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Salmonella typhimurium invasion induces apoptosis in infected macrophages

(bacterial invasion/programmed cell death/cytotoxicity)

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ABSTRACT **Invasive *Salmonella typhimurium* induces dramatic cytoskeletal changes on the membrane surface of mammalian epithelial cells and RAW264.7 macrophages as part of its entry mechanism. Noninvasive *S. typhimurium* strains are unable to induce this membrane ruffling. Invasive *S. typhimurium* strains invade RAW264.7 macrophages in 2 h with 7- to 10-fold higher levels than noninvasive strains. Invasive *S. typhimurium* and *Salmonella typhi*, independent of their ability to replicate intracellularly, are cytotoxic to RAW264.7 macrophages and, to a greater degree, to murine bone marrow-derived macrophages. Here, we show that the macrophage cytotoxicity mediated by invasive *Salmonella* is apoptosis, as shown by nuclear morphology, cytoplasmic vacuolization, and host cell DNA fragmentation. *S. typhimurium* that enter cells causing ruffles but are mutant for subsequent intracellular replication also initiate host cell apoptosis. Mutant *S. typhimurium* that are incapable of inducing host cell membrane ruffling fail to induce apoptosis. The activation state of the macrophage plays a significant role in the response of macrophages to *Salmonella* invasion, perhaps indicating that the signal or receptor for initiating programmed cell death is upregulated in activated macrophages. The ability of *Salmonella* to promote apoptosis may be important for the initiation of infection, bacterial survival, and escape of the host immune response.**

Salmonella typhimurium causes a self-limiting gastroenteritis in humans and typhoid-like systemic disease in mice. *S. typhimurium* entry into cultured epithelial cells is associated with dramatic host cell membrane ruffling (1, 2) and subsequent intracellular survival. *S. typhimurium* also invades murine M cells overlying the Peyer's Patch lymphoid follicles with associated membrane ruffling (3). Following invasion, the M cell is destroyed and the bacteria gain access to the subepithelial lymph tissue and the lamina propria, where they encounter macrophages, dendritic cells, lymphocytes, and neutrophils. Many laboratories have investigated the *S. typhimurium*–macrophage interaction *in vitro* (4) and found that *S. typhimurium* replicate in macrophage-like cell lines and survive in spleenic-derived macrophages from susceptible mice strains (5). Recently it was shown that *S. typhimurium* is cytotoxic to macrophages 14 h subsequent to infection. Noncytotoxic mutants, selected at 48 h postinoculation, were located in *ompR*, a gene belonging to a family of two-component regulators (6).

In this study, we demonstrate that RAW264.7 and murine bone marrow-derived macrophages (BMM) invaded by *S. typhimurium* show clear manifestations of apoptosis and that mutant *S. typhimurium* incapable of inducing host cell membrane ruffling fail to induce apoptosis. We conclude that invasion of macrophages by *S. typhimurium* through a specific pathway associated with membrane ruffling signals the mammalian cell to undergo programmed cell death.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The mouse-virulent *S. typhimurium* strain SL1344 (1), mutant derivatives of SL1344, *Salmonella typhi* (Table 1), and *Escherichia coli* strains were grown in a modified Luria–Bertani (LB) broth (1% bacto-tryptone (Difco)/0.5% bacto-yeast extract (Difco)/1.75% sodium chloride) or on LB agar (GIBCO). Strains were grown standing at 37°C overnight as described (7). The next day the culture was diluted and grown to late logarithmic/early stationary phase standing at 37°C. To obtain stationary phase bacteria, modified LB broth was inoculated with a single colony and was grown with aeration for 18–19 h. Where stated, bacteria were opsonized in 50% normal mouse serum for 15 min at 37°C.

Cell Culture and Isolation of BMM. Monolayers for bacterial invasion were prepared by seeding 2.5×10^5 cells into each well of a 24-well plate. RAW264.7 cells, a murine monocyte-macrophage cell line (ATCC TIB71), were grown in DMEM containing 10% fetal calf serum, 1 mM glutamine, and 1 mM sodium pyruvate. BMM were isolated as described (8). Monolayers for quantitating bacterial cytotoxicity were prepared by seeding 10^5 macrophages into each well of a 96-well plate.

