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Internalized *Listeria monocytogenes* modulates intracellular trafficking and delays maturation of the phagosome

Carmen Alvarez-Dominguez, Richard Roberts and Philip D. Stahl*

Department of Cell Biology and Physiology, Washington University, School of Medicine, St Louis, MO 63110, USA

*Author for correspondence

SUMMARY

Previous studies have shown that early phagosome-endosome fusion events following phagocytosis of *Listeria monocytogenes* are modulated by the live organism. In the present study, we have characterized more fully the intracellular pathway of dead and live *Listeria* phagosomes. To examine access of endosomal and lysosomal markers to phagosomes containing live and dead *Listeria*, quantitative electron microscopy was carried out with intact cells using internalized BSA-gold as a marker to quantify transfer of solute from endosomal and lysosomal compartments to phagosomes. To monitor the protein composition of phagosomal membranes and to quantify transfer of HRP from endosomes and lysosomes to phagosomes, highly enriched phagosomes containing live and dead *Listeria* were isolated. Enriched phagosomal membranes were used for western blotting experiments with endosomal and lysosomal markers. In this study, we used a listeriolysin-deficient mutant, *Listeria*$^{hly-}$, that is retained within the phagosome following phagocytosis. Western blotting experiments indicate that early endosomal markers (mannose receptor, transferrin receptor) and key fusion factors necessary for early events (NSF, α/β-SNAP) but not late endosomal markers (cation dependent mannose 6-phosphate receptor) or lysosomal proteins (cathepsin D or lamp-1) accumulate on the live-*Listeria* phagosomal membranes. On the contrary, phagosomes containing dead-*Listeria* are readily accessible by both endocytic and lysosomal markers. Studies with radiolabeled dead- and live-*Listeria*$^{hly-}$ indicate that, following phagocytosis, degradation of the live microorganism is substantially delayed. These findings indicate that dead-*Listeria* containing phagosomes rapidly mature to a phagolysosomal stage whereas live-*Listeria*$^{hly-}$ prevents maturation, in part, by avoiding fusion with lysosomes. The data suggest that by delaying phagosome maturation and subsequent degradation, *Listeria* prolongs survival inside the phagosome/endosome assuring bacterial viability as a prelude to escape into the cytoplasm.

Key words: *Listeria*, Trafficking, Phagocytosis

INTRODUCTION

Phagocytosis is a complex receptor mediated process characterized by particle binding, internalization and transport to lysosomal or degradative compartments deep within the cell. A key component of phagocytosis is phagosome maturation which appears to require extensive remodelling of the phagosomal membrane. Phagosome maturation, once considered a unidirectional and simple process where phagosomes fuse directly with lysosomes to form phagolysosomes, is now known to be due to a series of intracellular membrane fusion and budding events. Phagosome maturation and transport also require the participation of the cytoskeleton (Blocker et al., 1996; Desjardins et al., 1994a; Garcia-del Portillo and Finlay, 1995). The dynamic exchange of phagosomal membrane and content with other intracellular compartments includes the delivery of hydrolytic enzymes and proton pumps, which is accompanied by a decrease in intraphagosomal pH (reviewed by Berón et al., 1995a). Until recently (Sturgill-Koszychi et al., 1994; Alvarez-Dominguez et al., 1996; Garcia-del Portillo and Finlay, 1995) virtually all studies on phagosome maturation have been performed with model particles or dead microorganisms whose final fates along the phagocytic route appear to be similar, the phagolysosome (Mayorga et al., 1991; Rabinowitz et al., 1992; Bouvier et al., 1994; Desjardins et al., 1994a; Berón et al., 1995b; Oh and Swanson, 1996). However, it has been known for some years that certain live microorganisms interfere with one or more of these processes by a variety of strategies that may allow the pathogen to survive and in some cases to multiply (Moulder, 1991; Garcia-del Portillo and Finlay, 1996). Organisms such as *Coxiella burnetti* or *Leishmania* ssp. (Maurin et al., 1992; Russell et al., 1992), thrive in an acidic compartment. In other cases, microorganisms attenuate the acidic pH of the compartment in which they reside: e.g. *Mycobacterium avium*, *Legionella pneumophila*, or *Toxoplasma gondii* (Crowle et al., 1991; Sturgill-Koszycki et al., 1994; Horwitz and Maxfield, 1984; Sibley et al., 1985). Other intracellular pathogens have developed mechanisms to lyse the phagosomal membrane and escape to the cytoplasm: e.g. *Trypanosoma cruzi* or *Listeria monocytogenes* (Tardieux et al., 1992; Portnoy et al., 1988). Finally, some pathogens appear to have developed mechanisms to modulate the redis-

tribution of endosomal and lysosomal markers. For example, *Salmonella typhimurium* containing phagosomes appear to fuse with compartments containing lgp-proteins, bypassing compartments containing CD-M6P (cation-dependent mannose 6-phosphate receptor) or CI-M6P (cation-independent mannose 6-phosphate receptor) which are normally encountered along the endocytic route. The peculiarity of this compartment was also revealed by the recent finding that *Salmonella* phagosomes have an acidic pH (4.0-5.0; Rathman et al., 1996) contrary to previous reports that suggested a delay in phagosomal acidification (Alpuche-Aranda et al., 1992). This suggests that pathogens may have the capacity to selectively fuse with vesicles carrying different markers, possibly sub-sets of vesicles derived from the same compartment, thereby creating a unique phagosomal environment that allows for survival (Garcia-del Portillo and Finlay, 1995).

Listeria monocytogenes (LM) is a facultative intracellular pathogen that, upon internalization by phagocytes, remains within the phagosome for short periods before escaping to the cytoplasm (after ~30 minutes; Tilney and Portnoy, 1989). During this time, phagosomes are presumed to mature via multiple fusion events with other compartments. Using an in vitro fusion assay we have previously reported that live-LM^{hly-} (listeriolysin-deficient mutant) upregulate fusion with early endosomes by accelerating the delivery of rab5 and NSF to the phagosomal membranes; dead-LM phagosomes accumulate neither rab5 nor NSF and do not show accelerated fusion (Alvarez-Dominguez et al., 1996). In this report we have characterized the process by which a live non-hemolytic mutant, live-LM^{hly-}, upregulates membrane fusion events and controls intracellular fate. This experimental model has been particularly useful because both bacteria, live-nonhemolytic mutant LM^{hly-} (mutant retained inside the phagosome) and dead-heat-killed (LM^{hly-}); are retained in intracellular vesicular compartments. Therefore, it was possible to analyze the hypothesis that dead and live bacteria may travel by different itineraries inside macrophages. We present evidence suggesting that the live microorganism prevents phagosome maturation by selectively accelerating fusion with endosomes and delaying, if not preventing, fusion with lysosomes.

MATERIALS AND METHODS

Biological reagents

J774E-clone, a mannose-receptor positive macrophage-cell line, was grown as described by Pitt et al. (1992). β-Glucuronidase was purified and DNP-derivatized according to a methodology previously described (Diaz et al., 1988). Horseradish peroxidase (HRP) was obtained from Pierce. Protein was measured as described (Bradford, 1976) using bovine serum albumin as standard.

Antibodies

Monoclonal antibodies (mAbs) used include: 4F11, a mouse $IgG_{2a\kappa}$ mAb specific for the COOH terminal of canine rab5, recognizes mouse rab5 (Qiu et al., 1994); 6E6, a mouse IgG mAb that recognizes the D1-D2 domains of NSF (~76 kDa) (gift from Sidney W. Whiteheart, University of Kentucky, Lexington, KY); CI77.1, a mouse IgG mAb that recognizes brain and kidney α/β-SNAP (~32-34 kDa; gift from R. Jahn, Yale University, New Haven, CT); 1G11, rat mAb that recognizes mouse lamp-1 (~110-120 kDa) (gift from H. Rosen, Merck Laboratories); RI7 217.1.4, a rat mAb that recognizes mouse transferrin receptor (CD71) (~95 kDa) (Caltag Laboratories, San Francisco, CA); a11, a mouse mAb that recognizes bovine vacuolar H^+-ATPase, E subunit (~31 kDa) (gift from S. Gluck, Washington University, St Louis, MO). Polyclonal antibodies include: rabbit antiserum to mouse mannose receptor (~180 kDa); affinity purified rabbit anti-mouse cathepsin D (~53 kDa, immature form; and ~43 kDa, mature form) (both antibodies were obtained by immunizing rabbits with purified proteins); rabbit anti-mouse CD-M6P receptor (~46 kDa) (gift from W. Sly, St Louis University, St Louis, MO); all the secondary antibodies were peroxidase conjugated and were obtained from Gibco or Jackson Immunoresearch Laboratories.

Bacteria

The nonhemolytic *Listeria monocytogenes* mutant strain used in this study (DP-L2161) (LM^{hly-}) (Jones and Portnoy, 1994) derived from the wild-type strain (10403S) was kindly provided by D. A. Portnoy (University of Pennsylvania, Philadelphia, PE). Live bacteria and heat-killed bacteria (dead-LM) were prepared as previously described (Alvarez-Dominguez et al., 1996).

