Salmonella Stimulate Macrophage Macropinocytosis and Persist within Spacious Phagosomes

By Celia M. Alpuche-Aranda,* Esther L. Racoosin, Joel A. Swanson, and Samuel I. Miller*

From the *Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts 02114; and the †Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, 02115

Summary

Light microscopic studies of phagocytosis showed that Salmonella typhimurium entered mouse macrophages enclosed in spacious phagosomes (SP). Viewed by time-lapse video microscopy, bone marrow-derived macrophages exposed to S. typhimurium displayed generalized plasma membrane ruffling and macropinocytosis. Phagosomes containing Salmonella were morphologically indistinguishable from macropinosomes. SP formation was observed after several methods of bacterial opsonization, although bacteria opsonized with specific IgG appeared initially in small phagosomes that later enlarged. In contrast to macropinosomes induced by growth factors, which shrink completely within 15 min, SP persisted in the cytoplasm, enlarging often by fusion with macropinosomes or other SP. A Salmonella strain containing a constitutive mutation in the phoP virulence regulatory locus (PhoP c) induced significantly fewer SP. Similar to Yersinia enterocolitica, PhoP+ bacteria entered macrophages in close-fitting phagosomes, consistent with that expected for conventional receptor-mediated phagocytosis. These results suggest that formation of SP contributes to Salmonella survival and virulence.

Salmonellae are facultative, intracellular bacteria that cause systemic diseases including enteric (typhoid) fever, bacteremia, and gastroenteritis in humans and animals (1). Studies of the pathophysiology of human and mouse typhoid fever suggest that the ability to survive within macrophages is essential to pathogenesis (2-5). Though little is known of the mechanisms by which Salmonella survive after phagocytosis, they persist within membrane-bound phagosomes (6).

Recent studies indicate that bacterial gene expression essential to pathogenesis is linked with phagocytosis. The bacterial PhoQ (sensor-kinase) and PhoP (phosphorylated transcriptional activator) proteins specifically detect the macrophage vacuole environment and promote transcription of pag (phoP-activated gene) genes, which promote virulence in mice and survival within macrophages (7, 8, 9). Maximal induction of pag transcription occurs 3-6 h after Salmonella phagocytosis by macrophages, coincident with acidification of the phagosome to a mean pH of 4.9 (9). Phagosomes containing S. typhimurium are delayed and attenuated in acidification, relative to phagosomes containing dead bacteria, and yet it is the eventual phagosome acidification that results in increased transcription of virulence (pag) genes (9).

The considerable delay in pag expression indicates that Salmonella face two different tasks inside the macrophage phagosome. They must detect some feature of the phagosomal environment as a signal for gene expression and also survive inside the phagosome long enough to respond to this signal with pag expression. One may therefore distinguish two questions: first, what features of the phagosomal environment as a signal for gene expression and also survive inside the phagosome long enough to respond to this signal with pag expression? It is this second question that we address here.

The PhoP regulon also includes approximately 15 proteins that are repressed by a phoP locus mutation that results in constitutive pag transcription (phenotype PhoP+) (10). Some of these genes, termed prg (phoP-repressed gene) loci, are envelope proteins (11). PhoP+ Salmonella are also attenuated for mouse virulence and survival within macrophages (10). The delay between bacterial ingestion and pag expression predicts that a switch from prg to pag transcription occurs as the Salmonella-containing phagosome matures and acidifies. Thus, the proteins encoded by prgs may allow survival in the early phagosome, whereas those encoded by pags are required later.

We noticed during our measurements of pH that phagosomes containing bacteria were 3–6 μm in diameter (9). These

Abbreviations used in this paper: DM10F, DMEM with 10% FCS; FDx10, fluorescein-dextran of average molecular weight 10,000; M-CSF, macrophage-colony stimulating factor; NMS, normal mouse serum; pag, phoP-activated genes; prg, phoP-repressed genes; RB-BSA, Ringer’s buffer/2% bovine serum albumin; SP, spacious phagosomes; TBS, Tris-buffered saline.
unusually large or spacious phagosomes (SP) were similar in size to macropinosomes, endocytic vesicles formed from plasma membrane ruffles that appear in response to PMA or macrophage colony-stimulating factor (M-CSF) (12, 13). Because cell surface ruffling has been implicated in Salmonella invasion of epithelial cells we postulated that formation of the SP in macrophages involves Salmonella induction of macropinocytosis (14–17). In this work, video and fluorescence microscopy have been used to examine the morphology of S. typhimurium entry and persistence inside macrophages.

