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Live *Salmonella* Modulate Expression of Rab Proteins to Persist in a Specialized Compartment and Escape Transport to Lysosomes*

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We investigated the intracellular route of *Salmonella* in macrophages to determine a plausible mechanism for their survival in phagocytes. Western blot analysis of isolated phagosomes using specific antibodies revealed that by 5 min after internalization dead *Salmonella*-containing phagosomes acquire transferrin receptors (a marker for early endosomes), whereas by 30 min the dead bacteria are found in vesicles carrying the late endosomal markers cation-dependent mannose 6-phosphate receptors, Rab7 and Rab9. In contrast, live *Salmonella*-containing phagosomes (LSP) retain a significant amount of Rab5 and transferrin receptor until 30 min, selectively deplete Rab7 and Rab9, and never acquire mannose 6-phosphate receptors even 90 min after internalization. Retention of Rab5 and Rab18 and selective depletion of Rab7 and Rab9 presumably enable the LSP to avoid transport to lysosomes through late endosomes. The presence of immature cathepsin D (48 kDa) and selective depletion of the vacuolar ATPase in LSP presumably contributes to the less acidic pH of LSP. In contrast, proteolytically processed cathepsin D (M_r 17,000) was detected by 30 min on the dead *Salmonella*-containing phagosomes. Morphological analysis also revealed that after uptake by macrophages, the dead *Salmonella* are transported to lysosomes, whereas the live bacteria persist in compartments that avoid fusion with lysosomes, indicating that live *Salmonella* bypass the normal endocytic route targeted to lysosomes and mature in a specialized compartment.

Phagocytosis is an important process of host defense against invading microorganisms that involves their binding to the cell surface, internalization, and subsequent targeting to lysosomes for degradation. Many pathogenic microorganisms modulate this central process to survive in the phagocytes (1). Phagosomes fuse with different intracellular vesicles and recruit various factors like hydrolytic enzymes, proton pumps, etc. (2). The docking and fusion of the vesicles are regulated by small GTP-binding proteins of Rab family (3–6). Specific Rab proteins in active GTP-bound form localized on a particular vesicle regulate the assembly of the docking complex, thereby ensuring the specificity of the membrane fusion. How intracellular pathogens modulate the expression of these proteins to avoid or induce specific interactions of phagosomes with other vacuolar compartments is largely unknown.

Morphological observations using immunofluorescence and electron microscopy show that phagosomes containing inert particles or dead microorganisms appear to follow the endocytic route culminating in fusion with lysosomes (7). Live organisms, however, use various strategies to survive and proliferate inside phagocytes (1). For instance, *Coxiella burnetti* (8) and *Leishmania* (9) survive in the acidified phagosomes, whereas *Mycobacterium tuberculosis*, *Legionella pneumophilia*, and *Toxoplasma gondii* survive and proliferate in the vacuolar compartments that do not mature into phagolysosomes (1, 10, 11). In contrast, *Trypanosoma cruzi* (12), *Shigella flexneri* (13), and *Listeria monocytogenes* (14) lyse the phagosomal membranes to escape into the cytoplasm.

The pathogenesis of typhoid fever is related to the ability of *Salmonella* sp. to survive in phagocytes (15), but the mechanism by which *Salmonella* modulate their intracellular survival remains to be established. There are conflicting reports regarding the maturation of *Salmonella*-containing phagosomes. For instance, it has been reported that *Salmonella* prevent phagosome-lysosome fusion (16), whereas other studies indicate lysosomal targeting (17, 18). Confocal microscopic studies indicated that LSP[1] bypass the mannose 6-phosphate receptor (M6PR)-positive compartment that is normally encountered along the endocytic route to lysosome (19). However, it is largely unknown how LSP modulate their intracellular trafficking. Recently, it has been shown that *Mycobacterium*-containing phagosomes do not acquire Rab7 and *Listeria*-containing phagosomes modulate the fusion with early endosomes in a Rab5-dependent process (20, 21).

In the present investigation, we attempted to delineate the intracellular route of live or dead *Salmonella* by purifying phagosomes at various times after uptake and determining their contents of compartment specific markers. We demonstrate that *Salmonella* bypass the normal endocytic route by modulating the expression of different Rab proteins and persist in a specialized compartment of low acidity.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all reagents were obtained from Sigma. Tissue culture supplies were obtained from the Life Technologies, Inc. *N*-Hydroxysuccicinimidobiotin, avidin-horseradish peroxidase (avidin-HRP), avidin, and bicinchoninic acid reagents were purchased

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[1] The abbreviations used are: LSP, live *Salmonella*-containing phagosome; MP6R, mannose 6-phosphate receptor; DSP, dead *Salmonella*-containing phagosome; HRP, horseradish peroxidase; PNS, postnuclear supernatant; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid; SB, solubilization buffer; TGN, trans-Golgi network.

from Pierce. Colloidal gold particles (20 nm) were purchased from Sigma and conjugated with mannosylated bovine serum albumin by standard procedure. ECL reagents were procured from Amersham Pharmacia Biotech. Other reagents used were of analytical grade.

Antibodies—Monoclonal antibody 4F11, a mouse IgG_{2ak} monoclonal antibody specific for the COOH terminus of mouse Rab5, and an affinity purified Rabbit polyclonal antibody that recognizes COOH-terminal domain of Rab7 were generously provided by Dr. A. Wandinger-Ness (University of New Mexico, Albuquerque, NM). A rabbit polyclonal anti-Rab5 antibody was received as a gift from Dr. J. Gruenberg (EMBL, Heidelberg, Germany). Dr. Suzanne Pfeffer (Stanford University, Stanford, CA) kindly provided anti-Rab9 antibody. Antibody against CD-M6PR was a kind gift from Dr. W. Sly (St. Louis University, St. Louis, MO). Anti-Lamp1 (ID4B) antibody was a generous gift from Dr. David Russell (Washington University, St Louis, MO). Monoclonal antibody against *Salmonella* LPS ($P_5C_6D_1$) was obtained from Dr. Ayub Qadri (National Institute of Immunology, New Delhi, India). Yeast anti-vacuolar ATPase antibody that also recognizes mouse protein was a kind gift from Dr. Andrea Jahraus (EMBL, Heidelberg, Germany). Anti-Rab18 and anti-transferrin receptor antibodies were purchased from Calbiochem and Zymed Laboratories Inc., respectively. Anti-cathepsin D and all the second antibodies labeled with HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells—J774E clone, a mannose receptor-positive macrophage cell line was kindly provided by Dr. Philip D. Stahl (Washington University School of Medicine, St. Louis, MO). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and gentamycin (50 μg/ml) and were grown at 37 °C in a 5% CO_2, 95% air atmosphere.

