

## Characterization of *Salmonella*-Induced Cell Death in Human Macrophage-Like THP-1 Cells

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***Salmonella* strains are facultative intracellular pathogens that produce marked cytopathology during infection of host cells. Different forms of cytopathic effects have been associated with the virulence systems encoded by the two *Salmonella* pathogenicity islands (SPI-1 and SPI-2) and the *spv* locus. We used *Salmonella enterica* serovar Dublin to investigate the induction of cytopathology during infection of the human macrophage-like cell line THP-1. Analysis of host cells by flow cytometry using a fluorescent terminal deoxynucleotidyltransferase dUTP-biotin nick end labeling (TUNEL) assay revealed that 70% of THP-1 cells showed DNA fragmentation after 4 h of infection, increasing to greater than 90% by 5.5 h. Moreover, the results showed that gentamicin-killed or chloramphenicol-treated bacteria did not induce DNA fragmentation. Serovar Dublin strains with mutations in SPI-1, SPI-2, or *spvB* induced these cytopathic effects similar to wild-type bacteria. In contrast, a mutation in the *phoP* regulatory gene abolished DNA fragmentation in the TUNEL assay. Caspase-3 activation was detected during *Salmonella* infection of THP-1 cells, but caspase-8 and caspase-9 activities were not found. However, inhibition of caspase-3 did not block *Salmonella*-induced DNA fragmentation. These results identify a previously undetected apoptotic effect in *Salmonella*-infected cells that is dependent on *phoP* gene function.**

*Salmonella enterica* comprises a group of bacteria that produce a variety of diseases, ranging from self-limited enteritis to fatal, disseminated infections (23, 25, 26). The hallmarks of *Salmonella* pathogenesis are bacterial invasion and intracellular infection, processes that require the expression of a number of bacterial virulence loci. *Salmonella enterica* strains encode two distinct type III protein secretion systems (TTSS) that function in each of these two phases of the pathogenic process (6, 11, 30). These two secretion systems deliver bacterial virulence proteins into the cytoplasm of the host cell to modulate key signal transduction processes. The system encoded by *Salmonella* pathogenicity island I (SPI-1) is required for invasion of the bacterium into intestinal epithelial cells, while systemic infections and intracellular accumulation of *Salmonella* are dependent on the function of SPI-2. Furthermore, a critical regulon controlled by the two-component PhoP/PhoQ system is essential for the intracellular survival and replication of *Salmonella* strains (5, 21). In addition, the *spv* locus, carried on virulence plasmids by certain serovars of the *S. enterica* subspecies I lineage, greatly enhances the systemic virulence of these strains (10). The *spv* virulence phenotype is primarily due to the SpvB protein, an ADP-ribosyl transferase that modifies actin monomers and prevents polymerization, leading to loss of the F-actin cytoskeleton in infected cells (17).

*Salmonella* possesses a variety of mechanisms to produce cytopathic effects in infected host cells. *Salmonella* induces apoptosis in intestinal epithelial cells by a process that involves invasion mediated by the SPI-1 effectors, but that also requires

the functions of SPI-2 and the *spv* locus (24). Considerable work has focused on the cell death produced by *Salmonella* infection of macrophages. Experimental conditions, including the growth phase of the bacteria and treatment with opsonins such as normal serum, have a profound effect on the cytotoxic response of macrophages infected with *Salmonella*. Use of nonopsonized bacteria grown under conditions that maximize expression of SPI-1 leads to the rapid death and lysis of macrophages in a process that resembles necrosis as well as programmed death (2). This rapid cytotoxicity is mediated by the activation of caspase-1 by the SPI-1 effector protein SipB, but the detailed mechanism of cell death has not been established (13). A similar reaction has been described in dendritic cells (29). In caspase 1-deficient macrophages, SipB can activate caspase-2, with subsequent activation of caspase-3, -6, and -8, release of cytochrome *c* from mitochondria, and cytopathology with features of apoptosis (15). SipB also induces the formation of autophagic vesicles containing mitochondrial and endoplasmic reticulum components in caspase-1-deficient cells (12). A different cytotoxicity is seen when *Salmonella* is grown under conditions that repress expression of SPI-1, particularly if the bacteria are also opsonized with normal serum. Macrophages infected with these bacteria exhibit delayed cytopathology beginning about 12 h after infection (18, 19, 28). SipB and SPI-1 function are not required but SPI-2 is essential, and the process displays features of apoptosis, including nucleosome and DNA fragmentation. The *Salmonella* effectors mediating this delayed cytopathology have not been identified, although the SpvB protein appears to be required in human monocyte-derived macrophages (3, 17, 18).