Materials and Methods

**Reagents.** DMEM, FCS, penicillin/streptomycin, and Luria broth were obtained from GibCO BRL (Gaithersburg, MD). Fluorescein-dextran of average molecular weight 10,000 (FD x 10) was obtained from Molecular Probes, Inc. (Eugene, OR).

**Bacterial Strains.** In this study, all Salmonella strains except SL3261 were derived from the virulent S. typhimurium (14028s; American Type Culture Collection, Rockville, MD). Virulence-attenuated mutants or S. typhimurium SL3261 were derived from the virulent S. typhimurium strain CS022 amaraA (18), SL3261 amaraA (18), CS022 pho-24 (10), CS015 pili:2:hisd4:Cm (8), and JS41 DEL2911[bio-105]1501 araG] (a gift of Dr. J. Slanch, University of Illinois-Urbana, Urbana, IL). Bacteria were killed by incubation at 65°C for 30 min; death was confirmed by plating for colony-forming units. Yersinia enterocolitica was isolated by the Clinical Microbiology laboratory of Massachusetts General Hospital. All strains were grown overnight in Luria broth at 37°C with shaking to stationary growth phase.

**Cell Cultures.** Bone marrow-derived macrophages were obtained from femurs of BALB/c or C3H/HeJ mice and cultured in DMEM with bone marrow medium (30% L cell-conditioned medium, 20% heat-inactivated FCS, and 50% DMEM) as described previously (19).

**Opsonization.** 2–3 x 10^6 bacteria/ml in 0.9% NaCl (normal saline; NS) was mixed with 10% normal mouse serum (NMS) and incubated for 30 min at room temperature. Bacteria were washed twice with NS before addition to macrophages. Bacteria were also opsonized by incubation in NS containing a 1:1,000 dilution of rabbit IgG antibody to Salmonella "0" antigen B (Fisher Scientific Co., Pittsburgh, PA). Opsonization with human recombinant mannose-binding protein (a gift of Alan B. Ezekowitz, Children’s Hospital of Wisconsin, Milwaukee, WI) and human recombinant mannose-binding protein (a gift of Alan B. Ezekowitz, Children’s Hospital of Wisconsin, Milwaukee, WI) was performed as previously described (20).

**Time-Lapse Video Microscopy.** BALB/c or C3H/HeJ bone marrow-derived macrophages were plated at a density of 2 x 10^3/25-mm diameter cover slip and cultured overnight in DMEM with 10% FCS (DM10F) without antibiotics or growth factors. Macrophages were washed twice with Ringer’s buffer/2% bovine serum albumin (RB-BSA: 155 mM NaCl, 5 mM KCl, 2 mM CaCl_2, 1 mM MgCl_2, 2 mM NaH_2PO_4, 10 mM Heps, and 10 mM glucose, pH 7.2, + 2% BSA). Video images were collected using a Dage NC-66X video camera mounted onto the microscope. Video images were collected at one frame/s using an optical disc recorder (Panasonic) to turn the 100 mm film (Kodak) using a Freeze Frame video recorder (Polaroid).

For most assays, coverslips were assembled on the microscope and a field containing one to four macrophages was recorded for ~2 min before adding bacteria. The camera was stopped, the top coverslip of the chamber was removed, and 1 ml of RB-BSA was removed and replaced with 1 ml of the same buffer containing bacteria. The ratio of macrophages to bacteria was 1:100. The top coverslip was replaced to continue the recording, which proceeded for 5–30 min.

