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Shigella flexneri Is Trapped in Polymorphonuclear Leukocyte Vacuoles and Efficiently Killed

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We examined the bactericidal activity of polymorphonuclear leukocytes (PMN) against an invasive wild-type strain of *Shigella flexneri* (M90T) and a plasmid-cured noninvasive derivative (BS176). Both *Shigella* strains, as well as a rough strain of *Escherichia coli*, were killed with similar efficiencies by intact inflammatory PMN in room air and under N_2 (i.e., killing was O_2 independent). Bacterial killing by PMN extracts was substantially inhibited by antibodies to the bactericidal/permeability-increasing protein (BPI). Whereas wild-type *Shigella* escapes from the phagosome to the cytoplasm in epithelial cells and macrophages, wild-type *Shigella* was trapped in the phagolysosome of PMN as visualized by electron microscopy. The efficient killing of *Shigella* by PMN suggests that these inflammatory cells may not only contribute initially to the severe tissue damage characteristic of shigellosis but also ultimately participate in clearance and resolution of infection.

Shigellae are invasive bacteria that cause dysentery, a disease of considerable public health importance, particularly in the developing world, where it is often fatal for young children. Bacterial invasion of the colonic epithelium leads to severe inflammation which, together with bacterial dissemination, generates abscesses and ulcerations (12, 30). This tissue damage accounts for the clinical manifestation of dysentery, which is a severe form of bloody diarrhea (15).

Shigellosis is fatal when it causes extreme dehydration; otherwise, it is a self-limiting disease. How shigellosis is resolved is unknown. One possibility is that the mobilization of polymorphonuclear leukocytes (PMN) during inflammation provides, at sites of bacterial invasion, professional cytotoxic cells capable of bacterial killing and clearance (30). In fact, shigellosis patients have a higher ratio of immature to mature PMN counts in peripheral blood than patients infected with other enteric pathogens like salmonellae, *Campylobacter* spp., or *Escherichia coli*, indicating a very dramatic recruitment of PMN from peripheral blood to the infection site during shigellosis (1).

Typically, bacteria encountering PMN are engulfed within a phagosome that fuses with intracellular granules to form a phagolysosome. In the phagolysosome, the bacteria are exposed to enzymes, antibacterial polypeptides, and a progressively reduced pH. Major microbicidal substances include enzymes that create reactive forms of reduced oxygen and noncatalytic cytotoxic proteins and peptides. These cytotoxic polypeptides, which are mainly cationic and kill bacteria independently of oxygen, include the bactericidal/permeability-increasing protein (BPI), defensins, and members of protease and cathelicidin protein families (9, 10).

A crucial property in the pathogenesis of shigellae is the microorganism's capacity to invade the cytoplasm of eukaryotic cells. After phagocytosis, *Shigella* breaks the phagocytic vacuole and escapes into the cytoplasm of the cell (5, 15). Phagosome escape depends on the expression and secretion of the invasion plasmid antigen (Ipa)B, -C, and -D (16). These genes, as well as a type III secretion apparatus, are encoded in a 220-kb plasmid. Strains that have been cured of this plasmid are completely nonpathogenic (20).

Because *Shigella* can escape from the phagocytic vacuole in epithelial cells and macrophages and live free in the cell's cytoplasm, it is very different in its intracellular behavior from other enteric pathogens such as salmonellae and *E. coli* (5). However, the fate of *Shigella* within PMN has not been carefully studied. In this study, we show that wild-type *Shigella* does not escape from the phagolysosome of PMN but rather is trapped there and efficiently killed by O_2-independent mechanisms. The ability of PMN to trap and kill invasive shigellae within the phagolysosome suggests that these cells are likely to play an important role in the resolution of dysentery.

MATERIALS AND METHODS

Bacteria. The wild-type *S. flexneri* serotype 5 strain M90T, its plasmid-cured, noninvasive, noncytotoxic derivative, BS176 (31), and the rough *E. coli* strain J5 have been described previously (13). Bacterial strains were grown overnight at 37°C. *S. flexneri* strains were grown in tryptic soy broth, and *E. coli* was grown in nutrient broth. Overnight cultures were subcultured in the corresponding medium for 2 h, washed in phosphate-buffered saline (PBS), and adjusted to the appropriate concentration prior to the experiment.

