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Flagella Help *Salmonella typhimurium* Survive Within Murine Macrophages

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In this study, we evaluated how flagella enhance the pathogenicity of *Salmonella typhimurium* in strain C57BL/6J mice. When mice were infected orally with flagellated or nonflagellated *S. typhimurium*, equivalent numbers of bacteria colonized the gastrointestinal tracts of the animals, but the number of flagellated organisms increased faster once colonization began in the spleens and livers. To evaluate this differential rate of *Salmonella* growth, the rate of blood clearance, and the kinetics of net multiplication of salmonellae in splenic tissue after intravenous challenge, the two groups of mice were compared. We found that clearance of bacteria from the blood was the same for flagellated or nonflagellated strains. However, the number of flagellated bacteria in the spleen increased logarithmically until the death of the animals, whereas the number of nonflagellated salmonellae increased only slightly. In contrast, both flagellated and nonflagellated strains grew exponentially in the spleens of mice pretreated with silica, a macrophage toxic agent. In an in vitro macrophage assay, flagellated salmonellae survived longer than nonflagellated organisms. These results indicate that flagella either protect *S. typhimurium* from the intracellular killing mechanisms of murine macrophages or that flagella enhance the ability of *S. typhimurium* to multiply within murine macrophages.

The pathogenesis of the disease that occurs in mice infected with *Salmonella typhimurium* mimics typhoid fever in humans. When mice are challenged orally with *S. typhimurium*, the bacteria penetrate the gastrointestinal mucosa, invade the lamina propria, and enter the circulation. As a consequence of the primary bacteremia, the salmonellae are trapped within the liver and spleen. Inbred mice homozygous for the murine chromosome 1 allele *Ity*^{s} are unable to control the net replication of *S. typhimurium* within the reticuloendothelial cell system (RES) organs, and, after a secondary bacteremia, the animals die. Less than 10 bacteria of a virulent *S. typhimurium* strain are sufficient to kill an *Ity*^{s} mouse after intravenous (i.v.), subcutaneous, or intraperitoneal challenge. By contrast, *Ity*^{r} mice are usually able to contain the net growth of *S.typhimurium* in splenic and hepatic tissues and can survive parenteral challenge with up to 10^4 virulent bacteria. The effector cell for *Ity* expression is the macrophage (8, 9); *Ity*^{s} macrophages do not kill dividing salmonellae as efficiently as do *Ity*^{r} macrophages. The initial response of mice to infection with *Leishmania donovani* (2), *Mycobacterium bovis* BCG (16), and perhaps *Mycobacterium lepraemurium* (3) as well are also regulated by the same or a closely linked chromosome 1 gene.

As reported elsewhere (4), *Ity*^{s} mice of the C57BL/6J strain were infected orally, intraperitoneally, or i.v. with several different isogenic pairs of *S. typhimurium*. Each member of the pair differed only in the *fla* or *mot* genes. The results of the studies demonstrated that flagella are required for the full expression of the virulence of *S. typhimurium* after oral or parenteral infection. The purpose of this investigation was to use the previously developed isogenic pairs of *S. typhimurium* to learn how flagella contribute to the pathogenicity of *S. typhimurium* in *Ity*^{s} mice.

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MATERIALS AND METHODS

Bacterial nomenclature. Two or more bacterial strains differing only in the state of a single gene are designated an isogenic pair or isogenic partners. The phenotype with the appropriate superscript of each strain appears in parentheses below when this will aid in comprehension. The relevant phenotype designations in this study are Fla (flagella) and Mot (motility). The phenotype Mot$^-$ indicates nonmotile although flagellated, whereas Mot$^+$ indicates motile and flagellated.

Bacteria. The strains of *S. typhimurium* used in this study are listed in Table 1. Motility of all strains was tested throughout the various in vivo and in vitro assays in the motility agar described elsewhere (4). Motility tests were performed on colonies of bacteria grown from challenge inocula, spleens of moribund mice, and macrophage lysates.

Mice. C57BL/6J mice were supplied by Jackson Laboratory, Bar Harbor, Maine. The animals were kept on a cycle of 12 h of light and 12 h of darkness, with free access to food and water. Both male and female mice were used and were 6 to 8 weeks of age at the time of experimentation.

