicle. Future experiments will determine whether any of these models is correct.

We also showed, by use of specific monoclonal antibodies and observing characteristic nuclear morphology, that the cells undergoing apoptosis are macrophages and T and B cells (Fig. 6) and confirmed the apoptotic morphology by transmission electron microscopy (Fig. 7). It is very rare to locate bacteria within apoptotic cells, even in in vitro-infected macrophages, probably because the dying cell membranes become leaky and bacteria escape from their host before the apoptotic morphology is evident. Therefore, it remains to be determined whether the induction of B- and T-cell apoptosis in the course of *Shigella* infection is directly or indirectly caused by bacterial invasion. In vitro studies with purified populations of B and T cells will show whether shigellae can cause apoptosis upon invasion or whether cell death is initiated by factors released by other cells, possibly macrophages. Furthermore, the release of cytokines after T- or B-cell apoptosis might also play an important

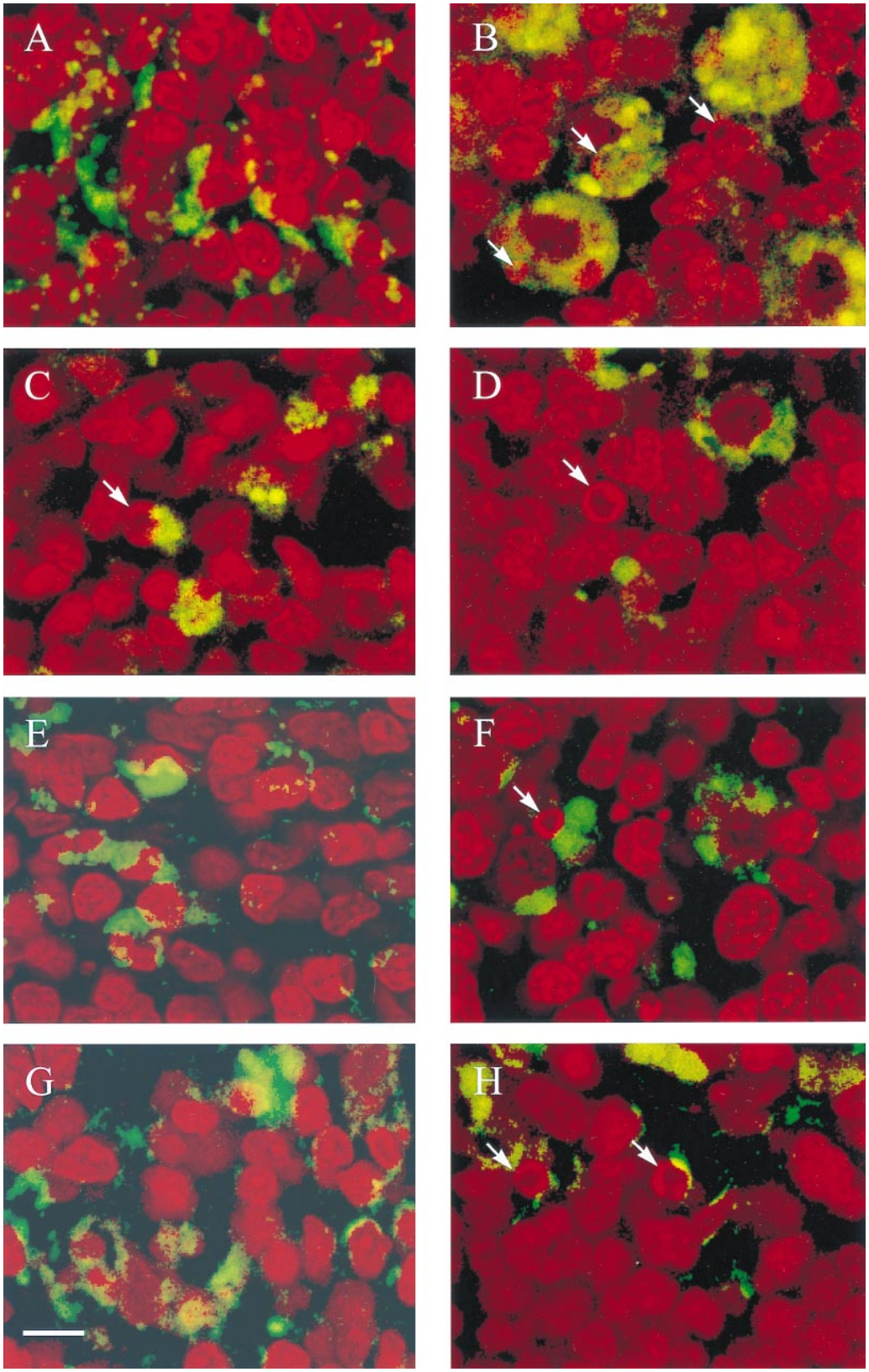

FIG. 6. Identification of apoptotic cells. Rabbit ligated ileal loops were infected with either the avirulent strain BS176 (A, E, and G) or the virulent strain M90T (B, C, D, F, and H) of *S. flexneri* and at 8 h postinfection, the tissues were fixed, embedded in paraffin, sectioned, and processed for immunofluorescence against different cell markers (shown in green) and with the DNA-binding dye propidium iodide (shown in red). The tissue sections were scanned in a confocal microscope, and after filtering, the optical sections were reconstructed in three-dimensional projections. Lymphoid follicles were stained with the rabbit macrophage marker RAM-11 (A to D), anti-CD4 (E and F), or anti-IgA (G and H). (A) BS176-infected tissue. Labeled cells showing normal nuclear morphology are visible. (B) M90T-infected tissue. Large labeled cells containing numerous apoptotic nuclei (arrows) are very prominent. (C) M90T-infected tissue. An arrow indicates labeled cells with a single apoptotic nucleus. (D) M90T-infected tissue. An arrow indicates an apoptotic nucleus not labeled by RAM-11. (E) BS176-infected tissue. Labeled cells have normal nuclear morphology. (F) M90T-infected tissue. An arrow indicates labeled cells with an apoptotic nucleus. (G) BS176-infected tissue. Labeled cells have normal nuclear morphology. (H) M90T-infected tissue. Arrows indicate apoptotic nuclei. Bar, 10 μm.