Collection of PMN. Rabbit PMN were obtained from sterile inflammatory peritoneal exudates elicited in New Zealand White rabbits by injection of glycogen in physiological saline as described previously (13). Cells were collected 12 to 14 h later and resuspended in Hanks balanced salt solution with divalent cations (HBSS) without phenol red at a concentration of 10^8 PMN/ml or stored at −80°C as frozen pellets.

Acid extraction of PMN proteins. Frozen PMN were resuspended in chilled water and sonicated intermittently in a water bath sonicator in the presence of ice for 10 s at 40 W until no cells could be seen settling to the bottom of the tube. The sonicate was extracted with 0.16 N H_2SO_4 and kept on ice for 30 min with intermittent vortexing. After centrifugation at 23,000 × *g* for 20 min to sediment insoluble material, the recovered supernatant (sup II) was dialyzed against 1 mM Tris-HCl (pH 7.4) until the pH of the dialysate was ≥7.0 and stored at 4°C until used.

Incubation of bacteria with PMN: aerobic or anaerobic conditions. Typical incubation mixtures contained 10^7 freshly isolated PMN (added in 0.1 ml of HBSS), 10^7 bacteria (added in 10 μl of PBS), and RPMI 1640 (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% heated fetal calf serum (GIBCO-BRL), and 2 mM L-glutamine was added to bring the final volume to 0.5 ml. Bacteria were pretreated for 20 min at 37°C with 2% rabbit serum (in HEPES-buffered [pH 7.4] saline plus HBSS) to promote opsonization of the bacteria. This concentration of rabbit serum was not bactericidal. After pretreatment with serum, the bacteria were washed and resuspended in PBS (10^9/ml) and added last to the experimental samples. The cell suspensions were incubated at 37°C

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with shaking. At the indicated times, the incubation was stopped by diluting samples 100-fold with ice-cold PBS.

To create anaerobic conditions, cell suspensions (PMN in RPMI 1640 and bacteria in PBS) were injected into Vacutainer tubes and equilibrated at room temperature under N_2 as described previously (26, 27). Briefly, N_2 (purified; Ohio Medical Products, Madison, Wis.) flushing was accomplished via 21-gauge (inlet) and 18-gauge (outlet) needles inserted through the rubber stopper that seals these tubes. Flushing was carried out for 30 min. The bacteria were added via a syringe to tubes containing PMN. N_2 flushing was continued for the next 5 min before removing the needles. Samples were incubated at 37°C with shaking, and reactions were stopped as described above.

Preparation of samples for transmission electron microscopy. For electron microscopy, shigellae were incubated with PMN as described above, and at indicated time points, samples were taken and spun in a microcentrifuge at 1,000 × *g* for 5 min. The supernatant was removed, and the cells were fixed in ice-cold 4% paraformaldehyde and 1% glutaraldehyde in PBS. Further fixation was carried out in 1% osmium tetroxide, and after washing, the cells were stained with 1% uranyl acetate, embedded in Epon, thin sectioned, contrasted with OsO_4, and observed in a JEOL JEM-1200 EX II electron microscope.

Antibacterial activity of PMN protein extract. Bacteria (10^5 in 5 μl of PBS) were incubated in the presence of different amounts of PMN extract diluted in nutrient broth supplemented with phosphate-buffered (20 mM, pH 7.4) physiological saline. The total incubation volume was 0.1 ml. To determine the role of the BPI in the antibacterial activity of the PMN extracts, the extract was preincubated with 3% (vol/vol) goat serum (nonimmune or anti-BPI) for 15 min at room temperature, as described previously (25, 28), before the addition of bacteria. Samples were incubated for 1 h at 37°C with shaking. Reactions were stopped by diluting samples in ice-cold PBS. Bacterial viability was assessed in the presence or absence of bovine serum albumin (BSA) in nutrient agar as described below.