Kinetics of *S. typhimurium* growth in vivo. (i) Oral infection challenge. C57BL/6J mice that were deprived of water overnight were fed (by means of a blunt-tipped plastic microdiluter) 25 μl (1×10^8 to 2×10^8 CFU) of an 18-h Penassay broth (Difco Laboratories, Detroit, Mich.) culture of *S. typhimurium* strain St36 or St38. Care was taken not to scrape the mucosa of the mouths of the animals during the feeding process. At different intervals postinfection, four mice from each inoculum group were sacrificed by cervical dislocation, and ca. 200 μl of heart blood was rapidly drawn and then added to a tube of Penassay broth. The entire bowel (stomach to anus) and spleen of each mouse were aseptically removed and homogenized in a mortar with sterile sea sand (Sigma Chemical Co., St. Louis, Mo.). The bowels were rinsed with 10 ml of sterile saline (0.85% sodium chloride)

TABLE 1. *S. typhimurium* strains

Strain[a]	Description; phenotypic designation
St36	Nonflagellated isogenic partner[b] of St38; Fla$^-$
St38	Flagellated isogenic partner of St36; Fla$^+$
St49	Flagellated transformant of St36; Fla$^+$
St61	Flagellated,motile isogenic partner of St62; Mot$^+$
St62	Flagellated, nonmotile isogenic partner of St61; Mot$^-$
St63	Flagellated, motile isogenic partner of St64; Mot$^+$
St64	Flagellated, nonmotile isogenic partner of St63; Mot$^-$

[a] All strains were constructed as described elsewhere (4).
[b] Isogenic partner, One of a pair of strains which differ only in the state of a single gene.

before homogenization. Serial dilutions of each homogenate were made in sterile saline. The number of viable organisms per homogenate was determined by plating dilutions on tryptic soy agar (TSA; Difco); the TSA used for the whole-bowel homogenates contained 50 μg of tetracycline per ml, which in preliminary experiments was shown to completely prevent growth of antibiotic-sensitive, normal flora bacteria. Slide agglutination tests with rabbit anti-*S. typhimurium* serum were used to verify that TSA-tetracycline colonies were in fact *S. typhimurium*. The blood culture broths that were turbid at 24 h were subcultured on TSA-tetracycline to confirm that the cultures were positive for *S. typhimurium*.

(ii) Parenteral infection. C57BL/6J or CD-1 mice were injected i.v. with 0.5 ml of bacteria diluted in saline. For blood clearance studies, mice were numbered by ear punch and aseptically bled from the retroorbital plexus with a sterile 50-μl capillary pipette at 5, 15, and 60 min postinfection. For assessment of the kinetics of *S. typhimurium* growth in RES tissue, four or five mice were killed by cervical dislocation, and the spleens or livers were ground in a mortar with a pestle as previously described (11). Dilutions of blood or tissue samples were plated on TSA plates for colony enumeration.

Treatment of mice with silica. Mice were injected i.v. with 0.15 mg of silica per g of mouse weight, contained in RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) with 10% fetal bovine serum (FBS; GIBCO Diagnostics, Madison, Wis.). The silica, obtained from Whittacker, Clarke and Davis (Plainsfield, N.J.), was cleaned and prepared as previously described (1). Control mice received 0.2 ml i.v. of RPMI 1640 with 10% FBS. Silica-treated and control mice were

