

Spacious Phagosome Formation within Mouse Macrophages Correlates with *Salmonella* Serotype Pathogenicity and Host Susceptibility

CELIA M. ALPUCHE-ARANDA,^{1†} ERIC P. BERTHIAUME,² B. MOCK,³
JOEL A. SWANSON,² AND SAMUEL I. MILLER^{1*}

Infectious Diseases Unit, Massachusetts General Hospital, Boston, Massachusetts 02114¹;
Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115²;
and Laboratory of Genetics, National Cancer Institute, Bethesda, Maryland 20892³

Received 17 May 1995/Returned for modification 24 July 1995/Accepted 22 August 1995

Light microscopic studies indicated a correlation between the virulence for mice of different *Salmonella* serotypes and the ability to form or maintain spacious phagosomes (SP) within mouse macrophages. Although *Salmonella typhimurium* induced membrane ruffling, macropinocytosis, and SP formation in macrophages from BALB/c mice, serotypes which are nonpathogenic for mice produced markedly fewer SP. SP formation correlated with both serotype survival within mouse macrophages and reported lethality for mice. Time-lapse video microscopy demonstrated that the human pathogen *S. typhi* induced generalized macropinocytosis and SP formation in human monocyte-derived macrophages, indicating a similar morphology for the initial phases of this host-pathogen interaction. In contrast to bone marrow-derived macrophages from BALB/c mice, macrophages from *S. typhimurium*-resistant outbred (CD-1) and inbred (CBA/HN) mice did not initiate generalized macropinocytosis after bacterial infection and formed markedly fewer SP. These deficiencies were not due to the *Ity* resistance genotype of these mice, as macrophages from mice that were congenic except for the *Ity* locus demonstrated equal SP formation in response to *S. typhimurium*. The observation that *S. typhimurium*-resistant CD-1 and CBA/HN mice are deficient in the ability to form and/or maintain SP indicates that a variable host component is important for SP formation and suggests that the ability to induce or form SP affects susceptibility to *S. typhimurium*. When serotypes nonpathogenic for mice were used to infect BALB/c macrophages, or when CD-1 or CBA/HN mouse macrophages were infected by *S. typhimurium*, some of the SP that formed shrank within seconds. This rapid shrinkage suggests that SP maintenance is also important for *S. typhimurium* survival within macrophages. These studies indicate that both host and bacterial factors contribute to SP formation and maintenance, which correlate with *Salmonella* intracellular survival and the ability to cause lethal enteric (typhoid) fever.

Salmonellae infect a variety of vertebrate hosts and cause a broad spectrum of diseases, including gastroenteritis, bacteremia, and enteric (typhoid) fever (23). Typhoid fever is a serious systemic illness in which bacteria disseminate to the liver, spleen, bone marrow, and other organs rich in phagocytic cells. The ability of *Salmonella typhimurium* to cause mouse typhoid fever correlates with in vitro bacterial survival within cultured macrophages (9).

Serotype-specific bacterial factors are necessary for disease production and outcome. Some *Salmonella* serotypes have a broad host range and produce different diseases in different hosts. For example, *S. typhimurium* commonly causes gastroenteritis in humans and a disease similar to enteric fever in inbred mice (20). Other *Salmonella* serotypes such as *S. arizonae*, *S. pullorum*, and *S. typhi* exhibit very narrow host ranges. *S. typhi* infects only humans and other higher primates such as chimpanzees (8, 23). *S. pullorum*, a serotype adapted to fowl, rarely causes disease in humans, is rarely isolated from animals other than fowl, and requires massive ingestion to cause even mild gastroenteritis in humans (5, 33, 35). Neither *S. typhi* nor *S. pullorum* causes disease in inbred mice susceptible to *S.*

typhimurium (3, 28). *S. arizonae* is rarely isolated from animals other than reptiles, causes human disease only in immunosuppressed individuals, and has not been isolated from mice (27, 40). For serotypes that have host specificity for chickens and mice, this specificity has been shown to be a result of differential survival of serotypes of different pathogenicities within the liver and spleen rather than an inability of these organisms to cross mucosal barriers and systemically colonize a resistant animal (3).

In addition to the bacterial contribution to infection, host immune status can dramatically affect disease outcome (23). For example, patients with AIDS develop recurrent *Salmonella* bacteremias with serotypes that cause only self-limited gastroenteritis in immunocompetent individuals (36). The importance of immune status to enteric fever pathogenesis has been most convincingly demonstrated by studying the susceptibility of inbred mice to *S. typhimurium*. In part as a result of mutations that alter immune status, the susceptibility of inbred mice to *S. typhimurium* varies dramatically (15, 19, 34). One locus essential to this property, *Ity* (also known as *Bcg* and *Lsh*), also alters the susceptibility to other intracellular pathogens, including *Mycobacteria* and *Leishmania* species (19, 34). *Ity*^s mice are defective in restricting the growth of intracellular pathogens within macrophages. *Ity* contains a gene, *Nramp*, which is expressed in macrophages and encodes a protein with sequence similarity to a membrane transporter (38). In addition to *Ity*, other loci which contribute to mouse susceptibility to *S.*

* Corresponding author. Present address: Health Science Building, Rm. K140, Box 357710, University of Washington, 1959 N.E. Pacific St., Seattle, WA 98195.

† Present address: Departamento de Bacteriología Intestinal, Hospital Infantil de México, México City 06720, Mexico.

typhimurium have been identified. These include *Xid*, a locus within the major histocompatibility complex which controls B-lymphocyte function in CBA/HN mice (30), and *Lps^d*, a locus responsible for lipopolysaccharide hyporesponsiveness in C3H/HeJ mice (29, 39).

In addition to the data on inbred mouse susceptibility, studies of the pathophysiology of mouse and human typhoid fever suggest that the capacity of bacteria to survive within macrophages is essential to pathogenesis (16, 20, 23, 31). Recent morphologic studies of this bacterial-eucaryotic cell interaction have demonstrated that *S. typhimurium* stimulates diffuse membrane ruffling and macropinocytosis by macrophages from *Ity^s* and *Lps^d* mice (1). Bacteria enter mouse macrophages in loose-fitting organelles called spacious phagosomes (SP) (1). SP are often several micrometers in diameter and are morphologically similar to macropinosomes. They form either by closing of membrane ruffles into large fluid-filled vacuoles or by enlargement after phagocytosis. One mechanism of SP enlargement entails fusion with *S. typhimurium*-induced macropinosomes. The *S. typhimurium* molecules involved in SP formation remain to be identified. However, strains with a constitutive mutation in the *phoP* regulatory locus (phenotype Pho^{Pc}) exhibit a significantly reduced ability to induce formation of SP, indicating that a *phoP*-repressed gene(s) contributes to this process (1). Since such Pho^{Pc} mutants are defective in survival within macrophages and are attenuated for mouse virulence (24), this suggested that SP formation was an important mechanism of bacterial intracellular survival.