Bacterial Strains—The virulent *Salmonella typhimurium* strain was a clinical isolate from Lady Hardings Medical College (New Delhi, India) and was obtained from Dr. Vineeta Bal of National Institute of Immunology (New Delhi, India). Bacteria were grown overnight in LB at 37 °C with constant shaking (300 rpm). The bacteria were harvested in the stationary phase, washed twice in phosphate-buffered saline (PBS), and used in phagosome preparation. For preparation of the phagosomes containing dead bacteria, bacteria were first incubated at 65 °C for 45 min and subsequently fixed with 1% glutaraldehyde at 4 °C for 30 min (17). Complete loss of viability of the bacteria was confirmed by the absence of colony formation on LB agar plates.

Uptake of Live and Dead Salmonella by Macrophages—To determine the uptake of live and dead bacteria, *Salmonella* were grown overnight in LB and metabolically labeled with [^{35}S]methionine (22). Briefly, cells were washed three times with PBS and grown in methionine-free RPMI 1640 medium containing 1 mCi of [^{35}S]methionine with constant shaking (300 rpm) for 9 h at 37 °C. The cells were washed five times with PBS to remove unincorporated radioactivity. For uptake assay, live or dead [^{35}S]methionine-labeled *Salmonella* (1×10^7) were added to a 24-well plate containing J774E macrophages (1×10^6/well) and centrifuged at a low speed (2000 rpm, 5 min at 4 °C) to synchronize the infection. After incubation for different periods of time at 37 °C, the cells were washed five times with PBS containing 1 mg/ml bovine serum albumin to remove unincorporated bacteria. The cells were solubilized with 1% Triton X-100 to ascertain cell-associated radioactivity.

Preparation of Biotinylated Salmonella—*Salmonella* grown in LB as described previously were biotinylated for use as a phagocytic probe for the phagosomes using the standard method (23). Briefly, bacteria were incubated with *N*-hydroxysuccicinimidobiotin (0.5 mg/ml) in PBS-CM (10 mM PBS, pH 8 containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) for 1 h at 4 °C. Then the cells were sequentially washed with PBS and 50 mM NH_4Cl to quench excess free biotin and resuspended in PBS. Biotinylation did not affect viability as shown by the ability of the bacteria before and after biotinylation to form similar number of colonies on LB agar plate. An aliquot of live biotinylated bacteria was killed by heat treatment followed by glutaraldehyde fixation. Both killed and live bacteria bound same amount of avidin-HRP, indicating a similar density of biotin in both the preparations. To determine the biotinylated bacterial proteins in dead and live *Salmonella*, 1×10^7 bacteria were boiled in SDS sample buffer, and aliquots were run on SDS-PAGE. In both the preparations, multiple and essentially identical proteins were biotinylated to similar extents as indicated by Western blotting with avidin-HRP.

Assay for Transport to Lysosomes—J774E cells (1×10^6 cells) were incubated in the presence of avidin-HRP (200 μg/ml) for 60 min at 4 °C in cold HBSA (Hanks' balanced salt solution buffered to pH 7.4 with 10 mM HEPES, 10 mM TES, and 10 mg/ml bovine serum albumin) to allow binding. Avidin-HRP was internalized for 10 min at 37 °C, and cells were washed three times with HBSA to remove uninternalized avidin-HRP. Subsequently, avidin-HRP was chased for 80 min at 37 °C in the presence 1 mg/ml mannan for transport to lysosomes (24). After washing, cells were allowed to bind biotinylated live or dead *Salmonella* (1×10^7 cells) at 4 °C for 1 h. Cells were resuspended in prewarmed medium, and uptake was carried out for 5 min at 37 °C. Cells were washed three times to remove unbound bacteria by centrifugation at low speed ($300 \times g$ for 6 min). Uninternalized surface-bound biotinylated bacteria were quenched by free avidin (0.25 mg/ml). Cells were washed twice and chased for indicated time at 37 °C. The reaction was stopped by chilling on ice. The HRP-avidin-biotin bacterial complex was recovered by centrifugation ($10{,}000 \times g$ for 5 min) after solubilization of the cells in solubilization buffer (SB; PBS containing 0.5% Triton X-100 with 0.25 mg/ml avidin as scavenger). The enzymatic activity of avidin-HRP associated with the biotinylated bacteria was measured as fusion unit. Total activity measured after solubilizing the cells without avidin was about 6.8 ng of HRP/mg of cell protein. Background values corresponding to HRP activity associated with the bacteria, when the cells were chased at 4 °C and solubilized in avidin-containing SB, were about 3.5% (0.24 ng/mg of cell protein) of the total activity and were subtracted from all the values to determine specific fusion.