In the present study, we used the human-derived macrophage-like cell line THP-1 to identify bacterial genes and host cell caspase activities involved in *Salmonella*-induced cell death. We found that *Salmonella* proliferated in THP-1 cells

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and induced extensive DNA fragmentation by 6 h following infection with *S. enterica* serovar Dublin. Live bacteria with active protein synthesis were required to induce this cytopathology. Mutations in SPI-1 (*sipB*), SPI-2 (*ssaV* and *ssaJ*), and *spvB* did not decrease the induction of DNA fragmentation. However, a *phoP* mutant caused much less cell death, despite similar numbers of intracellular bacteria. Caspase-3 activation was detected during infection with strains inducing cell death, but DNA fragmentation was not affected by blocking caspase-3 activity.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Wild-type *S. enterica* serovar Dublin and mutant derivatives *ssaV::mTn5*, *ssaJ::mTn5*, *sipB::aphT*, *spvBmut1*, and *phoP::Tn10* (3, 5) were used in these studies. Bacteria were cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar with antibiotics as appropriate: kanamycin at 50 mg/liter or tetracycline at 10 mg/liter. To obtain stationary-phase bacteria for infection of THP-1 cells, LB broth was inoculated with a single colony and grown overnight with vigorous shaking. Bacteria were harvested by centrifugation, washed with Dulbecco's phosphate-buffered saline (PBS), and opsonized for 20 min at 37°C in 50% human fresh-frozen AB serum (SeraCare Life Sciences). Opsonized bacteria were diluted 10-fold in Hanks salt solution and used immediately for inoculation of THP-1 cells.

**Cell culture and infection.** THP-1 cells (27) were maintained at a density of  $2 \times 10^5$  to  $1 \times 10^6$  cells/ml in RPMI 1640 with L-glutamine and 10% heat-inactivated fetal bovine serum supplemented with 2-mercaptoethanol (55  $\mu$ M), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). One day prior to infection, the cells were harvested, washed, and resuspended in fresh medium without 2-mercaptoethanol, penicillin, or streptomycin. To induce differentiation into adherent macrophage-like cells, 12-*O*-tetradecanoylphorbol-13-acetate (PMA) was added to 20 ng/ml and the cells were seeded into culture wells at a concentration of  $7 \times 10^5$  cells/ml (27). The next day, medium and nonadherent cells were removed and replaced with fresh complete medium without antibiotics. After 2 hours, the cultures were infected with opsonized *Salmonella* strains at a multiplicity of infection of 10 bacteria/THP-1 cell. Culture plates were centrifuged at  $200 \times g$  for 5 min and incubated at 37°C for 30 min to allow phagocytosis to occur. Under these conditions, essentially all cells are infected with bacteria. The medium was then replaced with fresh medium containing gentamicin (20  $\mu$ g/ml) and incubated for the indicated times. The total cell population in the well was harvested by gentle scraping with a cell scraper. To enumerate viable bacteria, an aliquot of the harvested cell population was centrifuged, the macrophages were lysed by 0.5% deoxycholate in Dulbecco's PBS, and the bacteria were diluted and plated on LB agar as described elsewhere (18).

**Gentamicin and chloramphenicol treatment of the bacterial inocula.** To kill *Salmonella* strains with gentamicin, bacteria were cultured in LB broth with shaking as described above and grown to an optical density at 600 nm of 0.8. Gentamicin at 100  $\mu$ g/ml was added, and the incubation continued for one more hour. Bacterial viability determined by colony counts decreased to less than 0.01% of the original number under these conditions (18). Gentamicin-treated bacteria were opsonized and used directly to inoculate THP-1 cells. For the chloramphenicol treatment, bacteria were grown and opsonized normally. Bacteria and chloramphenicol (100  $\mu$ g/ml) were added simultaneously to THP-1 cells. After 30 min of phagocytosis, the medium was replaced with fresh medium without chloramphenicol but containing gentamicin (20  $\mu$ g/ml), and the infected cells were harvested at the indicated times.