To test the role of SP formation in intracellular survival and host-pathogen specificity, we have studied the ability of mouse macrophages to form SP when they are infected with *Salmonella* serotypes that do not induce typhoid fever in mice. In addition, we have examined SP formation in macrophages from *Salmonella*-resistant mice.

MATERIALS AND METHODS

Mice. Animals used in this work included BALB/c (*Ity^s*), C3H/HeJ (*Lps^d*), C57BL (*Ity^s*), CBA/HN (*Xid Ity^s*), SWR/J (*Ity^s*), DBA/2 (*Ity^s*), A/J (*Ity^s*), and CD-1 (outbred, *Ity^s*) mice, obtained from Charles River Breeding Laboratory (Wilmington, Mass.) and Jackson Laboratory (Bar Harbor, Maine). BALBc/II (*Ity^s*) and CD₂V₁/6 (*Ity^s*) mice congenic except for the *Ity* locus were bred at the National Cancer Institute, Bethesda, Md., as previously described (26).

Reagents. Dulbecco's minimal essential medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and Luria broth were obtained from GIBCO/BRL (Grand Island, N.Y.). Fluorescein-dextran with a molecular weight of 10,000 (FDx10) was obtained from Molecular Probes (Eugene, Oreg.), and 4',6'-diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Bacteria. The *S. typhimurium* strain used was ATCC 14028s. *S. typhi* and *S. enteritidis* strains were isolated by the Clinical Microbiology Laboratory of Massachusetts General Hospital from immunocompetent individuals with typhoid fever and diarrhea. *S. pullorum* χ 3544, originally isolated at the National Veterinary Service Laboratory, Ames, Iowa, as chicken isolate 3045, and *S. arizonae* χ 3425, a diphasic strain originally from the Centers for Disease Control and Prevention, Atlanta, Ga., serotype S.61:1,v:1,5, (7),3b=Ariz 26:23:30, were the gift of Roy Curtiss III, Washington University, St. Louis, Mo. This *S. arizonae* serotype has been isolated from reptiles and only rarely from sheep (40). *S. typhimurium* mutants defective in epithelial cell invasion were IB040 with *prgHI::Tnp_{phoA}* (4); CS451, a derivative of EE451 (18) constructed by P22 bacteriophage transduction of a 10-kb deletion of *hil* including *prgHIJK* and *org* (17, 32) into the wild-type strain ATCC 14028s; and CS120, containing *pagC64* (25). *S. typhimurium* strains derived from the parent SR11 include the wild-type virulent strain χ 3306 *gyrA1816*, χ 4115 containing *invA::cat* (11), and χ 3337, a derivative of χ 3306 cured of the 100-kb pStSR100 plasmid (13). All strains were grown overnight to stationary phase in Luria broth at 37°C with shaking before infection of macrophage cultures.

Mouse macrophages. Bone marrow-derived macrophages were obtained from the femurs of mice and cultured in DMEM with bone marrow medium (30% L-cell conditioned medium, 20% heat-inactivated FBS, and 50% DMEM with 1% penicillin-streptomycin) as previously described (1). Cells were harvested 6 days after extraction and plated in 24-well tissue culture dishes on 12-mm-diameter glass coverslips at a density of 7×10^4 cells per well for fluorescence

assay or on 25-mm-diameter coverslips for video microscopy. The macrophages were cultured overnight in DMEM containing 10% heat-inactivated FBS (DMEM-10F) without antibiotics. The experiments were performed the following day.

Human monocyte-derived macrophages. Human monocytes were obtained from the peripheral blood of volunteers by a modified method of Boyum adapted for Histopaque (Sigma) gradients (6). The monocyte layers obtained were adhered to plastic petri dishes for 2 h in RPMI 1640 medium with 10% FBS (RPMI-10F). Nonadherent cells were removed, and adherent cells were washed three times in phosphate-buffered saline (PBS) at 37°C and then allowed to differentiate for 6 days in RPMI-1640 medium with 10% human autologous serum (as a source of growth factors), 5% FBS, and 1% penicillin-streptomycin (RPMI-10HS-5F). The medium was changed every 48 h. Monocytes were allowed to differentiate for 6 days and then grown on plates for 12 to 24 h in RPMI-10F without human serum or antibiotics before use in experiments.

Infection of macrophages by salmonellae. Unopsonized and opsonized (with either 10% normal mouse serum or 10% autologous human serum without antibody to *S. typhi* O or H antigen) bacteria were added to macrophage cultures at a ratio of 10 to 20 bacteria per macrophage. Bacterial survival after phagocytosis was determined after addition of 8 μ g of gentamicin per ml to the extracellular medium. Macrophages were harvested and bacterial survival assays were performed as previously described (24). Under various times of chase in medium with gentamicin, triplicate sets of infected monolayers were lysed with 0.5% deoxycholate and the released bacteria were diluted and plated on Luria-Bertani agar to determine CFU.

Time-lapse video microscopy. Mouse bone marrow-derived macrophages and human monocyte-derived macrophages plated on 25-mm-diameter coverslips were washed twice with Ringer's buffer-2% bovine serum albumin (RB-BSA) warmed to 37°C. The coverslips were assembled in Sykes-Moore chambers, and bacterial infection was performed as previously described (1). The ratio of macrophages to bacteria was 1:100. Cells were observed with a 100 \times lens, N.A. 1.25, with phase-contrast optics, and images were collected by using a Dage NC-66X video camera mounted on the microscope. One field containing one to four macrophages was chosen, and the image was recorded before infection. After bacteria were added, the infected macrophages were recorded for 5 to 45 min. Video images were collected at one frame per second with a Panasonic optical disc recorder or a time-lapse VHS recorder. Video images were photographed with T-MAX 100 film (Kodak) by using a Polaroid Freeze Frame video recorder connected to the optical disc recorder.

