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Shigella flexneri IpaH$_{7.8}$ Facilitates Escape of Virulent Bacteria from the Endocytic Vacuoles of Mouse and Human Macrophages

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The behavior of *Shigella flexneri ipaH* mutants was studied in human monocyte-derived macrophages (HMDM), in 1-day-old human monocytes, and in J774 mouse macrophage cell line. In HMDM, strain pWR700, an *ipaH*$_{7.8}$ deletion mutant of *S. flexneri* 2a strain 2457T, behaved like the wild-type strain 2457T. This strain caused rapid host cell death by oncosis, and few bacterial CFU were recovered after incubation in the presence of gentamicin as previously described for 2457T-infected HMDM. However, analysis of bacterial compartmentalization within endocytic vacuoles with gentamicin and chloroquine indicated that more pWR700 than 2457T was present within the endocytic vacuoles of HMDM, suggesting that *ipaH*$_{7.8}$ deletion mutant transited more slowly from the vacuoles to the cytoplasm. In contrast to findings with HMDM, CFU recovered from pWR700-infected mouse J774 cells were 2 to 3 logs higher than CFU from 2457T-infected J774 cells. These values exceeded CFU recovered after infection of J774 cells with plasmid-cured avirulent strain M4243A1. Incubation with gentamicin and chloroquine clearly showed that pWR700 within J774 cells was mostly present within the endocytic vacuoles. This distribution pattern was similar to that seen with M4243A1 and contrasted with the pattern seen with 2457T. Complementation of pWR700 with a recombinant clone expressing *ipaH*$_{7.8}$ restored the intracellular distribution of bacteria to that seen with the wild-type strain. Strains with deletions in *ipaH*$_{4.5}$ or *ipaH*$_{9.8}$, however, behaved like 2457T in both HMDM and J774 cells. The distribution profile of pWR700 in 1-day-old monocytes was similar to that seen in J774 cells. Like infected J774 cells, 1-day-old human monocytes demonstrated apoptosis upon infection with virulent *Shigella*. These results suggest that a role of the *ipaH*$_{7.8}$ gene product is to facilitate the escape of the virulent bacteria from the phagocytic vacuole of monocytes and macrophages.

The roles of virulence genes, present on the large invasion plasmid of shigellae, have been determined by using specific gene mutations and complementation analysis (1, 4, 11, 36, 41, 42). Functional assays have included the use of epithelial cells and macrophages in tissue culture experiments (7, 26, 30, 36, 37, 43, 45), animal models of pathogenicity, biochemical analyses of protein complexes, and immunohistochemical techniques to localize individual bacterial virulence gene products within host cells (11, 19, 27, 32, 35). Key steps in pathogenesis include the formation of an IpaB-IpaC complex within the bacterial cell, their translocation to the surface of the bacteria by the Mxi/Spa accessory proteins, release of the complex upon contact with host cells, and triggering of a signaling pathway that results in the entry and dissemination of the bacteria both intra- and intercellularly (1, 11). Host cytoskeletal proteins such as actin and actin-binding proteins bind to a bacterial outer membrane protein, VirG, and provide the force for intra- and intercellular dissemination (15). The IpaB protein is critically involved during entry into epithelial cells as well as lysis of the phagocytic vacuole within epithelial cells and macrophages. Macrophage cell death after *Shigella* infection, following uptake within the mucosal lymphoid tissues, and subsequent entry into epithelial cells at its basolateral end is considered a key step during pathogenesis of shigellosis (7, 8, 11, 16, 18, 24, 45).

The roles of the multicopy *ipaH* genes, which are present on both the invasion plasmid and the chromosome, are unknown (5, 12, 40). Five copies of the *ipaH* genes present on the invasion plasmid (pWR100) of *Shigella flexneri* 5 strain M90T-W have been cloned and sequenced (12, 40). All five copies have similar carboxy-terminal halves. The amino-terminal end, while different in each copy, encodes a common leucine-rich repeat (LRR) motif seen in a diverse group of bacterial and eukaryotic proteins (2, 3, 10, 14, 17, 20, 34, 38, 40). pWR100 *ipaH* contains six copies of the 20-amino-acid repeat module, while *ipaH*$_{4.5}$ contains nine repeats in this area. Three of the five *ipaH* copies, *ipaH*$_{7.8}$, *ipaH*$_{4.5}$, and *ipaH*$_{9.8}$, encode proteins that are 58 to 65 kDa and react with infected sera on Western blots. The *ipaH*$_{7.8}$ and *ipaH*$_{4.5}$ copies are located adjacent to each other near the *ipaBCDA* loci on pWR100.

In human monocyte-derived macrophages (HMDM), virulent *Shigella*, containing the large invasion plasmid, causes a rapid cell death similar to oncosis (7, 8; C. M. Fernandez-Prada, D. L. Hoover, B. Tall, and M. M. Venkatesan, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. 52, p. 243, 1998). A similar infection in a mouse monocytic cell line J774 results in programmed cell death or apoptosis (45). Plasmid-cured avirulent strains in both types of macrophages remain enclosed within endocytic vacuoles and do not cause cell death, presumably due to the inability of the IpaB mutant to reach the cytoplasm. Interleukin-1β (IL-1β) is released from virulent

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TABLE 1. *S. flexneri* strains used in this study

Strain	Characteristic(s)	Source
M90T-W	Wild-type *S. flexneri* serotype 5	WRAIR[a]
M90T-55	Plasmid-cured derivative of M90T-W	WRAIR
SC403	Deletion mutant of the *ipaB* gene in M90T-W	Pasteur Institute
2457T	Wild-type *S. flexneri* serotype 2a	WRAIR
2457T-str	Streptomycin-resistant derivative of 2457-T	WRAIR
M4243A1	Plasmid-cured derivative of 2457-T	WRAIR
pWR700	*ipaH*$_{7.8}$ deletion in 2457-T	WRAIR
pWR710	*ipaH*$_{4.5}$ deletion in 2457-T	WRAIR
pWR720	*ipaH*$_{9.8}$ insertion mutation in 2457-T	WRAIR
pWR730	*ipaH*$_{4.5}$ deletion in pWR700	WRAIR
pWR740	*ipaH*$_{9.8}$ insertion mutant in pWR700	WRAIR
pWR750	*ipaH*$_{9.8}$ insertion mutant in pWR730	WRAIR
pWR701	pWR700 complemented with *ipaH*$_{7.8}$	WRAIR
pWR800	*ipaH*$_{7.8}$ mutation in M90T-W	WRAIR

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Shigella-infected HMDM and J774 cells (7, 8, 16, 45). In vivo, IL-1β, a potent inflammatory cytokine, is thought to play a significant role in triggering the cascade of events that ultimately leads to an intense inflammatory reaction and necrosis of the epithelial cells characteristic of shigellosis (35). In a recent study, epithelial cells infected with enteroinvasive *Escherichia coli* have been shown to undergo apoptosis (22).

Although incubation of HMDM and J774 cells with virulent *Shigella* results in two different types of cell death, the pathways used within these cells appear to be essentially similar. J774 is a propagating macrophage cell line, whereas HMDM are short-term cultures of human monocytes obtained from volunteers. Thus, investigational analysis with both cell types offers complementary opportunities for studying the role of bacterial proteins in macrophages. In this study, we describe the behavior of *ipaH* mutants in HMDM, mouse J774 macrophage cell line, and 1-day-old human monocytes. These data, together, suggest that *ipaH* facilitates the escape of the bacteria from the phagocytic vacuoles of these cells.