**Labeling of DNA fragmentation with fluorescein and flow cytometric analysis.** THP-1 cytopathology was evaluated by flow cytometric analysis of cellular DNA fragmentation. At the indicated times after infection, cells were harvested as described and collected by centrifugation at  $1,000 \times g$  for 15 min, washed once with PBS, and fixed, and the DNA fragments were end labeled with fluorescein (terminal deoxynucleotidyltransferase dUTP-biotin nick end labeling [TUNEL] assay) using a commercial kit (Oncogene Research Products). Suspended cells were analyzed for fluorescence by flow cytometry; positive and negative controls were used to set the gating to determine the percent TUNEL-positive cells. The percent positive cells was determined from the fraction of cells with significantly increased fluorescence compared to simultaneous uninfected control cells.

**Assay of caspase activity.** After infection with *Salmonella*, the THP-1 cells were collected by centrifugation at  $1,000 \times g$  for 15 min at room temperature and washed once with PBS. The cells were resuspended in lysis buffer at a density of  $10^7$  cells/ml and incubated on ice for 10 min. The cell debris was removed by

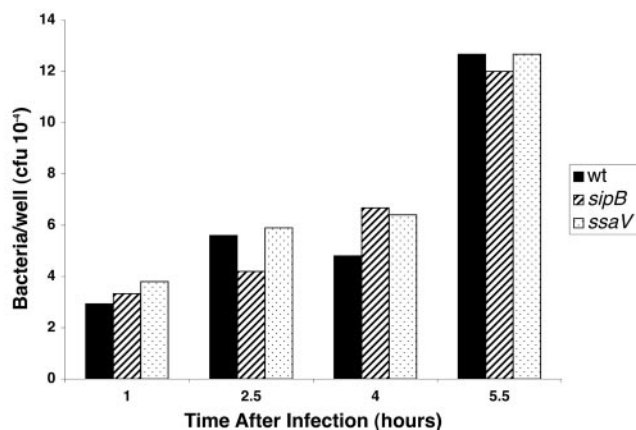


FIG. 1. Growth of *Salmonella* strains in THP-1 cells. Opsonized bacteria were used to infect cells as described, the cultures were harvested, and aliquots from each culture well were processed for viable organisms at the indicated times.

centrifugation at  $16,000 \times g$  for 5 min at 4°C, and the supernatant was used for the colorimetric assay of caspase-3, -8, and -9 activities using commercial kits (caspase-3 kit from Sigma and caspase-8 and -9 kits from Calbiochem). Protein concentrations were determined using the Bio-Rad protein assay according to the manufacturer's instructions.

#### RESULTS

**Growth of *Salmonella* strains in THP-1 cells.** Previous studies have shown that wild-type *S. enterica* serovar Typhimurium is able to proliferate in THP-1 cells (27). We found similar growth of wild-type serovar Dublin inside THP-1 cells, as shown in Fig. 1. In addition, we found no difference in the phagocytosis or growth of strains with mutations in SPI-1 (*sipB*) or in SPI-2 (*ssaV*). Studies with independent mutants in SPI-1 or SPI-2 confirmed these results (data not shown). We have also found no effect of SPI-2 on the early intracellular growth of serovar Typhimurium in THP-1 cells (L. Sly and D. Guiney, unpublished data). Furthermore, mutations in *spvB* and *phoP* did not affect the early proliferation of *Salmonella* in THP-1 cells. Overall, THP-1 cells represent a permissive environment for *Salmonella* growth.

**Cytopathology induced by *Salmonella* infection of THP-1 cells.** We examined the effects of *Salmonella* infection on DNA fragmentation in THP-1 cells as measured by flow cytometric analysis of TUNEL-stained cells. As shown in Fig. 2, the number of TUNEL-positive cells progressively increased with time after infection with wild-type serovar Dublin. Approximately 30% of cells were TUNEL positive after 1 hour of infection, increasing to nearly 100% by 5.5 h. The strains with mutations in *sipB* or *ssaV* also induced DNA fragmentation in THP-1 cells at the same level as wild type, and similar results were found with an *ssaJ* and an *spvB* mutant (data not shown). The induction of DNA fragmentation required live bacteria, since organisms killed by gentamicin treatment did not induce cell death, even after 24 h of infection (Fig. 3). Furthermore, transient inhibition of bacterial protein synthesis by simultaneous addition of bacteria and chloramphenicol to THP-1 cells, followed by removal of chloramphenicol after phagocytosis, reduced the cytopathic effect approximately 50% at 7 h postin-