Detection of SP by fluorescence microscopy. Macrophages plated on 12-mm-diameter glass coverslips and cultured overnight in DMEM-10F without antibiotics were infected with opsonized or unopsonized bacteria. The final condition was 20 bacteria per macrophage in a total volume of 500 μ l of DMEM-10F containing 1.5 mg of FDx10 per ml. After 30 min, the macrophages were washed five times with PBS at 37°C and incubated in 500 ml of DMEM-10F containing 8 μ g of gentamicin per ml (1). Ten, thirty, and sixty minutes after the addition of gentamicin, the macrophages were washed three times with warm PBS and fixed for 1 h with a modified form of paraformaldehyde-lysine-periodate fixative (75 mM lysine-HCl, 37.5 mM sodium phosphate, 10 mM NaIO₄, 2% paraformaldehyde, 4.5% sucrose, pH 7.2) (22). They were then washed three times with Tris-buffered saline (TBS)-sucrose (20 mM Tris-HCl, 150 mM NaCl [pH 7.5], 4.5% sucrose) at 37°C. Bacterial DNA and macrophage nuclei were stained in the presence of 0.1 mg of DAPI per ml diluted in TBS without sucrose for 5 min at room temperature. The coverslips were washed three times with TBS-sucrose and then were mounted for microscopy in a solution of 90% glycerol-10% PBS-1 mg of phenylenediamine per ml (37). SP quantification was determined on a Zeiss Photoscope III or a Leitz Laborlux-12 photoscope, with a 100 \times objective lens, N.A. 1.25, equipped with epifluorescence optics using fluorescein and UV filter sets. Each experiment was performed on three to seven different occasions, and duplicate samples were coded for blind assay of the results. At least 100 macrophages were examined for each experimental condition (50 macrophages per coverslip), and each macrophage was scored for the number of FDx10-labeled macropinosomes (phase-bright pinosomes containing FDx10), the total number of macrophage-associated bacteria stained by DAPI, and the number of SP. SP were defined as FDx10-labeled phagosomes in which the phagosomal membrane was not tightly apposed to the DAPI-stained bacteria, i.e., a volume of FDx10 surrounding the bacteria could be visualized (1). To determine the fraction of cell-associated bacteria that were intracellular, infected macrophages were fixed without permeabilization, total bacteria were detected by DAPI staining, and extracellular bacteria were detected by indirect immunofluorescence using antiserum to *S. typhimurium* O antigen. By this measure, >90% of the bacteria detected in such assays were intracellular after 10 min of gentamicin exposure (i.e., DAPI positive, antibody negative; data not shown).

Statistical analysis. Statistical analysis was performed by variance analysis and Mann-Whitney U test. *P* values of <0.05 were regarded as significant.

RESULTS

Fewer SP are induced by *Salmonella* serotypes nonpathogenic for mice. To test further the correlation between SP formation and pathogenesis, macrophages from BALB/c mice

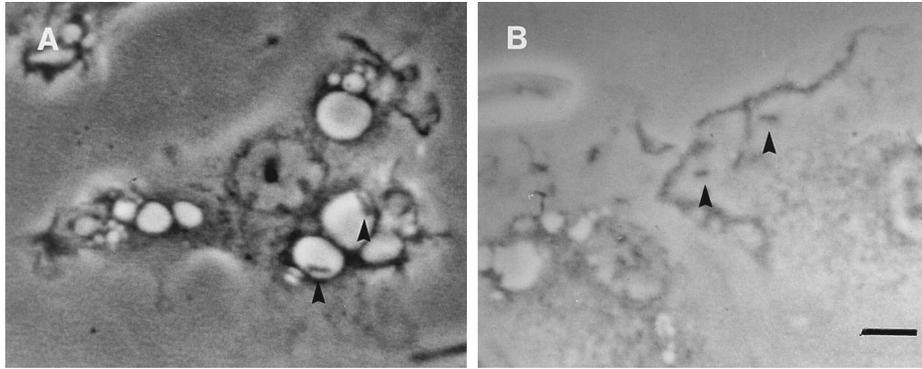


FIG. 1. Comparison of mouse macrophage phagosomes containing *S. typhimurium* and *S. typhi*. Representative images from time-lapse video recordings after 15 min of bacterial infection of macrophages are shown. Bacteria within phagosomes are indicated (arrowheads). Scale bar: 3 μ m.

were infected with *Salmonella* serotypes with different host specificities and SP formation was quantitated by fluorescence microscopy. As in earlier studies (1), *S. typhimurium* induced many SP that slowly diminished in number after the elimination of extracellular bacteria with gentamicin (Table 1). Other serotypes showed less effect; *S. typhi* and *S. arizonae* induced significantly fewer SP, and *S. pullorum* induced almost none (Table 1).

Infections of macrophages by these serotypes were also observed by time-lapse video microscopy. *S. typhi*- and *S. arizonae*-infected macrophages consistently made fewer SP than did macrophages infected with *S. typhimurium*. *S. typhi* and *S. arizonae* often entered macrophages in a manner expected for receptor-mediated phagocytosis, i.e., with a localized ruffle of the plasma membrane forming a very small phagosome. Even when SP were formed, they seemed to shrink faster than those formed by *S. typhimurium*, and in contrast to previous observations of antibody-opsonized *S. typhimurium* (1), the closely apposed phagosomes containing these organisms did not enlarge to form SP (Fig. 1). *S. pullorum*-infected macrophages displayed markedly less macropinocytosis, although an initial

burst of macropinocytosis was occasionally observed. Phagocytosis of *S. pullorum* occurred much less frequently than phagocytosis of *S. typhimurium*. Occasional small SP were observed, but most often *S. pullorum* entered macrophages via tightly apposed phagosomes.