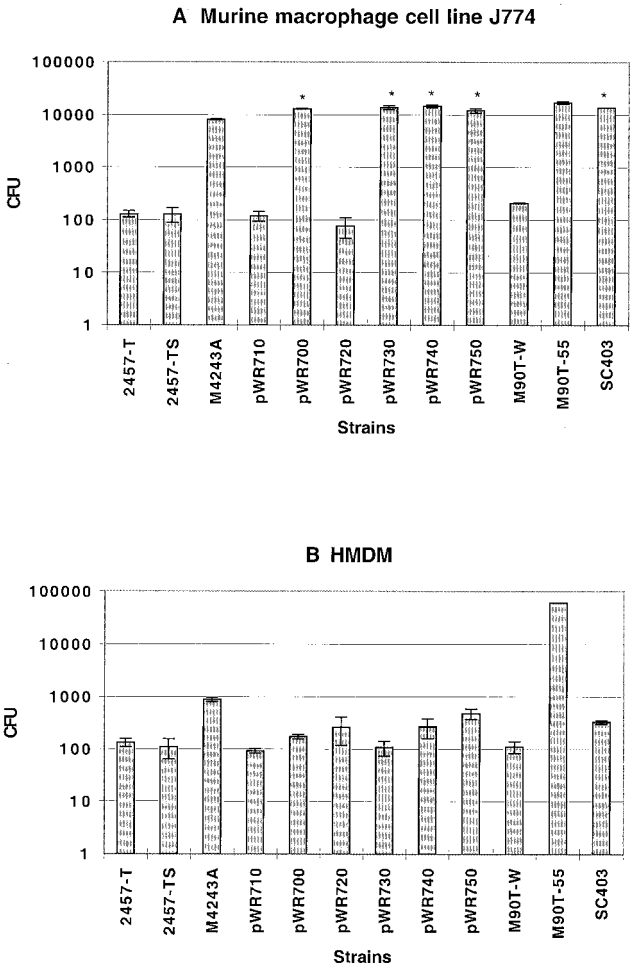

FIG. 1. Evaluation of infection of *ipaH* mutant strains in J774 cells (A) and HMDM (B). Bacteria were left in contact with macrophages for 30 min, washed with HBSS, and further incubated in gentamicin-containing medium for another 50 min. Macrophages were then washed and lysed. The numbers of viable bacteria were obtained by plating dilutions of the lysates on TSA plates. CFU represents the total number of bacteria in macrophage cell lysates. The characteristics of the strains are listed in Table 1. *, *P* value not significant compared to M90T-55. Error bars show means ± standard deviations.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study to infect macrophages are listed in Table 1. For all macrophage infections, overnight cultures of the bacterial strains were diluted 1:50 in 10 ml of Luria broth (Difco) and were incubated at 37°C until they reached the mid-log phase of growth. The bacteria were harvested and resuspended in 1 ml of Hanks balanced salt solution (HBSS; Gibco). Antibiotics (all from Sigma) were added, when indicated, at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 300 μg/ml.

Cell culture and macrophage/monocyte infections. Monocytes were isolated from citrated peripheral venous blood from healthy human volunteers by counterflow centrifugal elutriation, cultivated for HMDM in RPMI 1640 medium containing 10% heat-inactivated human AB serum (Sigma), 2 mM L-glutamine (Gibco), and macrophage colony-stimulating factor (10 ng/ml; a gift from Jay Stoudemire, Genetic Institute, Cambridge, Mass.), and incubated at 37°C in a humidified 5% CO_2 atmosphere for 7 to 10 days as described elsewhere (7, 8, 9).

Monocytes incubated in the same medium for only 1 day were used as the source of 1-day-old monocytes. The murine macrophage-like cell line J774 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, and penicillin-streptomycin (Gibco) in a humidified 5% CO_2 atmosphere at 37°C. Monocytes/macrophages were suspended in fresh medium in either 24-well culture plates, 6-well plates, or 100-mm-diameter tissue culture plates at a concentration of 10^6 cells/ml. The plates were washed to remove nonadherent cells before infection, and new medium without antibiotics was added. The cells were infected as described elsewhere (7, 8, 9). At selected intervals after infection, the medium was removed, and the macrophages/monocytes were washed and lysed with 0.1% Triton X-100. The numbers of viable bacteria were obtained by plating dilutions of the lysates on tryptic soy agar

TABLE 2. Severity rating of animals infected with wild-type and *ipaH* mutant strains of 2457T-str on day 6 postinfection

Strain	No. of eyes with severity rating of:			
	3	4	5	6
2457T-str				
Low dose	6	2	0	0
High dose	4	1	2	0
pWR700				
Low dose	1	1	6	0
High dose	0	0	4	4
pWR710				
Low dose	2	3	1	2
High dose	0	0	4	4
pWR730				
Low dose	0	0	6	2
High dose	0	2	6	0

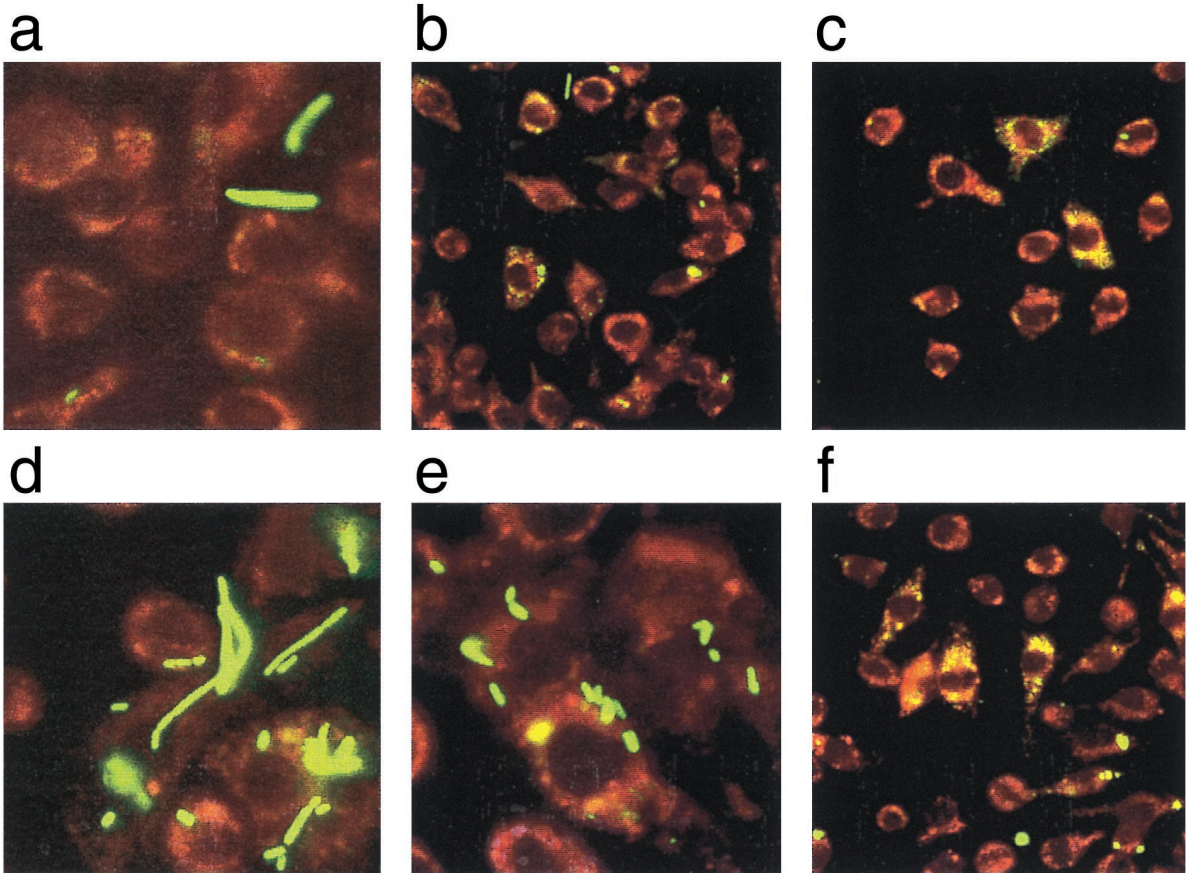

FIG. 2. Colocalization of *Shigella* with LAMP-1-containing vacuoles. J774 cells were infected for 10 min (a to c) or 30 min (d to f) with 2457T-GFP (a and d), pWR700-GFP (b and e), and M4243A-GFP (c and f). Cells were washed, stained with LAMP-1 antibody, and counterstained as described in Materials and Methods.

(TSA) plates. Colonies were counted after overnight incubation of the plates at 37°C.

Light microscopy analysis of infected macrophages and monocytes. Human or murine macrophages were seeded in tissue chamber slides (LabTek) and incubated at 37°C in a humidified 5% CO_2 atmosphere. At selected intervals after infection, the slides were washed and stained using a LeukoStat stain kit (Fisher), which is a modification of the Wright's stain technique. The slides were examined under a light microscope.

Confocal microscopy. J774 cells were seeded on coverslips and infected as described above, using *S. flexneri* strains containing a plasmid expressing GFPuv (Clontech Laboratories). At selected intervals after infection, the medium was removed and the macrophages were washed, fixed, and stained for LAMP-1 (CD107a) as instructed by the manufacturer (Molecular Probes). Briefly, macrophages were fixed in buffered paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). The coverslips were then incubated at 4°C in blocking buffer (PBS containing 2% goat serum), washed in PBS, and incubated in 1:20 dilution of anti-LAMP-1 (mouse immunoglobulin G1 monoclonal antibody [MAb]). After being washed in PBS, the coverslips were counterstained with a 1:20 dilution of Alexa 594 goat anti-mouse immunoglobulin G (heavy plus light chain) conjugate antibody. After a final wash in PBS, the coverslips were mounted in medium containing 0.1 M *n*-propyl gallate (to prevent photobleaching) in glycerol (59% [vol/vol])–gelatin (0.9% [wt/vol]) and visualized by confocal microscopy using a Zeiss 410 instrument equipped with a krypton-argon mixed-gas laser. For green fluorescent protein (GFP) visualization, a 488-nm laser line was used for illumination and emission was detected with a 510- to 545-nm bandpass filter and a dichroic mirror to reflect wavelengths below 510 nm and above 550 nm; Alexa 594-tagged antibodies were irradiated with 568-nm laser, and emission was detected using a >610-nm-long bandpass filter and a dichroic mirror to reflect wavelengths below 600 nm. Phagosome lysosome fusion was considered to take place if colocalization of LAMP-1 and bacteria was observed.