Preparation of highly-purified phagosomes

J774E-clone macrophages (10^8) were incubated with dead or live bacteria (10^9 CFU) (1 hour, 4°C) and centrifuged to synchronize the infection (2,000 rpm, 5 minutes, 4°C). Internalization was initiated by addition of prewarmed HBSA (Hanks' balanced salt solution with 10 mM Hepes and 10 mM TES, pH 7.4, supplemented with 1% BSA). After incubation at 37°C for 5 minutes; cells were washed in the cold with PBS-2 mM EDTA and chased at 37°C (for different times: 1, 10 or 25 minutes). Cells were sequentially washed at 4°C with HBSA, PBS-2 mM EDTA and HBE (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-KOH, pH 7.2) and homogenized (Pitt et al., 1991). (The percentage of bacteria internalized by cells ranged from 42-45%, for both dead- and live-LM. Homogenates were centrifuged at 400 ***g*** to eliminate nuclei and intact cells. The postnuclear supernatants (PNS) were quickly frozen in liquid nitrogen and stored at –80°C. Phagosomes were prepared as described by Alvarez-Dominguez et al. (1996) using a modification of a technique described by Sturgill-Koszycki et al. (1994)). Briefly, phagosomal fractions were obtained by rapidly thawing a phagosomal PNS aliquot, diluting it into HBE (1 ml) and centrifuging at 12,000 ***g*** for 10 seconds. The supernatant was kept at 4°C and the pellet resuspended in HBE was centrifuged again. The resulting supernatants were combined and centrifuged at 12,000 ***g*** for 6 minutes at 4°C. This pellet (phagosomal fraction) contained approximately 70% of internalized bacteria. The phagosomal fraction was resuspended in HBE-protease cocktail, filtered through 5 μM pore filters and applied directly to a 12% sucrose cushion. Following centrifugation, phagosomes were collected at the bottom of the tube. The percentage of phagosomes recovered in this highly purifed fraction was ~95% of those recovered in the phagosomal fraction with most of the phagosomes contained in intact vesicles (broken phagosomes were less than 3%), measured by the criterion that almost none of the surface biotinylated bacteria were able to bind to avidin-HRP after incubation in the absence of detergent. Percentages were similiar for both bacteria preparations, dead- or live-LM^{hly-}. Purity of the organelles was monitored by: (i) electron microscopy; and (ii) biochemical analysis using membrane and luminal markers. Plasma membrane contamination was assessed by the recovery of HRP in the purified phagosomes. Cells (previously infected with dead- or live-LM) were incubated with HRP at 4°C to allow binding to the mannose receptor (Lang and de Chastellier, 1985). The cells were extensively washed in the cold before homogenization and phagosome isolation. Cell surface-bound HRP recovered in the purified phagosomes was always less than 0.8% for both dead- and live-LM^{hly-} preparations. Less than 0.2% of the total galactosyltransferase, a Golgi marker (Desjardins et al., 1994b) was found in highly purified phagosomes (both dead- and live-LM^{hly-}). Endosomal and lysosomal contamination, respectively, was estimated by mixing an aliquot of phagosomal PNS (from a set of cells that had ingested dead- or live-LM for

the following times: 5 minute pulse, or 5 minute pulse and 1 minute, 10 minutes or 25 minutes chase) with an aliquot of endosomal or lysosomal PNS. Endosomal and lysosomal PNS were obtained from a different set of cells preincubated with DNP-β-glucuronidase (100 μg/ml) at 4°C and internalized for 5 minutes at 37°C (endosomal PNS), or 5 minutes uptake followed by 85 minutes chase (lysosomal PNS; Beron et al., 1995b). Purified phagosomes were then isolated as described above and the enzymatic activity was measured after immunoprecipitation of the DNP-β-glucuronidase onto anti-DNP coated plates, as previously described (Diaz et al., 1988). The percentage of endosomal or lysosomal contamination was estimated as the ratio of enzymatic activities found in the highly purified phagosome fraction (in the presence or absence of detergent) compared to the total activity estimated in the endosomal or lysosomal PNS (in the presence of detergent). Contamination of phagosome preparations with endosomes or lysosomes was never more than 0.30-0.35%, respectively. Integrity of the vesicles containing the endosomal/lysosomal PNS was estimated by two procedures: (i) total membranes were pelleted from PNS and the enzymatic activity was measured in pellets and supernatants (the supernatant enzymatic activity would correspond to lysed vesicles) after immunoprecipitation onto anti-DNP coated plates. This measurement records the percentage of activity associated with the membranes; and (ii) estimating the enzymatic activity immunoprecipitated onto the anti-DNP coated plates in the presence or absence of detergent. Vesicle lysis was never more than 5%. For analysis of phagosome composition, 30 μg of purified phagosomal proteins were added per lane. The proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with different monoclonal or polyclonal antibodies. After incubation with secondary antibodies conjugated with peroxidase, blots were developed by ECL (Amersham). Exposure times were always less than 2 minutes. The presence of *Listeria* proteins in each preparation of phagosomes was also determined using a western blot assay with a rabbit anti-LM antibody. The various preparations contained approximately equal amounts of bacterial proteins. Moreover, the protein patterns were similar. Bacterial proteins in the phagosome preparations were also quantitated by measuring the amount of protein bound biotin (~2.0-2.4 moles/mg protein). Bacteria were surface biotinylated prior to phagocytosis.

Detection of phagosome-endosome fusion and phagosome-lysosome fusion using BSA-gold as an endocytic tracer

J774E-clone cells were grown to confluence on 35 mm tissue culture dishes. For phagosome-endosome fusion assays, cells were first infected with 5×10^7 dead- or live-LM^{hly-} by centrifugation and incubated for different periods of time. For early time points (6 minutes phagocytosis), cells were infected with bacteria for 5 minutes and chased for 1 minutes. For intermediate times (15 minutes phagocytosis), cells were infected for 5 minutes and chased for 10 minutes. For later times (30 minutes phagocytosis), bacteria were infected for 5 minutes and chased for 25 minutes. The cells were then incubated for 10 minutes with BSA-gold (30 nm particle size). For phagosome-lysosome fusion, cells were first incubated for 90 minutes with BSA-gold (10 nm particle size) and chased overnight to assure lysosome labeling. Following infection as described above, the cells were washed once with PBS-2 mM EDTA to remove unbound bacteria, then (i) washed in cacodylate buffer and fixed in 1% glutaraldehyde/0.1 M Na cacodylate buffer for 15 minutes at 4°C, (ii) postfixed in 1.25% osmium tetroxide in the same buffer, and (iii) en bloc stained with 4% aqueous uranyl acetate. Samples were dehydrated in ethanol and embedded in overturned gelatin capsules containing Polybe 812. Cells were ultrathin sectioned and poststained in uranyl acetate and Reynolds lead (Diaz et al., 1989).

Transfer of endocytosed HRP to phagosomes as detected in highly purified isolated phagosomes

To follow HRP access to early phagosomes, J774E-clone cells (10^8) were infected with dead- or live-LM^{hly-} bacteria (10 bacteria per cell) for 5 minutes, washed and chilled on ice for 60 minutes with HRP (200 μg/ml) to allow binding to the cells. The cells were then allowed to internalize HRP for 5 minutes at 37°C, washed and chased for another 5 minutes. For HRP access to late phagosomes (30 minutes phagosomes and 10 minutes endosomes), cells were infected for 5 minutes with bacteria, washed, chased for 15 minutes and then chilled on ice. Cells were then incubated with HRP on ice for 60 minutes and then incubated at 37°C for 5 minutes plus 5 minutes chase. Following the above protocols, cells were chilled on ice, homogenized and phagosomes were isolated as described.

To assess transfer of HRP from HRP-loaded lysosomes to different aged phagosomes, cells were incubated with HRP (200 μg/ml) for 60 minutes on ice, warmed to 37°C for 5 minutes to internalize surface bound HRP followed by a 2 hour chase. The cells were then allowed to ingest dead- or live-LM^{hly-} for 5 minutes followed by different chase times: 1, 10 and 25 minutes. Following uptake and chase, cells were homogenized and phagosomes were isolated as described above.

Bacteria catabolism assay

The assay was performed as reported by Ziegler and Unanue (1981) and Harding and Unanue (1990) and modified by Cluff et al. (1990). Briefly, LM^{hly-} bacteria were grown overnight in BHI (Difco). After extensive washing, bacteria were resuspended in RPMI (minus methionine and cysteine) with 1 mCi of [^{35}S]methionine (ICN Trans^{35}S-label). The bacteria were incubated while rotating for 6 hours, washed until no radioactivity was detected in the supernatant and resuspended in PBS. Half of the preparation was incubated at 60°C for 1 hour to obtain dead-LM (^{35}S-dead-LM). Bacteria were washed and similar amounts of radioactive dead- and live-LM were added to the cells. In preparing the bacteria, loss of radioactivity was roughly equivalent for both dead- and live-LM preparation. Labeled bacteria incorporated 1-2 cpm/CFU. Dead-LM were also surface labeled with ^{125}I by the chloramine T method at 4°C (Allen et al., 1981). Live-^{35}S-LM^{hly-} and ^{125}I-dead-LM or ^{35}S-dead-LM were added (3×10^5 cpm/well) to 2×10^6 cells (J774E-clone cells) in 24-well plates, centrifuged to speed adherence and synchronize the infection and incubated for 20 minutes at 37°C to allow bacteria internalization. The cells were then extensively washed and incubated for different periods of time, the medium was removed and the cells were solubilized in 1% Triton X-100. Proteins were immediately precipitated from the medium or from cell lysates with 10% TCA on ice with 3% BSA as a carrier. Experiments were performed in triplicates.