Survival of *Salmonella* serotypes after phagocytosis by mouse and human macrophages correlates with virulence. Since differences in SP formation within mouse macrophages were noted, intracellular survival assays were performed to test whether decreased survival occurred early after phagocytosis. Consistent with a pathogenic role for SP formation, the survival of non-mouse pathogens was significantly reduced relative to that of *S. typhimurium*, shortly after phagocytosis (Fig. 2). These results were also consistent with the decreased numbers of intracellular bacteria determined after 1 h of gentamicin exposure (Table 1). Similar results indicating reduced SP formation by non-mouse-pathogenic *Salmonella* serotypes were also obtained with unopsonized bacteria (data not shown). Therefore, both the ability to cause lethal typhoid

TABLE 1. Induction of SP formation in BALB/c mouse bone marrow-derived macrophages infected by different *Salmonella* serotypes

Serotype	Chase time ^a (min)	Count (mean \pm SE) ^b			Index ^c
		SP	Total bacteria	Bacteria within SP	
<i>S. typhimurium</i>	10	256 \pm 9*	493 \pm 8	310 \pm 4*	0.52
	30	105 \pm 7*	504 \pm 6	127 \pm 6*	0.20
	60	39 \pm 7*	529 \pm 5	58 \pm 2*	0.07
<i>S. typhi</i>	10	81 \pm 8	371 \pm 7	100 \pm 7	0.20
	30	37 \pm 3	363 \pm 6	41 \pm 2	0.10
	60	3	276 \pm 7	5 \pm 1	0.01
<i>S. arizonae</i>	10	87 \pm 3	381 \pm 5	101 \pm 4	0.22
	30	27 \pm 2	408 \pm 7	37 \pm 2	0.06
	60	6	306 \pm 9	8	0.01
<i>S. pullorum</i>	10	12 \pm 1	169 \pm 2	15 \pm 2	0.07
	30	0	67 \pm 2	0	
	60	0	26 \pm 9	0	

^a Time of incubation in medium containing gentamicin.

^b The means and standard errors of three to seven blinded experiments in which 100 macrophages were counted are shown. Asterisks indicate that the numbers of SP induced by *S. typhimurium* are significantly greater ($P < 0.01$) than those for other *Salmonella* serotypes.

^c Obtained by dividing the mean number of SP by the mean number of intracellular bacteria.

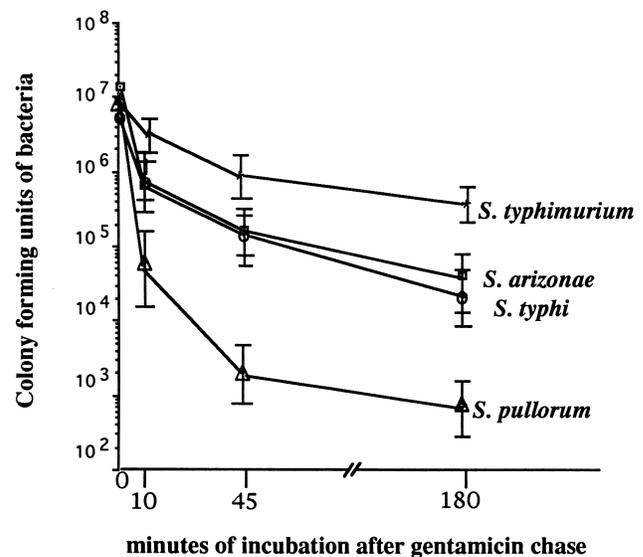


FIG. 2. Survival of *Salmonella* serotypes in bone marrow-derived macrophages from BALB/c mice. Bacterial CFU per well containing 5×10^5 macrophages (y axis) and the time after addition of gentamicin (x axis) are indicated. Results of a representative experiment (means and standard errors of the means) are shown. All values at 45 and 180 min were significantly different from the values for *S. typhimurium* ($P < 0.01$).

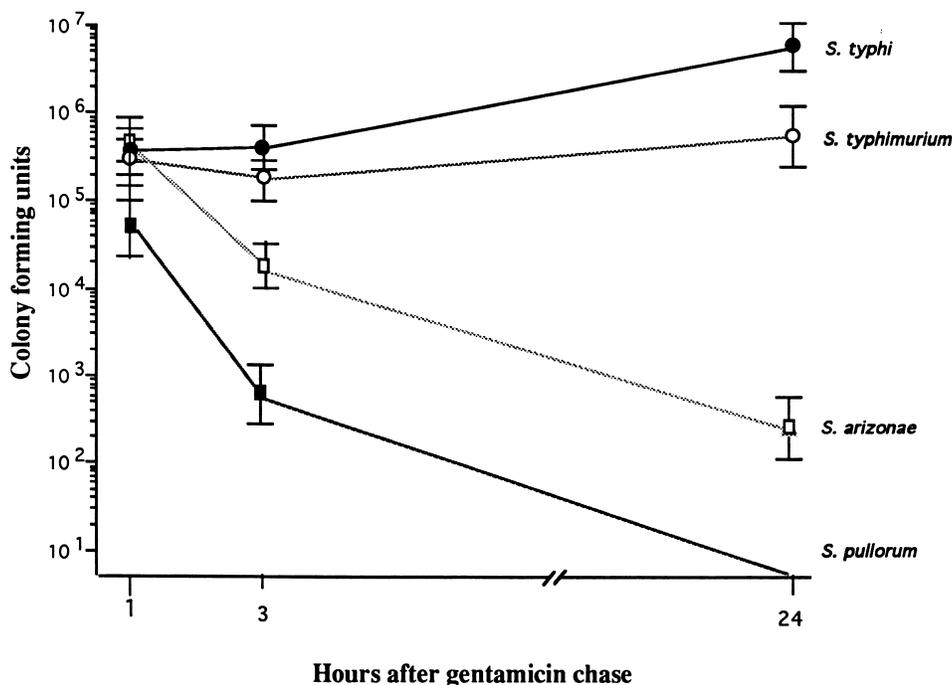


FIG. 3. Survival of *Salmonella* serotypes in human macrophages. Means and standard errors of the means are indicated. Bacterial CFU per well containing 5×10^5 macrophages (y axis) and the time after addition of gentamicin (x axis) are shown. All values at 24 h were significantly different from those for *S. typhi* ($P < 0.01$). At 3 h, values for *S. pullorum* and *S. arizonae* were significantly different from those for *S. typhimurium* and *S. typhi* ($P < 0.01$).

fever in mice and the ability to survive within macrophages early after phagocytosis correlated with the ability to induce SP.

The results in Fig. 3 also demonstrate that the survival of the common human pathogens *S. typhi* and *S. typhimurium* within human macrophages was significantly greater than that of the serotypes *S. arizonae* and *S. pullorum* during early intracellular infection (3 h after addition of gentamicin). Even though there was no difference in intracellular survival between *S. typhi* and *S. typhimurium* at early times, there was a significant increase in intracellular survival for *S. typhi* at later times. Therefore, these results demonstrate that the human pathogens survived better than the non-human pathogens within human macrophages and further suggest that the ability of *S. typhi* to survive within human monocytes correlates with the capacity to produce typhoid fever in humans.