Eukaryotic Cell Infections. Monolayers of macrophages were infected with bacteria at a 100:1 multiplicity of infection (moi) for RAW264.7 monolayer detachment assay and at a 10:1 moi for BMM monolayer detachment, as well as for bacterial invasion assays. To synchronize the infection of monolayers, the infected tissue culture plates were centrifuged at $165 \times g$ for 5 min. Following a 30-min incubation at 37°C (5% CO_2), fresh DMEM supplemented with 100 μg of gentamicin (Gm) per ml was added. Macrophage monolayers were incubated with added Gm for 90 min, washed with DMEM, lysed in 1% Triton X-100 for 10 min, and diluted with LB broth and dilutions of the suspension were plated on LB agar medium. To assess intracellular growth, the medium containing 100 μg of antibiotic per ml was replaced with DMEM supplemented with 10 μg of Gm per ml, and parallel cell cultures were assayed for viable bacteria at appropriate times after infection.

The effect of actin polymerization inhibition during macrophage infection was determined by treating macrophages with 10 μg of cytochalasin D (Sigma) per ml for 15 min at 37°C (5% CO_2) before bacterial infection and washed out following a 30-min incubation with or without bacteria. RAW264.7 macrophages were treated with 5 μM gliotoxin (Sigma) for 5 h as a positive control of apoptosis (9).

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Abbreviations: BMM, bone marrow-derived macrophages; LB, Luria–Bertani; moi, multiplicity of infection; Gm, gentamicin; TUNEL, terminal deoxytransferase-mediated dUTP nick end-labeling.

Table 1. Invasive *Salmonella* are cytotoxic for and induce apoptosis in the RAW264.7 macrophage cell line and BMM

Strain*	Relevant phenotype	Genotype	RAW264.7 macrophages		BMM	
			OD_{630}†	% apoptosis‡	OD_{630}†	% apoptosis‡
SL1344	Inv^+ Rep^+		0.05 ± 0.02	12.9 ± 2.8	0.09 ± 0.01	67.3 ± 6.7
P9G4	Inv^+ Rep^-		0.03 ± 0.03	9.1 ± 2.2	0.10 ± 0.00	72.9 ± 4.4
P9B3	Inv^+ Rep^-		0.04 ± 0.03	10.0 ± 1.6	0.10 ± 0.01	71.6 ± 3.1
P3A8	$Inv^{+/-}$ Rep^+		0.17 ± 0.09	ND	ND	ND
P7F8	$Inv^{+/-}$ Rep^+		0.16 ± 0.05	9.8 ± 1.4	ND	54.8 ± 9.0
BJ66	Inv^- Rep^+	*orgA*	0.53 ± 0.02	1.3 ± 0.24	0.45 ± 0.05	<1
P4H2	Inv^- Rep^+	*hilA*	0.77 ± 0.05	0.45 ± 0.12	0.42 ± 0.09	<1
P7G11	Inv^- Rep^+	*sipD*	0.71 ± 0.02	0.17 ± 0.04	0.47 ± 0.03	<1
S. typhi 200Ty	Inv^+ Rep^+		0.12 ± 0.03	5.2 ± 0.7	ND	53.2 ± 5.8
Shigella flexneri M90T			ND	8.8 ± 1.0	ND	ND
Gliotoxin			ND	7.4 ± 0.8	ND	ND
Uninfected			0.54 ± 0.17	0.82 ± 0.4	0.50 ± 0.07	<1

Inv^+, invasive; Rep^+, intracellular replication; Inv^-, noninvasive; Rep^-, intracellular replication deficient; and ND, not done.
S. typhimurium mutant strains are in SL1344 background.
†Mean OD_{600} values ± standard deviation from a representative 96-well tissue culture plate assay at 20 h postinfection with moi of 100 bacteria per RAW264.6 macrophage and 10 bacteria per BMM.
‡Mean values ± standard deviation from counting three different coverslips for macrophages stained positive for TUNEL reaction 2 h postinoculation, a minimum of 400 macrophages were counted per cover slip. 10:1 moi for Inv^+ and 100:1 moi for Inv^-.