Enzymatic reactions

The HRP assay was conducted in 96-well microplates (Costar, Co) using *o*-dianisidine as the chromogenic substrate (Gruenberg et al., 1989) and as previously reported (Alvarez-Dominguez et al., 1996). Each value was expressed as absorbence units per mg of protein. β-Glucuronidase activity was measured as previously described (Diaz et at., 1988) using 4-methyl-umbelliferyl-βD-glucuronide as substrate.

RESULTS

Endocytic access to live-LM^{hly-} and dead-LM phagosomal compartments with the marker BSA gold

We previously reported that the tight regulation of phagosome-endosome fusion exerted by rab5 is modulated by live-LM^{hly-}. Live-LM^{hly-} promotes the accumulation of rab5, a GTPase required for phagosome-endosome fusion, and fusion factors, such as NSF on the phagosomal membranes apparently as part of its strategy to increase the phagosome-endosome fusion rate.

In this report, we have further characterized the process by which live-LM^{hly-} manipulates the phagocytic pathway. BSA-gold was used as a pinocytic, fluid phase marker of the endosomal/lysosomal pathway, to examine the fusion of phagosomes containing live- and dead-LM with endosomes and lysosomes. In these experiments, cells were pulsed with live- or dead-LM for a total of 6, 15 or 30 minutes after which the cells were pulsed with BSA-gold for 10 minutes to follow endocytosis. The cells were then fixed and prepared for electron microscopy. Fig. 1 shows typical early phagosomes containing live (Fig. 1a) or dead (Fig. 1b) bacteria after 6 minutes uptake followed by a 10 minutes pulse of BSA-gold. Both examples show phagosomes positive for gold indicating fusion with early endosomes. Quantitation of the data from 50 cells (Fig. 2a) indicate most (98%) of the early live-LM^{hly-} phagosomes were BSA-gold positive. When the data were calculated as % of the total internalized BSA-gold present in live-LM^{hly-} phagosomes (Fig. 2b), we found 58% of the internalized BSA-gold in these phagosomes. Dead-LM phagosomes were much less accessible to an early endosomal marker. When examined at the same early time point, 67% of the dead-LM phagosomes were BSA-gold positive (Fig. 2a) and 36% of internalized BSA-gold localized to dead-LM phagosomes (Fig. 2b). Surprisingly, at later chase times (30 minutes of phagocytosis), as illustrated in Fig. 2a and b, live-LM^{hly-} phagosomes became less accessible to endocytic tracers whereas dead-LM phagosomes became more accessible to such markers. In Fig. 3a and b, cells were pulsed with live and dead bacteria, respectively, for a total of 15 minutes after which the cells were offered BSA-gold for 10 minutes. Both live- and dead-LM phagosomes (25 minute total time) were positive for endocytosed BSA-gold. However, compared to earlier time points, live-LM^{hly-} phagosomes were less accessible and dead-LM phagosomes were more accessible to the endosomal marker at this intermediate timepoint (Fig. 2a and b). At the third time point (Fig. 4a and b) (viz, 40 minutes phagosomes; 30 minutes of phagocytosis and 10 minutes of endocytosis), live-LM^{hly-} phagosomes (Fig. 4a) were often quite large containing multiple bacteria whereas phagosomes containing dead bacteria (Fig. 4b) contained fewer bacterial profiles per phagosome suggesting that the live preparations were undergoing phagosome-phagosome fusion. (Total bacteria per cell did not differ among the live and dead preparations.) Quantitation of BSA-gold found in phagosome profiles indicated that 58% of the 40 minute live-LM^{hly-} phagosomes were BSA-gold positive but they contained less than 16% of the internalized endocytic marker. Forty minute dead-LM phagosomes, on the other hand were very accessible to the endocytic marker; 95% were BSA-gold positive and these profiles contained 53% of the internalized BSA-gold. The accessibility of different aged phagosomes (16, 25 or 40 minutes) by an early endosomal marker (5-10 minutes) is summarized in Fig. 2. These data indicate that phagosomes containing both dead- or live-LM^{hly-} are able to fuse extensively with the endosomal compartment. However, the two compartments have different apparent fusogenic rates. Early live-LM^{hly-} containing phagosomes fuse readily with endosomes. However, with increasing time of chase, live-LM^{hly-} phagosomes lose their ability to fuse with the endosomal compartment. Thus, at the 30-40 minutes time point, live-LM^{hly-} phagosomes have virtually lost their ability to fuse with early endosomes. Dead-LM, on the other hand, enter a compartment which is increasingly accessible to an endocytic marker.

Live-LM phagosomes fail to fuse with lysosomes marked with BSA-gold

It is possible that an accelerated fusion rate with the endosomal compartment delays the fusion of live-LM^{hly-} phagosomes with the lysosomal compartment. In previous work, we speculated that the sequestration or immobilization of factors involved in early phagosome-endosome fusion may prevent phagosome maturation. Given the above, we examined the extent of fusion of dead-LM and live-LM^{hly-} phagosomes with the lysosomal compartment. As shown in Fig. 5a and b, live-LM^{hly-} phagosomes rarely fused with BSA-gold-marked lysosomes (see also Fig. 6a,c,e,g,h and i). Lysosomes were identified by incubating cells with BSA-gold for 90 minutes followed by an overnight chase. The cells were then offered live- or dead-LM for 6, 15 and 30 minutes after which the cells were fixed and prepared for electron microscopy. At the earliest time point, 58% of the dead-LM phagosomes were BSA-gold positive whereas only 5% of

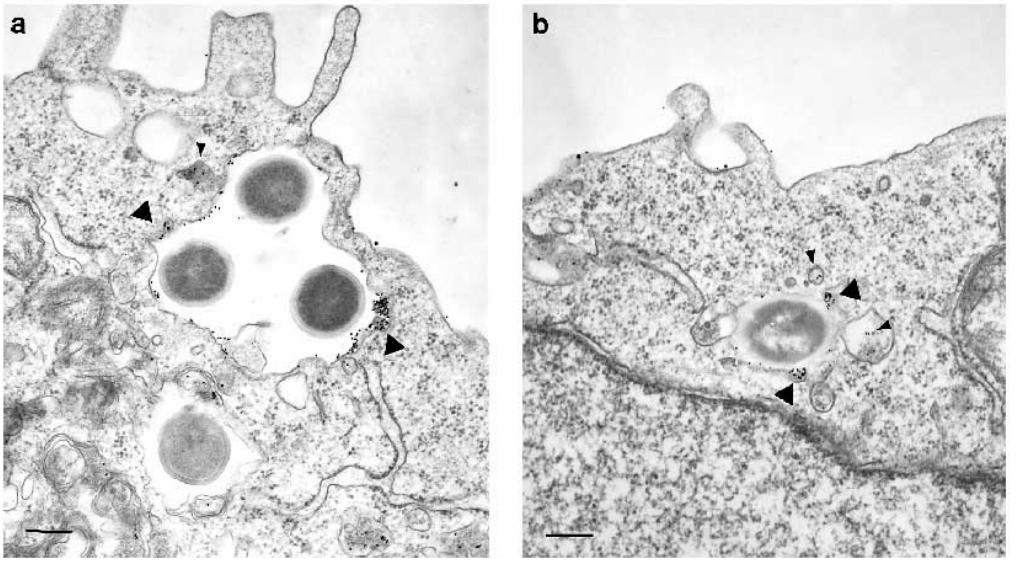

Fig. 1. Fusion of early phagosomes containing dead- or live-LM^{hly-} with early endosomes.
(a and b) Representative images showing localization of internalized BSA-gold in early live-LM^{hly-} or dead-LM phagosomes, respectively. Macrophages were infected with dead- or live-LM^{hly-} for 6 minutes, washed and then incubated with BSA-gold for 10 minutes. After washing in cacodylate buffer, cells were fixed and processed for EM as described in Materials and Methods. Large arrowheads indicate the presence of BSA-gold (30 nm) in the phagosomal compartment, while small arrowheads indicate the presence of BSA-gold in small endosomes adjacent to phagosomes. Bars, 0.256 μm.