Assay of growth inhibitory activity. The bactericidal activity of intact PMN or PMN extracts was determined by measuring the effect of PMN (extract) on bacterial colony-forming ability. From bacterial suspensions incubated with or without PMN (extract), aliquots were taken and serially diluted in sterile ice-cold PBS. A 30-μl aliquot of a diluted sample was transferred to 5 ml of molten (48°C) 1.3% (wt/vol) Bacto Agar containing 0.8% (wt/vol) nutrient broth and 0.5% (wt/vol) NaCl and poured into a petri dish. The high temperature of the agar causes disruption of PMN and release of intracellular bacteria. Where indicated, the agar was supplemented with 1 mg of BSA per ml to distinguish killed bacteria (no colony formation in nutrient agar plus BSA) from sublethally injured bacteria (colony formation in nutrient agar with but not without albumin). The agar was allowed to solidify, and the number of CFU was determined on the plates after incubation at 37°C overnight.

Assay of cell death by fluorescence microscopy. Samples of PMN and bacteria were prepared as described above and incubated for 3 h at 37°C. Aliquots of 50 μl (10^6 cells) were removed from the experimental sample and centrifuged at 1,000 × *g* for 5 min. After removal of the supernatant, the cells were resuspended in staining solution (5 μg of propidium iodide [PI] per ml of PBS supplemented with 10% glycerol) and examined under a fluorescence microscope. At least 500 cells were counted for each experimental sample, and the percentage of stained (dead) cells was determined.

RESULTS

Microbicidal action of PMN against *S. flexneri*. We compared the antibacterial activities of rabbit inflammatory PMN against three different strains: (i) the wild-type invasive *S. flexneri* strain M90T; (ii) the noninvasive plasmid-cured derivative of M90T, strain BS176; and (iii) *E. coli* J5, a rough strain that has been used extensively in studies of the antimicrobial function of PMN (13). Figure 1 shows that PMN efficiently kill both *Shigella* strains as well as *E. coli* J5. When 1 bacterium was added per PMN, more than 80% of M90T and BS176 were killed within 1 h. More than 95% of the bacteria were killed after 3 h of incubation. No differences in survival of M90T and BS176 were seen over a range of 0.1 to 10 bacteria/PMN. Figure 1 also shows that PMN have a similar antimicrobial activity against *E. coli* J5. *E. coli* J5 and both strains of *S. flexneri* are killed efficiently, although the *Shigella* strains are killed with slightly delayed kinetics.

Killing of *S. flexneri* by inflammatory PMN is O_2 independent. Intracellular killing of certain members of the family *Enterobacteriaceae* (e.g., *Salmonella typhimurium* and *E. coli*) by rabbit and human PMN is O_2 independent and preceded by sublethal alterations which render the bacteria incapable of forming colonies in nutrient agar unless the medium is supplemented with albumin (13). To determine whether killing of both virulent and avirulent shigellae by PMN occurred by similar mechanisms, the viabilities of the two *Shigella* strains and, for comparison, that of *E. coli* J5 were measured in nutrient agar with or without albumin after incubation of the bacteria with PMN in room air or under N_2. Figure 2 again shows that the antibacterial effects of PMN on the two *Shigella* strains and *E. coli* are closely similar. For each of the three different bacterial populations, <10% of the added bacteria could form colonies in nutrient agar after 2 h of incubation with the PMN in either room air or N_2. However, ca. 30% of the bacteria could form colonies in nutrient agar supplemented with albumin. Thus, after 2 h of incubation with PMN in either room air or N_2, ca. 70% of the added bacteria were killed and most of the remainder had suffered sublethal (but not yet lethal) injury. The close similarity in the effects on *E. coli* and on shigellae suggests that the PMN kill these bacteria by a similar mechanism(s).