Host Cell Viability. Macrophages on coverslips were infected with bacterial inocula that were adjusted so that the number of intracellular bacteria and the number of infected macrophages was similar for each strain at 30 min (10 SL1344 bacteria and 100 BJ66 bacteria per macrophage). Thirty minutes postinoculation, Gm was added to a final concentration of 100 μg/ml. The medium was removed and Live/Dead Eukolight Viability/Cytotoxicity reagent (Molecular Probes, Eugene OR) was added to the monolayer at various times thereafter. After a 10-min incubation, cells were fixed with 3.7% formaldehyde, washed with PBS, and permeabilized with 0.2% Triton X-100. Following permeabilization, cells were incubated with rabbit polyclonal antisera to *S. typhimurium*, then incubated with goat anti-rabbit fluorescein isothiocyanate-conjugated antibody (Sigma) and analyzed by fluorescence microscopy. The detection dyes calceinAM and EthidiumD-1 were excited at 485 nm. Live cells fluoresced a faint green with a 530-nm bandpass emission filter due to loss of dye upon fixation and permeabilization. Dead cells fluoresced red with a 590-nm long-pass emission filter. Cells (400–600 per coverslip) were counted and scored as either live or dead and for the presence of bacteria.

Cytotoxicity Assays in 96-Well Plates. RAW264.7 cells and BMM were infected with a moi of 100 and 10, respectively. At 18–20 h postinfection, surviving, adherent cells were fixed with 10% formaldehyde and stained with crystal violet. The absorption at wavelength 630 was read on a microplate reader (Bio-Tek Instruments) as a measure of host cell detachment due to cytotoxicity.

Preparation of Samples for Transmission Electron Microscopy. RAW264.7 cells were seeded onto coverslips and allowed to adhere overnight. Infected cells were prepared exactly as described (10). Serial sections were cut and examined with a Phillips model 201c transmission electron microscope (Phillips Electronic Instruments, Mahwah, NJ).

Assessment of Apoptosis by Fluorescence Microscopy. Macrophages infected with *S. typhimurium* were analyzed for the presence of DNA fragmentation using terminal deoxytransferase-mediated dUTP nick end-labeling (TUNEL reaction). The *In Situ* Cell Death Detection Kit for Fluorescein (Boehinger Mannheim) was used to label free 3′-OH termini of DNA fragments with fluorescein. At 2 h postinfection, cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and then overlayed with the TUNEL reagents. The cells were then incubated with polyclonal rabbit anti-*S. typhimurium* antiserum, followed by goat anti-rabbit 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated antibody (Vector Laboratories), stained with rhodamine phalloidin (Molecular Probes), and analyzed by fluorescence microscopy.

Statistical Analysis. Statistical analysis was performed using Student's two-tailed *t*-test for independent means.

RESULTS

Invasion and Intracellular Replication of *S. typhimurium* in RAW264.7 Cells. Wild-type, invasive *S. typhimurium* SL1344 enter epithelial cells (1) and RAW264.7 cells by a ruffling mechanism (10). In this study, we compared invasive and noninvasive *S. typhimurium* entry into RAW264.7 macrophages. Two hours postinfection, 6- to 10-fold higher numbers of invasive wild-type SL1344 bacteria were Gm-protected compared with the isogenic, noninvasive strain, BJ66. BJ66 is unable to induce ruffling and does not invade human epithelial cells (11), nor does it induce ruffling in macrophages (data not shown). Nonetheless, BJ66 cells were phagocytosed and protected from Gm at levels similar to *E. coli* HB101 (Fig. 1*a*). Similarly, SL1344 grown with aeration to late stationary phase, a growth condition known to induce a noninvasive phenotype (7), resulted in 10-fold lower numbers of Gm-protected bacteria recovered at 2 h (Fig. 1*a*).

We compared the level of intracellular replication in macrophages of invasive and noninvasive *S. typhimurium*. The average number of colony-forming units/well increased for wild-type bacteria over the first 8 h of infection and then plateaued (Fig. 1*b*). Surprisingly, BJ66 replicated at a slightly slower rate for the first 8 h, but exceeded the number of intracellular wild-type bacteria by 24 h. *E. coli* DH12α was phagocytosed by the RAW264.7 macrophages, but did not replicate over the 24 h assay. Thus, *S. typhimurium* that have entered the macrophage by a membrane ruffling mechanism or by phagocytosis were capable of replicating intracellularly. Yet the number of Gm-protected wild-type bacteria was not as great as the number of Gm-protected BJ66 at later times of infection. This observation led us to look more closely at the fate of infected macrophages.