Transmission electron microscopic (TEM) analysis of infection. At selected intervals following infection, monocyte/macrophage cell monolayers were washed with HBSS three times and prefixed with 4% paraformaldehyde–1% glutaraldehyde in 0.2 M sodium cacodylate buffer (SCB), pH 7.2, for 1 h at room temperature. The cells were then scraped off the tissue plate surfaces and placed in fresh prefixative and stored at 4°C for further processing. The samples were again washed three times with SCB and postfixed with 1% osmium tetroxide in SCB for 2 h as described elsewhere (8). The postfixed samples were further processed and embedded into Epon 812 (EPONATE 12; Ted Pella, Redding, Calif.). Ultrathin sections were made using a Leica Ultracut-S ultramicrotome. The sections were stained with uranyl acetate and lead citrate as described elsewhere (8) and were evaluated in a Philips 400 HM transmission electron microscope operating at an acceleration voltage of 80 kV.

LDH assays for measuring cytotoxicity. Monocytes/macrophages were seeded in 24-well plates and infected at a multiplicity of infection (MOI) of 30 bacteria/cell. Aliquots of the supernatants were collected and assayed for lactate dehydrogenase (LDH) release using a colorimetric Cytotox 96 kit (Promega Corp., Madison, Wis.) according to the manufacturer's instructions, with some modifications as described elsewhere (8, 9).

DNA fragmentation on agarose gels. Internucleosomal DNA fragmentation of infected monocytes/macrophages was measured as previously described (27, 28). The samples were electrophoresed on 1.2% agarose gels and stained with ethidium bromide as previously described (8, 9).

DNA analysis by flow cytometry. Macrophages were infected as described elsewhere (8, 9). After the last wash, nuclei from infected and uninfected macrophages were released from cells by treatment with 1% Triton X-100 in 0.1 M citric acid, stained with 10 μg of propidium iodide per ml, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) (8, 9).

Detection of levels of cytokines in the supernatant of infected macrophages. The release of cytokines in the culture supernatants of macrophages was measured at different times by using enzyme-linked immunosorbent assays (ELISA) for human or mouse IL-1β, tumor necrosis factor alpha (TNF-α), and human IL-10 and IL-12 as instructed by the manufacturer (Endogen). The reaction was stopped, and the intensity of the color was measured at 450 nm (correction wavelength was 570 nm) using a Titertek Multiscan ELISA reader as described elsewhere (7, 8, 9).

Sereny reaction in guinea pigs. 2457T-str containing a deletion in $ipaH_{7.8}$ (pWR700) was used to construct a second deletion in $ipaH_{4.5}$, generating the double-deletion mutant pWR730. Individual mutants of $ipaH_{4.5}$ (pWR710) and

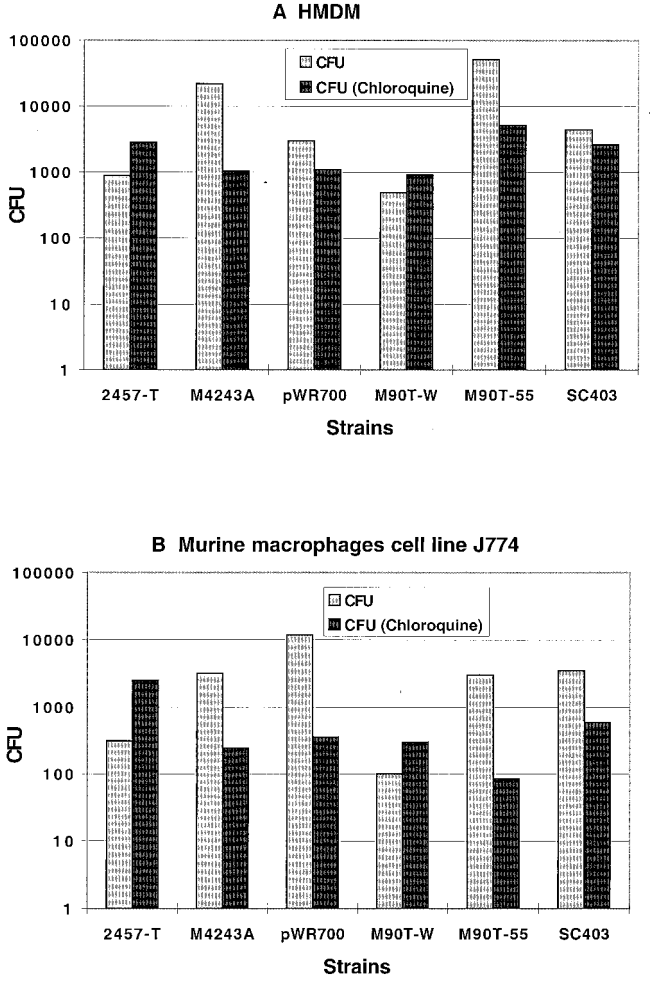

FIG. 3. Effect of chloroquine on the intracellular survival of *S. flexneri* strains in HMDM (A) and murine macrophage cell line J774 (B). The subcellular localization of bacteria was determined by testing the effect of chloroquine (0 or 2.5 mg/ml) on bacterial recovery. Characteristics of the bacterial strains are listed in Table 1.

ipaH$_{9.8}$ (pWR720) were also constructed in 2457T-str. The *ipaH* mutants were checked in invasion assays before administration into guinea pig eyes. Overnight plate growths of cells were harvested in PBS, 2.5×10^8 (low dose) to 10^9 (high dose) were inoculated into individual eyes of guinea pigs, and eyes were observed for development of keratoconjunctivitis as previously described (13). Diseased eyes were rated as follows: 0, no disease or mild irritation; 1, mild conjunctivitis; 2, keratoconjunctivitis without purulence; 3, fully developed keratoconjunctivitis with purulence; 4, eyes as in 3 but unusually swollen; 5, eyes as in 4 but with additional unusual inflammation around eyelids; 6, eyes as in 5 but with additional purulence and discharge. The unusual reaction seen with the *ipaH* mutants made it necessary to extend the normal rating scheme for the Sereny reaction as described previously (13).

Statistical analysis. Statistical analysis was done by Student's *t* test using the INSTAT statistical analysis package (Graph Pad Software, Inc., San Diego, Calif.). Significance was a P value <0.05.

RESULTS

Behavior of IpaH deletions in the Sereny reaction. Deletions of *ipaH*$_{7.8}$ alone (pWR700) or in combination with *ipaH*$_{4.5}$ (pWR730) (Table 1) in 2457T resulted in normal invasion of HeLa cells comparable to the wild-type strain. However, administration of pWR700 and pWR710 into guinea pig eyes resulted in a significantly exacerbated Sereny reaction; the eyes were considerably more swollen and redder, and they appeared highly irritated, with more inflammation than in the reaction seen with 2457T-str alone. Table 2 gives a representative example of one experiment with four guinea pigs (eight eyes) for each strain at each dose. Four such experiments were carried out with similar results. In the early stages of the disease, animals receiving the mutant strains often exhibited copious production of tears and had unusual redness and swelling around the eye and in the lower lid. The degree of inflammation, redness, and puffing of the eyes remained enhanced with the double mutant strain pWR730.

Evaluation of infection of *ipaH* mutant strains in HMDM and J774 cells. In J774 cells, infection with pWR700 (Table 1), pWR730 (deletion in both *ipaH*$_{7.8}$ and *ipaH*$_{4.5}$ [Table 1]), pWR740, and pWR750 (containing three deletions, including one in *ipaH*$_{7.8}$ [Table 1]) resulted in greatly increased recovery of CFU compared to the wild-type strain 2457T in a gentamicin-based assay (Fig. 1A). These CFU values were similar to those seen upon infection with plasmid-cured avirulent strains M4243A1 and M90T-55 or the *ipaB* mutant strain SC403 (Table 1). Strains pWR710 and pWR720, containing mutations only in the *ipaH*$_{4.5}$ and *ipaH*$_{9.8}$ genes, respectively, behaved like the wild-type strain. A similar contrast in CFU recovery values between pWR700 and 2457T could not be demonstrated in HMDM since CFU of *ipaH*$_{7.8}$ mutants released from HMDM were similar to the values seen with 2457T infection (Fig. 1B). As has been previously described, strain-dependent differences in CFU recovery were observed between M4243A1 and