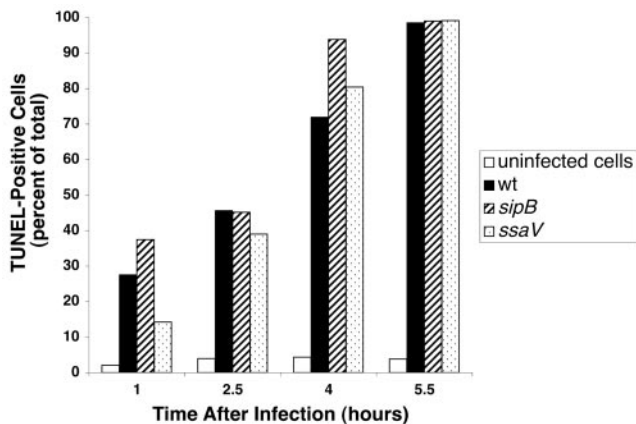


FIG. 2. Cytopathology caused by *Salmonella* strains in THP-1 cells. Cells from the same experiment as in Fig. 1 were processed for cytopathology in a fluorescent TUNEL assay as described in the text.

fection (Fig. 4). This treatment did not affect phagocytosis or the viability of the infecting bacteria, although growth of the chloramphenicol-treated organisms was delayed as expected (data not shown).

**Dependence of apoptosis on the *Salmonella phoP* gene.** In contrast to the results with mutants in SPI-1, SPI-2, and *spvB*, we found that a *phoP* mutant was unable to induce DNA fragmentation in THP-1 cells. As shown in Fig. 5A, very low levels of TUNEL-positive cells were found after infection with the *phoP* mutant. The *phoP* mutant produced less than 10% positive cells even after 4 and 5.5 h of incubation, conditions that induced close to 100% positive cells with the other strains. As shown in Fig. 5B, comparable numbers of bacteria for all the strains were recovered from infected cells at both time points, excluding the possibility that the *phoP* result was due to lower numbers of intracellular organisms.

**Caspase activation during *Salmonella* infection of THP-1 cells.** We determined whether caspase-3, -8, or -9 was activated by *Salmonella* infection by using biochemical assays of lysates from infected cells. As shown in Fig. 6, caspase-3 activity in-

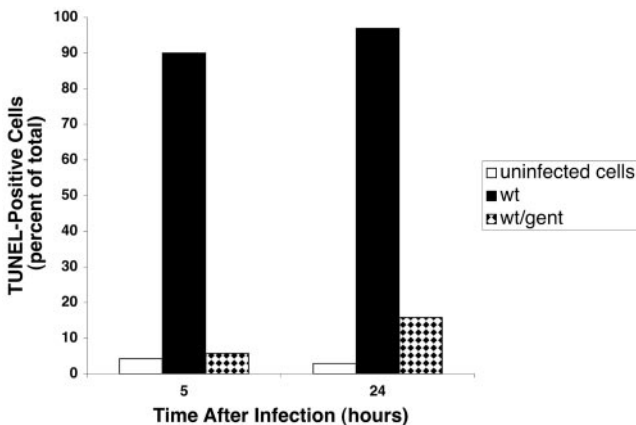


FIG. 3. Effect of pretreatment of the bacterial inoculum with gentamicin. Equal numbers of untreated and gentamicin-treated wild-type *Salmonella* bacteria were used to infect THP-1 cells, and the cytopathology was determined at the indicated times.

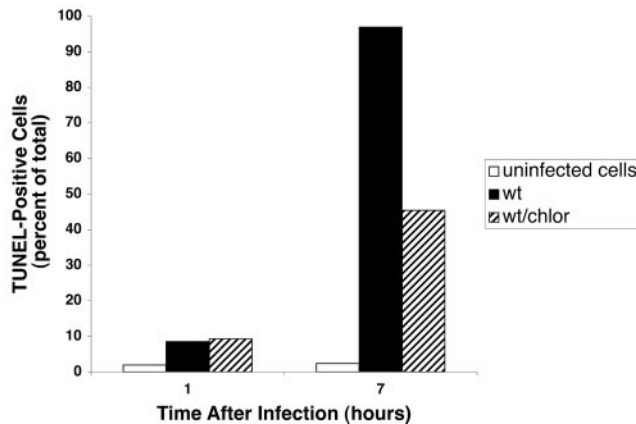


FIG. 4. Effect of chloramphenicol treatment at the time of bacterial infection of THP-1 cells. Cultures were treated with chloramphenicol during the time of phagocytosis as described in the text. The chloramphenicol was then removed and gentamicin was added to kill extracellular bacteria. Cytopathology was measured in a TUNEL assay at the indicated times.