**Detection of SP by Fluorescence Microscopy.** 5–10 x 10^4 macrophages from BALB/c or C3H/HeJ mice were plated on 12-mm glass coverslips and cultured overnight in DM10F without antibiotics. 10 bacteria/macrophage in 500 μl of DM10F containing 2 mg/ml of FD x 10. After 30 min, macrophages were washed 5 times with PBS and incubated in DM10F containing 8 μg/ml gentamicin at 37°C. At different times, macrophages were washed 3 times in PBS, and fixed for 1 h in a pH 7.2 solution of 75 mM lysine-HCl, 37.5 mM sodium phosphate, 10 mM NaIO_4, 2% paraformaldehyde, 4.5% sucrose, and 0.25% sodium periodate (21). After fixation, macrophages were washed three times with Tris-buffered saline/sucrose (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 4.5% sucrose) at 37°C. Coverslips were stained for 5 min at room temperature with 0.1 μg/ml DAPI diluted in TBS. The coverslips were washed 3 times with TBS/sucrose and mounted for microscopy in 90% glycerol, 10% PBS, and 1 mg/ml phenylenediamine (22).

**Results**

Salmonellae Stimulate Macropinocytosis and Persist in SP. Macrophages observed by time-lapse video microscopy displayed generalized plasma membrane ruffling and macropinocytosis within 2 min exposure to S. typhimurium (Fig. 1, A and B). Bacteria transiently contacted both ruffling and nonruffling regions of macrophage membranes, and were occasionally caught within membrane folds and ruffles and internalized (Fig. 2). The nascent phagosome was a bacterium enclosed within a 2–6-μm vacuole, morphologically indistinguishable from the macropinosomes forming nearby. Phagosomes were often large enough that bacteria could swim freely within the enclosed space (Figs. 1, B and C, and 2, B–D). In addition, some phagosomes enlarged by fusion with other phagosomes or with macropinosomes (Figs. 1, B and C, and 2, A–D). Many phagosomes remained spacious for the 35-min duration of the recording (Fig. 1), and although some shrank...
S. typhimurium 14028s was opsonized with 1:1,000 dilution of IgG anti-LPS, 10% MNS or no opsonization. SP indicates the number of SP counted per 80 macrophages. Bacteria indicates the number of bacteria counted per 80 macrophages.

SP formation under different experimental conditions. SP were visualized by allowing BALB/c-derived macrophages to internalize S. typhimurium in the presence of fluorescein dextran (FD × 10). After 30 min of infection plus an additional 10 min of incubation in gentamicin to kill extracellular bacteria, macrophages were fixed and stained with DAPI. The SP formation, total number of bacteria within SP, and total number of bacteria per macrophage were scored in a blind test. The percentage of bacteria in SP were determined as the number of bacteria within SP divided by the total number of intracellular bacteria counted. Each experimental condition was repeated on at least three different occasions. A representative experiment is shown.

<table>
<thead>
<tr>
<th>Condition</th>
<th>SP</th>
<th>Bacteria</th>
<th>Percent bacteria in SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unopsonized</td>
<td>118</td>
<td>256</td>
<td>64</td>
</tr>
<tr>
<td>Anti-LPS IgG</td>
<td>178</td>
<td>600</td>
<td>55</td>
</tr>
<tr>
<td>NMS (complement)</td>
<td>161</td>
<td>359</td>
<td>51</td>
</tr>
</tbody>
</table>

S. typhimurium stimulation of macropinocytosis and SP formation. Selected frames from a time-lapse videomicroscopy sequence of Salmonella phagocytosis by BALB/c bone marrow–derived macrophages. Macrophages are shown before (A), and 5 min (B) or 25 min (C) after infection with S. typhimurium. Membrane ruffling (asterisks) and macropinocytosis (arrows) began within 3 min of addition of S. typhimurium (B). Two of the SP indicated in C, formed from the ruffling region of the macrophage indicated by the asterisks in B. One SP in C (uppermost arrow) formed by fusion of smaller SP, shown in B.
Figure 2. *S. typhimurium* phagocytosis within plasma membrane ruffles. Selected time-lapse videomicroscopic images of *S. typhimurium* phagocytosis by bone marrow-derived macrophages. B and C show a fusion event between two SP. Elapsed time in minutes is indicated at the bottom of each frame. Arrows track the progress of one SP.

to enter via smaller phagosomes (Fig. 3, E–G). Remarkably, during the several minutes following entry, these smaller phagosomes enlarged by fusion with macropinosomes (Fig. 3, F–H). The number of SP formed after 40 min of infection with IgG-opsonized bacteria was equal to that formed by other opsonization conditions, further indicating that phagosomes containing IgG-opsonized bacteria enlarged after formation.