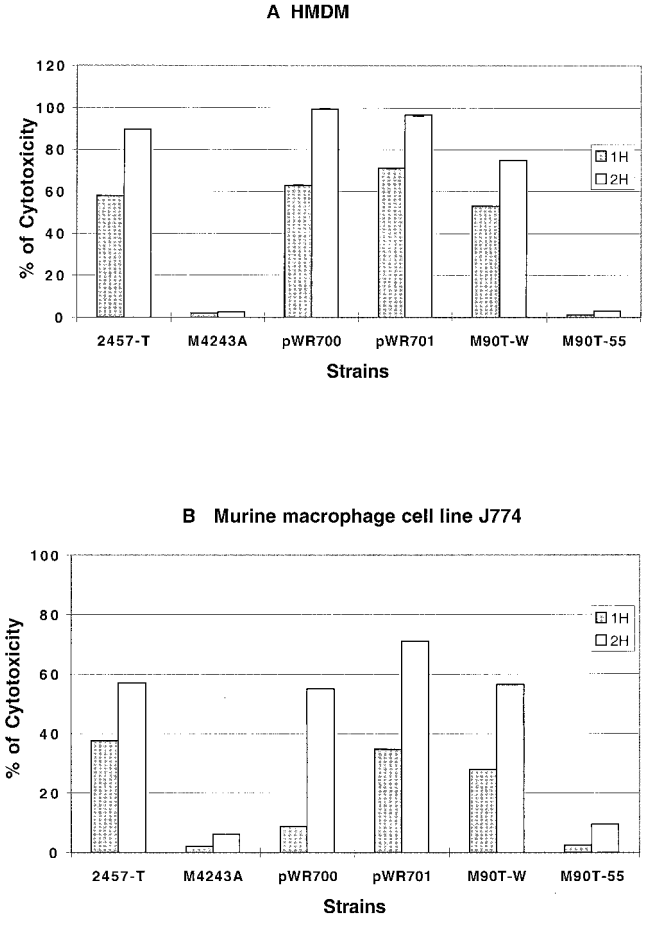

FIG. 4. Evaluation of cytotoxicity by LDH release of *S. flexneri* strains in HMDM (A) and J774 cells (B).

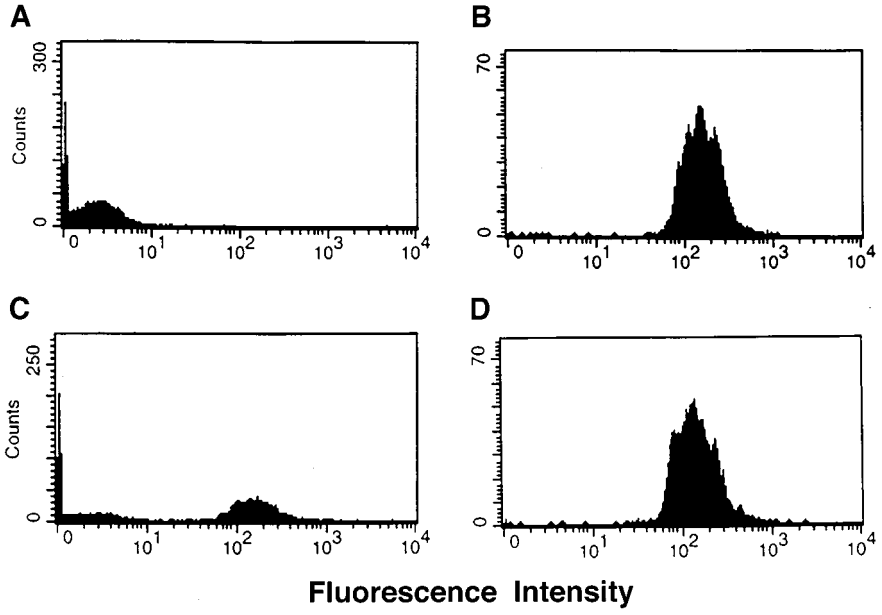

FIG. 5. DNA analysis by flow cytometry. J774 cells incubated with *Shigella* strains were lysed, and the released nuclei were labeled with propidium iodide and analyzed on a FACScan flow cytometer. Cells were infected with 2457T (A), M4243A1 (B), or pWR700 (C) or were noninfected (D).

M90T-55 (7, 8). Most of the experiments described below were carried out with the $ipaH_{7.8}$ single mutant pWR700.

Since bacteria were taken up by the macrophages within endocytic vacuoles, it was pertinent to determine the vacuolar compartment where the bacteria localized. *Shigella* strains were transformed with a plasmid carrying GFP and shown to remain both invasive and fluorescent within HeLa cells. J774 cells infected with GFP-*Shigella* were stained with LAMP-1 antibodies at different times after infection. After development with secondary antibodies, the cells were evaluated by confocal microscopy (Fig. 2). At 10 min after infection and subsequent staining, very few J774 cells infected with 2457T-GFP could be seen with internalized bacteria, while a few brightly staining green bacteria were seen outside the infected cells. This was more evident at 30 min of infection and staining (Fig. 2a and d). In contrast, at similar times after infection, many more cells with internalized bacteria colocalized within LAMP1-containing compartments were seen within J774 cells infected with either pWR700-GFP or M4243-GFP (Fig. 2b, c, e, and f). Colocalization was assessed by the presence of a bright yellow stain where the green bacteria superimposed on areas also stained with the red LAMP-1 protein. At 30 min after infection, a few green bacteria were seen outside the cells in pWR700-infected J774 cells but not in J774 cells infected with the avirulent strain (Fig. 2e and f). These experiments clearly indicated that after uptake, *Shigella* colocalized with LAMP-1 in acidic compartments.

Effect of chloroquine on the intracellular survival of *S. flexneri ipaH* mutant strains in HMDM and J774 cells. Since *Shigella* was shown to colocalize within acidic vacuoles, gentamicin alone and in combination with chloroquine was used to quantitate the *ipaH* mutants within macrophages after intracellular uptake. At moderate concentrations, tissue culture cells are impermeable to gentamicin. Gentamicin is therefore used in tissue culture invasion assays to kill extracellular bacteria. CFU recovered from infected cells lysed after gentamicin treatment represents the sum of intracellular bacteria present within the phagocytic vacuoles as well as freely in the cell cytoplasm. Chloroquine, on the other hand, enters eukaryotic cells, becomes concentrated within endosomes, and kills bacteria that may be present within these organelles. Thus, CFU recovered after treatment of infected cells with both gentamicin and chloroquine represent intracellular bacteria that are located freely in the cytoplasm, outside of the endosomes. No detectable differences were observed in the recovery of 2457T or M90T-W from either J774 cells or HMDM, in the presence of gentamicin alone or in the presence of both gentamicin and chloroquine. This finding indicates that virulent *Shigella* rapidly exits from the endocytic vacuole and is largely present freely in the macrophage cytoplasm. In contrast, the recovery of plasmid-cured strains M90T-55 and M4243A1 as well as IpaB mutant strain SC403 was more than a log higher in the presence of gentamicin alone than in the presence of the two drugs. This indicates that, in contrast to wild-type Shiga, the plasmid-cured strains are contained predominantly within the endocytic vacuoles (Fig. 3). The recovery of $ipaH_{7.8}$ mutant pWR700 from J774 cells in the presence of gentamicin alone or in the presence of gentamicin and chloroquine is similar to the distribution pattern seen with M4243A1 or M90T-55. Thus, pWR700, like the plasmid-cured strains, was trapped within the endocytic vacuoles. Although this distribution pattern seen with J774 cells was not obvious in infected HMDM (Fig. 3B), the absolute numbers of CFU indicated that more pWR700 than 2457T was present within the endocytic vacuoles of infected HMDM.

Characteristics of infection of macrophages with *ipaH* mutant. The release of LDH in culture supernatants after infection is used as a measure of cytoxicity (7, 8, 45, 46). Virulent *Shigella* releases LDH from infected macrophages, presumably with the aid of the IpaB protein after the bacteria exit from the endocytic vacuole (45). Infection of J774 cells and HMDM with M90T-W, 2457T, and pWR700 resulted in a time-dependent release of LDH activity (Fig. 4). In HMDM, maximum LDH release with all three strains occurred within 2 h of incubation, while in similarly infected J774 cells, maximal LDH release took longer (Fig. 4). Although little to no difference