Macrophages infected with SL1344 detached from the monolayer over time, whereas BJ66-infected macrophages did not display any significant cytotoxicity. As the two isogenic strains differ in their capacity to induce host cell ruffles and to replicate intracellularly, we addressed the nature of host cell death.

Invasive *S. typhimurium* Are Cytotoxic to the Macrophages. *S. typhimurium* cytotoxicity was examined using a method to measure macrophage intracellular esterase activity and plasma membrane integrity. This fluorescence-based method of as-

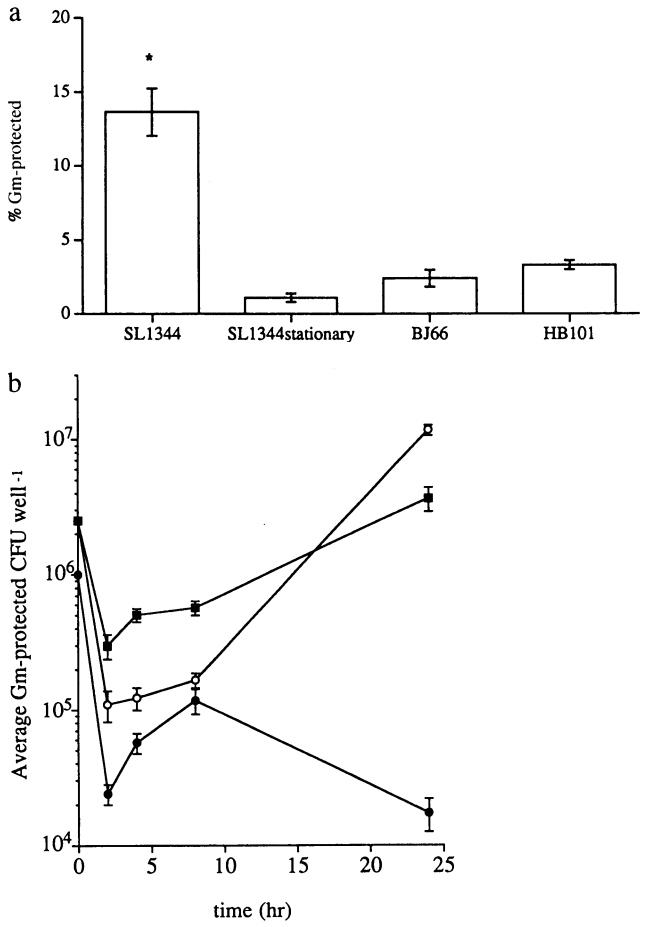

FIG. 1. Invasion and replication or survival in RAW264.7 macrophages. (*a*) percentage of input values that are Gm-protected at 2 h postinfection. *, $P = 0.0215$ for invasive SL1344 compared with noninvasive BJ66. (*b*) Gm-protected colony-forming units recovered at 2, 4, 8, and 24 h. ■, SL1344; ○, BJ66, ●, DH12α. moi, 10 bacteria per macrophage.

sessing cell viability can be used in place of trypan blue dye exclusion and ^{51}chromium release (12).

The kinetics of *S. typhimurium*-induced cytotoxicity was followed over a 2-h period. Eight percent of the adherent RAW264.7 macrophages infected with SL1344 were dead at 60 min after infection (Fig. 2). The percent of dead macrophages increased to 18.4% and 26.3% at 90 and 120 min, respectively. The vast majority of dead macrophages contained bacteria

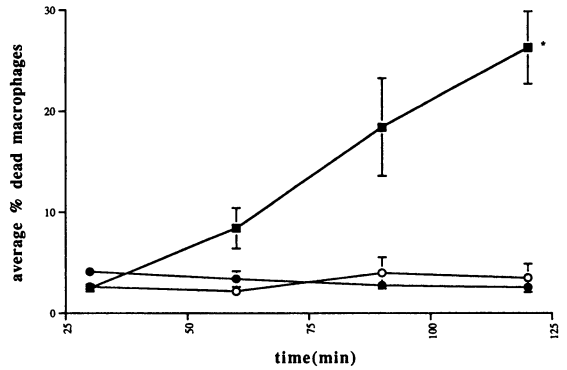

FIG. 2. Average percentage of dead macrophages at 30, 60, 90, and 120 min postinfection as scored by counting the number of macrophages with EthidiumD-1 bound to the DNA. ■, SL1344, moi 10 bacteria per macrophage; ○, BJ66, moi 100 bacteria per macrophage; and ●, uninfected. *, $P = 0.0223$ at 120 min.