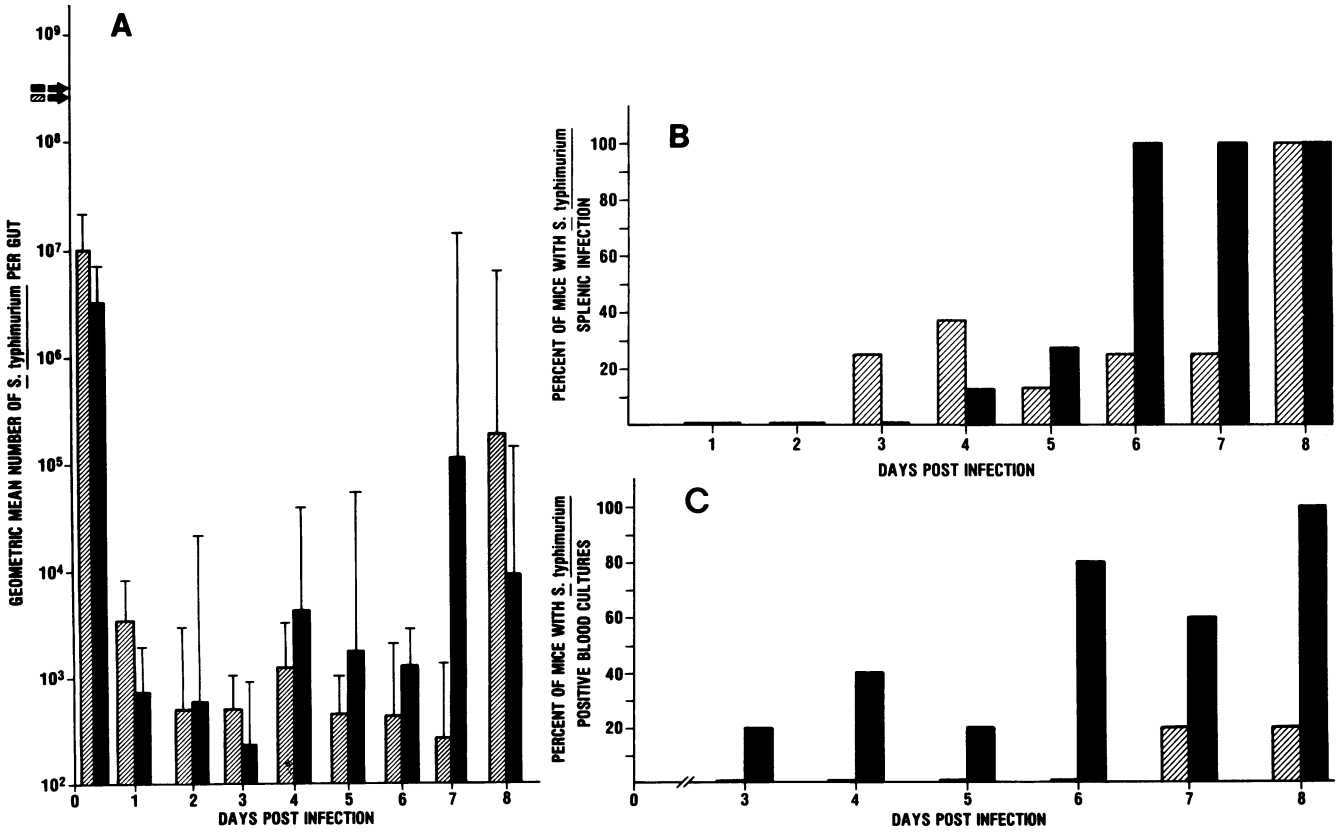

FIG. 1. Kinetics of *S. typhimurium* strains St36 (Fla$^-$ (▨)) and St38 (Fla$^+$) (■) after oral inoculation of 3×10^8 bacteria per mouse. Four mice inoculated with each strain were sampled at each time point. (A) Geometric means +2 standard errors of the mean (SEM) of viable counts in the guts of mice infected with each bacterial strain. Before homogenization of the intestine, four to seven Peyer's patches per gut were pooled and tested for viable salmonellae. On days 1 through 4, the average number of St36 or St38 per Peyer's patch pool was $\leq 10^2$. On days 5 through 8, one or two of four Peyer's patch pools from mice infected with either St36 or St38 contained between 2×10^2 and 3×10^4 organisms. (B) Percentages of spleen cultures positive for *S. typhimurium* from mice inoculated with each bacterial strain. (C) Percentages of blood cultures positive for *S. typhimurium* from mice inoculated with either bacterial strain.