***S. typhi* organisms are within SP after phagocytosis by human macrophages.** To establish further correlations between SP formation and host-pathogen interactions, human peripheral blood-derived macrophages were infected with *S. typhi* and *S. typhimurium* and examined for SP formation by time-lapse video microscopy. Within the first 5 min after *S. typhi* contact, human macrophages displayed intense ruffling and vigorous macropinocytosis followed by *S. typhi* endocytosis (Fig. 4A). SP formation was very similar to that induced in mouse macrophages by *S. typhimurium*. Human macrophages infected by *S. typhimurium* also showed some membrane ruffling, macropinocytosis, and SP formation (Fig. 4B). Although quantification of the differences between these serotypes was not possible because of the thickness of the human macrophages, *S. typhi* appeared to induce more extensive macropinocytosis than did *S. typhimurium*. These experiments demonstrated that the human pathogens induced SP formation in human macrophages.

Decreased ability to form or maintain SP by *S. typhimurium*-resistant outbred (CD-1) and inbred (CBA/HN and DBA/2) mice. Strains of mice differ in their susceptibility to *S. typhimurium* infection (15). The ability to induce SP could be an indicator of host susceptibility. The 50% lethal dose (LD_{50}) for *S. typhimurium* infections is 10,000 to 1,000,000 times greater for *Ity^r* mice, such as A/J, DBA/2, CBA/HN, and CD-1 mice, than for BALB/c and other *Ity^s* mice (15, 34). Moreover, macrophages from CD-1 mice kill *S. typhimurium* approximately 50 to 100 times more efficiently than do macrophages from *Ity^s* mice such as BALB/c and C57BL (data not shown). We therefore tested the ability of macrophages from *Ity^s* and *Ity^r* mice to form SP after *S. typhimurium* infection. Macrophages obtained from CD-1 and CBA/HN mice formed markedly fewer SP than did macrophages from BALB/c and C57BL mice (Table 2). In

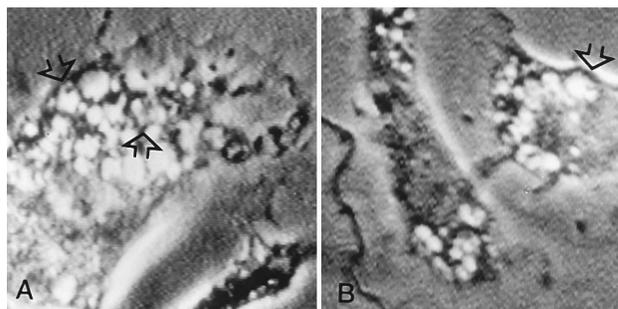


FIG. 4. Induction of macropinocytosis and SP formation in human macrophages by salmonellae. Representative images from time-lapse video microscopy of a human macrophage 10 min after *S. typhi* (A) and *S. typhimurium* (B) infection are shown. Bacteria within SP in an area of membrane ruffling are indicated (arrows).

TABLE 2. Induction of SP formation within macrophages from mice with different *S. typhimurium* susceptibilities

Mouse line	Count (mean \pm SE) ^a			Index ^b	Relevant genotype ^c	LD ₅₀ ^d
	SP	Total bacteria	Bacteria within SP			
BALB/c	288 \pm 6	452 \pm 8	275 \pm 5	0.64	<i>Ity</i> ^s	<10
BALB/cII	260 \pm 10	450 \pm 6	267 \pm 4	0.57	<i>Ity</i> ^s	<10
C57BL	240 \pm 8	405 \pm 5	210 \pm 4	0.59	<i>Ity</i> ^s	<10
C3H/HeJ	195 \pm 11	296 \pm 7	265 \pm 3	0.50	<i>Ity</i> ^r <i>Lps</i> ^d	<10
SWR/J	225 \pm 14	373 \pm 4	192 \pm 3	0.60	<i>Ity</i> ^r	10 ⁵
CD ₂ V ₁ /6 ⁻	231 \pm 6	423 \pm 6	232 \pm 4	0.54	<i>Ity</i> ^r	10 ⁵
DBA/2	115 \pm 18*	374 \pm 12	119 \pm 2*	0.30	<i>Ity</i> ^r	10 ⁵
A/J	150 \pm 9	281 \pm 6	174 \pm 4	0.50	<i>Ity</i> ^r	10 ⁶
CBA/HN	28 \pm 4**	336 \pm 6	35 \pm 1**	0.08	<i>Ity</i> ^r <i>Xid</i>	10 ⁷
CD-1	38 \pm 2**	395 \pm 7	41 \pm 1**	0.09	<i>Ity</i> ^r	>10 ⁶

^a SP formed, total number of macropinosomes, total number of bacteria, and total number of bacteria in SP per 100 macrophages were determined in blinded experiments after 10 min of incubation in medium with gentamicin. The means and standard errors of three experiments are presented. Asterisks indicate that the macrophages formed significantly fewer SP (*, $P < 0.05$; **, $P < 0.01$).

^b Determined as described for Table 1.

^c Genes known to affect susceptibility to *S. typhimurium*.

^d LD₅₀s are derived from the literature (15, 26, 28–30).

addition, macrophages obtained from DBA/2 mice formed or maintained SP less efficiently than those from BALB/c mice (Table 2). Furthermore, time-lapse video microscopy of CD-1 and CBA/HN macrophages indicated little macropinocytosis or SP formation in response to *S. typhimurium* infection (data not shown). CD-1, CBA/HN, and DBA/2 mice carry the *Ity*^r allele, and this in part explains their resistance to *S. typhimurium* infection. Therefore, to test whether *Ity* was important for *S. typhimurium* induction of SP, macrophages from BALB/c mice congenic except for the *Ity* locus were evaluated for SP formation. As can be seen in Table 2, macrophages from congenic CD₂V₁/6⁻ (*Ity*^r) and BALB/cII (*Ity*^s) mice (26) formed equal numbers of SP. This indicated that the *Ity*^r allele is not sufficient to explain the lack of SP formation in macrophages from CD-1 and CBA/HN mice. In addition, the phenotype of DBA/2 mice, intermediate between those of BALB/c and CBA/HN mice, correlated with mouse sensitivity to *S. typhimurium*, since the resistance of DBA/2 is intermediate between those of BALB/c and CBA/HN mice (15).