(>90%). In striking contrast, the noninvasive strain, BJ66, did not cause macrophage cytotoxicity beyond that seen in uninfected controls, even though >95% of the macrophages were infected (Fig. 2).

We analyzed a panel of different *Salmonella* mutants and species for their host cell cytotoxicity. In these experiments, macrophage cytotoxicity was measured by the degree of macrophage detachment from 96-well plates at 20 h. As shown in Table 1, the invasive phenotype correlated with detachment of macrophages from the monolayer. Mutants selected on the basis of their inability to invade epithelial cells did not cause detachment of macrophages by 20 h. We next determined whether the cytotoxicity we observed was related to bacterial entry by a membrane ruffling mechanism or to subsequent bacterial intracellular replication. Two invasive strains that induced membrane ruffling but could not initiate intracellular replication, P9G4 (13) and P9B3 (B.R., unpublished data) were as cytotoxic as the wild-type SL1344 strain (Table 1).

Although *S. typhi* is a human pathogen, it is still capable of invading and replicating within the RAW264.7 murine macrophage cell line, and it can enter murine M-cells with membrane ruffling (10). However, typhoid bacilli cannot cause disease in animals other than humans. Yet *S. typhi* was cytotoxic for RAW264.7 macrophages and BMM (Table 1).

Macrophage cytotoxicity was not unique to the macrophage cell line, RAW264.7. Indeed, a striking degree of cytotoxicity was observed in primary BMM cell cultures, even at mois that were 10-fold lower than in the RAW264.7 macrophage infections (Table 1).

Salmonella Invasion Induces Apoptosis in Macrophages. To discern the nature of the *Salmonella*-induced cytotoxicity, we examined SL1344-infected RAW264.7 cells by transmission electron microscopy. Within 2 h postinfection, many of the infected cells displayed intense perinuclear chomatin aggregation, cytoplasmic vacuolization, and maintenance of organelle structure, which is characteristic of cells undergoing apoptosis (Fig. 3*a*; refs. 14 and 15). In contrast, cells infected with BJ66 had a normal appearance, although they contained intracellular bacteria within vacuoles (Fig. 3*b*).

A characteristic of apoptosis is the cleavage of DNA of the dying cell at the internucleosomal regions, resulting in multimers of 180–200 bp (16). The DNA in macrophages infected with SL1344 was cleaved into multimers of 180–200 bp, whereas DNA from macrophages infected with BJ66 was uncleaved. The pattern of DNA cleavage in macrophages invaded by SL1344 was similar to that in cells treated with gliotoxin, a toxin that is known to induce macrophage apoptosis (data not shown; ref. 9). We used the TUNEL reaction (17) to measure DNA fragmentation and quantitate *S. typhimurium*-induced apoptosis of RAW264.7 and BMM infected with 10 invasive bacteria and 100 noninvasive bacteria per macrophage. By 2 h postinfection with SL1344, 12.9% of RAW264.7 macrophages (Fig. 4) and 70% of BMM were undergoing apoptosis. Macrophages infected with nonruffling mutants exhibited no more apoptosis than uninfected controls (Table 1). The invasive, replication-deficient mutants, P9G4 and P9B3, also induced apoptosis in RAW264.7 and BMM (Table 1). A mutant, P7F8, that demonstrated intermediate levels of invasion of epithelial cells (B.R., unpublished data) and macrophage cytotoxicity also induced apoptosis. *S. typhi* also induced apoptosis in RAW264.7 cells, albeit at a lower level than SL1344 (5.2% at 2 h).