The Number of SP within Macrophages Diminished Over Time. Time-lapse video microscopy indicated that SP shrank more slowly than M-CSF-induced macropinosomes, which usually become undetectably small within 10–15 min of formation (23). We therefore quantified SP and macropinosomes at different times after the addition of gentamicin to culture medium to eliminate extracellular bacteria. After 10 min, 58% of internalized bacteria were in SP and infected macrophage cultures had 15 times as many macropinosomes as macrophages infected with dead bacteria (data not shown). However, after 45 min of gentamicin treatment the percentage of bacteria in SP declined to 29%, and by 3.5 and 5.5 h after the removal of extracellular bacteria <10% of bacteria were in SP (Table 3). We have observed bacteria in SP 24 h after infection (data not shown). Thus, although most SP shrink, they do so much more slowly than macropinosomes, and some persist in the macrophage cytoplasm for many hours.

Defective Induction of Macropinocytosis and SP Formation by *S. typhimurium* phoP-constitutive Mutants. If SP formation is necessary for pathogenesis and intracellular survival of *Salmonella*, then some virulence-defective strains should have reduced ability to induce SP. Testing a number of strains with reduced survival within macrophages, we identified one strain deficient in SP formation and macropinocytosis (Tables 2 and 3). This strain, phenotype PhoP+, contains a mutation in the *phoP* coordinate regulatory locus, that confers a constitutive state of *pag* expression and *prg* repression (10). All other *S. typhimurium* strains tested formed SP; including strains deleted for *phoP*, as well as purine and aromatic amino acid auxotrophs. When compared to wild type bacteria (Table 2, and Fig. 4, A and B) only PhoP+ bacteria induced significantly fewer SP (Tables 2 and 3, and Fig. 4, C and D). PhoP+ organisms formed no more macropinosomes then did...
PhoP Mutants of S. typhimurium Are Deficient in Induction of SP Formation Compared with Other Avirulent S. typhimurium Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>No. SP/80 macrophages</th>
<th>Percent of bacteria in SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td>Wild type</td>
<td>184</td>
<td>62</td>
</tr>
<tr>
<td>CS015</td>
<td>PhoP null</td>
<td>163</td>
<td>65</td>
</tr>
<tr>
<td>CS022</td>
<td>PhoP constitutive</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>CS003</td>
<td>Purine auxotroph</td>
<td>125</td>
<td>49</td>
</tr>
<tr>
<td>SL3261</td>
<td>Aromatic amino acid auxotroph</td>
<td>169</td>
<td>63</td>
</tr>
<tr>
<td>JS41</td>
<td>Rough, biotin, and aromatic amino acid auxotroph</td>
<td>118</td>
<td>45</td>
</tr>
<tr>
<td>14208s</td>
<td>Heat-killed</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

See legend to Table 1.

Heat-killed bacteria, approximately eightfold fewer than that induced by wild type organisms. Consistent with the decreased formation of SP altering intracellular survival, gentamicin-resistant colony-forming units of PhoP<sup>+</sup> bacteria were significantly reduced in the first hour after phagocytosis when compared to wild type organisms (data not shown). PhoP<sup>+</sup> revertants isolated after selection within mice formed SP as efficiently as wild type organisms indicating that defective SP formation was correlated with this phenotype (data not shown).