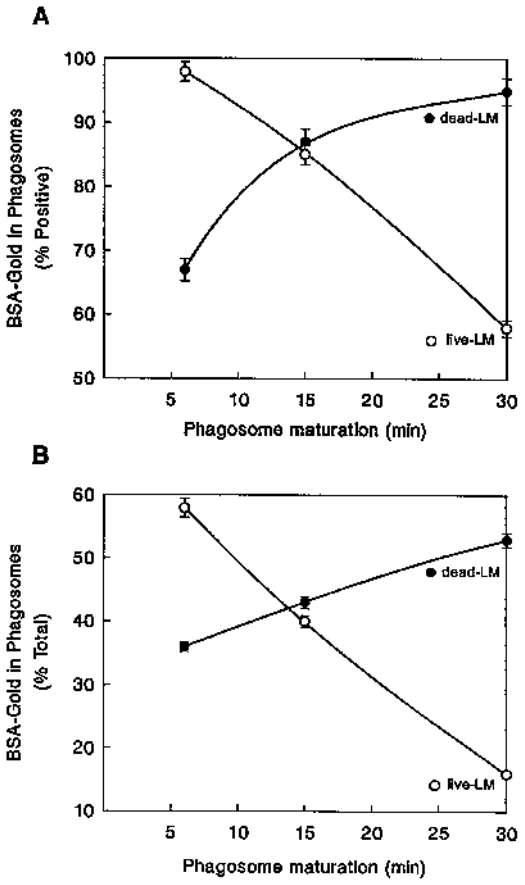

Fig. 2. Distribution of the early endocytic marker, BSA-gold in phagosomes containing dead- or live-LM^{hly-}. Cells were infected with dead- or live-LM^{hly-} as described in Materials and Methods to obtain 6, 15 and 30 minute phagosomes. A total of 50 cells were examined per time point and the total number of gold particles per cell as well as the number of gold particles per phagosome was recorded. The percentage of phagosomes positive for gold particles was also quantified. (A) Summary of the data corresponding to phagosomes containing gold particles in cells infected for different times (6, 15 or 30 minutes) with dead- or live-LM^{hly-} and then offered BSA-gold for 10 minutes. Results are expressed as the percentages of phagosomes positive for BSA-gold ± s.d.
(B) Summary of the intracellular distribution of internalized BSA-gold. Results are expressed as the number of particles found in phagosomes compared with the total number of particles inside cells ± s.d. Filled circles represent data obtained with cells infected with dead-LM and open circles with live-LM^{hly-}.

the live-LM^{hly-} phagosomes were BSA-gold positive (Fig. 5a). In Fig. 5a, the data are presented as the fraction of internalized BSA-gold present in live-LM^{hly-} or dead-LM phagosomes. Dead-LM phagosomes contained 51% of the internalized BSA-gold whereas live-LM^{hly-} phagosomes contained only 2% of the internalized marker (Fig. 6a and b). At the intermediate time point, roughly similar findings were obtained (Fig. 5a and b) (also Fig. 6c and d). At the 30 minute time point, 76% of the dead-LM phagosomes were BSA-gold positive whereas the live-LM^{hly-} phagosomes were still only 17% positive for the BSA-gold lysosomal marker. At the latter time point, 70% of the internalized BSA-gold was found in dead-LM phagosomes while live-LM^{hly-} phagosomes contained only 10% of the lysosomal marker (see also Fig. 6e and f). Thus, live-LM^{hly-} phagosomes fail to fuse with lysosomal compartments. Electron micrographs (Fig. 6a-f) show examples of phagosome-lysosome fusion at different times following internalization of dead- and live-LM. Note the absence of BSA-gold localized to phagosomes in Fig. 6a,c and e. (It is present in other lysosomal structures as in g,h and i.) Dead-LM phagosomes fuse rapidly and extensively with BSA-gold marked lysosomes. (BSA-gold is indicated by arrowheads in Fig. 6b,d and f).

Access of endosomal and lysosomal markers to live-LM^{hly-} and dead-LM phagosomes: studies with isolated phagosomes

To further explore access to dead- and live-LM^{hly-} phagosomes to the endocytic pathway, cells were offered live- and dead-LM before a pulse of HRP (a receptor-mediated tracer, internalized via the mannose-receptor; Lang and Chastellier, 1985) accompanied by a chase period. Following the chase, the cells were lysed and phagosomes were purified by fractionation and gradient sedimentation. The isolated phagosomes were then analyzed for their HRP content. Two pulse chase protocols were employed as summarized in Table 1a-b. For all protocols, internalization of HRP was preceded by ligand binding at 4°C for 60 minutes. For short periods (Table 1a), cells were incubated with live- or dead-LM for 5 minutes. The cells were cooled, free bacteria were washed away and the cells were pulsed with HRP for 5 minutes (following a 60 minute, 4°C ligand prebinding). Following a 5 minute chase, the cells were lysed and phagosomes were isolated as above; 65% of the internalized HRP activity was recovered in both the live and the dead-LM phagosome preparations. A similar protocol was used to study the interactions of maturing endosomes with the forming phagosomes (cells were loaded first with HRP for 5 minutes

Table 1. Access of the endocytic marker HRP to LM phagosomes

Protocol	Dead-LM	Live-LM
LM→HRP→5 minutes*	65	65
LM→15 minutes→HRP→5 minutes†	80	34
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HRP→2 hours→LM→1 minutes‡	42	14
HRP→2 hours→LM→10 minutes‡	50	17
HRP→2 hours→LM→25 minutes‡	85	11

*J774E-clone cells were infected with dead- or live-LM^{hly-} for 5 minutes, washed and incubated at 4°C for 60 minutes with HRP (200 μg/ml) to allow binding to the mannose receptor. For internalization of HRP, cells were warmed to 37°C for 5 minutes, cooled on ice, washed and chased for another 5 minutes. After washing, cells were homogenized and purified phagosomes were isolated as described in Materials and Methods. HRP activity recovered in the phagosomes is compared to the total activity present in the PNS. Results are expressed as the fraction of HRP recovered in isolated phagosomes.

†Cells infected with dead- or live-LM^{hly-} for 5 minutes, washed, chased for 15 minutes were incubated with HRP (4°C) and followed by HRP internalization (5 minutes, 37°C), washing and 5 minutes chase. After homogenization and phagosome isolation, HRP activity was estimated as above.

‡Cells were incubated at 4°C with HRP to allow the binding, incubated for 5 minutes at 37°C, washed and chased for 2 hours. Cells were then infected with dead or live-LM^{hly-} for 5 minutes, chased for different periods of time: 1, 10 or 25 minutes, homogenized and phagosomes isolated as above. HRP activity was measured in the isolated phagosome preparation as above.

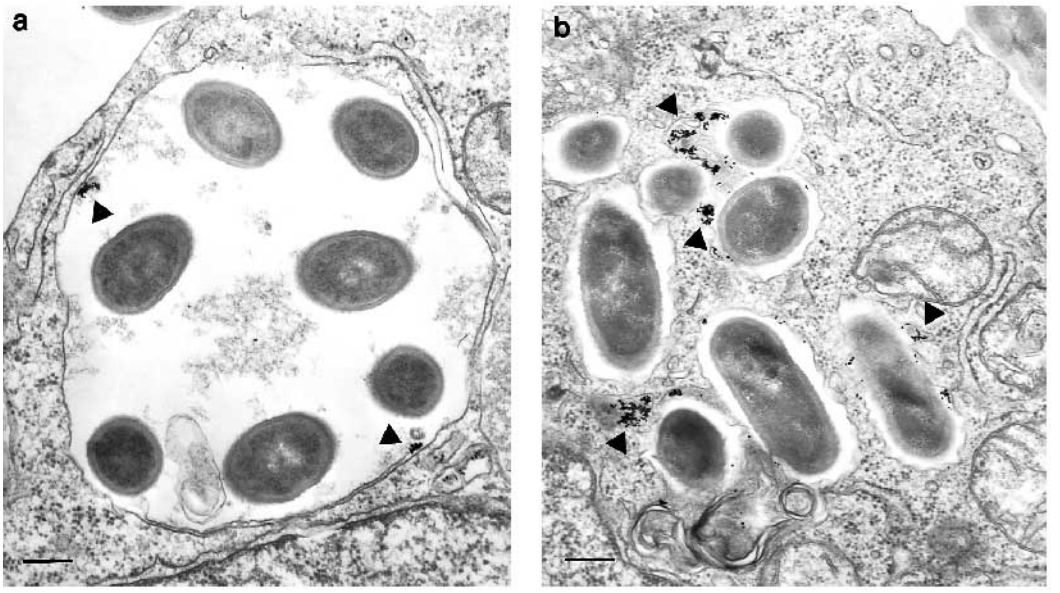


Fig. 3. Fusion of intermediate phagosomes containing dead- or live-LM with early endosomes. (a and b) Representative electron micrographs showing fusion between 15 minutes live-LM^{hly-} or dead-LM phagosomes, respectively, and endosomes. J774E clone cells were infected with dead- or live-LM for 15 minutes, washed and incubated with BSA-gold (30 nm) for 10 minutes at 37°C. The preparation was then washed, fixed and processed for EM. Arrowheads indicate the presence of BSA-gold (30 nm) in the phagosomal compartment reflecting fusion with early endosomes (10 minutes). Bars, 0.256 μm.

and then infected with bacteria for another 5 minutes). The results were similar although the interaction of early phagosomes with these early endosomes was slightly higher for the live-LM (92%) than for the dead-LM (55%). To examine later times, cells were pulsed with live-LM^{hly-} and dead-LM for 5 minutes. Bacteria were removed and the cells were incubated for 15 minutes at 37°C. The cells were then pulsed with HRP for 5 minutes as described above and then fractionated. As shown in Table 1c, 34% of the HRP activity was recovered in the live-LM^{hly-} phagosomal fraction whereas 80% of the HRP activity was recovered in the dead-LM phagosomal fraction. These data extend the morphological studies indicating that live-LM^{hly-} phagosomes fuse very rapidly with early endosomes but appear to lose the ability to fuse with time. Dead-LM phagosomes initially fuse slowly with endosomes but subsequently access a compartment (e.g. a phagolysosomal compartment) which is readily accessible by endocytic markers. To extend these findings, we checked the accessibility of a lysosomal marker to live- and dead-LM phagosomes after loading the lysosomal compartment with HRP (5 minutes pulse and 2 hours chase). Cells were pulsed for 5 minutes with dead- or live-LM^{hly-} and chased for 1, 10 or 25 minutes as above. Phagosomes were isolated and the HRP activity recovered in these phagosomes was quantified (Table 1d). Dead-LM phagosomes fuse extensively with HRP-loaded lysosomes at all time points with as much as 85% of the internalized HRP activity recovered in the dead-LM phagosomal fraction. However, as shown in Table 1, very little of the lysosomal HRP was transferred to the live-LM^{hly-} phagosomes.