Rapid shrinkage of SP. Time-lapse video microscopy of *S. typhi* and *S. arizonae* infections of BALB/c macrophages

showed mixed morphologies of phagosomes. Bacteria were observed in both SP and tightly apposed phagosomes. In general, many of the SP induced by these serotypes shrank more rapidly than those induced by *S. typhimurium* (Fig. 1 and 5A). The rare SP formed during *S. typhimurium* infection of macrophages obtained from CD-1 and CBA/HN mice also shrank quickly, sometimes within 10 s (Fig. 5B). These observations suggest that both bacterial and host factors maintain SP volume and that in the absence of either bacterial or host contributions SP rapidly shrink. This could occur either by transport across membranes into the cytoplasm or by release of fluid from the phagosome into the extracellular space.

***S. typhimurium* mutants defective in signaling epithelial cells form SP normally.** Our previous observations of defective SP induction by PhoP^c mutants suggested that PhoP-repressed gene products contribute to SP formation (1). Recently, it was found that one such PhoP-repressed locus, *prgH*, is essential to induce bacterium-mediated endocytosis by epithelial cells and that it is essential for the secretion of proteins required for epithelial cell invasion (4, 32). The process of entrance into cultured epithelial cell lines is morphologically similar to that observed for macrophages, in that organisms enter within membrane ruffles and can form and persist within large vacuoles (10, 12). Therefore, to determine whether the same bacterial products signal both eucaryotic cell types, several mutants defective in epithelial cell signaling were tested for the ability to induce SP. As demonstrated in Table 3, these mutants did not show dramatically different SP formation in comparison with wild-type bacteria. These results were consistent with prior results demonstrating normal survival of *prgH* mutants within macrophages (4). The fact that the megaplasmid-cured strain of *S. typhimurium*, χ 3337 (13), induced SP as well as its wild-type parent also demonstrates that genes contained on this plasmid are not required for induction of SP formation.

DISCUSSION

Previous work demonstrated that *S. typhimurium* persisted in a novel SP within mouse macrophages (1). This suggested that the ability to induce SP formation was a pathogenic mechanism for bacterial survival within macrophages. In support of this hypothesis, a regulatory mutant defective in intracellular survival, a *phoP* constitutive mutant, was found to be defective in induction of SP formation (1). The present report describes

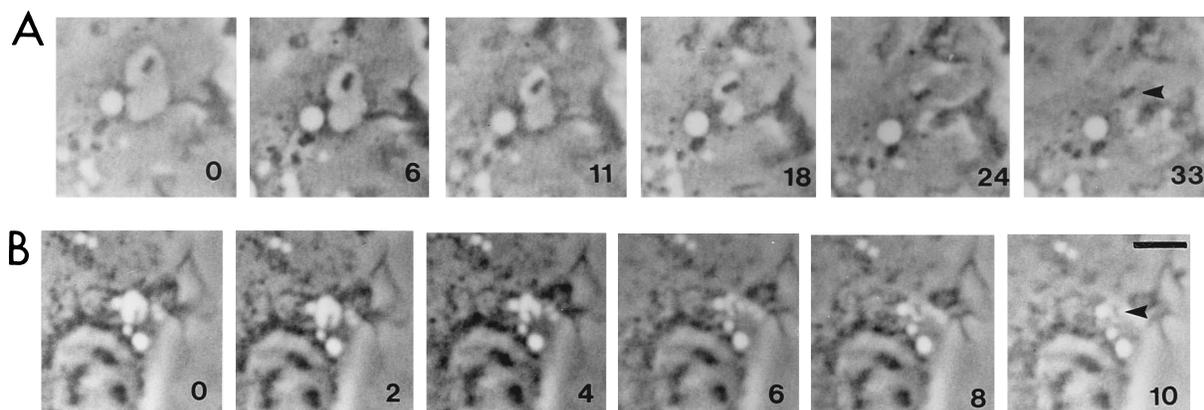


FIG. 5. Rapid shrinkage of SP induced in *S. typhimurium*-resistant mouse macrophages by nonpathogenic serotypes. Frames are from a time-lapse video microscopy sequence. The time (in seconds) is indicated in the lower right corner of each frame. (A) BALB/c mouse macrophage phagosomes containing *S. arizonae*. (B) CD-1 mouse macrophage phagosomes containing *S. typhimurium*. Scale bar: 3 μ m. Shrunken SP are indicated (arrowheads).

TABLE 3. Induction of SP formation by *S. typhimurium* mutants defective in epithelial cell invasion and cured of the megaplasmid

Strain name or description ^a	Count (mean \pm SE) ^b			Index ^c
	SP	Total bacteria	Bacteria within SP	
WT 14028	217 \pm 3	458 \pm 6	283 \pm 5	0.50
PhoP ^c	27 \pm 2*	175 \pm 3*	33 \pm 1*	0.15
PrgHIJK	232 \pm 3	449 \pm 6	272 \pm 3	0.52
Hil Δ	242 \pm 3	546 \pm 5	291 \pm 6	0.44
PagC64	289 \pm 3	585 \pm 4	301 \pm 6	0.49
InvA	184 \pm 7	418 \pm 6	183 \pm 3	0.44
WT SR11	232 \pm 5	525 \pm 10	328 \pm 6	0.44
Plasmid cured	255 \pm 0	523 \pm 7	308 \pm 5	0.48

^a Strains are as follows: PhoP^c, CS022; PrgH, IB040; Hil, CS451; PagC64, CS120; InvA, χ 4115; and plasmid cured, χ 3337. WT, strain with wild-type virulence properties.

^b SP formed, total bacteria, and bacteria in SP per 100 BALB/c macrophages were determined in blind experiments. The means and standard errors of at least three experiments are displayed.

^c Determined as described for Table 1.

further correlations between SP formation and bacterial virulence. *Salmonella* serotypes that are nonpathogenic for mice survived poorly within mouse macrophages and were defective in induction of SP formation and maintenance. Experiments with macrophages from outbred and inbred mice with different resistances to *S. typhimurium* indicated that a host component was essential to SP formation. The result that SP were formed or maintained more easily in susceptible mice suggested that SP formation may be a factor in host susceptibility to salmonellae.

The properties of *Salmonella* serotypes that determine host specificity seem likely to provide clues to pathogenic mechanisms. The diversity of interactions between salmonellae and their various hosts suggests that multiple factors contribute to serotype and host specificity. Serotype host ranges vary dramatically from narrow (*S. typhi*) to broad (*S. typhimurium*) (23). In addition, infection with broad-host-range serotypes results in different diseases, depending on the host infected. For example, *S. typhimurium* infection results in diarrhea in humans and enteric fever in mice (23). This work suggested that the signaling at the macrophage surface that results in SP formation is an important feature of serotypes that cause enteric fever in mice.