Preparation of Purified Phagosomes—Live or dead *Salmonella*-containing phagosomes were prepared using a method described previously (20, 25). J774E clone macrophages (1×10^8) were incubated with 1×10^9 bacteria at 4 °C for 1 h in HBSA, and bacterial infection was synchronized by centrifugation at low speed. Then the cells were shifted to prewarmed medium and incubated for 5 min at 37 °C. The uptake was stopped by the addition of ice-cold medium. Cells were washed three times to remove unbound bacteria by centrifugation at low speed ($300 \times g$ for 6 min). Subsequently, cells were resuspended in prewarmed medium and chased for different periods of time at 37 °C as indicated. Finally, cells were washed and resuspended (2×10^8 cells/ml) in homogenization buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2) and homogenized in a ball bearing homogenizer (26) at 4 °C. Homogenates were centrifuged at a low speed ($400 \times g$ for 5 min) at 4 °C to remove nuclei and unbroken cells. The postnuclear supernatants (PNS) were quickly frozen in liquid nitrogen and stored at −70 °C. To obtain the phagosomal fraction, the PNS was quickly thawed and diluted with homogenization buffer (1:3) and centrifuged at $12{,}000 \times g$ for 1 min in a microcentrifuge at 4 °C as reported earlier (26, 27). Subsequently, phagosomes were further purified using the protocol as described previously (11). Briefly, the phagosomal fractions were resuspended in 100 μl of homogenization buffer containing protease inhibitors and loaded on 1 ml of 12% sucrose cushion. Samples were centrifuged at 1,700 rpm for 45 min at 4 °C, and the purified phagosomes were recovered from the bottom of the tube. The viability of bacteria in the phagosomes was determined by selective lysis of the phagosomal membrane with SB followed by cultivation of bacteria in LB-agar plate. Live *Salmonella* remained viable under these conditions. To determine the percentage of intact phagosomes, the phagosome preparations were treated with avidin at 4 °C to quench the biotin accessible in broken phagosomes. Subsequently, the phagosomes were washed and solubilized in SB in the presence of avidin-HRP. The difference in the HRP activity in avidin-untreated and avidin-treated phagosomes was used to determine the content of intact phagosomes. The HRP activity associated with avidin-untreated solubilized phagosome preparation was taken as 100%. About 74% of DSP and 70% of LSP remained intact by this assay.

Characterization of Purified Phagosomes—The purity of the phagosomes was checked by biochemical analysis to determine the contamination with other cellular components. Plasma membrane contamination was measured as described previously using the mannose receptor as the plasma membrane marker (28). Briefly, the macrophages were allowed to internalize the bacteria for 5 min at 37 °C in HBSA and washed. Subsequently, the macrophages were incubated with HRP (500 μg/ml) for 30 min at 4 °C to bind to the mannose receptors on the cell surface and washed. These cells were used to prepare purified phagosomes as described above. No HRP activity was detected in the purified phagosomes, indicating no plasma membrane contamination.

To label the lysosomes, J774E cells were incubated with HRP for 30 min at 4 °C in HBSA, washed, and chased for 90 min (29). After allowing the washed cells to internalize bacteria for 5 min at 37 °C, phagosomes were prepared. Most of the HRP activity was detected in the lysosomal fraction containing about 97% of total β-galactosidase activity, and no HRP activity was detected in the purified phagosomes. We have also measured the β-galactosidase activity to determine the lysosomal contamination both in LSP and DSP. About 3% of the total β-galactosidase activity were detected in early (5 min) LSP and DSP, indicating no lysosomal contamination (29). In contrast, significantly higher β-galactosidase activity (12–15% of the total) was detected in

late DSP (90 min) compared with late LSP (about 3%), suggesting lysosomal targeting of the dead organisms.

The endosome contamination was determined by mixing an aliquot of PNS after bacterial uptake and an aliquot of PNS after 5 min of uptake of HRP at 4 °C (20). Phagosomes were purified, and endosomal contamination was measured as a percentage of HRP activity present in the phagosome compared with the total activity present in the PNS. Less than 0.2% of the HRP activity in the phagosomal fraction indicates the purity of the phagosome.

The galactosyltransferase activity (30) was measured to check the Golgi contamination using [^{3}H]UDP-galactose, which is found to be about 3% of the total activity in the purified phagosome. Similarly, no glucose 6-phosphatase (31) activity was detected in purified phagosomes, indicating that the phagosomes are free of endoplasmic reticulum and Golgi contamination.

Measurement of HRP Activity—The HRP activity was measured in a 96-well microplate (Costar Co.) using *o*-phenylenediamine as the chromogenic substrate (32). Briefly, the final pellet after the fusion reaction was resuspended in 20 μl of PBS and transferred to microplates. The reaction was initiated by adding 100 μl of 0.05 N sodium acetate buffer, pH 5.0, containing *o*-phenylenediamine (0.75 mg/ml) and 0.006% H_2O_2. After 20 min, the reaction was stopped by adding 100 μl of 0.1 N H_2SO_4, and absorbance was measured at 490 nm in an enzyme-linked immunosorbent assay reader.

Analysis of Phagosomal Composition—To analyze the phagosomal composition at different time points, 40 μg of purified phagosomes from each time point were run on SDS-PAGE, transferred to nitrocellulose membranes, and incubated with appropriate dilution of respective monoclonal or polyclonal antibodies. Subsequently, membranes were incubated with secondary antibodies conjugated with peroxidase, and blots were visualized by using ECL (Amersham Pharmacia Biotech).

Electron Microscopic Observation of Phagosome-Lysosome Fusion Using MBSA-Gold as a Lysosomal Marker—J774E cells (1×10^6 cells) were incubated in the presence of mannosylated bovine serum albumin (MBSA) conjugated with 20-nm colloidal gold (100 μg/ml) for 30 min at 37 °C in prewarmed HBSA to allow uptake. Cell were washed three times with HBSA and chased for 80 min at 37 °C in the presence of 1 mg/ml mannan to label the lysosomes (24). After washing, cells were allowed to bind live or dead *Salmonella* (1×10^7 cells) at 4 °C for 1 h. Cells were resuspended in prewarmed medium, and uptake was carried out for 10 min at 37 °C. Cells were washed twice to remove the uninternalized bacteria and chased for 60 min at 37 °C. The cells were washed five times with cold PBS and fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, washed, and postfixed with 1% osmium tetroxide in the same buffer. The cells were rinsed and dehydrated in ethanol and embedded in araldite (27). Thin sections were double stained with uranyl acetate and examined with an electron microscope.