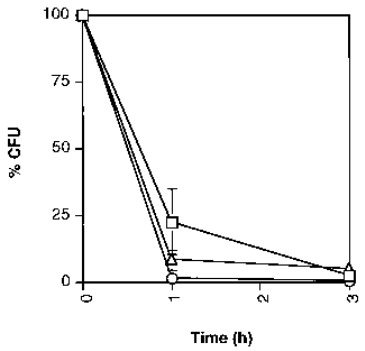

FIG. 1. Killing of both virulent and avirulent *Shigella* by PMN. Bacteria (*S. flexneri* M90T [squares] and BS176 [triangles] and *E. coli* J5 [circles]) were incubated in the presence or absence of freshly isolated rabbit inflammatory PMN at a 1:1 ratio. At the indicated times, bacterial viability was measured as indicated in Materials and Methods. Bacterial viability is expressed as percent CFU, where 100% represents the number of colonies formed at indicated time points in the absence of PMN (which increase during the experiment) and percent CFU is the percent survival in the presence of PMN. The results shown represent the mean (± standard error of the mean) of six independent experiments.

Sensitivity of shigellae to acid extracts of PMN: role of BPI. To further document the activity of O_2-independent bactericidal agents of PMN against both virulent and avirulent strains of *Shigella*, the effects of an acid extract of rabbit PMN on the two *Shigella* strains and *E. coli* J5 were compared. Figure 3 shows that the PMN extract exhibits potent antibacterial activity against each of the three bacterial populations. As in intact PMN, a portion of the treated bacterial population is still able to form colonies when the nutrient agar contains albumin, reflecting bacteria that are only sublethally injured (Fig. 3A to C). Because BPI appears to be the most prominent agent in PMN active against many gram-negative bacteria (3, 4, 28), we tested the effect of a serum containing neutralizing antibodies to BPI on the antibacterial activity of the crude PMN extract. Figure 3D to F shows that at lower doses of the extract, BPI antiserum but not normal serum substantially inhibited the activity of the PMN extract against both *Shigella* strains and *E. coli* J5. The lack of inhibitory effect of the antiserum at five-times-higher doses of extract may reflect either BPI-independent activity or BPI excess at this high dose of extract.

Invasive M90T does not escape the phagocytic vacuole of PMN. A few minutes after entry, the wild-type *Shigella* strain (M90T) lyses its phagocytic vacuole and gains access to the cell cytoplasm in both macrophages and epithelial cells. BS176,

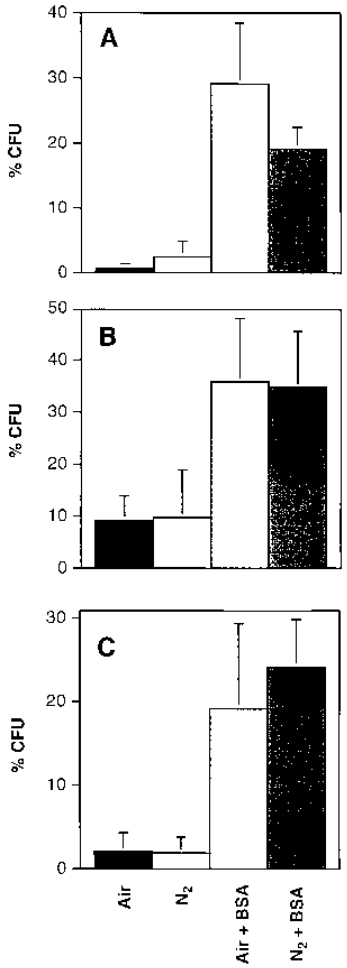

FIG. 2. *Shigella* killing by PMN is O_2 independent. Effects of incubation of *S. flexneri* M90T (A) and BS176 (B) and *E. coli* J5 (C) with PMN (1 bacterium/PMN) at 37°C for 2 h in room air or N_2 on bacterial colony-forming ability in nutrient agar with or without BSA (see Materials and Methods). Data shown represent the mean (± standard error of the mean) of three independent experiments.