Recent work has demonstrated that *Salmonella* serotype specificity for causing human gastroenteritis correlates with the ability to induce neutrophil migration across a cultured T84 epithelial cell monolayer from the basolateral to apical (luminal) surface (21). *Salmonella* mutants defective in induction of epithelial cells to endocytose bacteria by a ruffling mechanism were also defective in induction of neutrophil transmigration. This suggested that serotype specificity involved cell signaling at the epithelial cell surface that ultimately resulted in inflammation (diarrhea). In the present work, these mutants as well as mutants defective in the expression of a single PhoP-repressed operon (*prgHIJK*) essential to induction of bacterial endocytosis by epithelial cells (4, 32) all induced SP formation in macrophages. The induction of membrane ruffling and SP formation is morphologically different in epithelial cell lines and bone marrow-derived macrophages, and the efficiency of bacterial uptake is markedly greater in professional phagocytes. Transformed epithelial cell lines ruffle only where a bacterium is close to the membrane (10). In contrast, the membrane ruffling and macropinocytosis induced in macrophages are generalized responses, not restricted to one region

of the cell (1). Only a pleiotropic PhoP constitutive mutant has been demonstrated to be deficient in both SP formation and signaling to epithelial cells. The pleomorphic phenotype of this mutant may be due to the fact that it is deficient in the production of over 20 cellular proteins (4). In addition, the culture supernatants of this mutant contain markedly fewer peptide species (approximately 15) than wild-type bacteria, suggesting that this organism has a defect in secretion of soluble molecules that could interact with macrophages and epithelial cells (32). Mutants defective in the PhoP-repressed locus *prgH*, as well as many other mutants defective in signaling epithelial cells, show normal induction of SP formation and survival within macrophages. The *prgH* locus has recently been demonstrated to be an operon that encodes proteins similar to secretion determinants of *Shigella* and *Yersinia* spp. These determinants are required for the secretion of Yop and Ipa proteins, which promote pathogenesis by signaling eucaryotic cells (32). Analysis of the culture supernatants of *prgH* mutants indicates that they also have a secretion defect like that of PhoP^c bacteria, though many fewer peptide species (only five) are absent. These results as well as this analysis of SP formation indicate that some bacterial gene products that induce a response in epithelial cells are not required for SP formation in macrophages.

Our findings that CD-1 and CBA/HN mouse macrophages highly resistant to *S. typhimurium* do not form or maintain SP as well as macrophages from susceptible mice indicated that a host component was necessary for the induction of SP. It has long been known that various mutations that alter macrophage function alter mouse susceptibility to *S. typhimurium*. The best characterized of these mutations are in the *Ity* locus, which is also known as *Lsh* and *Bcg*. *Ity* genotype alters the susceptibility of a number of intracellular pathogens by alteration of a macrophage function (19, 34). A gene with mutations that could be responsible for this phenotype has been located on mouse chromosome 1 (*Nramp*) and is similar to a membrane transporter (38). It was possible that this protein could be located in the phagosome and alter SP formation. However, this work indicates that *Nramp* is not involved in SP formation. Numerous other genes that alter immune function have been demonstrated to alter mouse susceptibility to *S. typhimurium*, including those that alter lipopolysaccharide sensitivity and others linked to the major histocompatibility complex (29, 30, 34, 39). Previous work demonstrated that lipopolysaccharide responsiveness was not required for SP formation (1). This difference between highly resistant outbred mice and highly susceptible inbred mice could lead eventually to the identification of new genes important to mouse bacterial susceptibility, since it is possible that the ability to form SP can be identified in crosses between resistant and susceptible mice as a factor in mouse pathogen susceptibility.

In both infections of susceptible macrophages with nonpathogenic serotypes and infections of resistant macrophages with *S. typhimurium*, SP often shrank rapidly. Such vacuole shrinkage is not without precedent, as freshwater amoebae maintain contractile vacuoles that expel water rapidly as part of a mechanism for osmoregulation. These nonacidic vacuoles have associated calmodulin, proton pumps, and myosin IC (7, 14, 41). Contractile vacuoles fuse with the cell surface and contract, over approximately 90 to 100 s, with resultant expulsion of water. Contraction can be inhibited by intracellular antibodies against myosin IC (7). Physiologic and morphologic studies of the rapidly shrinking SP should define their relationship to the contractile vacuoles of unicellular organisms. These shrinking SP contrast with our previous observations of phagosome enlargement of adherent tight phagosomes formed

after *S. typhimurium* opsonization with specific antibody (1). The capacity of SP for enlargement and shrinkage suggests that the ability to maintain SP is an important element of this host-pathogen interaction.

We propose a three-stage model of *S. typhimurium*-macrophage interactions based upon this work and our previous work. First, PhoP-repressed genes induce macropinosytosis and SP formation (1). Second, SP are prevented from shrinkage or are enlarged by fusion with macropinosomes or other SP. Third, shrinkage and acidification of the phagosome with subsequent induction of PhoP-activated genes (2) allow survival in a shrunken, acidified phagolysosome.

ACKNOWLEDGMENTS

We thank Cathy Lee, Jorge Galan, Virginia Miller, Roy Curtiss III, and Paul Gulig for bacterial strains. We also thank María Ramirez-Aguilar for technical support.

This work was supported by grants AI34504 to S.I.M. and AI 35950 to J.A.S. from the National Institutes of Health. J.A.S. is an Established Investigator of the American Heart Association. C.M.A.-A. is supported by a grant from the Rockefeller Foundation.