### Table 3. SP Decreased Over Time

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chase time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>Bacteria in SP</td>
<td>135 ± 2.3</td>
</tr>
<tr>
<td>Total number of bacteria</td>
<td>231 ± 1.4</td>
</tr>
<tr>
<td>Percent in SP</td>
<td>58</td>
</tr>
</tbody>
</table>
| PhoP<sup>-</sup>
| Bacteria in SP | 25 ± 1.4 | 8 ± 1 | 4 ± 1.4 | 0 |
| Total number of bacteria | 136 ± 3.1 | 128 ± 1.4 | 60 ± 2.9 | 42 ± 1.4 |
| Percent in SP | 18 | 6 | 7 | – |

30 min after infection the coverslips containing macrophages infected with Salmonella were washed with PBS and chased with medium containing 8 μg/ml gentamicin. The chase time indicates the time of incubation in medium containing gentamicin before infected macrophages were sampled. 80 macrophages per coverslip were analyzed. Each macrophage was quantitated for the total number of enclosed bacteria and the number of bacteria within SP. The mean and standard error of three different experiments are displayed above.


and SP formation was visualized by video microscopy. This increased the number of PhoP" mutants internalized, but did not increase the number of macropinosomes or percentage of SP formed (data not shown). Since PhoP" mutants are repressed in prg expression these results implicate prg products in SP formation.

Macrophage LPS Response Is Not Essential for SP Formation. S. typhimurium contains LPS, which can stimulate pinocytosis in macrophages derived from BALB/c but not C3H/HeJ mice, which are LPS resistant (24). To determine if S. typhimurium LPS was inducing macropinocytosis, Salmonella phagocytosis was examined in macrophages derived from C3H/HeJ mice. SP formed equally well in macrophages from these mice (data not shown). In contrast, when heat-killed bacteria were used as a source of LPS, neither SP nor macropinocytosis were induced. A galE mutant of S. typhimurium, deficient in production of the polysaccharide side chain of LPS, also formed SP as efficiently as wild-type bacteria (Table 2). The O polysaccharide side chain of LPS extracted from PhoP" and wild type S. typhimurium have similar chain length (Behlau, I., and S. I. Miller, unpublished observations). These results indicated that bacterial LPS was not primarily responsible for the S. typhimurium–induced macropinocytosis.

Discussion

Salmonella Entry. Phagocytosis of Salmonella typhimurium by macrophages was unconventional both in the mechanism of entry and the morphology of the phagosome. Rather than generating a localized membrane response close to the bacterium, Salmonella stimulated macropinocytosis over a large portion of the macrophage surface. Bacteria that contacted the macrophage surface were sometimes caught as the membrane ruffles closed to form an intracellular vesicle. Although this would seem an inefficient mechanism of invasion, the process resulted in considerable uptake of bacteria. Uptake was enhanced by opsonization, apparently because it increased binding of bacteria to macrophages. It did not appear that more conventional mechanisms of phagocytosis were suppressed, because IgG-opsonized bacteria often entered via close-fitting phagosomes. Instead capture by already forming SP was a more rapid process than entry by conventional phagocytosis.

S. typhimurium invasion of epithelial cells also been associated with membrane ruffling (14, 17). Indeed, entry into both macro- phages and epithelial cells could occur by a single mechanism. However, in epithelial cells membrane ruffles are observed only in the local area adjacent to the bacterium (14, 17). In macrophages, generalized membrane ruffling and macropinocytosis of Salmonella was observed within minutes of exposure to bacteria and in areas without overt bacterial adhesion. This suggests that a soluble factor is produced by Salmonella or the infected macrophage that stimulates ruffling.

Formation and Maintenance of SP. Perhaps more important than the stimulation of macropinocytosis was the formation and persistence of SP. In part, this was an obvious consequence of entry via macropinocytosis. But even close-fitting phagosomes that formed around IgG-opsonized bacteria enlarged by fusion with macropinosomes and eventually formed SP (Fig. 3, E–H). Unlike conventional macropinosomes, SP persisted in the cytoplasm, sometimes enlarging considerably by fusing with macropinosomes or other SP. Indeed, continued induction of macropinocytosis may be required for maintenance of SP volume.