Markers present on phagosomal membranes. Differential composition of phagosomes containing dead-LM or live-LM^{hly-}

To further characterize dead- and live-LM^{hly-} phagosomes, equal amounts of phagosomal proteins were separated by SDS-PAGE and western blotting experiments were carried out on

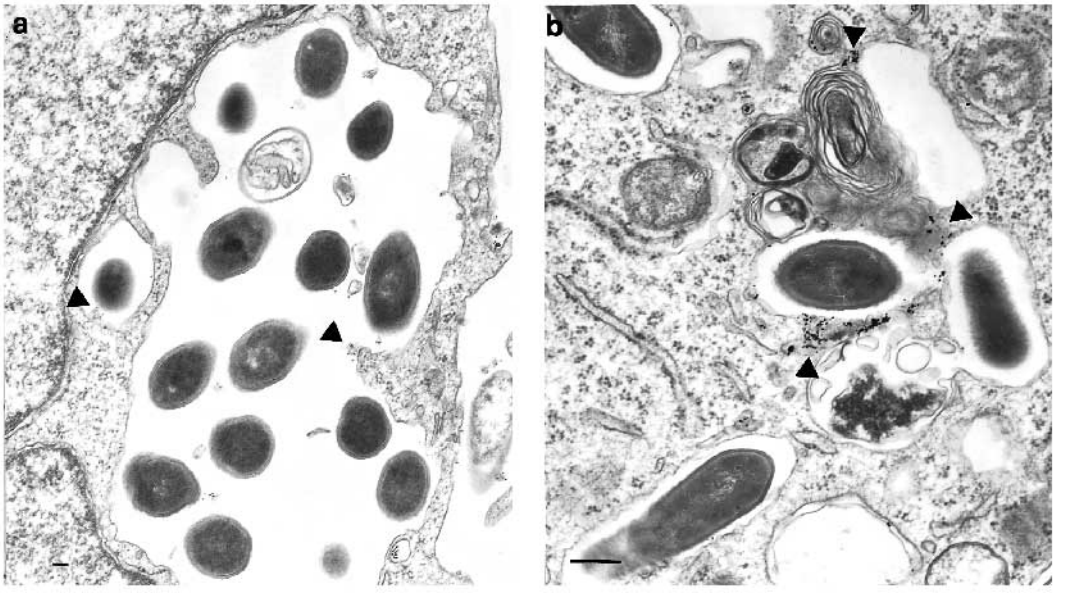


Fig. 4. Fusion of late phagosomes containing dead- or live-LM^{hly-} with early endosomes. (a and b) Representative electron micrographs showing fusion between 30 minute live-LM^{hly-} or dead-LM phagosomes, respectively and early endosomes. J774E clone cells were infected with dead- or live-LM^{hly-} for 30 minutes, washed and incubated with BSA-gold (30 nm) for 10 minutes at 37°C. The cells were washed again, fixed and processed for EM. Arrowheads indicate the presence of BSA-gold (30 nm) in the phagosomal compartment, reflecting fusion with early endosomes (10 minutes). Bars: 0.345 μm (a); 0.256 μm (b).

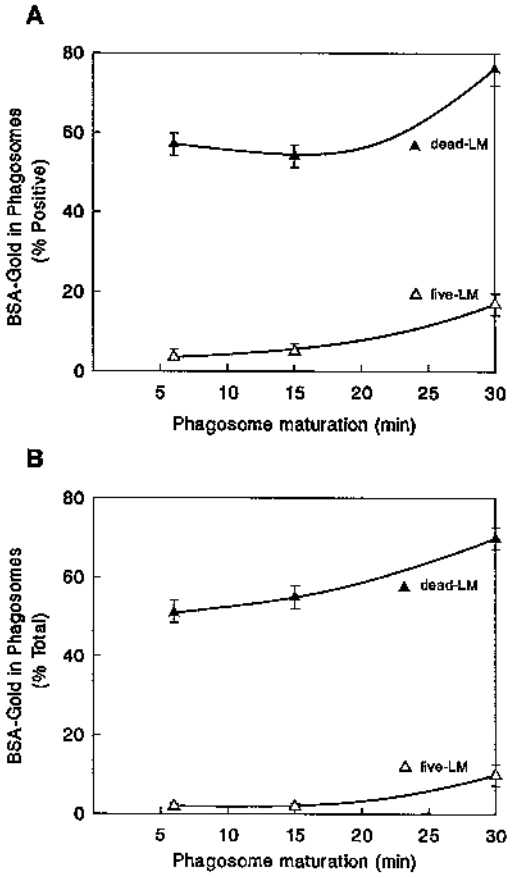


Fig. 5. Phagosome-lysosome fusion. Fusion of different 'aged' phagosomes containing dead- or live-LM^{hly-} with lysosomes. J774E-clone cells were incubated with BSA-gold (10 nm) for 90 minutes at 37°C, washed and chased overnight. Cells were then infected with dead- or live-LM for different times (6, 15 or 30 minutes). After washing in cacodylate buffer, cells were fixed and processed for EM. (A) The number of phagosomes containing gold particles in cells infected for different times (6, 15 or 30 minutes) with dead- or live-LM^{hly-}. Results are expressed as the percentages of phagosomes positive for BSA-gold ± s.d. Quantification was performed as in Fig. 2. (B) The fraction of internalized BSA-gold particles found in different 'aged' phagosomes of cells infected with dead- or live-LM^{hly-}. Results are expressed as the percentage of particles detected in the phagosomes compared with the total number of particles inside the cells ± s.d. Filled triangles represent data obtained with cells infected with dead-LM and open triangles with live-LM^{hly-}.

different 'aged' phagosomes with antibodies to endosomal and lysosomal markers. Control experiments were carried out using western blotting and a rabbit anti-LM antibody to confirm that similar amounts of bacterial proteins were loaded per lane for all the phagosome preparations (Fig. 7c). As shown in Fig. 7a, phagosomes loaded with live-LM^{hly-} bacteria are enriched in mannose receptor (ManR) and transferrin-receptor (TfR) at all points along the phagocytic route. The dead-LM containing phagosomes, however, were virtually devoid of ManR, and nearly so of TfR, at early times (6 and 15 minutes). At later times both markers were readily detectable (Fig. 7a), especially at 30 minutes. The late endosomal marker, CD-M6P receptor, was detectable only on phagosomes loaded with dead-LM, at 15 minutes with decreasing levels at 30 minutes. Live-LM^{hly-} loaded phagosomes showed only low amounts of CD-M6P receptor at late time points e.g. at 30 minutes (lower western blot in Fig. 7a). The lysosomal membrane protein lamp-1 (~110-120 kDa; Fig. 7b) was detected in variable amounts in all phagosomes containing dead-LM bacteria; however, it was undetectable on live-LM^{hly-} loaded phagosomes. The mature form of cathepsin D (~43 kDa in mouse macrophages), which corresponds to the lysosomal form (Diment and Stahl, 1985; Diment et al., 1988), was detectable in dead-LM-loaded phagosomes at 6 minutes. Increased levels were detected with 15 and 30 minute samples. However, the mature form was absent from early live-LM^{hly-} loaded phagosomes with low levels detectable at 30 minutes. Interestingly, the amount of the immature form of cathepsin D (~53 kDa; i.e. the form that can be found in endosomes) (Diment et al., 1988) present in phagosomes containing live-LM^{hly-} was lower than that found in dead-LM phagosomes. In general, dead-LM phagosomes shared both lysosomal and endosomal markers whereas live-LM^{hly-} phagosomes displayed characteristics of an endosomal rather than a lysosomal compartment. These data suggest that the live microorganism prevents phagosome maturation, in part, by preventing delivery of lysosomal enzymes such as cathepsin D. Interestingly, when we checked for the presence of the vacuolar H^+-ATPase on isolated phagosomes (Fig. 8), we readily detected the 31 kDa E-subunit of the ATPase both in dead-LM and live-LM^{hly-} loaded phagosomes. These data, along with other studies with fluorescent acid sensitive dyes (data not shown) indicate that both dead- and live-LM^{hly-} phagosomes are acidic.

Membrane associated proteins involved in vesicle fusion events were examined by western blotting. Rab5 is required for fusion between phagosomes and early endosomes (Alvarez-Dominguez et al., 1996) and rab7 may be required for fusion between late-endosomes and phagosomes (Desjardins et al., 1994a). Fusion factors such as NSF and SNAPs are known to be required for phagosome-endosome fusion (Mayorga et al., 1991; Berón et al., 1995a; Alvarez-Dominguez et al., 1996). Extending earlier work with rab5, western blotting experiments were carried out to identify NSF and SNAP in phagosomes containing both dead-LM or live-LM^{hly-}. NSF and α/β-SNAPs (Fig. 9) were present on phagosomes loaded with live- and dead-LM. Live-LM^{hly-} phagosomes contained higher amounts of NSF and SNAP at earlier times compared with phagosomes loaded with dead-LM. However, NSF and α/β-SNAP appear to be released from the live-LM^{hly-} phagosomes at later times (e.g. 30 minutes in Fig. 9). Phagosomes loaded with dead-LM behave somewhat differently. NSF and α/β-SNAP are nearly undetectable at early time points but their levels increase with time.