creased from 1 to 4 h following infection with wild-type *Salmonella* as well as with the *sipB* and *ssaV* mutant. At 4 h, caspase-3 activity was approximately fivefold higher in cells infected with the wild-type strain compared to uninfected con-

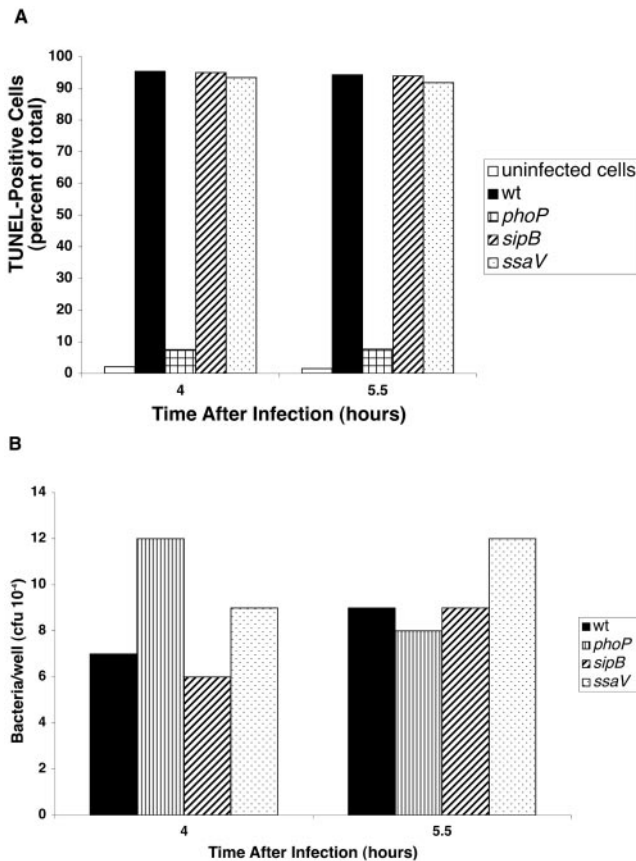


FIG. 5. Effect of a *phoP* mutation on the cytopathology induced by *Salmonella* strains. A. Cells processed for TUNEL assay. B. Viable bacteria recovered from the same culture wells.



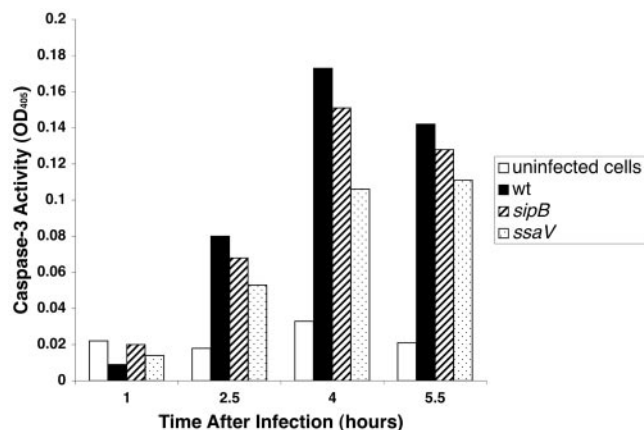


FIG. 6. Caspase-3 activity in lysates from *Salmonella*-infected THP-1 cells. Cultures were infected as described in the text and harvested for caspase-3 assays at the indicated times.

trols. No major differences were found between caspase-3 activation by the wild type and the *sipB* or *ssaV* mutants. Infection with the *phoP* mutant gave results similar to uninfected cells (data not shown). However, we did not find significant activation of caspase-8 or caspase-9 in the same infected cells that displayed caspase-3 activation (Fig. 7). To investigate whether *Salmonella*-induced DNA fragmentation is dependent on caspase-3 activation, we used the caspase-3 inhibitor Z-DEVD-FMK. Although Z-DEVD-FMK was effective in lowering caspase-3 activity in treated cells (Fig. 8A), the inhibition of caspase-3 did not affect the extent of DNA fragmentation (Fig. 8B). This result indicates that *Salmonella*-induced cell death in THP-1 cells resembles other systems in which caspase inhibitors are unable to prevent DNA fragmentation (1, 4, 12).