Salmonella epithelial cell invasion is inhibited by cytochalasin D, a compound that affects actin polymerization (18). We confirmed that cytochalasin D inhibited *S. typhimurium* entry into RAW264.7 macrophages. The number of recoverable Gm-protected SL1344 was reduced 100- to 200-fold at 2 h. Apoptosis induced by SL1344 invasion was markedly inhibited by pretreatment of macrophages with cytochalasin D, reducing the percentage of macrophages positive for the TUNEL

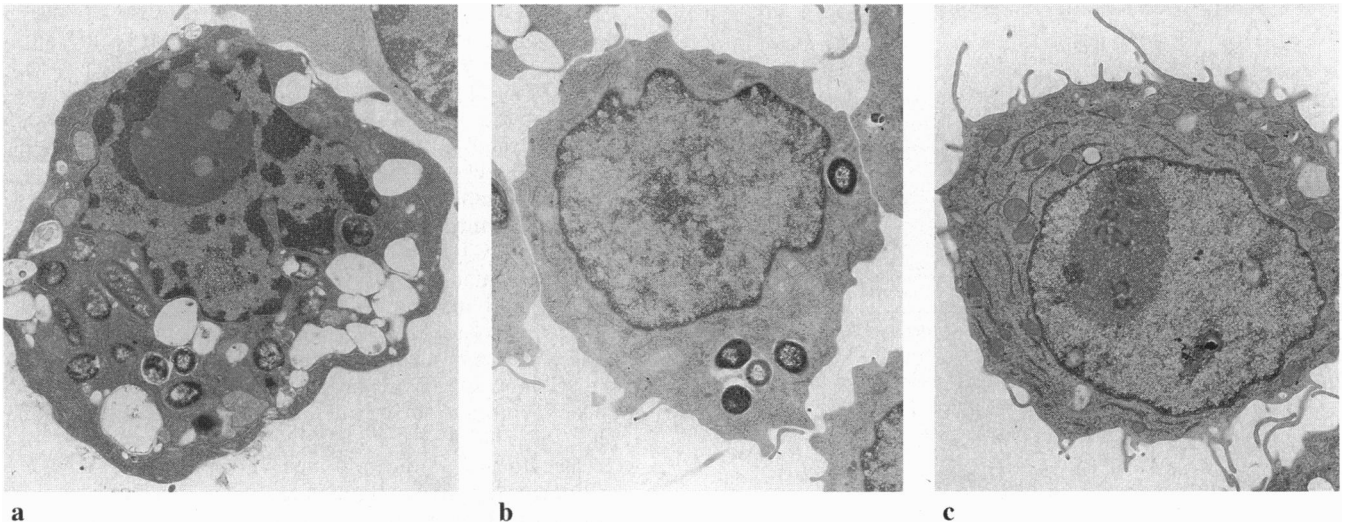

FIG. 3. Transmission electron micrographs of RAW264.7 macrophages infected with moi of 100 bacteria per macrophage. (*a*) SL1344 2 h postinoculation, (*b*) BJ66 2 h postinoculation, and (*c*) uninfected. (×7000.)

reaction at 2 h and 6 h postinoculation to background levels, 1% and 2.5%, respectively. Thus, *Salmonella* must enter host cells but not necessarily replicate intracellularly to induce apoptosis.

Salmonella-induced macrophage apoptosis correlated with bacterial entry by a membrane ruffling mechanism. An additional mechanism of macrophage entry occurs when the bacteria are coated with complement via CR3. Invasive SL1344 opsonized with normal mouse serum still induced membrane ruffling in RAW264.7 macrophages, similar to unopsonized SL1344 as seen by visualization of actin filaments with rhodamine phalloidin. SL1344 opsonized with NMS were taken up more efficiently by macrophages, yet the percentage of cells undergoing apoptosis at 2 h was the same as unopsonized SL1344 (Table 2). Opsonized BJ66 and noninvasive, stationary phase SL1344 also entered macrophages more efficiently but still did not induce apoptosis of RAW264.7 macrophages at 2 h (Table 2). Twenty hours after infection of RAW264.7 and BMM with a noninvasive mutant, the number of macrophages showing apoptosis remained the same as uninfected controls despite high levels of intracellular replication within the RAW264.7 macrophages (data not shown). We concluded that, although complement-coated bacteria entered macrophages more efficiently and thus increased the intracellular numbers of bacteria, this mechanism of entry and increased bacterial load did not induce apoptosis, nor did it block the *Salmonella*-mediated mechanism of inducing macrophage apoptosis associated with membrane ruffling.