REFERENCES

- Alpuche-Aranda, C. M., E. L. Racoosin, J. A. Swanson, and S. I. Miller. 1994. *Salmonella* stimulate macrophage macropinosytosis and persist within spacious phagosomes. *J. Exp. Med.* **179**:601–608.
- Alpuche-Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller. 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophages phagosomes. *Proc. Natl. Acad. Sci. USA* **89**:10079–10083.
- Barrow, P. A., M. B. Huggins, and M. A. Lovell. 1994. Host specificity of *Salmonella* infection in chickens and mice is expressed in vivo primarily at the level of the reticuloendothelial system. *Infect. Immun.* **62**:4602–4610.
- Behlau, I., and S. I. Miller. 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J. Bacteriol.* **175**:4475.
- Blaser, M. J., and L. S. Newman. 1982. A review of human salmonellosis. I. Infective dose. *Rev. Infect. Dis.* **4**(Suppl. 6):1096–1106.
- Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):77–89.
- Doberstein, S. K., I. C. Baines, G. Wiegand, E. D. Korn, and T. D. Pollard. 1993. Inhibition of contractile vacuole function *in vivo* by antibodies against myosin-I. *Nature (London)* **365**:841–843.
- Edsall, G., S. Gaines, M. Landy, W. D. Tigertt, H. Sprinz, R. J. Trapani, A. D. Mandel, and A. S. Benenson. 1960. Studies of infection and immunity in experimental typhoid fever. 1. Typhoid fever in chimpanzees orally infected with *Salmonella typhosa*. *J. Exp. Med.* **112**:143–166.
- Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. USA* **83**:5189.
- Francis, C. L., M. N. Starnbach, and S. Falkow. 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol. Microbiol.* **6**:3077–3087.
- Galan, J. E., and R. Curtiss III. 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate cultured cells. *Proc. Natl. Acad. Sci. USA* **86**:6383–6387.
- García-del Portillo, F., and B. B. Finlay. 1994. *Salmonella* invasion of non-phagocytic cells induces formation of macropinosomes in the host cell. *Infect. Immun.* **62**:4641–4645.
- Gulig, P. A., and R. Curtiss III. 1987. Plasmid-associated virulence of *Salmonella typhimurium*. *Infect. Immun.* **55**:2891–2901.
- Heuser, J., Q. Zhu, and M. Clarke. 1993. Proton pumps populate the contractile vacuoles of *Dictyostelium* amoebae. *J. Cell Biol.* **121**:1311–1327.
- Hormaeche, C. E. 1979. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology* **37**:311–318.
- Jenkin, C. R., and B. Benacerraf. 1960. In vitro studies on the interaction between mouse peritoneal macrophages and strains of *Salmonella* and *E. coli*. *J. Exp. Med.* **112**:403–420.
- Jones, B. D., and S. Falkow. 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. *Infect. Immun.* **62**:3745–3752.
- Lee, C. A., B. D. Jones, and S. Falkow. 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. *Proc. Natl. Acad. Sci. USA* **89**:1847–1851.
- Lissner, C. R., R. N. Swanson, and A. D. O'Brien. 1983. Genetic control of the innate resistance of mice to *Salmonella typhimurium*: expression of the *Ity* gene in peritoneal and splenic macrophages isolated in vitro. *J. Immunol.* **131**:3006–3013.
- Mackaness, G. B., R. V. Blanden, and F. M. Collins. 1966. Host-parasite relations in mouse typhoid. *J. Exp. Med.* **124**:573–583.
- McCormick, B. A., S. I. Miller, D. Carnes, and J. L. Madara. 1995. Trans-epithelial signaling to neutrophils by salmonellae: a novel virulence mechanism for gastroenteritis. *Infect. Immun.* **63**:2302–2309.
- McLean, I., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22**:1077–1080.
- Miller, S. I., E. L. Hohmann, and D. A. Pegues. 1995. *Salmonella* (including *Salmonella typhi*), p. 2013–2033. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*. Churchill Livingstone, Inc., New York.
- Miller, S. I., and J. J. Mekalanos. 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. *J. Bacteriol.* **172**:2485–2490.
- Miller, V. L., K. B. Beer, W. P. Loomis, J. A. Olson, and S. I. Miller. 1992. An unusual *pagC::TnpA* mutation leads to an invasion- and virulence-defective phenotype in salmonellae. *Infect. Immun.* **60**:3763–3770.
- Mock, B. A., D. L. Holiday, D. P. Cerretti, S. C. Darnell, A. D. O'Brien, and M. Potter. 1994. Construction of a series of congenic mice with recombinant chromosome 1 regions surrounding the genetic loci for resistance to intracellular parasites (*Ity*, *Lsh*, and *Bcg*), DNA repair responses (*Rep-1*), and the cytoskeletal protein villin (*Vil*). *Infect. Immun.* **62**:325–328.
- Noskin, G. A., and J. T. Clarke. 1990. *Salmonella arizonae* bacteremia as the presenting manifestation of human immunodeficiency virus infection following rattlesnake meat ingestion. *Rev. Infect. Dis.* **12**:514–517.
- O'Brien, A. D. 1982. Innate resistance of mice to *Salmonella typhi* infection. *Infect. Immun.* **38**:948–952.
- O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campell, R. P. MacDermott, and S. B. Formal. 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *J. Immunol.* **124**:2–24.
- O'Brien, A. D., I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1979. Susceptibility of CBA/N mice to infection with *Salmonella typhimurium*: influence of the x-linked gene controlling B lymphocyte function. *J. Immunol.* **123**:720–725.
- O'Brien, A. D., I. Scher, and S. B. Formal. 1979. Effect of silica on the innate resistance of inbred mice to *Salmonella typhimurium* infection. *Infect. Immun.* **25**:513–520.
- Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller. 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol. Microbiol.* **17**:169–181.
- Pomeroy, B. S., and K. V. Nagaraja. 1991. Fowl typhoid, p. 87–99. In B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder (ed.), *Diseases of poultry*. Iowa State University Press, Ames.
- Skamene, E., and C. E. Pietrangeli. 1991. Genetics of the immune response to infectious pathogens. *Curr. Opin. Immunol.* **3**:511–517.
- Snoeyenbos, G. H. 1991. Pullorum disease, p. 73–86. In B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder (ed.), *Diseases of poultry*. Iowa State University Press, Ames.
- Sperber, S. J., and C. J. Schleupner. 1987. Salmonellosis during infection with the human immunodeficiency virus. *Rev. Infect. Dis.* **9**:925–934.
- Swanson, J. A., A. B. Bushnell, and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA* **84**:1921.
- Vidal, S. M., D. Malo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for *Bcg*. *Cell* **73**:469–485.
- Watson, J., K. Kelly, M. Largen, and B. A. Taylor. 1978. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. *J. Immunol.* **120**:422–424.
- Weiss, S. H., M. J. Blaser, F. P. Paleologo, R. E. Black, A. C. McWhorther, M. A. Asbury, G. P. Carter, R. A. Feldman, and D. J. Brenner. 1986. Occurrence and distribution of serotypes of the *arizona* subgroup of *Salmonella* strains in the United States from 1967 to 1976. *J. Clin. Microbiol.* **23**:1056–1064.
- Zhu, Q., and M. Clarke. 1992. Association of calmodulin and an unconventional myosin with the contractile vacuole complex of *Dictyostelium discoideum*. *J. Cell Biol.* **118**:347–358.