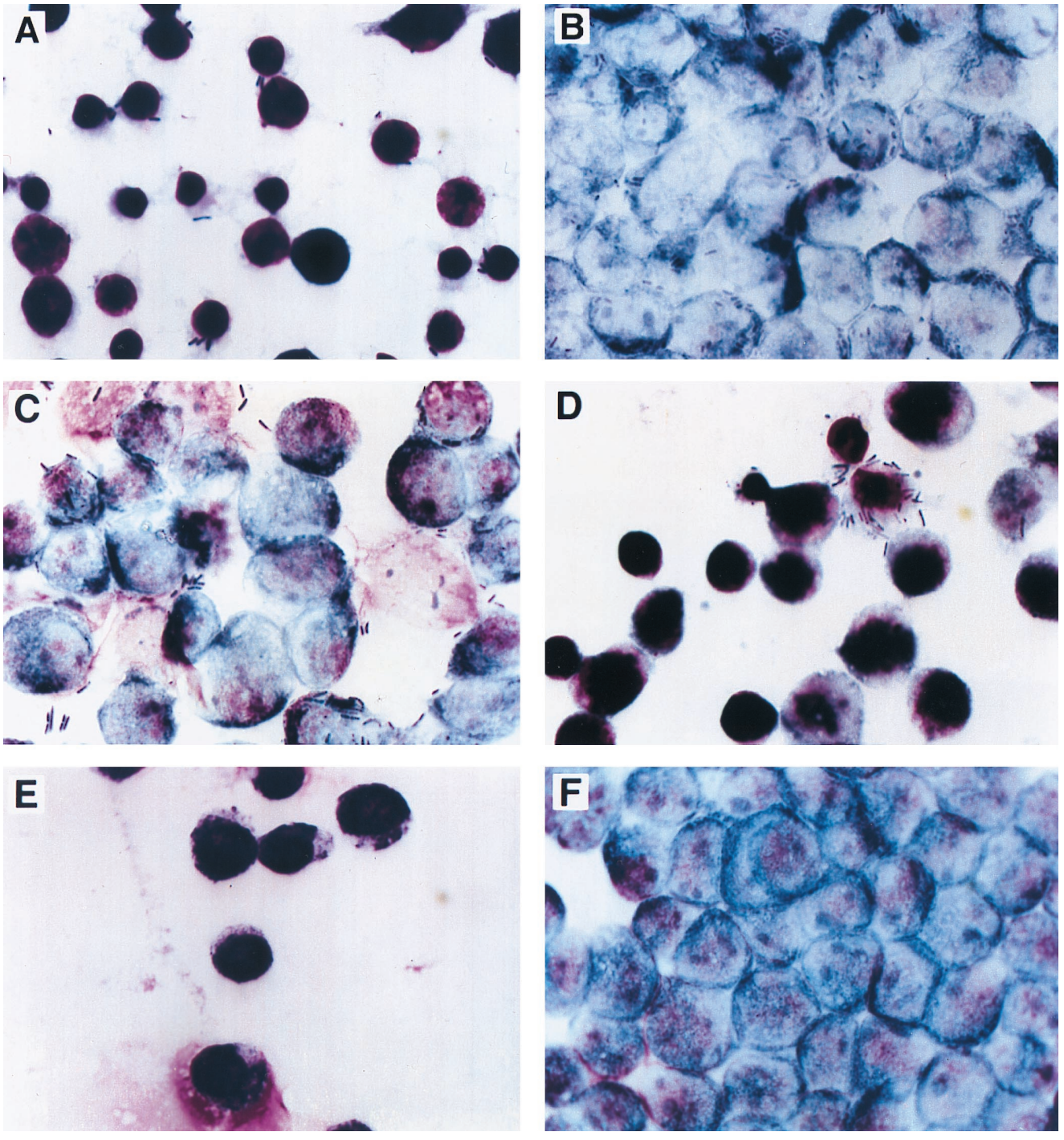

FIG. 6. Light microscopic analysis of J774 cells infected with *S. flexneri* strains. Bacteria were left in contact with macrophages for 30 min and then treated with gentamicin-containing medium for another 50 min (A to C) or 2 h (D). Macrophages were stained with a modified Wright's stain after infection with 2457T (A), M4243A1 (B), or pWR700 (Δ*ipaH*) (C and D). (E) J774 cells cultured in serum-free medium for 6 h showing apoptotic cells; (F) noninfected macrophages. Magnification, ×1,000.

was observed in the kinetics of LDH release from HMDM infected with either pWR700 or 2457T, substantially less LDH was released from pWR700-infected J774 cells at 1 h after infection compared to 2457T-infected cells. Eventually, however, both infections in J774 cells result in cell death since both pWR700 and 2457T contain the IpaB protein. These results suggest that pWR700 slows down the rate of cell death in J774 cells, presumably because it escapes more slowly from the endocytic vacuole than 2457T. These observations were further substantiated by flow cytometric analysis and microscopy described below.

Flow cytometry of J774 cells infected with 2457T for 2 h (Fig. 5A) showed a prominent hypodiploid peak of nuclei indicating cell death by apoptosis; in contrast, nuclei from M4243A1-

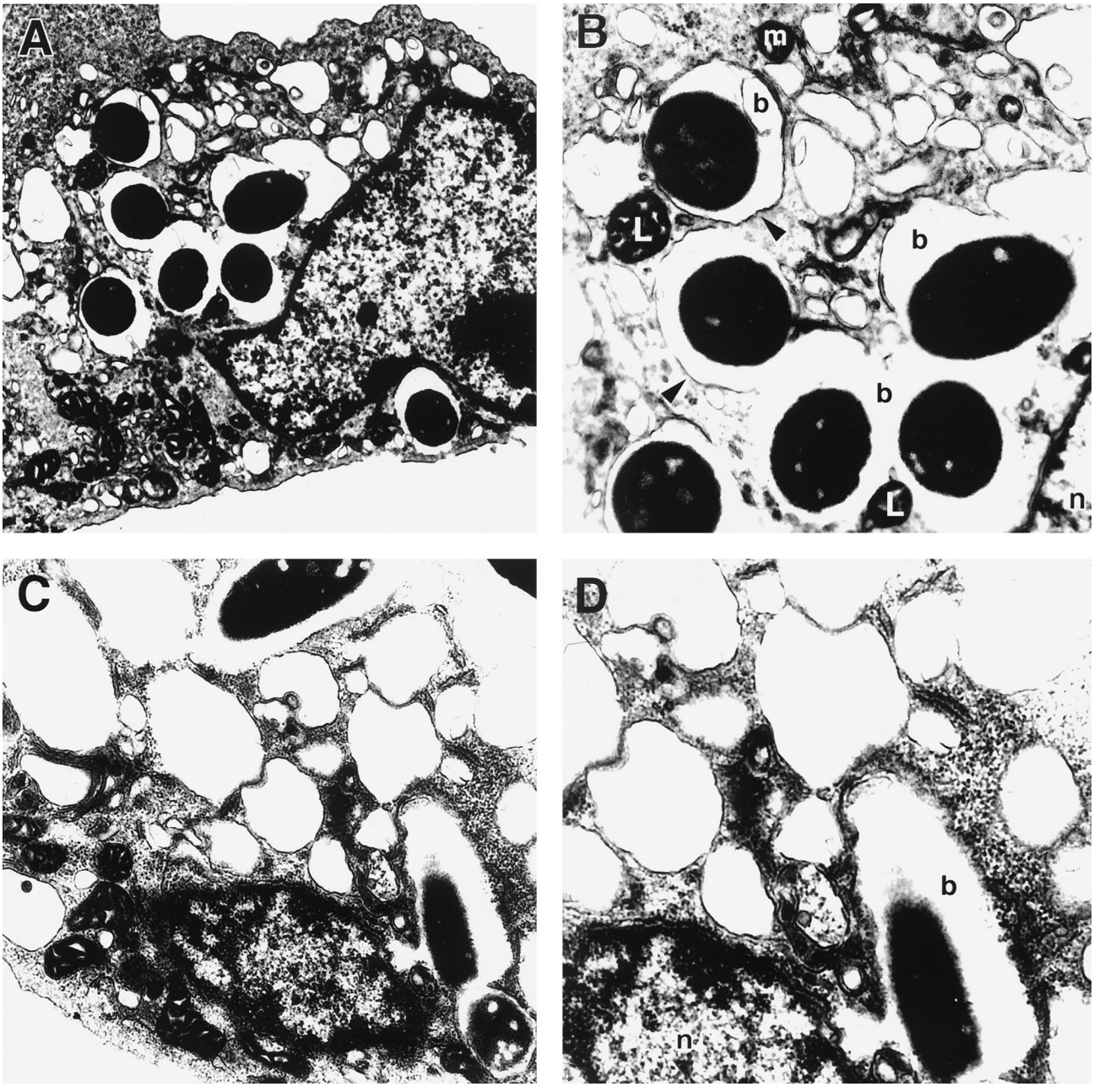

FIG. 7. TEM analysis of J774 cells infected with pWR700 (A and B) and 2457T (C and D) at 30 min postinfection. Magnifications: ×12,000 (B and D) and ×7,000 (A and C).

infected J774 cells (Fig. 5B) retained their normal phenotype (Fig. 5D). Under the same assay conditions, however, pWR700-infected J774 cells showed a less pronounced hypodiploid nuclear peak and a greater fraction of nuclei that retained the normal phenotype (Fig. 5C).