Immunolocalization of Dead Bacteria in the Lysosomes—Lysosomes of the J774E cells were labeled with MBSA conjugated with 20-nm colloidal gold, and the cells were infected with dead or live *Salmonella* as described above. Cells were washed twice to remove the uninternalized bacteria and chased for 90 min at 37 °C. The cells were washed twice and fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS, pH 7.2, for 20 min at 4 °C. Cells were washed, dehydrated in ethanol, and embedded in LR White resin. Ultrathin sections of the LR White-embedded cells were blocked with 3% casein in 0.001% Tween 20 in PBS for 1 h at 37 °C. Sections were washed five times with PBS-Tween 20 and incubated with anti-*Salmonella* LPS antibody (1:50) for 30 min at 37 °C. Sections were washed five times in similar manner, and they were incubated with protein A-conjugated with 5-nm colloidal gold for 30 min at 37 °C to allow the detection of primary antibody binding sites on *Salmonella*. Finally, the cells were stained with uranyl acetate and viewed in a transmission electron microscope (Jeol 1200 EX 11).

RESULTS AND DISCUSSION

Uptake of Live and Dead Salmonella by Macrophages—Because the intracellular trafficking of phagosomes containing dead or live *Salmonella* might depend on their rate of uptake by the macrophages, we determined the rate of uptake of live *Salmonella* by the macrophages in comparison with dead *Salmonella*, which will be targeted to the lysosome like any other inert particles (28). When J774E macrophages were incubated with metabolically labeled live or dead *Salmonella* at 37 °C for different periods of times, the rates of uptake of both preparations of *Salmonella* were essentially the same and reached a steady state plateau at about 20 min (Fig. 1). Thus, the number

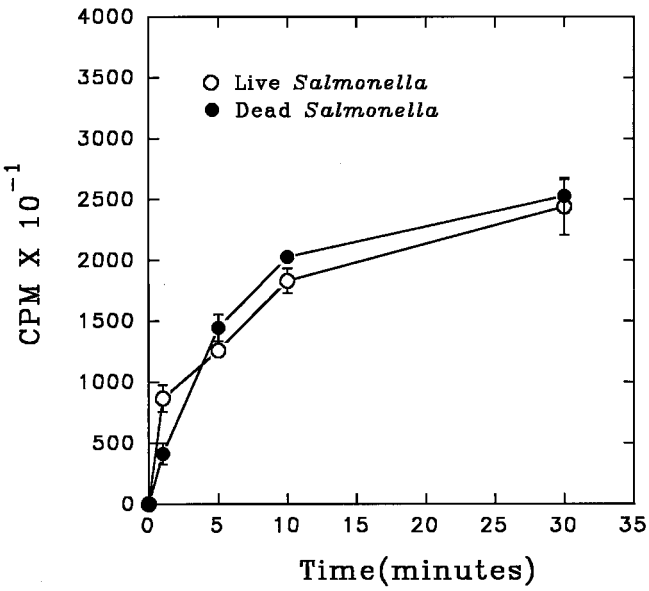

FIG. 1. **Uptake of [^{35}S]methionine-labeled live or dead *Salmonella* by macrophages.** Dead or live [^{35}S]methionine-labeled *Salmonella* (1×10^7) were added to a 24-well plate containing 1×10^6 J774E macrophages, and the infection was synchronized by low speed centrifugation at 4 °C as described under "Experimental Procedures." After incubation for the indicated time periods at 37 °C, cells were washed extensively to remove unbound bacteria and solubilized with 1% Triton X-100. An aliquot was used to determine radioactivity in a β-counter, and the results were expressed as the means ± S.D. of three determinations. Specific activity of live and dead *Salmonella* were 0.8 cpm/bacteria.

of live or dead *Salmonella* associated with macrophages at different time points was identical (Fig. 1). Previous studies have shown that *Salmonella* enter into the nonphagocytic cells by a membrane ruffling mechanism and that noninvasive mutant organism is unable to induce its uptake (33). In contrast, Rathman *et al.* (34) reported that uptake of noninvasive mutant organism in phagocytes is probably mediated through a host cell-directed mechanism such as lectinophagocytosis, as opposed to pathogen-induced membrane ruffling. This is consistent with our finding that both live and dead bacteria labeled with calcein and ethidium homodimer, respectively, are internalized by macrophages with similar efficiency (data not shown).

Live Salmonella Inhibit Transport to Lysosome—To quantify the transport of the live or dead *Salmonella* from the early compartment to the lysosomes, cells were preloaded with avidin-HRP and chased for 90 min to label the lysosomes (24). Subsequently, cells were pulsed with live or dead biotinylated bacteria at 37 °C for a short period of time (5 min) to restrict their entry to the early compartment followed by a chase. At the indicated times the formation of bacteria biotin-avidin-HRP complex was measured to determine the transport of the *Salmonella* to lysosomes. The results presented in Fig. 2 show that dead *Salmonella* co-localized with avidin-HRP-preloaded lysosomes within 45 min, and maximum fusion was observed within 90 min. In contrast, live *Salmonella* did not form complexes with avidin-HRP even after 90 min. These results were further supported by the electron microscopic observations shown in Fig. 3 in which macrophages were preloaded with MBSA conjugated with 20-nm colloidal gold to label the lysosomal compartment. The results presented in Fig. 3 (*a* and *b*) show that live *Salmonella* were found in a distinct vesicular compartment that was well separated from the 20-nm gold-containing compartment. In contrast, dead bacteria after 60