Other mechanisms of maintaining SP volume are possible. Salmonella may produce molecules that inhibit the shrinkage of phagosomes that normally follows their formation. This may occur by altering macrophage transport proteins, by filling the phagosome with an osmotically active solute, or by altering the phagosome interactions with other endocytic organelles. Regarding the latter possibility, we have found that Salmonella-containing phagosomes merge with the lysosomal compartment, albeit somewhat slowly. The lysosomal membrane glycoprotein, IgPA, arrives in SP within 40 min of their formation, but complete delivery of the lysosomal enzyme cathepsin L to all phagosomes is delayed by >30 min (Alpuche-Aranda, C., J. A. Swanson, and S. I. Miller, unpublished observations). It therefore appears that the SP is likely a phagolysosome, and that SP are not maintained by altering endocytic vesicle traffic.

Correlations between the PhoP Regulon and SP. One pleiotropic Salmonella regulatory mutant defective in survival within macrophages was deficient in stimulating macropinocytosis and SP formation. PhoP" bacteria were observed to enter macrophages in a fashion typical of receptor-mediated phagocytosis and to induce less macropinocytosis. This defect in SP formation was specific for PhoP" bacteria and was not seen with several other mutants defective in virulence or survival within macrophages, including PhoP null mutants. This specificity for PhoP" bacteria implicates prg-encoded proteins in the stimulation of macropinocytosis and SP formation. Because PhoP" bacteria are attenuated for survival within macrophages this result also suggests that SP formation may be necessary for Salmonella survival. Most likely it is survival within early phagosomes that is enhanced by SP formation. Salmonella entry or SP formation may depend on expression of prg, and in a later phase, shrinkage and acidification of SP <5.0 results in a switch to pag expression. Consistent with this model, the timing of pag expression correlated with a significant reduction in the number of SP. In addition, PhoP" mutants, are defective in survival early after phagocytosis while PhoP null mutants begin to show a defect only 4–6 h after macrophage infection.

The PhoP" strain has another remarkable property in that it is highly immunogenic when used as a live vaccine (10). As few as 15 organisms provide protection against 5 logs greater wild type bacteria. Lacking further data, one can only speculate that this immunogenicity results from a deficiency in SP formation. Indeed, the normal delay in pag expression may allow Salmonella time to reach or create an intracellular compartment removed from the organelle traffic normally involved in antigen presentation.

Role of the SP in Pathogenesis. How could SP formation protect bacteria from killing by macrophages? First, the macro- phage's ability to kill ingested organisms may require a close-
fitting phagosome, and a small lumenal volume, in order to obtain sufficiently high concentrations of toxic compounds. The SP could dilute such compounds, thus allowing Salmonella to initially survive. When combined with delay in acidification of this phagosome (9), the bacterium may create a tolerable intracellular environment. Second, a gradual exposure to an acidic environment may allow the organism time to express other genes necessary for survival.

Abshire and Neidhardt (25) have recently identified two Salmonella populations that exist within macrophages: one static and another replicating. Because macrophages often show a mixture of tight and spacious phagosomes, SP may contain the replicating population of Salmonella.

Our results indicate that some SP shrink while others are maintained for hours. How can we explain the heterogeneity of SP persistence given genetic identity among the bacteria? One possibility is that survival is a cooperative enterprise. Extracellular bacteria may stimulate generalized macrophagocytosis and maintain intracellular bacteria within SP. Persistence would then be modulated by initial SP size and subsequent organelle fusion events with variations in the number of extracellular bacteria affecting SP size. If the ability of SP to fuse with other SP or macropinosomes is a random event, then the more macropinosomes formed the greater the chance for organelle fusions and SP persistence.

We are grateful to Dr. John Heuser for helpful discussions and for sharing unpublished video microscopy of Listeria entry into macrophages.

This work was supported by National Institutes of Health grants AI-30479 and AI-00917 (to S. I. Miller) and by an American Cancer Society research grant (to J. A. Swanson). J. A. Swanson is an Established Investigator of the American Heart Association. C. M. Alpuche-Aranda was supported by the Hospital Infantil de Mexico and the Universidad Nacional Autonoma de Mexico.

Address correspondence to Dr. Samuel I. Miller, Infectious Disease Unit, Massachusetts General Hospital, Fruit Street, Boston, MA 02114.

Received for publication 12 August 1993 and in revised form 7 October 1993.

References