Light microscopy (Fig. 6) and TEM (Fig. 7) analysis of infected J774 cells further substantiated the observation that mutation in *ipaH*$_{7.8}$ resulted in a lower rate of exit from the endocytic vacuoles into the cytoplasm. While nuclei from J774 cells incubated for 1 h with 2457T looked uniformly condensed (Fig. 6A), nuclei from pWR700-infected cells, incubated under the same conditions, were only slightly contracted (Fig. 6C) and appeared more similar to cells infected with M4243A1 (Fig. 6B) or uninfected cells (Fig. 6F). At longer times of incubation, however, pWR700-infected cells demonstrated the morphology seen after infection with 2457T (Fig. 6D). Shortly after infection, more pWR700 bacteria were observed inside membrane-bound vacuoles than were observed with 2457T infection (Fig. 7A and B). Lysosomes were observed fused to the phagocytic vacuoles containing pWR700 bacteria (Fig. 7A and B), and some bacteria were seen in the process of division. Under the same conditions of incubation, 2457T was mostly observed free in the cytoplasm of infected macrophages, although bacteria were occasionally seen inside vacuoles (Fig. 7C and D). J774 macrophages infected with 2457T or pWR700 showed changes in nuclear morphology such as perinuclear

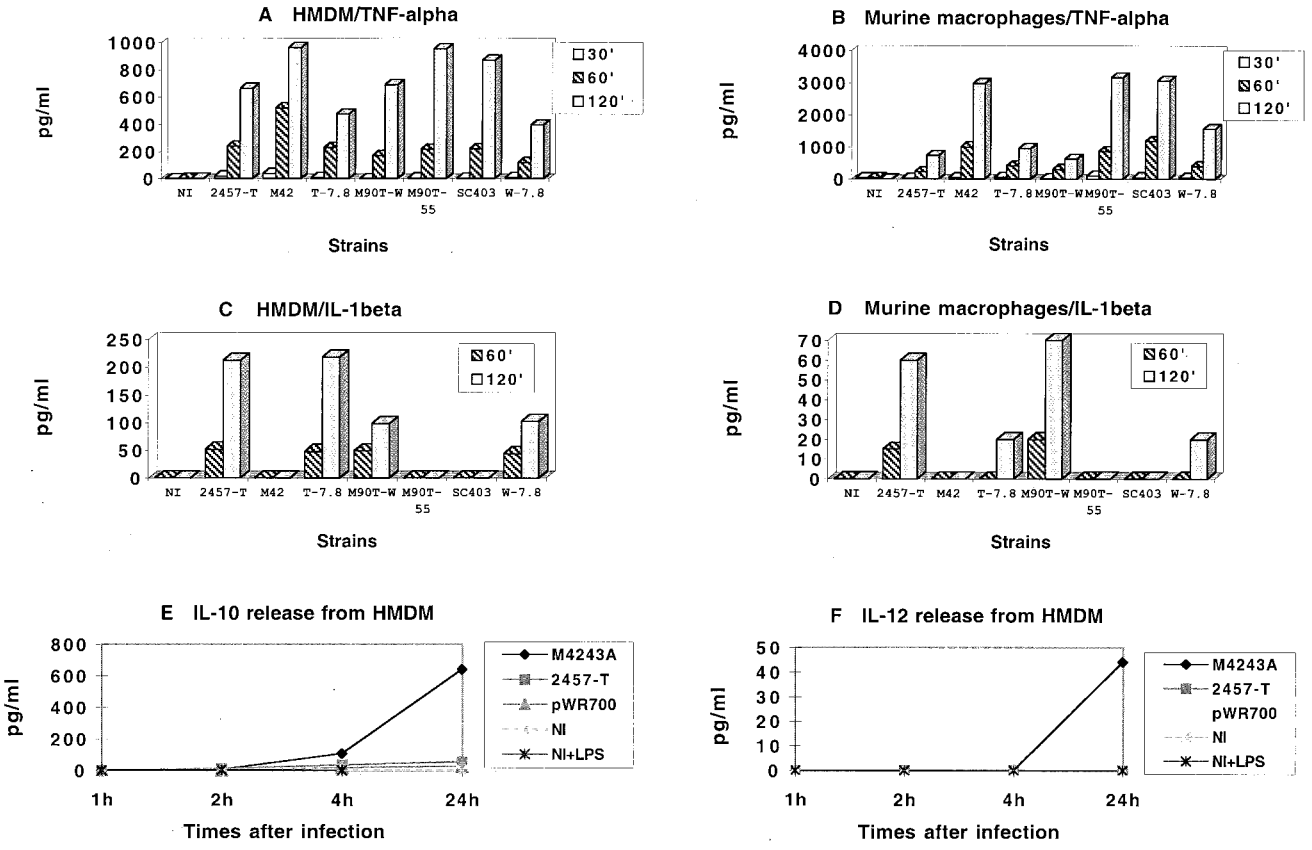

FIG. 8. (A to D) TNF-α (A and B) and IL-1β (C and D) release into the supernatants of HMDM (A and C) and murine macrophages (B and D). Supernatants were collected at 30, 60, and 120 min postinfection and tested for cytokine production by ELISA. (E and F) IL-10 (E) and IL-12 (F) release into culture supernatants of HMDM. Supernatants were collected at 1, 2, 4, and 24 h postinfection. Characteristics of the bacterial strains are listed in Table 1. NI, not infected.

chromatin aggregation, an early characteristic of cells undergoing apoptosis (Fig. 7). These infected macrophages were also highly vacuolated and had intact plasma membranes, resembling apoptotic cell death described previously (7, 8, 45).

In both HMDM and J774 cells, the levels of TNF-α and IL-1β increased with increased times of *Shigella* infection. In general, *Shigella*-infected HMDM released proportionately less TNF-α and more IL-1β than J774 cells infected with the same strains (Fig. 8A to D). IL-1β release from both types of macrophages was detected only in cells incubated with IpaB-expressing strains such as M90T-W, 2457T, pWR700, and pWR800 (Fig. 8C). More IL-1β levels were observed in HMDM infected with 2457T than M90T-W, but this strain-dependent difference was not observed with murine macrophages (Fig. 8C and D). While TNF-α release was unaffected by the *ipaH*$_{7.8}$ mutation in both types of macrophages, the IL-1β levels released from pWR700-infected J774 cells were consistently threefold lower than those infected with 2457T or M90T-W (Fig. 8D). This difference could not be observed with HMDM. An interesting finding in this regard was the detection of IL-10 and IL-12 late in infection from culture supernatants of HMDM infected with avirulent *Shigella* strains (Fig. 8E and F). This activity could not be seen with macrophages treated with lipopolysaccharide (LPS) alone.

Characteristics of *Shigella* infection in 1-day-old human monocytes. Since the behavior of *ipaH* mutants in J774 could not be reproduced in HMDM, 1-day-old human monocytes were used as host cells to investigate the behavior of *Shigella* strains. Infection of these monocytes with wild-type and *ipaH*$_{7.8}$ mutant strains in the presence of gentamicin or gentamicin and chloroquine clearly indicate that a greater proportion of the *ipaH* mutants were present within endocytic vacuoles as compared to 2457T or M90T-W (Fig. 9A). Although the CFU recovery was more than 1 log lower than the values seen for plasmid-cured strains M4243A1, M90T-55, or the *ipaB* mutant SC403, the distribution pattern of the *ipaH* mutant in the presence of these drugs was similar to that for plasmid-cured or *ipaB* mutants (Fig. 8A). In monocytes, LDH release after infection with virulent *Shigella* was much slower than in HMDM. Only 25% of maximal LDH activity was released from 1-day-old monocytes after 4 h of incubation with virulent strains infected at the same MOI as with HMDM (Fig. 9B). Furthermore, pWR700-infected human monocytes infected for the same period of time yielded twofold less LDH than monocytes infected with 2457T. Cytotoxicity was only partially restored in a complemented strain pWR701. No LDH activity was detected with plasmid-cured strains (Fig. 9B).

To determine the mode of cell death in human monocytes after *Shigella* infection, nuclei from infected monocytes were subjected to DNA fragmentation analysis on agarose gels (Fig. 10). One-day-old monocytes infected with 2457T, M90T-W, or pWR700 for 4 h showed evidence of DNA fragmentation suggestive of apoptosis. Fragmentation of DNA was also observed in infected J774 cells but not in HMDM infected with the same strains (Fig. 10). These observations were further substantiated by both light microscopy (Fig. 11) and TEM (Fig. 12). Light microscopy indicated that 1-day-old monocytes infected with 2457T for 2 h had a greater proportion of cells showing apop-