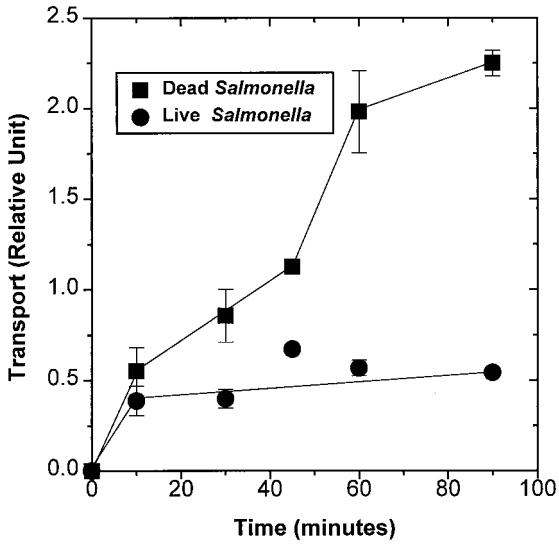

FIG. 2. **Intracellular transport of live or dead *Salmonella* to the lysosomes.** J774E macrophages were preloaded with the avidin-HRP and chased for 90 min to label the lysosomes. Subsequently, cells were pulsed with live or dead biotinylated *Salmonella* at 37 °C for a short period of time (5 min) to restrict their entry to the early compartment and incubated for the indicated times at 37 °C. At the indicated times, the cells were lysed by SB containing avidin as scavenger as described under "Experimental Procedures." HRP activity associated with bacteria-biotin-avidin-HRP complex was measured to determine the transport of the *Salmonella* to lysosomes. Each point represents the mean ± S.D. from three independent experiments.

min of chase at 37 °C were colocalized with 20-nm gold- labeled compartment (Fig. 3, *c* and *d*). After 90 min of chase the morphology of the dead bacteria was not well preserved, suggesting possible degradation of the dead bacteria. The presence of the dead bacteria was confirmed by treating the sections with an antibody against *Salmonella* LPS followed by protein A conjugated with 5-nm gold particles for visualization. Extensive colocalization of the 5-nm gold particles was observed with 20-nm gold particles, suggesting the targeting of the dead organisms to the lysosomes (Fig. 4, *c* and *d*). In contrast, live bacterial morphology were well preserved after 90 min of chase and not colocalized with 20-nm gold particles, demonstrating that live bacteria survive in a specialized compartment and thereby avoid lysosomal degradation (Fig. 4, *a* and *b*). It is pertinent to mention that more than 80% of the DSP colocalized with 20-nm gold particles, whereas less than 3% of LSP contain 20-nm gold particles.

Live Salmonella Bypass the Endocytic Route and Mature into a Specialized Compartment—To delineate the intracellular route of *Salmonella* in macrophages, we purified phagosomes at different time points after internalization of live or dead *Salmonella* (20, 25). The biochemical characterization of the phagosomes demonstrated that these phagosomes are free of endosome, lysosome, Golgi, and endoplasmic reticulum contamination. These phagosomes were used to determine the presence of compartment specific markers like transferrin receptor, M6PR, as well as LAMP1. Transferrin receptor serves as a specific marker for the early endosome because it has been demonstrated not to travel beyond 5 min (35, 36). The data in Fig. 5 show that only early (5 min) DSP is positive for transferrin receptor. No transferrin receptor was detected in DSP purified after 5 min of internalization, suggesting their further maturation. In contrast, transferrin receptors persisted with LSP until 30 min and subsequent loss of transferrin receptor indicated that live *Salmonella* might be transported to a different endocytic compartment.

Two classes of M6PRs (CD-M6PR, molecular mass of 46 kDa, and CI-M6PR, molecular mass of 215 kDa) involved in the transport of lysosomal enzymes serve as specific markers for the late endosomal/prelysosomal compartments (37–39) and are not present in mature lysosomes (40). The data presented in Fig. 5 are consistent with the previous finding (19) that LSP did not acquire CD-M6PR, suggesting that live *Salmonella* might either prevent the fusion of LSP with M6PR-containing vesicles from the trans-Golgi network (TGN) or selectively remove M6PR. This could be a mechanism to prevent the accumulation of the lysosomal enzymes in live *Salmonella*-containing vesicles, which might be detrimental for the invading organisms. In contrast, we detected significant amounts of CD-M6PR on DSP at 30 min but not at later time points, indicating their further maturation to the lysosomal pathway after a transient stay in the late endosomal compartment. Phagosomes containing latex bead or *Leishmania mexicana*, which are destined to fuse with lysosomes, are also found to have increasing amounts of M6PRs (9, 41, 42). Thus, the virtual absence of transferrin receptor in 60 and 90 min LSP indicates their transit from the early compartment, but a lack of M6PR on purified phagosomes containing live organisms suggests that LSP do not acquire the properties of the late endosomal compartment.

We have also compared the expression of the lysosomal protein LAMP1 in DSP and LSP at different time points. The results presented in Fig. 5 show the presence of LAMP1 on DSP at all time points, but the LAMP1 expression on LSP is detected only after 30 min. Moreover, the overall expression of LAMP1 is relatively more on DSP than on LSP. Our results have also shown that live *Salmonella* bypass the late compartment and do not transport to lysosomes, thus the retention of the lysosomal marker LAMP1 on matured LSP may be due to the fusion of LSP with LAMP1-containing vesicles from the TGN. This is consistent with earlier observations that phagosomes containing *Mycobacterium* (25) or *Salmonella* (43) retain LAMP1 from the early to late time points. LAMP1 trafficking from the TGN to lysosomes is postulated to be mediated through two different routes (44, 45). One subset of LAMP1 is delivered directly from the TGN to lysosomes through endosomes without appearing on the plasma membrane. It is also possible that some of the LAMP1 appearing on the cell surface is transported back to early endosomes and progress to lysosomes via late compartments. Thus, the retention of LAMP1 in live bacteria-containing phagosomes that are not targeted to lysosomes is possibly due to ubiquitous distribution of LAMP1 in all endocytic compartments (44, 45) or may be due to the fusion of LSP with LAMP1 containing vesicles from the TGN (11).