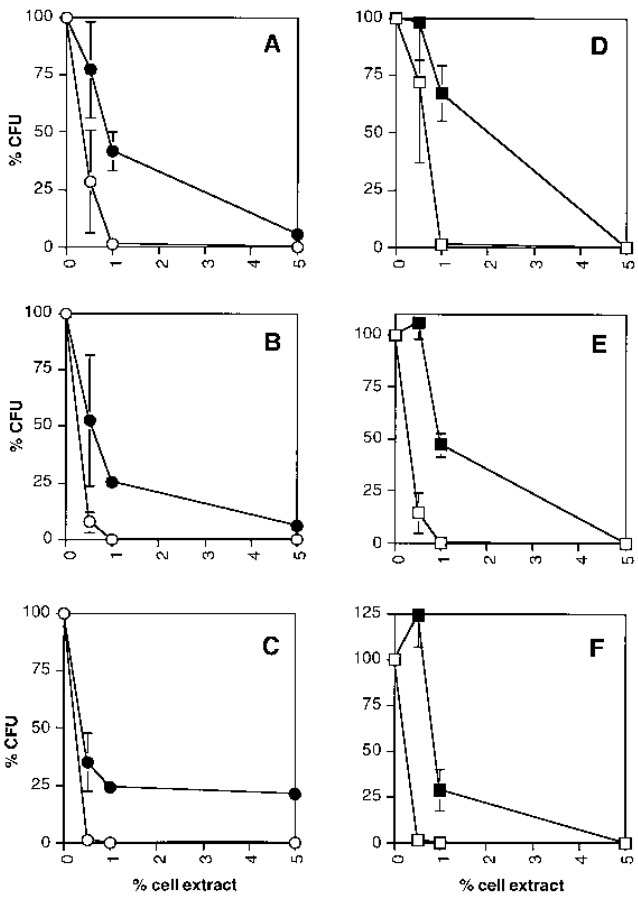

FIG. 3. Antibacterial activity of crude acid extracts of rabbit PMN toward *S. flexneri*: role of BPI. *Shigella* strains M90T (A and D) and BS176 (B and E) and *E. coli* J5 (C and F) were incubated with different concentrations of crude acid extract of rabbit PMN and plated on molten nutrient agar with (closed circles) or without (open circles) BSA. In panels D, E, and F, the buffered sup II was preincubated for 15 min with 3% (vol/vol) normal goat serum (open squares) or goat anti-BPI serum (closed squares) prior to the addition of bacteria. After 1 h of incubation at 37°C, the samples were diluted and plated on molten nutrient agar without BSA.

however, is not able to invade nonphagocytic cells and remains in the phagocytic vacuoles in macrophages (8, 31). The fact that M90T, BS176, and *E. coli* J5 were killed by PMN with similar efficiencies by apparently similar mechanisms suggested that in PMN, M90T may not be able to escape from the phagocytic vacuole. To test this hypothesis, we determined the subcellular localization of the bacteria by transmission electron microscopy. Figure 4A shows noninvasive strain BS176 within the vacuolar compartment of inflammatory PMN cells. Strikingly, invasive strain M90T is also contained in phagocytic vacuoles in PMN cells at 15 (Fig. 4B and C), 30 (Fig. 4D), and 60 (Fig. 4E) min after infection. In Fig. 4C, the double membrane of the gram-negative bacterium and the surrounding vacuolar membrane are clearly visible. We examined 51 BS176-infected and 45 M90T-infected cells, and in each case, bacteria were found inside the phagocytic vacuoles. After 15 min, the majority of bacteria had a partially preserved double membrane and a largely intact morphology. More severe bacterial destruction is apparent at later time points, and after 60 min, bacterial damage is very extensive, corresponding to lethal injury (4).

Effect of virulent and avirulent strains of *Shigella* on PMN viability. Virulent, but not avirulent, strains of *Shigella* cause apoptosis in macrophages (31). To test whether *S. flexneri* induces cell death in PMN cells, we incubated them with or without bacteria and assayed for the uptake of PI. PI uptake into the nucleus of cells is a measure of cell membrane degeneration, a relatively early event in cell death (22, 23). The results presented in Fig. 5 show that incubation of PMN with either M90T or BS176 as well as *E. coli* J5 induces PI uptake by 20 to 25% of the PMN after 3 h of incubation with the bacteria. Thus, gene products encoded in the *Shigella* virulence plasmid were not needed for this effect. Interactions of PMN and shigellae differ from that of shigellae and macrophages in the fate of both the ingested bacteria and of the host cell.