## DISCUSSION

*Salmonella* enters macrophages and replicates within a vacuolar compartment (16). When entry is mediated primarily through the SPI-1-encoded invasion mechanism, the SipB pro-

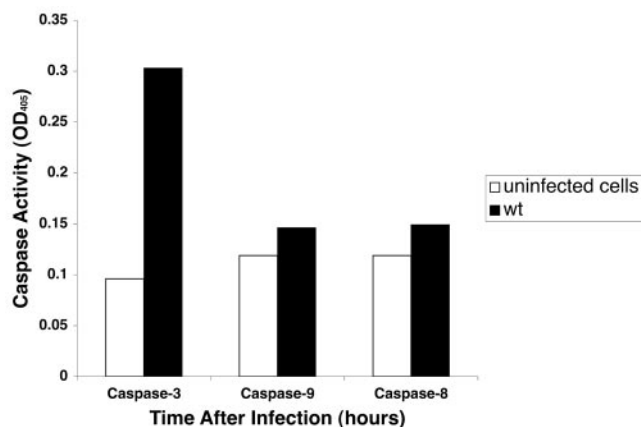


FIG. 7. Caspase-8 and -9 activities in lysates from *Salmonella*-infected THP-1 cells. Cultures were harvested 5 h after infection and processed for caspase-8 and -9 assays compared to caspase-3 activity in the same lysate.

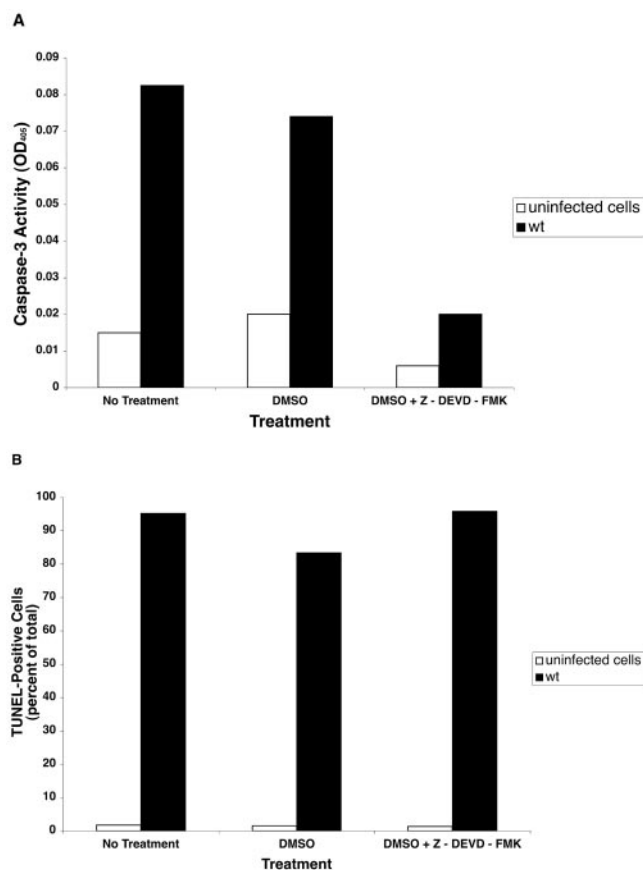


FIG. 8. Effect of the caspase-3 inhibitor Z-DEVD-FMK on *Salmonella*-induced DNA fragmentation. THP-1 cells were preincubated for 1 hour with 12  $\mu$ M Z-DEVD-FMK in 0.6% dimethyl sulfoxide (DMSO), or the DMSO vehicle alone, and then infected with wild-type *Salmonella* serovar Dublin. After 5 h, the cells were harvested and processed for caspase-3 activity in cell lysates (A) and by FACS analysis for DNA fragmentation (B).