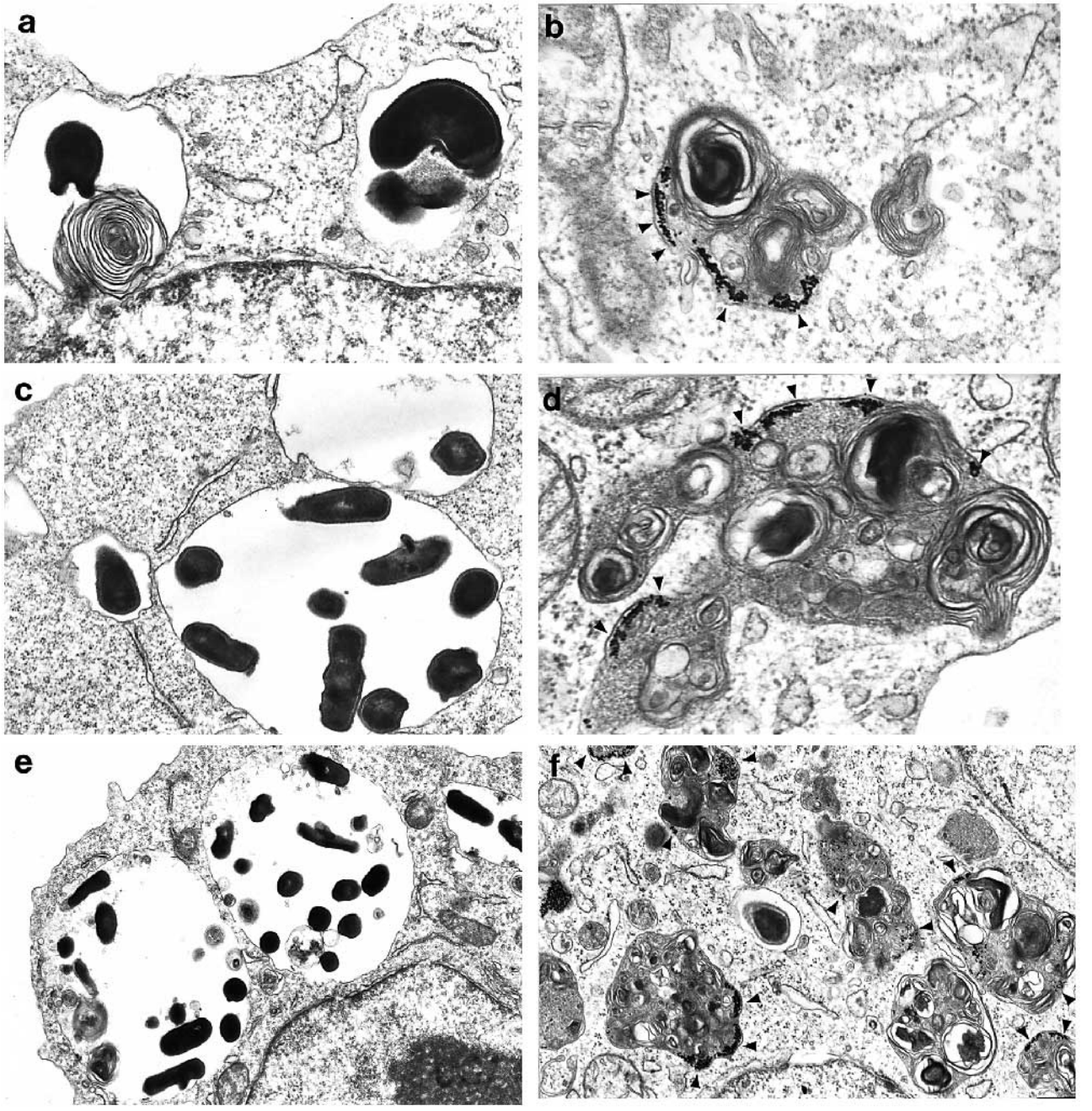
Degradation of live- and dead-LM following phagocytosis

A prediction of delayed delivery of lysosomal hydrolases to live-LM^{hly-} phagosomes is delayed degradation. To check this point, we performed catabolism experiments with ^{35}S- or ^{125}I-labeled dead-LM using a procedure developed by Cluff et al. (1990) to follow for dead- and live-LM. Fig. 10 shows the degradation of ^{125}I-labeled dead-LM and ^{35}S-labeled live-LM, following 20 minutes of internalization. The rate of catabolism for live-LM is substantially different from that observed with dead bacteria (catabolism experiments were also carried out with ^{35}S-dead-LM and similar rates were found). With both preparations, TCA-soluble material released to the medium

increased with time although the extent of degradation, as reflected in the release of radioactivity, was substantially less for the live preparation. Thus, catabolism of the live bacteria is clearly blunted. Correspondingly, TCA-precipitable material associated with the cells infected with live-LM^{hly-} decreases more slowly compared with cells infected with dead-LM bacteria. These data suggest that although some bacterial proteolysis occurs in the live-LM^{hly-} infected macrophages, the rate of bacterial degradation is retarded.

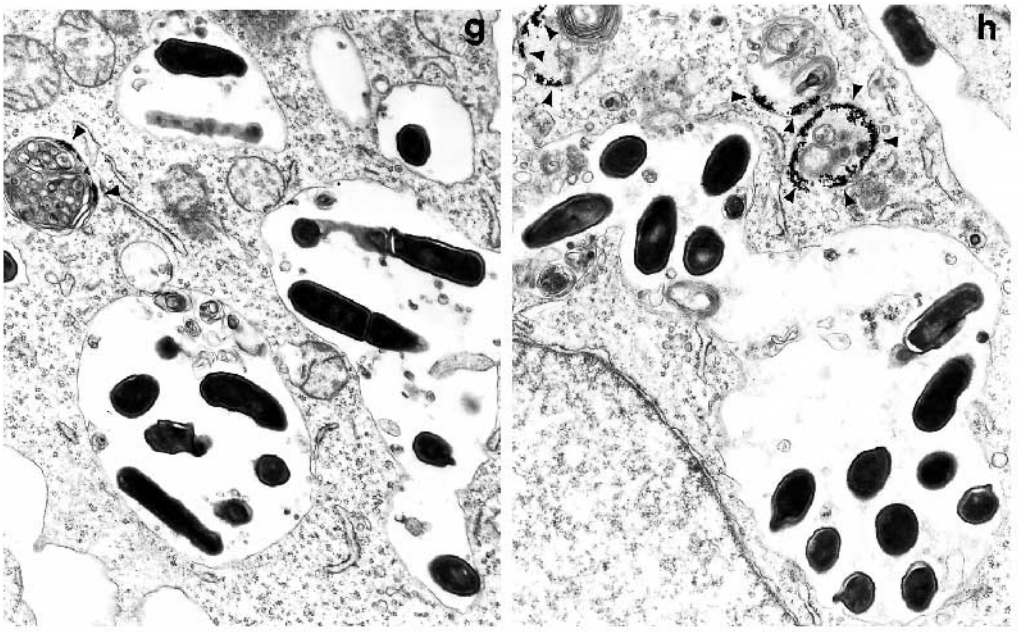
DISCUSSION

We have previously shown that early phagosome-endosome fusion events following phagocytosis of LM are modulated by the small GTPase, rab5. Interestingly, live-LM bacteria upregulate phagosome-endosome fusion in vitro (Alvarez-Dominguez et al., 1996). The increase in phagosome-endosome fusion displayed by live-LM^{hly-} correlates with the delivery of rab5 and fusogenic factors, such as NSF, to the phagosomal membranes. In the present study, we have characterized more fully the intracellular pathways taken by dead- and live-LM^{hly-} phagosomes. We followed the time course and the extent of fusion of phagosomes, containing dead- or live-LM^{hly-}, with endosomes and lysosomes. We used quantitative electron microscopy, in intact cells using internalized BSA-gold as a marker and we highly enriched phagosomes containing live- and dead-LM. The latter has allowed us to analyze phagosomes for various soluble and membrane components



and to quantify transfer of HRP from endosomes and lysosomes to phagosomes. Enriched phagosomal membranes were also used to carry out western blotting experiments with endosomal and lysosomal markers. This experimental model, using a nonhemolytic mutant of LM, LM^{hly-} (a mutant retained inside the phagosome), as live-bacteria and heat-killed LM as dead-bacteria has several advantages. First, both bacteria enter the cell identically, as far as we can ascertain. Thus, they begin their intracellular journey from similar starting points. Second, unlike its virulent counterpart LM^{hly+}, LM^{hly-} stays within the vacuolar apparatus. Thus, membrane lysis is avoided. Third, since live-LM^{hly-} is unable to grow in macrophages, both live-LM^{hly-} and dead-LM are ultimately degraded by the host cell.