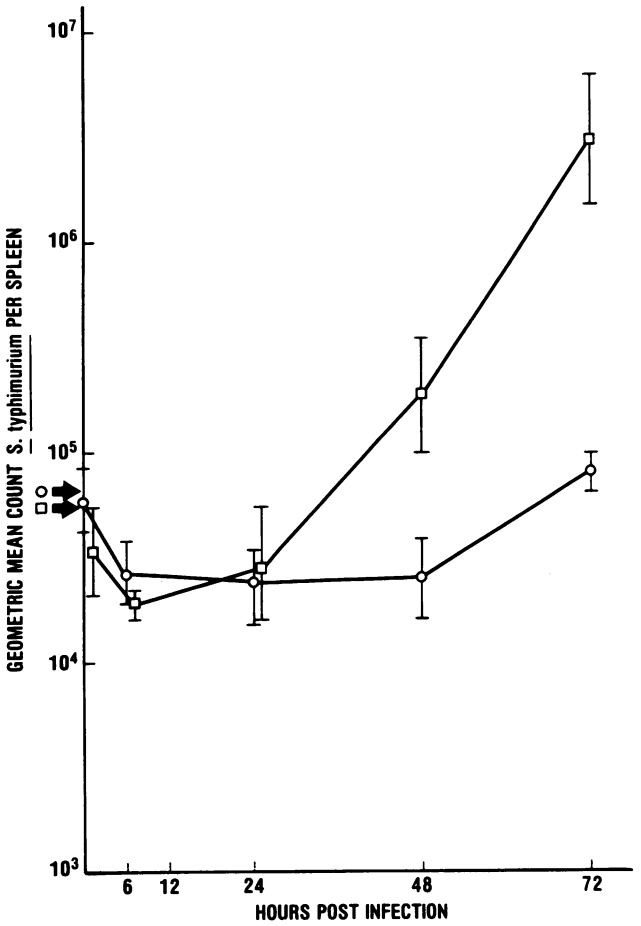

FIG. 2. Viable counts in the spleens of C57BL/6J mice after intravenous inoculation with 6.5×10^4 *S. typhimurium* strain St36 (Fla^-) (○) or strain St38 (Fla^+) (□) per mouse. Each point represents the geometric mean count of four spleens ±2 SEM.

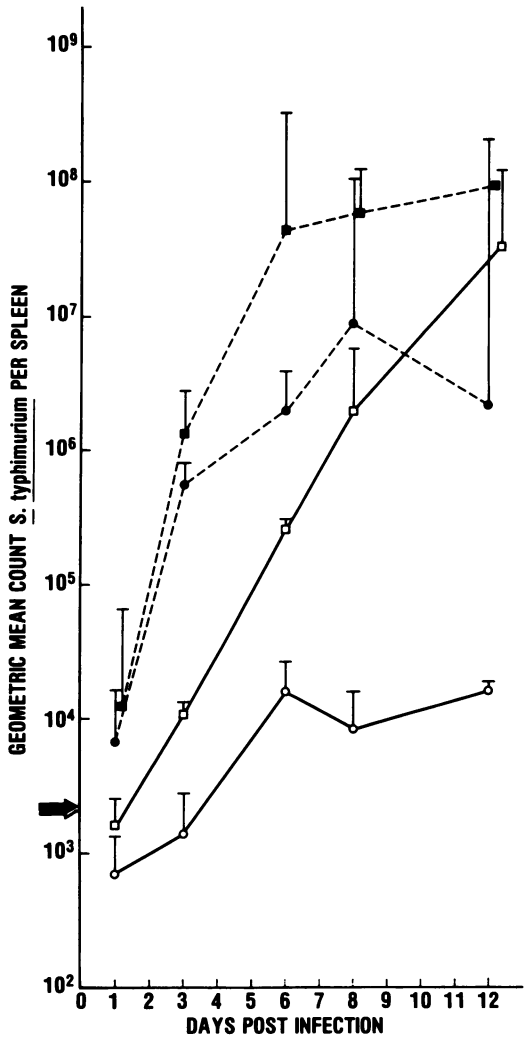

FIG. 3. Effect of silica (0.15 mg/g of mouse weight) on viable spleen counts from C57BL/6J mice after i.v. inoculation of *S. typhimurium* strain St36 (Fla^-) (●) or strain St38 (Fla^+) (■). Control mice were pretreated with RPMI and then challenged with strain St36 (○) or strain St38 (□). The geometric means of viable counts +2 SEM are given.

challenged i.v. 48 h later with *S. typhimurium*. Animals were sacrificed at days 1, 3, 6, and 8 postinfection, and the spleens were removed and aseptically homogenized as detailed above.