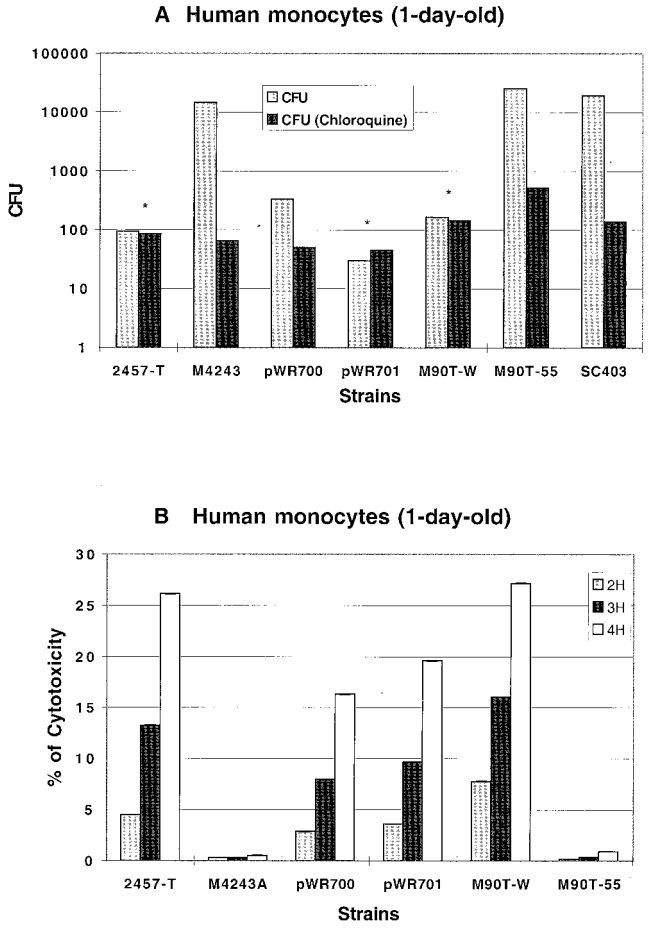

FIG. 9. (A) Effect of chloroquine on the intracellular survival of *S. flexneri* strains in 1-day-old monocytes. (B) Evaluation of cytotoxicity by LDH release assay in 1-day-old monocytes infected with *S. flexneri* strains. *, *P* value not significant comparing CFU recovered in the presence of gentamicin and gentamicin plus chloroquine. Error bars show means ± standard deviations.

totic, condensed nuclei than monocytes infected with pWR700 for the same length of time. Again, these observations were indicative of delayed exit from endocytic vacuoles in the absence of the $ipaH_{7.8}$ gene (Fig. 11B and C). TEM analysis of 2457T-infected 1-day-old monocyte nuclei clearly showed characteristic features of apoptosis, including compacted nuclei and loss of intracellular organelle morphology (Fig. 12).

DISCUSSION

The role of *Shigella ipaH* genes during pathogenesis remains unclear. Mutations in *ipaH* alone or in both $ipaH_{7.8}$ and $ipaH_{4.5}$ do not affect invasion in HeLa cells or plaque assay, indicating that these genes are not critical for the initial entry or dissemination of the bacteria within epithelial cells. However, these mutations induce an exaggerated Sereny response in guinea pig eyes, suggesting that $ipaH_{7.8}$ may play a role in modulating the inflammatory response elicited by infection. Whether this observation equates to a physiological response in the colon of a natural host, such as humans and primates, remains to be determined. It is hoped that testing of *ipaH* mutants in a primate model may shed some light on the physiological role of these genes during pathogenesis.

Whether *ipaH* interacts with other bacterial or host proteins remains to be determined. The presence of a characteristic LRR region at the amino-terminal end of each *ipaH* gene classifies it as a member of the larger superfamily of LRR-containing proteins which include bacterial, plant, and vertebrate proteins (for reviews, see references 3 and 6). In a database search of proteins that are likely to fold into a parallel beta helix, 50% belonged to proteins with sequences containing LRRs (14). The high level of sequence conservation in the LRR superfamily indicates that the LRR region is likely to be of structural and/or functional significance and may involve protein-protein interactions (3). The functional role of the LRR region is clearly different for different proteins. While *ipaH* does not appear to have a role in invasion of epithelial cells, a functional analysis of internalin A, a surface protein from the bacterial pathogen *Listeria monocytogenes*, demonstrates that the amino-terminal region, encompassing the LRR and interrepeat regions, is necessary and sufficient to promote bacterial entry into cells expressing its receptor E-cadherin (23). Other LRR-containing bacterial proteins whose functions are less clear are the *Yersinia* YopM protein, which like *ipaH* shows heterogeneity (2, 6), and the more recently described hypothetical 60.5-kDa protein Y4FR from *Rhizobium* sp. strain NGR234 (10). The gene (*bspA*) encoding a cell surface-associated protein of *Bacteroides forsythus* contains 14 complete repeats of 23 amino acid residues that show partial homology to LRR motifs. BspA binds strongly to fibronectin and fibrinogen in a dose-dependent manner and inhibits the binding of *B. forsythus* cells to these extracellular matrix components. It has been speculated that BspA mediates the binding of bacteria to extracellular matrix components and clotting factors. This binding may be important in the colonization of the oral cavity by this bacterium (38). Several eukaryotic proteins which play critical roles in immune responses to infection and inflammation also contain LRR repeats; these include monocyte cell surface molecule CD14, human RP105 protein, which is specifically expressed on mature B cells and has an important regulatory role in B-lymphocyte function, and members of the proteoglycan family (34). It is believed that the LRRs in these proteins function in protein-protein interaction, cell adhesion, and cellular signaling. It is tempting to speculate that IpaH, by virtue of its LRR domain, competes as a ligand with host LRR-containing proteins that play critical roles in host defense to infection.

Shigella infection induces apoptosis in 1-day-old monocytes in vitro compared to HMDM, where the cell death after *Shi-*

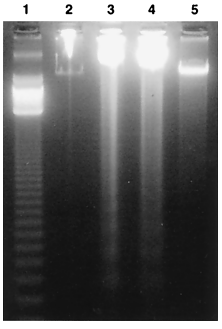

FIG. 10. DNA fragmentation assay on agarose gel. DNA was isolated from human monocytes infected with different *Shigella* strains. The DNA was electrophoresed on a 1.2% agarose gel for 3 h at 100 V. DNA was isolated from monocytes infected with 2457T (lane 3), pWR700 (lane 4), and M4243A1 (lane 5). Lane 2 represents DNA extracted from noninfected monocytes; lane 1 contains a 123-bp DNA ladder molecular weight marker (GIBCO-BRL, Gaithersburg, Md.).

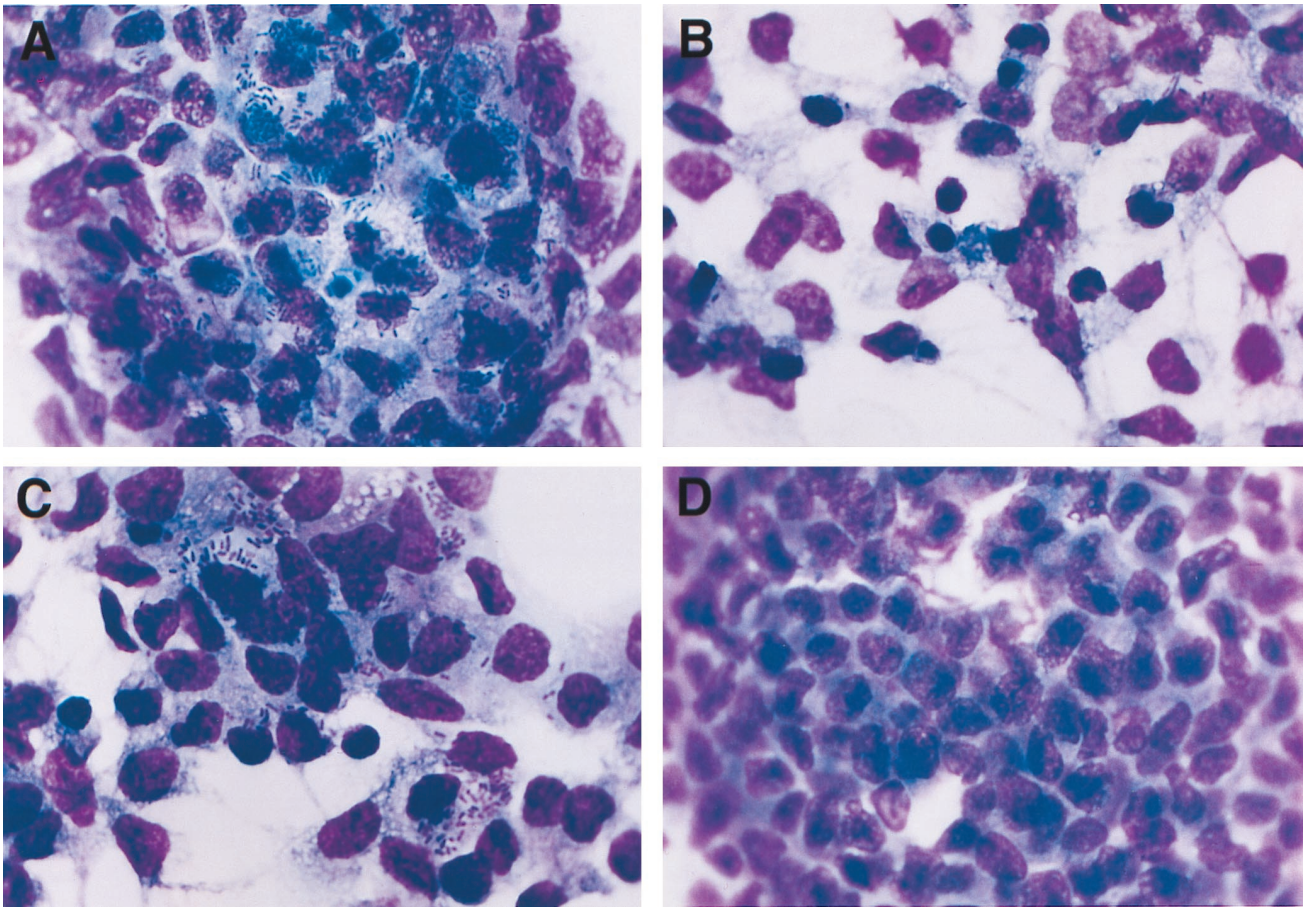

FIG. 11. Light microscopic analysis of human monocytes infected with *S. flexneri* strains. Bacteria were left in contact with monocytes for 30 min followed by gentamicin treatment for up to 4 h. Monocytes were stained with a modified Wright's stain after infection with M4243A1 (A), 2457T (B), and pWR700 (C). (D) Noninfected monocytes. Magnification, ×1,000.