Our data indicate that dead- and live-LM^{hly-} phagosomes display strikingly different behaviors with respect to their interactions with endosomes and lysosomes. Similar results were obtained irrespective of whether a fluid phase marker (data with BSA-gold) or a receptor-mediated endocytosis marker (data with HRP-transfer) was used. Dead bacteria remain at all times in compartments that are highly accessible to markers present in both endosomes and lysosomes. Live-LM^{hly-} phagosomes behave quite differently. Initially, at the very early time points (6 minutes), live-LM^{hly-} phagosomes are highly fusogenic with endosomes and accessible to endocytic markers. However, with increasing time of chase (30 minutes), live-LM^{hly-} phagosomes rapidly lose their highly fusogenic properties and become inaccessible to endocytic markers. The fusion of live-LM^{hly-} phagosomes with the lysosomal compartment remains very low at all times. Our hypothesis is that live-LM^{hly-} phagosomes effectively delay maturation, possibly by sustaining and accelerating very early their fusion with endosomes. One of the consequences of delayed maturation is that fusion with the lysosomal compartment is retarded or prevented. When the highest fusion rates are recorded (6 minutes after bacteria ingestion) live-LM^{hly-} promotes the translocation and retention of small GTPases such as rab5 (Alvarez-Dominguez et al., 1996) and NSF and α/β-SNAP, key proteins required for early fusion events with endosomes. With



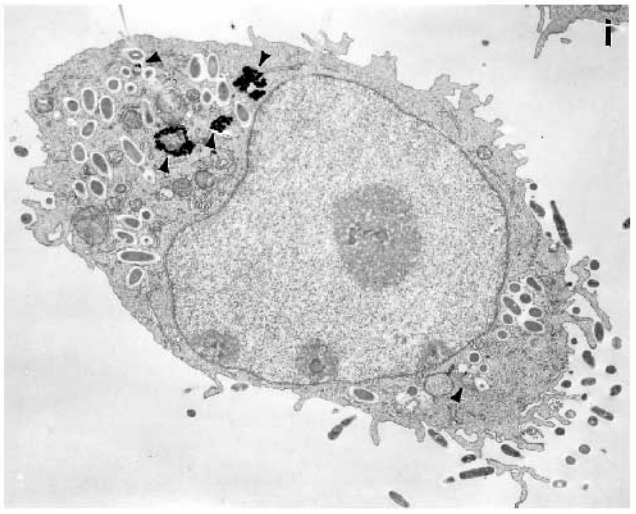


Fig. 6. Distribution of a lysosomal marker BSA-gold in phagosomes containing dead- or live-LM^{hly-}. Macrophages were incubated with BSA-gold (10 nm) at 37°C for 90 minutes, washed and then chased overnight to label the lysosomal compartment. Cells were then infected with live (a,c,e) or dead-LM (b,d,f) for different periods of time (6, 15 and 30 minutes). The cells were then washed, fixed and processed for EM. (a and b) Fusion of 6 minute phagosomes with lysosomes containing BSA-gold, (c and d) to 15 minutes phagosomes and (e and f) to 30 minutes phagosomes. Arrowheads indicate the presence of lysosomes with BSA-gold fused with dead-LM phagosomes. (g,h, i) Cells infected with live-LM for 15, 30 and 6 minutes, respectively, showing no detectable gold in the phagosomes, while present in other lysosomal structures, indicated by arrowheads. Bars: 0.345 μm (a,b,c,d); 0.256 μm (e and f), 0.526 μm (g and h); and 2 μm (i).

time of chase, NSF and α/β-SNAP are lost from the live-LM^{hly-} phagosomes and this is correlated with a decrease in the apparent fusion with endosomes. Earlier studies show that the GTPases rab5 and rab7 are retained (Alvarez-Dominguez et al., 1996) on live-LM^{hly-} phagosomes. The retention of GTP-binding proteins and perhaps other fusion factors on live-LM^{hly-} phagosomes may be part of a strategy to delay phagosome maturation. (The presence of a factor, such as SNAP may or may not correspond to increased fusion activity. For example, the ATPase activity of NSF has been shown to regulate the exit of SNAP from the membranes (Mayer et al., 1996; M. I. Colombo, S. W. Whiteheart and P. D. Stahl, unpublished results). Thus the presence of SNAP might indicate impaired NSF ATPase activity and therefore decreased fusion). Delayed maturation of live-LM^{hly-} phagosomes is also supported by the composition studies performed with purified dead- and live-LM^{hly-} phagosomes. Characteristic endosomal markers such as ManR and TfR are enriched on early live-LM^{hly-} phagosomes while only barely detectable on dead-LM phagosomes. This difference in endosomal markers may reflect the relative phagosome-endosome fusion rates for each phagosome type. Phagosomes loaded with live-LM^{hly-} fused with endosomes more extensively at early time points compared with dead-LM phagosomes. It is also possible that the live bacteria are transported from this early compartment to a different endosomal compartment (perhaps a recycling compartment; Ghosh and Maxfield, 1995), where early markers such as ManR or TfR could be sequestered) with reduced fusogenicity with endosomes and lack of fusion with lysosomes. Dead-LM phagosomes, on the other hand, are increasingly accessible with time of chase to endocytosed tracers. The enrichment of TfR suggests that the late dead-LM phagolysosome is not an ordinary lysosomal compartment. The existence of an unusual lysosomal compartment was also stressed by Rabinowitz et al. (1992) who showed that latex beads enter a tubulo-vesicular compartment that has a complex structure which contains lamp, rab7 and CI-M6PR, although the latter was restricted to certain regions of the phagolysosome. Moreover, Harding and Geuze (1992) using heat-killed LM as a reagent to follow antigen processing, also postulated that this tubulo-vesicular compartment could be accessible via

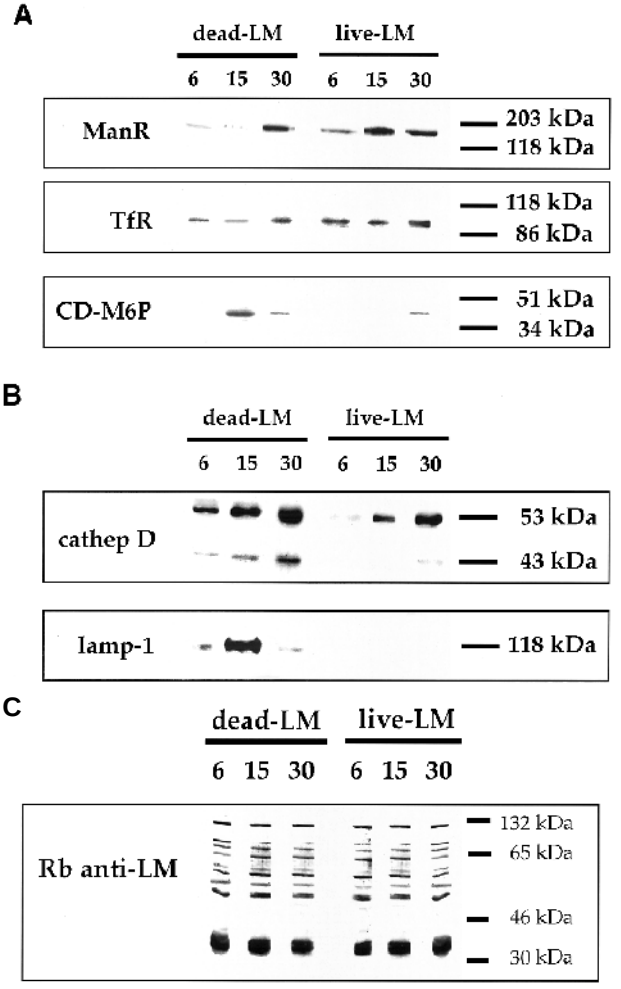


Fig. 7. Analysis of endosomal and lysosomal markers in phagosomes containing dead- or live-LM^{hly-}. (A) Western blot analysis for ManR, TfR and CD-M6P in phagosomes loaded with dead- or live-LM^{hly-}. Phagosomal proteins (30 μg per lane) were separated by SDS-PAGE, transferred to membranes, incubated with primary and HRP-conjugated secondary antibodies and detected by ECL. Results from western blots are representative of 4 independent preparations. (B) Western blot detection of cathepsin D and lamp-1 in the same phagosomes as in A. Results are representative of 5 preparations. Arrows indicate the two forms of cathepsin D, a higher molecular mass inmature form (~53 kDa) and a lower molecular mass mature form (~46 kDa). (C) Bacterial proteins from the same phagosomal preparations as in A and B. Proteins were analyzed by SDS-PAGE (30 mg per lane). After transferring to membranes, they were incubated with rabbit anti-M antiserum (Difco) followed by HRP-conjugated goat anti-rabbit and developed by ECL.

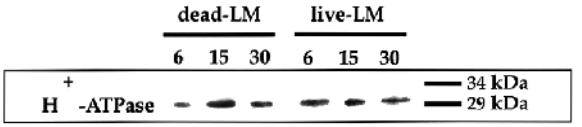


Fig. 8. Analysis of the vacuolar H$^+$-ATPase in isolated phagosomes: 30 μg of phagosomal proteins per lane were separated in SDS-PAGE, transferred and incubated with primary (a11) and HRP-conjugated secondary antibodies with detection by ECL as in Fig. 7. Results are representative of 3 different preparations.