In vitro assay for *S. typhimurium* infection. The in vitro assay was a modification of that described by Lissner et al. (8) for use with splenic or peritoneal macrophages. Because we find that splenic macrophages are difficult to obtain and more readily killed by manipulation, resident peritoneal macrophages were selected as prototype macrophages for these studies. Briefly, the resident peritoneal cells of C57BL/6J mice were harvested and seeded into 24 well plates (Costar Data Packaging Corp., Cambridge, Mass.). To obtain macrophage monolayers, 200 μl of the cell suspensions that contained ca. 2×10^6 cells diluted in RPMI with 10% FBS and 100 μg of gentamicin per ml (harvest medium) was dispensed as a droplet into the centers of the wells. The plates were left in a laminar flow hood for 90 min, and the cells were then overlaid with 500 μl of harvest medium. After overnight incubation in RPMI with 10% FBS, the macrophages were infected (at the predetermined optimum ratio of 20 bacteria per adherent cell) with 500 μl of bacteria that had been opsonized by incubation for 45 min in RPMI 1640 with 10% fresh, normal C57BL/6J serum. After a 50-min infection period, all wells were washed three times with Dulbecco phosphate-buffered saline (pH 7.4). Cells were lysed with 0.5% sodium desoxycholate, and dilutions were plated on TSA. Adherent cells were released from the plastic by lidocaine treatment and counted in a hemacytometer for use in determining the numbers of bacteria per macrophage. The remaining wells were overlaid with maintenance medium (RPMI with 5% FBS and 5 μg of gentamicin per ml). The MIC for the bacterial strains was 2.5 μg of gentamicin per ml in RPMI with 5% FBS. Cell lysates were prepared at 1, 4, and 24 h postinfection.

Radiolabeling of bacteria. *S. typhimurium* strains St36 and St38 were radiolabeled by incorporation of [^{3}H]leucine. For these radiolabeling experiments, overnight cultures of St36 and St38 grown in minimal M-9 medium (10) supplemented with 40 μg/ml each of histidine, leucine, and cysteine were diluted 10-fold in fresh supplemented M-9 and allowed to enter log phase, as determined spectrophotometrically. The log-phase salmonellae were diluted in M-9 medium supplemented with histidine and cysteine to a concentration of ca. 10^7 bacteria per ml and 100 μCi of [^{3}H]leucine (Amersham Corp., Arlington Heights, Ill.) was added for 1 h at 37°C with

shaking. Salmonellae were washed three times by centrifugation at 1,000 × *g* to remove free ^3H. The in vitro macrophage assay was performed with bacteria radiolabeled on the day of experimentation. Samples from the fluids above the macrophage monolayers and samples from the macrophage lysates were suspended in Ready-Solv liquid scintillant (Beckman Instruments, Inc., Fullerton, Calif.), and radioactivity was determined in a model LS7500 liquid scintillation counter (Beckman).

RESULTS

Kinetics of *S. typhimurium* multiplication and survival within bowels, spleens, and blood of orally infected mice. To compare the in vivo multiplication of flagellated and nonflagellated salmonellae, C57BL/6J mice were orally infected with 3×10^8 *S. typhimurium* strain St36 (Fla^-) or St38(Fla^+), and the numbers of viable bacteria per whole bowel or the percentage of animals with salmonellae in their spleen and blood was determined at days 0 through 8 postinfection. There were no statistically significant differences in the numbers of flagellated or nonflagellated organisms found in the gut over the 8-day course of infection (Fig. 1A). Moreover, both the flagellated and nonflagellated strains eventually spread to the RES as indicated by spleen cultures positive for *S. typhimurium* (Fig. 1B) in 100% of infected animals by day 6 (St38) or day 8 (St36) of challenge. However, only the flagellated strain, St38, was detected in the blood of animals during the first 6 days after infection (Fig. 1C), and only a minority of St36-fed mice displayed positive blood cultures even by day 8 postinoculation. Once the bacteria reached the spleens of the animals, the mice infected with the flagellated strain eventually died (5/5 deaths), whereas the animals infected with the nonflagellated salmonellae survived (0/5 deaths).