gella infection occurs by oncosis. From the studies described here, it is clear that the characteristics of infection of monocytes in vitro are different from those of HMDM in vitro. Our previous reports have indicated that a time-dependent differentiation of human monocytes into macrophages in in vitro studies is an important factor affecting the mode of cell death occurring after *Shigella* infection (8; Fernandez-Prada et al.). A more recent report has indicated that *S. flexneri* can induce apoptosis or oncosis in U397 cells depending on their differentiation state (30). These observations may have physiological relevance since the initial interaction of the pathogen probably occurs with the resident, activated, macrophages in the lymphoid follicles of the colonic epithelium. The macrophages are killed quickly by oncosis, allowing the bacteria to subsequently escape into the adjacent epithelial cells. In the process, inflammatory mediators are released, setting up the cascade of events that ultimately leads to polymorphonuclear leukocyte infiltration at the mucosal lumen, necrosis of the epithelial layer, and resolution of the infection. In vivo, infiltrating monocytes at sites of bacterial infection may be at different stages of activation, and this heterogeneity may explain why only a subset of monocytes/macrophages in tissue sections of patients with shigellosis showed apoptotic nuclei (18). It is known that fresh human monocytes, cultured in the absence of serum, LPS, and growth factors, readily undergo apoptosis within 48 h (27, 28). Apoptosis can also be induced by the expression of Fas and Fas ligand in these cells. However, upon cultivation with serum, LPS, growth factors, and cytokines, these human monocytes readily differentiate and become activated (HMDM) such that expression of Fas and Fas ligand fails to induce apoptosis (21). The molecular mechanisms involved in activation-induced survival signals in monocytes remain generally uncharacterized. Both rapid down-regulation at the mRNA level of caspase-8/FLICE, the most apical protease in the death receptor pathway, as well as induction of Bfl-1, an antiapoptotic member of the Bcl-2 family, have been implicated (32). LPS-treated monocytes are resistant to the apoptotic action of Fas. Under these conditions, LPS did not down-regulate Fas but inhibited the Fas-dependent elevation of ROI. Therefore, monocytes appear to have a protective mechanism that can interfere directly with the Fas-induced pathway of cell suicide (39). There may be other differences between monocytes and macrophages related to rates of bacterial multiplication, rates of entry and exit from endocytic vacuoles, and rates of undergoing cell death in tissue culture experiments. It may be pertinent here that in an effort to investigate the molecular mechanisms of programmed cell death in human T cells, MAbs to dying cells have been developed. One of these MAbs, antiporimin, efficiently induces a unique type of cell death in Jurkat cells which is very similar to oncosis and is distinct from complement-dependent cytolysis or complement-independent apoptosis (44).

The *ipaH*$_{7.8}$ mutant escaped slowly from the endocytic vacuole in both J774 cells and 1-day-old monocytes. The main difference between virulent *Shigella* infection of 1-day-old monocytes and J774 cells is in the overall recovery of CFU of

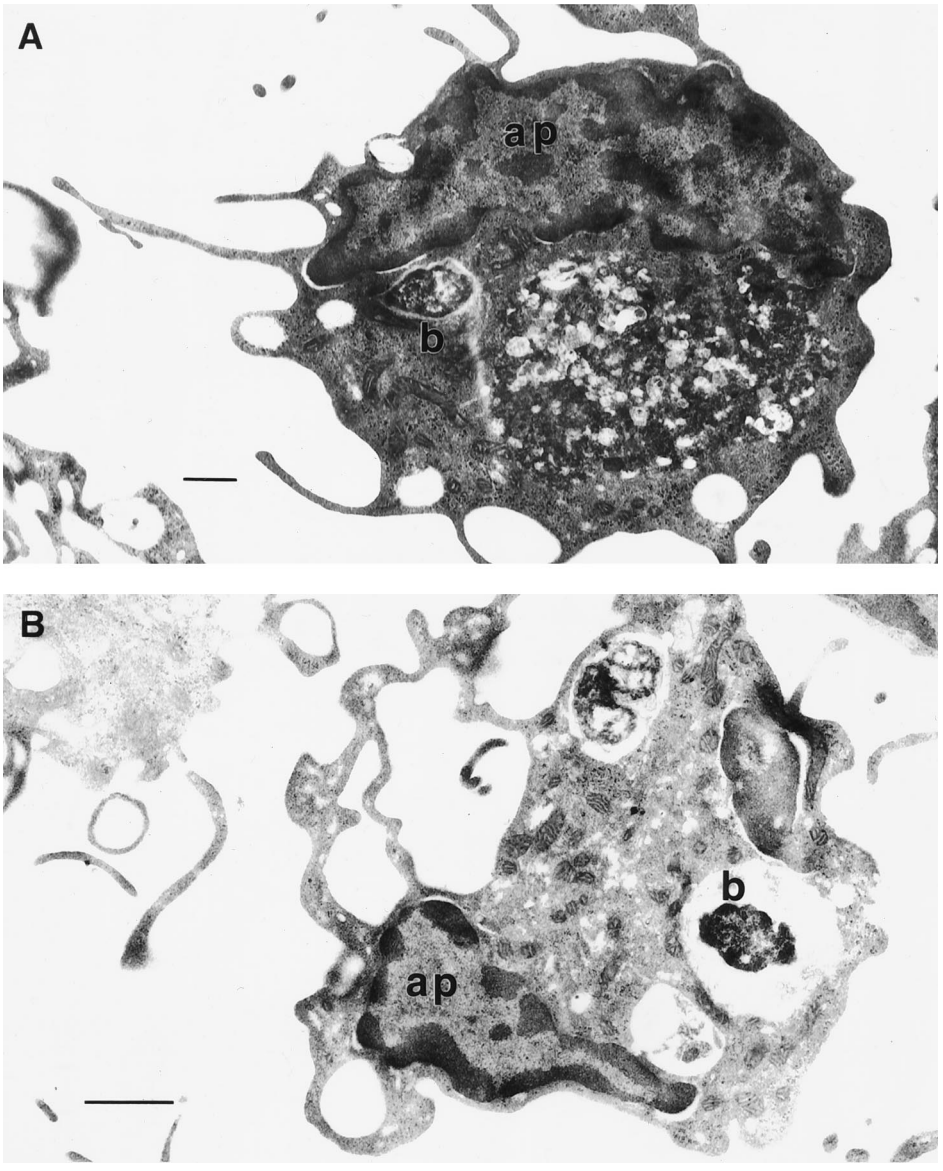

FIG. 12. TEM of *Shigella*-infected human monocytes. Human monocytes were infected for 30 min followed by 4 h in gentamicin-containing medium with 2457T (A) and pWR700 (B). Bar markers: 1 μm. b, bacteria; ap, apoptotic nuclei; n, normal nuclei.

the mutant strain after infection. However, this could be partly related to the fact that 1-day-old monocytes in tissue culture represent a heterogeneous population of cells. It is not certain from the experiments described here whether all of the 1-day-old monocytes are at the same stage of differentiation and what percentage of them are undergoing spontaneous apoptosis. Cell sorting by expression of surface markers leading to a more homogeneous population of cells may shed light on these differences.

It is not clear why the role of *ipaH* mutants is not so easily demonstrable in HMDM although CFU recovered after incubation of HMDM in the presence of gentamicin alone and gentamicin and chloroquine do seem to indicate that a greater number of the *ipaH* mutants were within endosomes compared to the wild-type strain. It is possible that the *ipaH* gene plays a bigger role in monocytes than macrophages. These results also suggest that functions of some bacterial genes such as *ipaH* may be better assayed in vitro in cells such as J774, where the events occurring after infection in vitro are slower than in HMDM, where the cells die rapidly by oncosis. The interactions between immune effector cells and bacterial pathogens in vivo occur in a complex microenvironment rich in cytotoxic inflammatory mediators and reactive free radical species (39). In vitro, the bacterium-host interactions will be determined by several experimental variables, which include purity and phenotype specificity of the cell studied, species and tissue origin of the cell, adherence to surfaces, presence of LPS, native or recombinant cytokines, and inhibitors, serum concentrations, and other sensitivities and specificities of the assays. These variables will also affect the manner in which bacterial proteins register their functions.

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