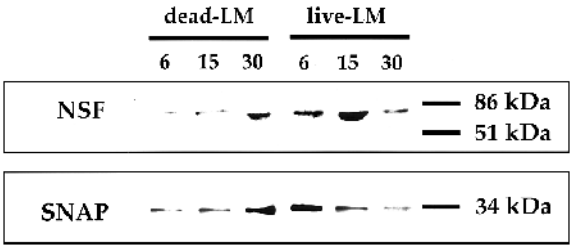


Fig. 9. Presence of fusogenic factors in phagosomes containing dead- or live-LM^{hly-}. Phagosomes containing dead- or live-LM^{hly-} were isolated after different infection times: 6, 15 or 30 minutes, at 37°C. Phagosomal proteins (30 μg per lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Antibodies 6E6 and CI77.1 were used to detect NSF and α/β-SNAP, respectively. HRP-conjugated secondary antibodies were separately incubated with the blots and detected by ECL. Results are representative of 4 different preparations.

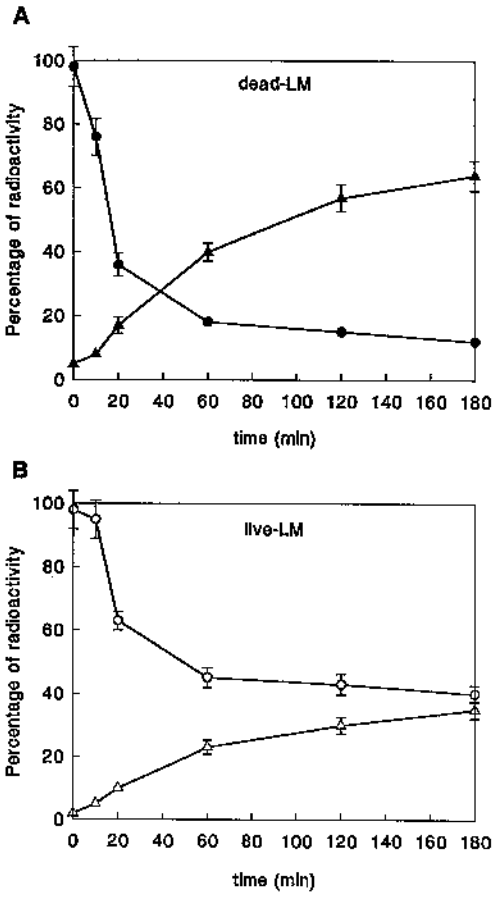


Fig. 10. Degradation of live- and dead-LM in J774-E macrophages. (A) The catabolism of internalized ^{125}I-dead-LM (experiments were also performed with ^{35}S-labeled dead-LM) and (B) of ^{35}S-live-LM by macrophages. Live-LM^{hly-} bacteria were metabolically labeled with Trans^{35}S-label. Heat-killed ^{35}S-labeled dead-LM were prepared as described in Materials and Methods. Labeled dead-LM (^{125}I) (filled symbols) or live-LM^{hly-} bacteria (^{35}S) (open symbols) were added to the macrophages (5×10^6), centrifuged for 5 minutes and incubated at 37°C for 20 minutes to allow phagocytosis. The macrophages were then washed and incubated for the indicated periods (after the 20 minute pulse) at 37°C. The medium was removed and the cells were solubilized in 1% Triton X-100. TCA precipitation was used to separate intact bacterial proteins (TCA precipitable) (○, ● symbols) and low molecular mass catabolites (TCA soluble) (△, ▲ symbols) from the cells and medium, respectively. Input of ^{125}I dead-LM was 200,000 cpm and the percentage of bacteria internalization (corresponding in the graphic as time 0) was 45%, which corresponds to 92,000 cpm. Input value for ^{35}S-live-LM^{hly-} was 346,000 cpm and percentage of internalization was 44% corresponding to 155,600 cpm at time 0 (similar numbers were obtained with ^{35}S-dead-LM). Data represent the mean of triplicates ± s.d.

endocytosis, via traffic through the TGN and via delivery of materials from late lysosomes. The dead-LM late phagosomal compartment described in this manuscript may well be similar to the tubulo-vesicular compartment described by Harding and Geuze (1992). Moreover, although the immature endosomal form of cathepsin D can be detected in phagosomes containing live-LM^{hly-}, less is present than in dead-LM phagosomes. Correspondingly, the mature lysosomal form of this enzyme is not present in the live-LM^{hly-} loaded phagosomes. This indicates that the maturation of the two types of phagosomes is different and suggests that a mechanism may exist to prevent the targetting of newly synthesized lysosomal enzymes to the live-LM^{hly-} loaded phagosomes. Recent work by Aniento et al. (1996) as well as by van Weert et al. (1995) suggests that acidification is required for early to late endosomal transport. The lack of delivery lysosomal enzymes to these phagosomes is not due to a rise in intraphagosomal acidity. First, readily detectable levels of the H^+-ATPase E-subunit are present on phagosomes containing both dead- and live-LM^{hly-}. Second, as previously reported for the pathogenic bacteria, LM^{hly+}, LM seems to reside in an acidic compartment (de Chastellier and Berche, 1994); preliminary experiments using a fluorescent weak base amine (lysotracker) that accumulates in acidic compartments suggest that both live-LM^{hly-} and dead-LM phagosomes are in an acidic environment (C. Alvarez-Dominguez, R. Roberts and P. D. Stahl, unpublished results). While both types of phagosomes are acidic, the lack of hydrolytic activity accessing this phagosomal compartment clearly affects the extent of bacterial degradation. The low levels of lysosomal enzyme activity may be due to impaired targeting of newly synthesized hydrolases and to inhibition of fusion of immature phagosomes with pre-existing lysosomal compartments. Taken together these results indicate that live-LM^{hly-} modulates the phagocytic machinery. We speculate that the mechanism may involve early enhanced delivery and/or retention of key cytosolic factors, required for phagosome-endosome fusion events, to the phagosomal membranes. As a consequence, fusion with endosomes is accelerated while fusion with prelysosomes and lysosomes is prevented. In this regard, the recent findings by Collins et al. (1997), that live-LM phagosomes exclude the lysosomal targeting molecule, annexin I while it is clearly present on dead-LM phagosomes indicate that the live bacteria is active in regulating its intracellular destination.

Additional work is necessary but it is possible that the retention of rab5 and possibly rab7 coupled with the loss of NSF and α/β-SNAP constitute some part of a signal for inhibition of phagosome-lysosome fusion. Thus, live-LM^{hly-} phagosomes do not mature and accumulate endosomal markers such as ManR and TfR. However, they are devoid of lysosomal markers such as cathepsin D and lamp-1. Finally, delayed phagosomal maturation clearly provides the bacteria with a mechanism for degradation thereby prolonging intracellular survival.

From our work, we speculate that intracellular survival of *Listeria* is enhanced by the manipulation of intracellular trafficking mechanisms. Retention of the bacteria in intracellular compartments whose properties are less 'lysosomal' would provide an attractive phagosomal environment from which to escape to the cytosol. We speculate that this would increase the survival of the organism and therefore the success of intracellular infection. The efficiency with which LM escapes to the cytoplasm appears to be critical since, depending of the macrophage, most of the internalized LM actually remains inside the phagosome (de Chastellier and Berche, 1994; Portnoy et al., 1992b). Much work by numerous investigators has been carried out on the mechanisms of killing of *Listeria* following phagocytosis. Multiple mechanisms are involved in intracellular killing including the oxidative burst and the pro-

duction of oxygen metabolites, the production of nitrogen intermediates and the delivery of lysosomal enzymes to the phagolysosome (Beckerman et al., 1993; Gregory and Wing, 1993). Each of these mechanisms may vary from one macrophage state to another. Cell lines differ substantially from primary macrophages stimulated with interferon γ with respect to the mechanisms of killing. In all of the mechanisms outlined, the nature and extent of intracellular trafficking following phagocytosis may have important consequences on the success of killing. Just as delayed maturation of the phagosome may be important in providing the bacteria with a non-hostile environment from which to enter the cytoplasm, delayed or altered vesicular trafficking may be equally important in blunting or delaying an oxidative burst, abrogating the production of nitrogen metabolites or preventing the delivery of lysosomal enzymes, each of which represents a key component of the host defense cascade for intracellular killing. We believe our results with LM^{hly-} bacteria can be extended to their pathogenic counterparts (Kuhn et al., 1988; Alvarez-Dominguez et al., 1995). Clearly, hemolysin is the key factor for escape to the cytosol, as described in several reports (Portnoy et al., 1992b; Bielecki et al., 1990). Our results and those of others suggest that there may be other factors involved. In fact, our previous findings highlight the ability of live-LM^{hly-} to accelerate fusion of phagosomes with the endosomal compartment, a process clearly independent of hemolysin (Alvarez-Dominguez et al., 1996). Unfortunately, the inability to isolate phagosomes containing live-LM^{hly+} bacteria, particularly at later times, precludes characterization of these phagosomal compartments. Initial experiments at the EM level with the live pathogenic LM (live-LM^{hly+}) indicate that the intracellular pathway taken is quite similar to the LM^{hly-} mutant (C. Alvarez-Dominguez and P. D. Stahl, this manuscript as well as unpublished results; Kuhn et al., 1988; Alvarez-Dominguez et al., 1995). The factors produced by the organism to alter vesicular trafficking in the host cell are under study.

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