Kinetics of net *S. typhimurium* multiplication in spleens of i.v.-infected mice. Differential net growth of flagellated and nonflagellated *S. typhimurium* in the murine RES was noted in one oral infection study in which the geometric mean number of *S. typhimurium* per spleen was monitored (data not shown). To confirm this observation, the kinetics of net *S. typhimurium* St36 and 38 multiplication were followed over a short interval. For studies of the first 72 h after infection, C57BL/6J mice were injected i.v. with 6×10^4 to 7×10^4 flagellated or nonflagellated CFU diluted in saline, and their spleens were aseptically homogenized at 2, 5, 24, 48, and 72 h after inoculation. There was no difference in the number of spleen-associated bacteria until 48 h postinfection (Fig. 2). At that time, the number of viable St38 (Fla^+) organisms was sevenfold higher than at 24 h, whereas the number of viable St36 (Fla^-) organisms remained the same from 24 to 48 h postinfection. At 72 h after challenge, the number of viable salmonellae in spleens of St38-infected animals increased 16-fold over the 48-h count, whereas there was only a threefold increase in the number of viable St36 organisms during the same time period. The difference between the viable counts of St36 and St38 at 48 and 72 h postinfection was statistically significant ($P < 0.05$) as calculated by the Student's unpaired *t* test.

To ascertain whether St36 was potentially as virulent as St38, we treated C57BL/6J mice with silica, a macrophage toxic agent (14), before infection. The silica treatment, when

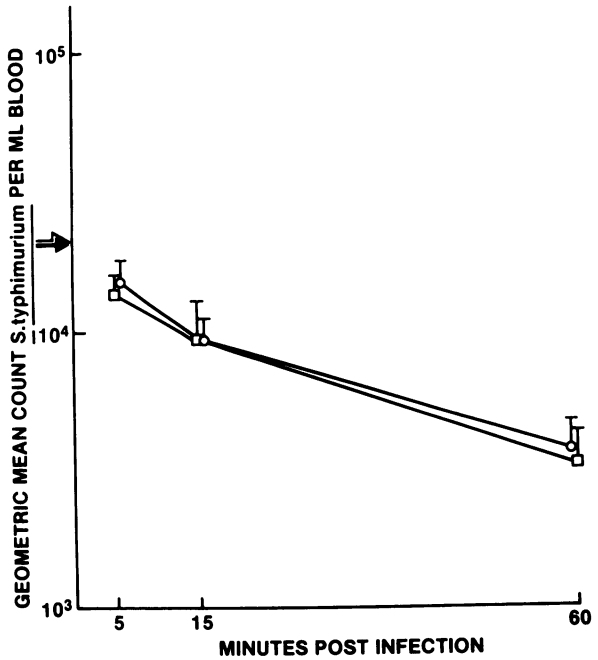

FIG. 4. Viable counts in the blood of C57BL/6J mice after i.v. inoculation with 2×10^4 *S. typhimurium* strain St63 (Mot^+) (□) or St64 (Mot^-) (○). The geometric means of viable counts +2 SEM are given.