tein triggers cell cytotoxicity through several mechanisms involving activation of caspase-1, caspase-2, and mitochondrial autophagy, leading to loss of macrophage viability within a few hours of infection (12, 13, 15). The significance of this cytotoxicity for the pathogenesis of *Salmonella* infection is unclear. This process may play a role during infection of intestinal macrophages, and caspase-1 is required for efficient dissemination of infection from the bowel in the murine model (14, 22). However, SipB and other components of the SPI-1 TTSS are not required for virulence in systemic disease in mice (7). A second form of cytopathology is seen in macrophages when bacteria enter by phagocytosis, the mechanism likely to account for intracellular infection during the systemic phase of disease (23). Bacteria taken up by phagocytosis proliferate over an extended period of time and reach high intracellular numbers. Cytopathology appears later in the infection and cell membrane function remains intact, as evidenced by the continued protection of bacteria from extracellular gentamicin (18). Cell death is manifested by DNA fragmentation before membrane integrity is compromised, one of the key features of apoptosis. In various macrophage systems, the SPI-2 TTSS and

the *spvB* virulence gene have been implicated in this delayed cytopathology (18, 28).

We describe here a cytopathic effect induced by *Salmonella* in THP-1 cells that differs from both of the previously described processes. In THP-1 cells, opsonized *Salmonella* is phagocytized and rapidly proliferates. The uptake and early intracellular growth are not dependent on SPI-1, SPI-2, *spvB*, or *phoP* functions. This finding suggests that the *Salmonella*-containing vacuole in THP-1 cells is a permissive environment that does not require the function of specific *Salmonella* virulence factors for intracellular growth to be initiated. Previous work from our group has shown that induction of phagocyte NADPH oxidase activity by vitamin D<sub>3</sub> is required to restrict the growth of *Salmonella* in THP-1 cells (27). In the present study, we have found that *Salmonella* infection induces DNA fragmentation in the vast majority of infected cells within 6 h. This cytopathology requires active bacterial protein synthesis, making it unlikely that the process is due only to the interaction of preexisting bacterial structural components with THP-1 cells. In fact, only a transient inhibition of bacterial protein synthesis with chloramphenicol at the time of phagocytosis is sufficient to reduce DNA fragmentation in infected cells, implying that proteins synthesized after phagocytosis by growing *Salmonella* are required to induce cytopathology.

Analysis of *Salmonella* mutants indicates that the virulence functions specified by SPI-1, SPI-2, or *spvB* are not involved in the induction of DNA fragmentation in THP-1 cells. This system allowed us to demonstrate a requirement for *phoP* that could not be detected using other macrophage systems. In murine macrophages, *phoP* is required for intracellular survival of *Salmonella* (5, 21). Therefore, it is difficult to assess an independent role for *phoP* in cytopathology. In primary human macrophages, *phoP* is required for the cytopathology mediated by SpvB, again preventing assessment of an independent role for *phoP* (D. Guiney, unpublished data). However, in THP-1 cells, the *phoP* mutant grew as well as the wild-type strain in the first few hours, although it did not produce cell death. Since PhoP is the response regulator of the two-component regulatory system using PhoQ as the sensor protein, these results suggest that one or more PhoP-regulated proteins are required for the induction of DNA fragmentation in THP-1 cells. PhoQ appears to sense low Mg<sup>2+</sup> levels in the *Salmonella*-containing vacuole following phagocytosis (8). Our finding of the requirement for bacterial protein synthesis to induce DNA fragmentation is consistent with a model in which PhoP induces the synthesis of one or more proteins that promote cytopathology in THP-1 cells following entry of *Salmonella* strains. The PhoP-dependent effect appears to activate caspase-3 and DNA fragmentation in parallel without caspase-8 or -9 involvement (Fig. 2 and 6 to 8), in contrast to the classical extrinsic and intrinsic apoptosis pathways proceeding through caspase-8 or -9 to caspase-3 as the executioner initiating DNA fragmentation (20). It is significant that the *phoP*-mediated effect on cytopathology in THP-1 cells does not require the SPI-2 TTSS. A previous study defined a *phoP*-dependent phenotype involving inhibition of phagosome fusion that also did not require SPI-2 function (9). Taken together, these results suggest that *phoP* controls *Salmonella* virulence factors that affect phagocyte physiology and can be

secreted by either the SPI-1 or SPI-2 TTSS or, alternatively, do not require these transport systems.

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