Role of Rab Proteins in Regulation of Intracellular Trafficking of Live Salmonella—Transport of phagosomes to lysosomes is of interest particularly with regard to the mechanism by which pathogenic microorganisms survive inside phagosomes by interfering with membrane fusion. Recent studies have shown that Ras-related Rab-GTPases regulate membrane fusion during intracellular trafficking (3–6). Rab proteins specifically localized on an intracellular compartment mediate specific vesicular transport by controlling vesicle docking and fusion (5, 46, 47). A number of Rab proteins, *e.g.* Rab4, Rab5, Rab7, Rab11, and Rab18, have been found to localize on the early endocytic compartment, indicating that early endocytic compartment is highly complex with multiple functions. Rab4 is involved in the recycling from the early endosomes to the plasma membrane (48), and Rab11 regulates the recycling from the perinuclear endodosomes (49). Rab9 regulates the traffic from the trans-Golgi network to the lysosomes through late

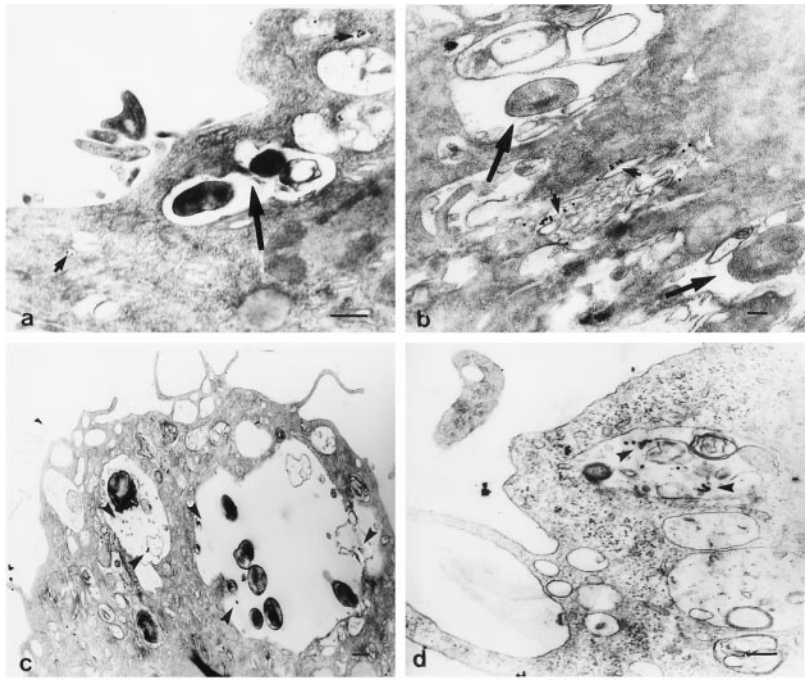

FIG. 3. **Fusion of live or dead *Salmonella* containing late phagosomes with lysosome.** J774E cells (1×10^6 cells) were incubated for 30 min at 37 °C to internalize MBSA conjugated with 20-nm colloidal gold (100 μg/ml) and chased for 80 min at 37 °C to label the lysosomes. Subsequently, cells were pulsed with live (*a* and *b*) or dead (*c* and *d*) *Salmonella* at 37 °C for a short period of time (10 min) to restrict their entry to the early compartment followed by a chase for 60 min as described under "Experimental Procedures." Cells were washed, fixed, and processed for electron microscopy. *Arrowheads* indicate the presence of MBSA-gold (20 nm) in the phagosomes containing dead *Salmonella* (*c* and *d*) reflecting the fusion with lysosomes. *Big arrows* indicate the live *Salmonella*-containing phagosomes (*a* and *b*), and *small arrows* indicate the presence of MBSA-gold (20 nm), which is separated from the live *Salmonella*-containing phagosomes. *Bars*, 200 nm.

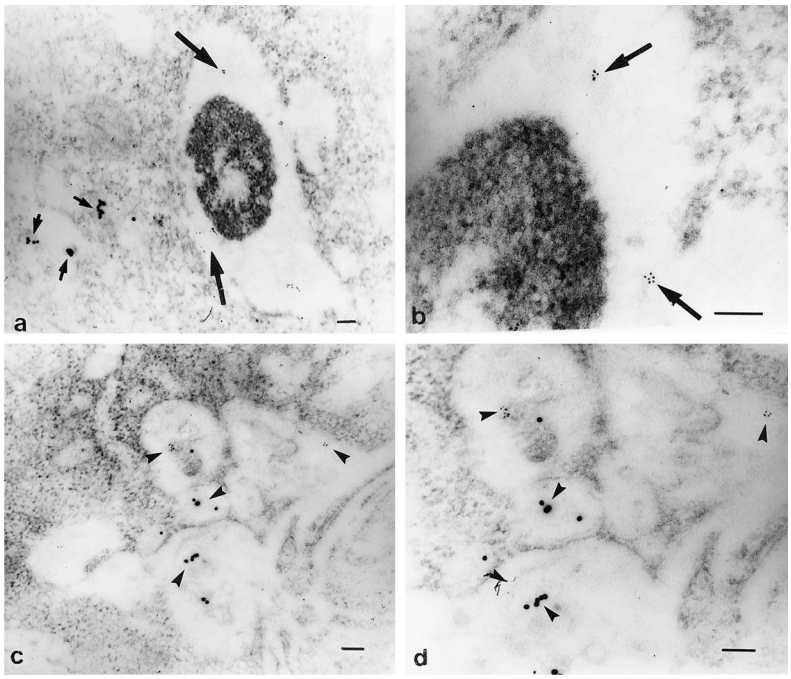

FIG. 4. **Immunolocalization of dead bacteria in the lysosomes.** Lysosomes of the J774E cells were labeled with MBSA conjugated with 20-nm colloidal gold, and the cells were infected with dead or live *Salmonella* as described under "Experimental Procedures." Cells were washed twice to remove the uninternalized bacteria and chased for 90 min at 37 °C. The cells were washed twice and processed for immunogold labeling using anti-*Salmonella* antibody as described under "Experimental Procedures." *Arrowheads* indicate the presence of MBSA-gold (20 nm) and 5-nm gold particles in the phagosomes containing dead *Salmonella* (*c* and *d*) reflecting the fusion with lysosomes. *Big arrows* indicate the presence of 5-nm gold particles in live *Salmonella* containing phagosomes (*a* and *b*), which is well separated from 20-nm gold-containing vesicles as indicated by *small arrows*. *Bars*, 100 nm.

endosomes (50), and Rab7 is involved in the transport from the early to the late compartment (51–54). Rab5 is involved in the transport from the plasma membrane to the early compartment as well as in homotypic fusion among early endosomes (55–58). Recent studies have shown that fusion of endocytic vesicles with phagosomes containing inert particles requires fusion proteins like Rabs (27, 41, 59, 60) and *N*-ethylmaleimide-sensitive factor (24, 26, 41). How intracellular pathogens alter the function of these proteins to avoid or induce the specific interactions of phagosomes with other vacuolar compartments is not clear. To understand the mechanism of intracellular trafficking of *Salmonella*, we compared the expression of different endocytic Rabs, *viz.* Rab5, Rab7, Rab9, and Rab18, on purified LSP or DSP prepared at different time points after internalization.