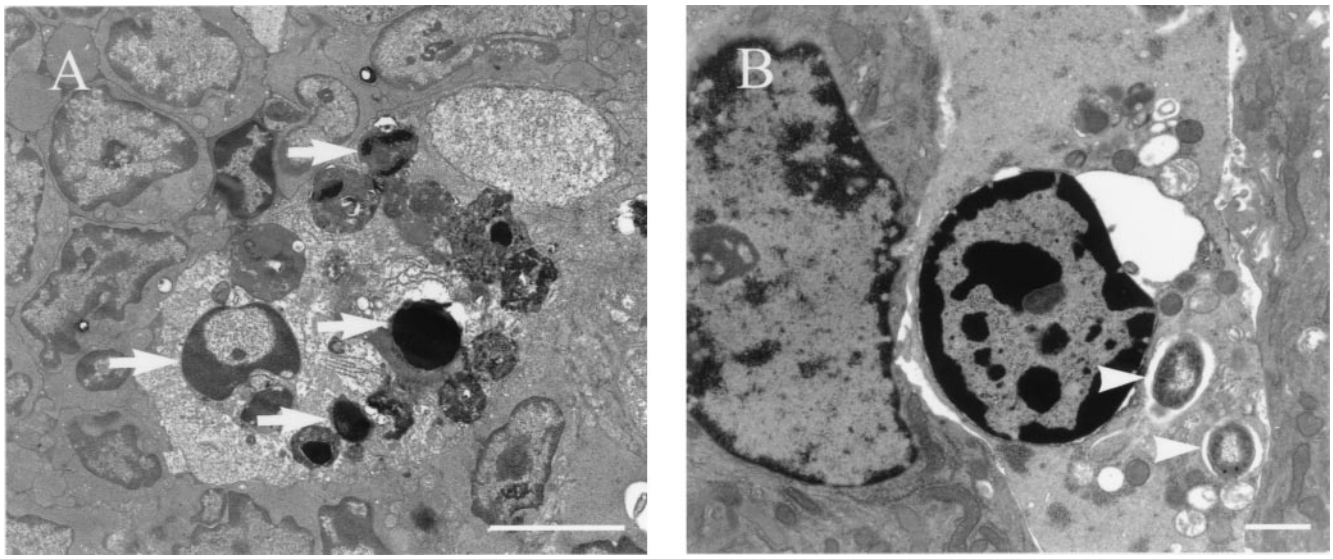

FIG. 7. Transmission electron microscopy of follicles infected with *S. flexneri*. Rabbit ligated ileal loops infected with the virulent strains of *S. flexneri*. At 8 h postinfection, the tissues were fixed, processed, and sectioned for transmission electron microscopy. (A) Large cells containing numerous apoptotic nuclei (arrows), similar to cells shown in Fig. 4B, are very prominent. Bar, 5 μm. (B) Cell with apoptotic morphology. Bacteria are present in the cytoplasm (arrowheads). Bar, 1 μm.

role in the pathogenesis of dysentery. It is also interesting to speculate that the deletion of these cells from the mucosal immune system might cause difficulties in immunizing against shigellosis with live attenuated vaccine strains.

The results presented here show that virulent *Shigella* strains induce apoptosis in vivo in the rabbit ileal loop model. *Shigella* infections are restricted to humans and some nonhuman primates. The rabbit ileal loop faithfully reproduces the histopathology of shigellosis (14, 17) and is likely to provide an accurate model, at least for the initial stages of the infection.

We have previously shown that during the apoptotic process, activated macrophages release IL-1 but not IL-6 or tumor necrosis factor (22). Furthermore, blocking the effects of IL-1 with IL-1ra abrogates the inflammatory response during *Shigella* infection (16). On the basis of the results presented in this study, we conclude that induction of macrophage apoptosis by shigellae is likely to be a crucial factor in initiating the pathogenesis of dysentery in vivo. The probable sequence of events in the initial stages of shigellosis consists of (i) translocation of shigellae from the lumen to the lamina propria of the colon by M cells, (ii) infection of macrophages and T and B cells in the lymphoid nodules, and (iii) induction of macrophage apoptosis, with the concomitant release of IL-1, which in turn initiates the acute inflammatory response. The additional role of cytokines and chemokines released by apoptotic lymphocytes remains an open question.

Attenuation of *Shigella*'s capacity to induce apoptosis represents a key target in the design of potential live vaccines against shigellosis.

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