DISCUSSION

S. typhimurium host cell invasion into M cells *in vivo* and into cultured epithelial and macrophage cell lines *in vitro* is associated with dramatic cytoskeletal changes that appear as membrane ruffles. Following passage through the epithelium of the Peyer's Patch, virulent *Salmonella* strains encounter an array of host immune cells. Several studies have established that *S. typhimurium* survival and replication within macrophages is essential for survival (4). Mutants that are unable to survive within cultured macrophages are less virulent (19). We began our investigation to determine if *S. typhimurium* that

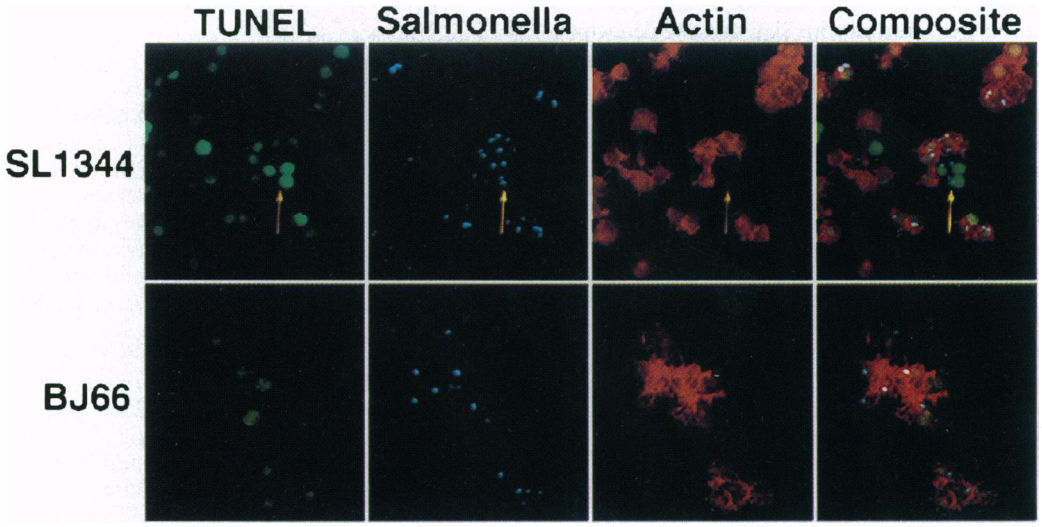

FIG. 4. TUNEL reaction in infected RAW264.7 macrophages. Infections with 10 SL1344 bacteria per macrophage and 100 BJ66 bacteria per macrophage for 2 h are shown. TUNEL reaction was used to label 3′-OH termini with fluorescein. α-Salmonella primary antibody and α-rabbit-7-amino-4-methylcoumarin-3-acetic acid (AMCA) secondary antibody were used to visualize bacteria in Hoechst filter. Rhodamine phalloidin was used to label actin filaments. Images of epifluorescence were scanned into Adobe PHOTOSHOP and aligned to make a composite. Arrow indicates an infected macrophage positive for TUNEL reaction.

Table 2. Apoptosis in RAW264.7 macrophages is dependent on *S. typhimurium* invasion and not complement-mediated entry

Strain	Opsonin	% inoculum Gm-protected at 2 h	% apoptosis*
SL1344	None	21.5 ± 3.6	12.9 ± 2.8
SL1344	NMS	56.5 ± 5.3	13.0 ± 2.7
BJ66	None	5.9 ± 1.3	1.3 ± 0.2
BJ66	NMS	30.4 ± 2.8	1.0 ± 0.6
SL1344 stationary phase	None	1.4 ± 0.2	0.2 ± 0.1
SL1344 stationary phase	NMS	28.1 ± 2.8	0.3 ± 0.1

NMS, normal mouse serum.
*Mean values ± standard deviation from counting three different cover slips for macrophages stained with TUNEL at 2 h. A minimum of 400 cells were counted per cover slip. 10:1 moi.

contained specific mutations in epithelial cell invasion would still invade macrophages normally and replicate intracellularly. *S. typhimurium* SL1344 invaded RAW264.7 macrophages at 10-fold higher levels than phenotypically noninvasive, stationary-phase SL1344 or a noninvasive SL1344 mutant. Although both invasive and noninvasive bacteria replicated intracellularly, macrophage cytotoxicity was only observed with invasive bacteria.