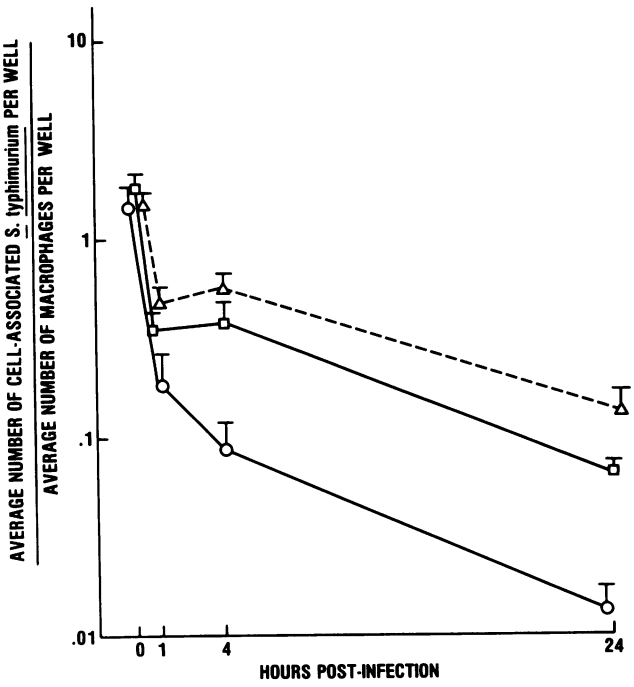

FIG. 5. Resident peritoneal macrophages from C57BL/6J mice were infected in vitro with *S. typhimurium* strain St36 (Fla^-) (○), St38 (Fla^+) (□), or St49 (Fla^+) (△). Each point represents the average ratio +2 SEM of cell-associated bacteria per macrophage. The bacterial counts were averaged from four separate culture dish wells per time point, and the macrophage numbers were averaged from two infected culture dish wells per time point. The actual mean number of bacteria per well (and mean number of macrophages per well) for each bacterial strain at time zero and 1, 4, and 24 h after infection, respectively, were as follows: St36, 1.5×10^5 (1×10^5), 1.4×10^4 (6.6×10^4), 5.5×10^3 (6.25×10^4), 5.8×10^2 (4.3×10^4); St38, 1.2×10^5 (6.3×10^4), 1.9×10^4 (5.4×10^4), 1.7×10^4 (4.4×10^4), 2.4×10^3 (3.4×10^4); St49, 1.3×10^5 (9.1×10^4), 2.7×10^4 (5.8×10^4), 2.3×10^4 (4.1×10^4), 3.5×10^3 (3.8×10^4).

compared with the diluent treatment, significantly altered the growth kinetics of both bacterial strains in the C57BL/6J spleens (Fig. 3). *S. typhimurium* counts from animals pretreated with diluent and then given St36 increased slowly over the first 6 days postinfection and then reached a plateau. By contrast, the geometric mean number of *S. typhimurium* strain St36 from spleens of mice pretreated with silica increased logarithmically with time, and the difference was not, in fact, statistically significant, at any time point, from the geometric mean number of *S. typhimurium* strain St38 per spleen of mice pretreated with silica. St38 also grew exponentially in the spleens of diluent-treated mice, but the net growth of St38 in murine spleens was even more rapid when animals were pretreated with silica (Fig. 3). At 6 weeks postinfection, only mice given diluent and challenged with St36 were still alive. By contrast, mice in the other three treatment groups (silica and St36, silica and St38, and diluent and St38) were dead by day 13. Thus, silica treatment of the host compromised its natural defenses to flagellated and nonflagellated *Salmonella* infection.

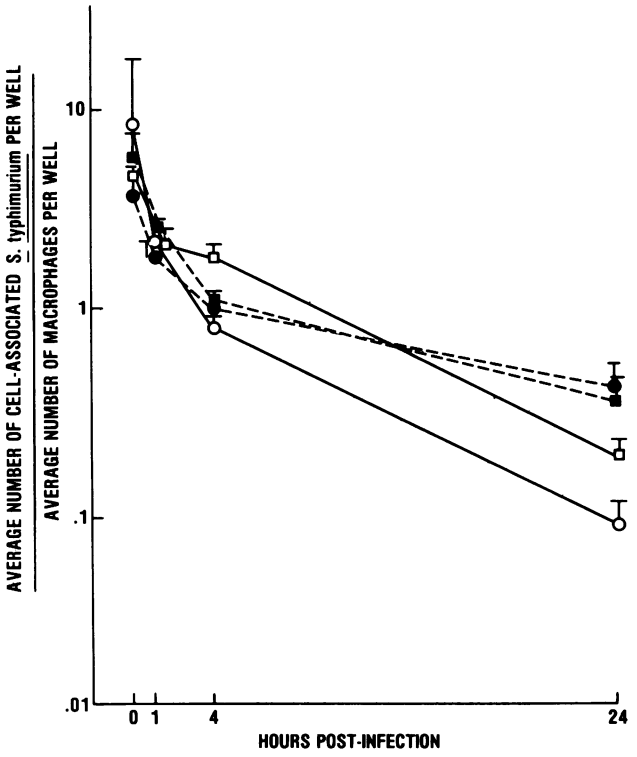

FIG. 6. Resident peritoneal macrophages from C57BL/6J mice were infected in vitro with *S. typhimurium* strains St36 (Fla^-) (○), St38 ((Fla^+) (□), St61 (Mot^+) (■), or St62 (Mot^-) (●). Each point represents the average ratio +2 SEM of cell-associated bacteria per macrophage. The bacterial counts were averaged from four separate culture dish wells per time point, and the macrophage numbers were averaged from two infected culture dish wells per time point. The actual mean number of bacteria per well (and mean number of macrophages per well) for each bacterial strain at time zero and at 1, 4, 24 h after infection, respectively, were as follows: St36, 7.2×10^5 (8.3×10^4), 1.1×10^5 (5.2×10^4), 2.8×10^4 (3.3×10^4), 2.3×10^3 (2.6×10^4); St38, 4.2×10^5 (9.2×10^4), 1.1×10^5 (5.1×10^4), 5.0×10^4 (2.7×10^4), 5.6×10^3 (3.0×10^4); St61, 3.8×10^5 (6.5×10^4), 9.2×10^4 (4×10^4), 3.7×10^4 (3.5×10^4), 8.3×10^3 (2.1×10^4); St62, 3.4×10^5 (8.8×10^4), 8.5×10^4 (4.3×10^4), 4.2×10^4 (2.5×10^4), 1.2×10^4 (3.2×10^4).