DISCUSSION

While great progress has been made in understanding the mechanisms of invasion employed by *Shigella*, much less is known about host defense mechanisms against this pathogen. In this report, we investigate the bactericidal activity of PMN against *Shigella*. We demonstrate that both invasive and non-

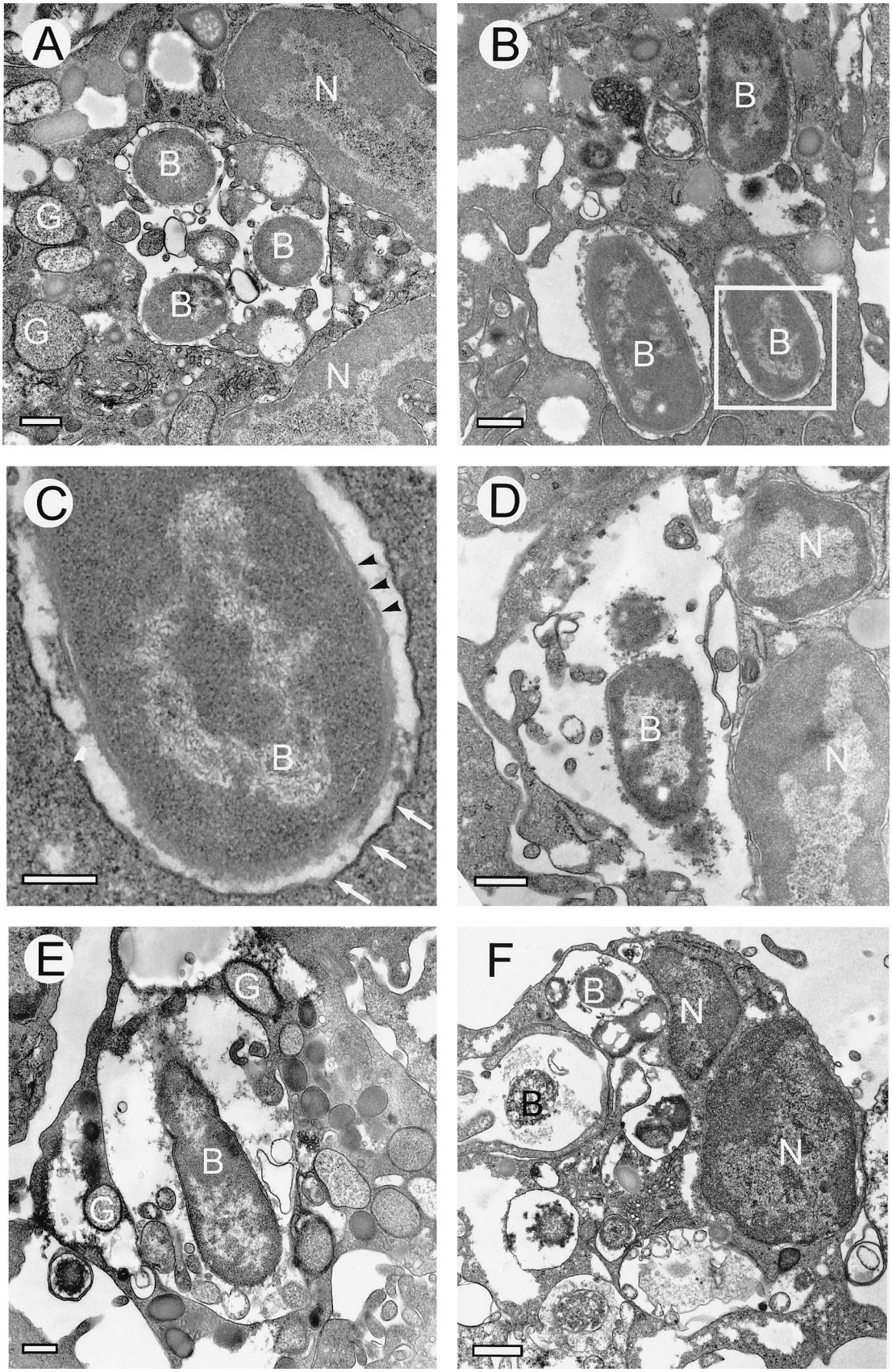

FIG. 4. Transmission electron microscopy of rabbit PMN infected with *Shigella*. PMN infected with *Shigella* were fixed after incubation at 37°C with BS176 for 30 min (A), M90T for 15 min (B), M90T for 30 min (D), M90T for 30 min (E), and M90T for 60 min (F). Panel C shows the bacterium outlined in panel B at a higher magnification. Both bacterial strains are clearly contained within vacuoles. Further signs of degeneration are evident at 30 and 60 min. B, bacteria; N, nucleus; G, granules. In panel C, white arrows indicate the membrane of the phagocytic vacuole and black arrowheads indicate the double membrane of bacteria. Bars: panels A, B, and D, 0.5 μm: panel C, 2 μm; panel F, 1 μm.

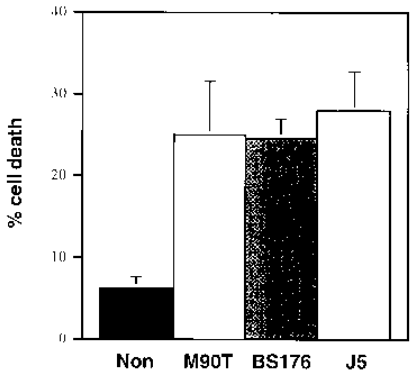

FIG. 5. Effect of gram-negative bacteria on PMN cell death. PMN were incubated with bacteria at a 1:1 ratio with the indicated strains in standard incubation media for 3 h. The cultures were stained with PI in PBS without fixation. Only dead cells take up PI. The figure represents the percentage of cells stained with PI. One hundred percent is defined as the total number of cells counted in three different fields (at least 500 cells). The results are expressed as the mean (± standard error of the mean) of at least four different experiments for *S. flexneri* strains and two experiments for *E. coli* J5.