As shown in Fig. 6, LSP recruit more of early acting Rab5 than DSP at 5 min. Subsequently, significant amount of Rab5 was not detected on DSP, indicating their maturation toward later compartment. Similar results were obtained with phagosomes containing latex beads that are destined to fuse with lysosomes (41, 42). In contradiction, recent studies by the same group and others (21, 60) reported retention of Rab5 on phagosomes containing latex beads through the late stages as well. No satisfactory explanation was offered for the apparent contradiction. The processing and fate of phagocytic particles has been shown to depend on the nature and the size of the particles (61). It is therefore possible that the size difference between *Salmonella* (2–4 μm) and the latex bead (1 μm) modulates the levels of Rab5 and other factors on the phagosomes resulting in targeting to the lysosomes with differential effi-

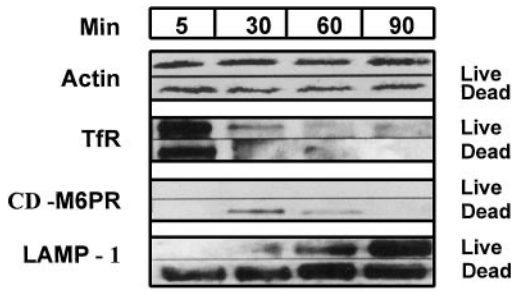

FIG. 5. **Analysis of endosomal and lysosomal markers in phagosomes containing live or dead *Salmonella*.** Western blot analyses were carried out for the detection of actin, transferrin receptor, CD-M6PR, and LAMP1 in phagosomes purified at different time points containing live or dead *Salmonella*. Proteins from purified phagosomes (40 μg/lane) were separated by SDS-PAGE, transferred to membrane, and incubated with the appropriate dilution of specific antibodies followed by HRP-conjugated secondary antibody and visualized by ECL. Results from Western blots are representative of three independent preparations.

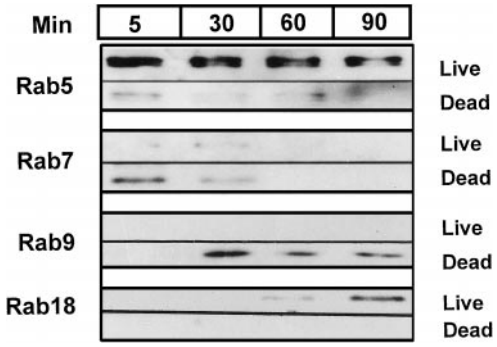

FIG. 6. **Modulation of live or dead *Salmonella* trafficking by Rab proteins.** Western blot analysis was carried out for the detection of Rab5, Rab7, Rab9, and Rab18 in phagosomes purified at different time points containing live or dead *Salmonella*. Proteins from purified phagosomes (40 μg/lane) were separated by SDS-PAGE, transferred to membrane, and incubated with the appropriate dilution of specific antibodies followed by HRP-conjugated secondary antibody and visualized by ECL. Results from Western blots are representative of three independent preparations.

ciency. In our studies, comparing the intracellular trafficking of live and dead *Salmonella* obviated the possible effect of particle size. Furthermore, our results with DSP are consistent with the findings of Mordue and Sibley (62) that dead *Toxoplasma*-containing phagosomes also rapidly deplete Rab5. In contrast to the DSP, LSP appeared to recruit and retain Rab5 over long periods of chase (90 min), indicating that LSP effectively promotes the fusion with early endosomal compartment to delay their transport to lysosomes. The virtual absence of transferrin receptor after 30 min and retention of Rab5 on LSP suggest that live *Salmonella* are transported to a different endosomal compartment where the Rab5 is sequestered. These results are consistent with the earlier report that *Salmonella* survive in relatively large membrane-bound vesicles (63). It could be due to the Rab5-mediated fusion of LSP with early endosomal compartment because it has been shown that overexpression of GTPase-defective mutant of Rab5 led to the appearance of unusually large endocytic vesicles (64). Rab proteins in GTP form activate the SNARE and trigger the vesicle fusion. Subsequently, GTPase-activating protein increases the GTPase rate of the Rab protein, converting it into its GDP form, and triggers the release of the Rab to the cytosol by GDI (65). Thus, the retention of Rab5 on the LSP may be due to the inhibition of the Rab5-GTPase-activating protein activity by the live organism. A recent study by Hardt *et al.* (33) has shown that SopE, a protein secreted by the *Salmonella,* stimulates GDP to GTP nucleotide exchange of several Rho GTPases. This result indicates the possibility that a similar protein secreted by the *Salmonella* may be regulating the nucleotide exchange of Rab5. Our observation that live bacteria containing phagosomes retain Rab5 on the phagosomal membrane and thereby probably retard their transport to lysosomes is also consistent with recent findings with listeriolysin-defective mutant of *Listeria* and *Mycobacterium* (20, 21).

Rab7, located on the late compartment, functions downstream of Rab5 and regulates the transport between early to late endosomes/lysosomes (51–54). Accordingly, we looked for the expression of Rab7 on purified LSP or DSP. Western blot analysis with specific antibodies showed that DSP is enriched in Rab7 even 5 min after and retains Rab7 until 30 min (Fig. 6), suggesting rapid transport of dead *Salmonella* to late endocytic compartment as well as their exit from the late compartment after 30 min. In contrast, live *Salmonella* selectively deplete the Rab7 from the phagosomal membrane and therefore inhibit their transport to the later endocytic compartments. Rab7 serves as a targeting signal for the transport from the early to late lysosomal compartment, and the low pH of the lysosomal compartment mediates the killing of the invading microorganisms by lysosomal hydrolases. Selective depletion of Rab7 from LSP presumably enables the *Salmonella* to escape their targeting to the Rab7-dependent lysosomal pathway, contributing to their survival inside the phagocytes. These results further supported our observation (Fig. 5) that LSP never acquired M6PR, characteristic of the late endocytic compartment.