Invasive *S. typhimurium* caused macrophage programmed cell death or apoptosis, whereas noninvasive mutant strains did not. Blocking entry of invasive *S. typhimurium* into RAW264.7 macrophages by inhibiting actin polymerization with cytochalasin D abolished programmed cell death, which supports our conclusion that entry by a membrane ruffling mechanism triggers the initiation of apoptosis. The entry process, not subsequent replication, triggers a signal transduction pathway within the macrophage that induces programmed cell death. The mechanism of *Salmonella* entry into mammalian cells is not known, but it is thought to initiate entry by a rac- and rho-independent pathway (20). During invasion, *S. typhimurium* triggers an increase in several host cell second messengers, such as intracellular calcium levels, phospholipase A_2 activity, and leukotriene production, as well as enhanced protein kinase activity (21, 22). These second messengers may play a role in the activation of programmed cell death. The exact signal transduction mechanisms of apoptosis are not known and can vary depending on cell type and external stimulus, but elevation of intracellular calcium levels has been described after T-cell receptor ligation, which is mediated by enzymatic protein tyrosine kinase activity and tyrosine phosphorylation in thymocytes undergoing apoptosis (23).

The activation state of the macrophages plays a major role in the response of the *Salmonella* invasion. Surprisingly, 70% of the BMM exhibited apoptosis 2 h after infection with an invasive *S. typhimurium*. Indeed, RAW264.7 macrophages activated by treatment with interferon gamma and lipopolysaccharide (24), show a greater level of apoptosis 2 h after infection with invasive *S. typhimurium* (data not shown). Similar increases in cytotoxicity of lipopolysaccharide-stimulated murine macrophages induced by *Shigella flexneri* invasion have been demonstrated (25). Thus, the level of expression of an intracellular signal for apoptosis or a surface molecule involved in signaling the macrophage programmed cell death machinery appears to be upregulated in activated macrophages. Fas receptor and tumor necrosis factor receptor, both members of the family of receptors that includes nerve growth factor receptor, are upregulated in interferon-γ and lipopolysaccharide-activated macrophages (26, 27). When Fas receptor and tumor necrosis factor receptor are stimulated with ligand or cross-linking antibody, they trigger apoptotic cell death by a mechanism that has yet to be elucidated (28, 29).

A previous study demonstrated *Salmonella choleraesuis* induced apoptosis in proteose peptone-elicited peritoneal macrophages when incubated in the presence of neutralizing anti-interleukin 10 antibody (30). Accompanying this increase in cell death was an associated increase in tumor necrosis factor type α and interleukin 1 release, suggesting the infected macrophages were dying from autocrine suicide. Normally in the absence of neutralizing antibody, interleukin 10 protects the infected macrophages from apoptosis. We are currently investigating the roles of interleukin 10 and tumor necrosis factor type α in our tissue culture system.

Several other pathogenic bacteria and toxins isolated from pathogenic bacteria have been shown to induce apoptosis in various immune cells (31). *Corynebacterium diphtherae*, *Shigella flexneri*, *Bordetella pertussis*, and *Listeria monocytogenes* produce toxins that can cause programmed cell death under certain conditions in specific cell types, including macrophages (31–33). Not only must *S. flexneri* be intracellular to induce cell death, it also must escape the vacuole, and *ipaB* must be present for this induction (34).

It is clear that *S. typhimurium* must also actively enter RAW264.7 macrophages to induce apoptosis and that the mode of entry influences the signal to initiate programmed cell death in macrophages. The *sipEBCDA* genes from *S. typhi* exhibit extensive sequence similarities to the effectors of *Shigella* entry into epithelial cells encoded by the virulence plasmid-borne *ipa* operon, and it has been shown that *sipB* and *sipE* can complement a *Shigella* non-invasive *ipaB* mutant (35). The structural and functional conservation of the Sip and Ipa proteins suggests that *Salmonella* and *Shigella* entry processes are promoted by similar effectors. We demonstrated that a *S. typhimurium sipD* mutant is unable to induce apoptosis. Our data suggest that perhaps these two pathogenic bacteria share a common mechanism of induction of apoptosis in macrophages. Although *S. flexneri* must escape the vacuole to induce apoptosis, *S. typhimurium* does not.

The induction of programmed cell death *in vivo* may play a role in aiding *Salmonella* evasion of the immune system. Apoptosis in macrophages before the macrophage can synthesize proinflammatory cytokines would aid in the establishment of infection. Or perhaps the infected macrophage induces programmed cell death as a defense against this assault. The role of apoptosis in the pathogenesis of salmonellosis is currently under investigation.

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