invasive strains of *Shigella* are killed efficiently by rabbit inflammatory peritoneal PMN (Fig. 1). Bactericidal activity against *Shigella* is comparable to toxicity against a well-studied strain of *E. coli* that is readily killed by the (nonoxidative) antimicrobial arsenal of the PMN (4, 13). *Shigella* killing by PMN did not require opsonization with *Shigella*-specific antibodies, suggesting that PMN killing is part of the innate immunity against dysentery.

Previous studies have strongly suggested that BPI, a specific antibacterial component of PMN (4, 13), plays an important role in the intracellular action of these cells against *E. coli* and many other gram-negative bacteria (13). Several findings presented in this study are consistent with a similar role for BPI in the intracellular action of the PMN against *S. flexneri*. (i) Intracellular killing of *Shigella* is not diminished by O_2 depletion (Fig. 2), indicating that nonoxidative antibacterial mechanisms of PMN are sufficient for intracellular killing of *Shigella*. The presence of reactive oxidant scavenging systems like superoxide dismutase (*sodB*) (6) may render *Shigella* relatively resistant to O_2-dependent cytotoxic systems of the PMN. (ii) Inhibition of growth (i.e., colony formation) of ingested bacteria includes effects that are reversible by albumin, a hallmark of the action of purified BPI and of PMN action against BPI-sensitive gram-negative bacteria (4, 11, 13, 14). (iii) Killing of *Shigella* by crude extracts of PMN enriched in the cell's nonoxidative antibacterial machinery is substantially inhibited by neutralizing BPI antibodies (Fig. 3D to F). BPI-dependent antibacterial activity may either reflect direct and independent effects of BPI or include actions of other antibacterial proteins in PMN (e.g., p15s and defensins) in concert with BPI (11, 25). The fact that high doses of these extracts produce antibacterial effects that are neither reversible by albumin (Fig. 3A and B) nor blocked by BPI antiserum (Fig. 3D to F) may mean that at these high doses, BPI-independent antibacterial effects can also be exerted.