Late endosomal compartments are highly enriched in Rab9, which is involved in the transport of lysosomal enzymes from the TGN to lysosomes through late endosomes (50). To understand how live *Salmonella* modulate the trafficking of lysosomal enzymes, we looked for the expression of Rab9 on LSP and DSP. Consistent with this we detected Rab9 predominantly present on 30 min and later DSP (Fig. 6), indicating active transport of lysosomal enzymes through the Rab9-dependent M6PR-mediated pathway (66). In contrast, absence of Rab9 on LSP (Fig. 6) indicates a lack of transport of lysosomal enzymes to the LSP, suggesting that survival of live *Salmonella* depends on their ability to reside in a compartment that does not acquire lysosomal enzymes, which is further supported by the absence of M6PR on LSP (Fig. 5).

The absence of transferrin receptor and M6PR on matured LSP (60 min onward) indicates their transport from the early endosomal compartment to a different compartment bypassing the endocytic route. To understand the nature of the compartment, we compared the expression of Rab18. The function of Rab18 is not clearly known, but it appeared to localize in the endocytic structure underlying the apical plasma membrane in polarized cells, suggesting their role in endocytosis/recycling (67). The presence of Rab18 and the absence of transferrin receptor on LSP at 90 min (Fig. 6) indicate that live *Salmonella* finally exit from the early endosomal compartment and mature in a special compartment that retains Rab5 and Rab18.

Live Salmonella-containing Phagosomes Retain Cathepsin D in Its Immature Form—The lysosomal hydrolase cathepsin D is synthesized as a 51–55-kDa precursor protein in TGN and transported to the early endosomal compartment (pH 6), where the precursor protein is cleaved from the amino terminus resulting in a 48-kDa intermediate form. Subsequently, the enzyme is further transported to lysosomes (pH 4.5–5), where it matures into 31- and 17-kDa proteases by an internal cleavage (68, 69). Therefore, the processing status of cathepsin D could

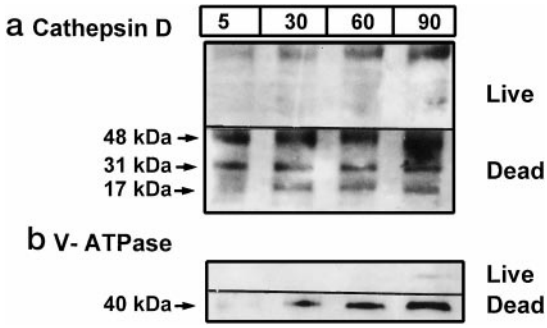

FIG. 7. **Live *Salmonella*-containing phagosomes retain cathepsin D in its immature form.** To determine the pH of the phagosomes containing dead or live *Salmonella*, purified phagosomes at different time points were analyzed for the presence of cathepsin D (*a*) and vacuolar ATPase (*b*). Proteins from purified phagosomes (40 μg/lane) were separated by SDS-PAGE, transferred to membrane, incubated with the appropriate dilution of specific antibodies followed by HRP-conjugated secondary antibody, and visualized by ECL. Cathepsin D in the 90-min phagosome containing live *Salmonella* is predominantly in its proenzyme form and lacks detectable expression of vacuolar ATPase. Results from Western blots are representative of three independent preparations.

serve as an indicator of the pH of the intracellular compartment in which *Salmonella* reside. Fig. 7*a* demonstrates the presence of 48-kDa procathepsin D in LSP for 90 min, whereas mature 31- and 17-kDa forms of cathepsin D started appearing from 30 min. Immature cathepsin D is also detected in *Mycobacterium*-containing phagosomes even after 9 days (11). Moreover, when the same *Mycobacterium*-containing phagosomes were incubated with 50 mM acetate buffer, pH 4.5, for 10 min, the mature forms of cathepsin D were detected, suggesting that the pH of the phagosomes is critical for controlling the maturation of cathepsin D (11). Taken together with these results, our results indicate that the pH of the phagosomes containing live *Salmonella* is less acidic.

Recent studies have shown that the process of phagosome maturation is complex and requires extensive remodulation of the phagosomal membrane (7, 41). To understand how live *Salmonella* modulate the pH of the intracellular vesicles, we looked for the expression of vacuolar H^+-ATPases in LSP (70). The results presented in Fig. 7*b* show that LSP have relatively less expression of the vacuolar ATPase in comparison with DSP, suggesting that live *Salmonella* selectively deplete the vacuolar ATPases from the phagosomal membrane to maintain a relatively high intravacuolar pH. Similar depletion of the vacuolar ATPase has been reported in live *Mycobacterium*-containing phagosomes (11). Moreover, the presence of vacuolar ATPase on DSP is consistent with previous reports on trafficking of phagosomes containing latex bead (43) or dead *Listeria* (71). These results indicate that an active process mediated by live bacteria may selectively eliminate this membrane component, which is responsible for the acidification of the vacuolar compartment. Our results thus show that *Salmonella* modulate the expression of vacuolar ATPases to generate a relatively less acidic vesicular environment essential for their survival.

In conclusion, our results represent the first documentation that live *Salmonella* modulate the expression of various Rabs (*e.g.* Rab5, Rab7, Rab9, and Rab18) on the phagosomes to reside in a specialized low acidity compartment devoid of active lysosomal enzymes and transferrin receptors but that retain Rab5 and Rab18. It appears that success in thus altering the intracellular trafficking pattern could be the mechanism of survival of *Salmonella* in the pathophysiology of enteric fever. Further studies are in progress to determine the interaction of live *Salmonella*-containing phagosomes with other intracellular compartments.

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