Whatever the precise nature of the mechanism of killing of *Shigella* by PMN, our findings strongly suggest that both virulent and avirulent *Shigella* strains are killed in a manner that is essentially the same as the killing of other members of the family *Enterobacteriacae*. This is a striking finding since one of the hallmarks of *Shigella* infections is the ability of wild-type invasive bacteria to escape the phagolysosome, the site in which the PMN unleashes antibacterial agents against ingested bacteria. However, as revealed in electron micrographs, in PMN even wild-type *Shigella* is retained in phagolysosomes (Fig. 4). These results stand in sharp contrast to *Shigella* infections of epithelial cells, where 90% of invasive bacteria escape to the cytosol within 15 min after ingestion (21). What accounts for the very different fate of wild-type *Shigella* in PMN than in these other host cells is unknown. An important difference between these cells is the presence in PMN but not macrophages or epithelial cells of potent antibacterial proteins active against gram-negative bacteria such as shigellae (e.g., BPI). Ingested BPI-sensitive gram-negative bacteria (e.g., *E. coli* J5) suffer outer envelope injury almost immediately after ingestion by PMN (3, 4). Alterations of the outer envelope of ingested *Shigella* are also manifest soon after ingestion (Fig. 4B and C) and could include inactivation of the bacterial invasion system before the bacteria can break out of the vacuole. Other differences may also exist within the microenvironment of the phagocytic vacuole that make escape of *Shigella* highly efficient in macrophages and epithelial cells but not in PMN.

Wild-type *Shigella* induces apoptosis in macrophages but not in epithelial cells (30) nor, as shown in this study, in PMN (Fig. 5). IpaB is the plasmid-encoded gene product in *Shigella* that is necessary and sufficient to trigger apoptosis in macrophages (2, 29). This protein must be secreted directly into the cytoplasm of macrophages, where it binds to interleukin-1β-converting enzyme or a closely related cysteine protease and induces apoptosis (2, 31). Thus, the absence of bacterium-directed apoptosis in PMN could be explained by the intravacuolar localization of *Shigella* and the inability of IpaB to reach the cytoplasm. Alternatively, PMN could lack the appropriate cytosolic target for IpaB. The expression of interleukin-1β-converting-enzyme-related proteases in PMN has not been investigated.

Alterations in PMN membrane integrity leading to increased uptake of PI were observed after *Shigella* infection. However, unlike the induction of macrophage apoptosis, increased PI staining of PMN accompanied phagocytosis of both virulent and avirulent *Shigella* strains and of *E. coli* J5 as well (Fig. 5). Thus, this is a relatively nonspecific effect, induced by heat-killed bacteria as well (24), possibly reflecting membrane and/or metabolic alterations induced in PMN during phagocytosis.

In contrast to our findings, Renesto and coworkers (19) recently demonstrated that wild-type *Shigella*, but not a plasmid-cured derivative, promotes PMN adherence and degranulation, suggesting, therefore, that Ipa invasins are necessary for PMN activation. These effects were seen, however, at bacterium/PMN ratios of 100 to 1,000:1 and not at the lower ratios examined in this study. Similarly, Gbarah et al. showed that human granulocytes were ineffective killers of *Shigella* without opsonization at high bacterium/PMN ratios (7). Whether in vivo there is sufficient invasion and intraepithelial multiplication of *Shigella* before mobilization of PMN to achieve these high bacterium/PMN ratios is uncertain. If so, it is possible that early in infection, the effects of the Ipa products on PMN further exacerbate the inflammatory damage provoked by PMN, whereas later, the higher concentrations of PMN accumulated produce lower bacterium/PMN ratios and favor the bactericidal action of the PMN that we have described.

The work of Perdomo et al. (17, 18) has elegantly demonstrated that PMN play a crucial role in disrupting the epithelial barrier as they migrate out into the lumen of the colon. Blocking of PMN migration decreases both the inflammatory response and the number of bacteria in Peyer's patches of infected animals. Here we propose that inflammatory PMN also

play an important role in the resolution of shigellosis by killing the bacteria. Thus, in bacillary dysentery, PMN may have a pivotal role both in the onset of the disease and in the ultimate eradication of the infectious agent.

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