Clearance of *S. typhimurium* from blood of i.v. infected mice. The effect of flagella on uptake of *S. typhimurium* by the RES of i.v.-infected mice was assessed. Both bacterial strains were cleared from the blood at similar rates. By 60 min, 89% of the nonflagellated St36 organisms had been cleared from the blood, and 85% of the flagellated St38 organisms had been cleared from the blood of infected animals. There were no statistically significant differences in the numbers of bacteria per ml of blood between the two strains of bacteria at 5 and 60 min postchallenge (data not shown).

The blood clearance of strains St63 (Mot^+) and St64 (Mot^-) was also assessed by using the same method. Again, there were no statistically significant differences between the two strains in the number of salmonellae per milliliter of blood at 5, 15, and 60 min postchallenge (Fig. 4).

In vitro activity of flagellated and nonflagellated salmonellae in macrophages. The differential fate of isogenic Fla^+ and Fla^-, as well as of isogenic Mot^+ and Mot^-, strains in C57BL/6J macrophages was assessed by an in vitro assay (8). Strains St36 (Fla^-), St38 (Fla^+), St49 (Fla^+), and the isogenic pair St61 (Mot^+) and St62 (Mot^-) were used to infect C57BL/6J peritoneal macrophages. The average number of cell-associated salmonellae at 0, 1, 4, and 24 h postinfection was determined. Extracellular fluids were also sampled for viable bacteria at each time point and found to contain <10% of the total cell-associated bacterial counts. Moreover, light-microscopic examination of fluids subjected to centrifugation in a cytospin and then stained with the Giemsa reagent indicated that the viable bacteria were in fact contained within macrophages that had lifted off the plastic. The difference in the ratios of the average number of viable flagellated and nonflagellated salmonellae per macrophage at time zero was not statistically significant ($P > 0.05$) (Fig. 5 and 6). At 24 h postinfection, the numbers of cell-associated, flagellated salmonellae were higher, to a statistically significant degree ($P < 0.05$), than the numbers of cell-associated nonflagellated bacteria. These findings suggest that nonflagellated salmonellae are killed faster in vitro than are flagellated salmonellae.

Uptake of *S. typhimurium* by macrophages in vitro. To compare the relative in vitro uptake of flagellated and nonflagellated *S. typhimurium* by C57BL/6J macrophages, we performed a radiolabel study. Strains St36 and St38 were radiolabeled by [^{3}H]leucine incorporation, suspended in 10% normal C57BL/6J mouse serum, and used to infect resident peritoneal macrophages in vitro. A ratio of 50 bacteria per macrophage was used to ensure accurate counting of radioactivity. Lysates were prepared at time zero, and the cell-associated radioactivity was measured. There was no statistically significant difference ($P > 0.05$) in cell-associated or protein-associated radioactivity between the two flagellated strains (Table 2). This finding is consistent with the in vivo blood clearance results which indicated that the rates of uptake of flagellated and nonflagellated *S. typhimurium* from the blood of the animals were the same.

DISCUSSION

In these studies, the contribution of flagella and motility to the virulence of *S. typhimurium* in the murine typhoid model was investigated both in vivo and in vitro. After oral infection of mice with *S. typhimurium*, the numbers of flagellated and nonflagellated salmonellae that colonized the guts (Fig. 1A) or Peyer's patches (see the legend to Fig. 1) of mice were not significantly different. Moreover, intestinal

TABLE 2. Uptake of radiolabeled *S. typhimurium* by mouse macrophages in vitro[a]

Strain	MOI[b]	Uptake[c] (mean cpm ±2 SEM) per:	
		Macrophage	μg of lysate protein[c]
St36	50:1	0.010 ± 0.002	87.1 ± 12.2
St38	50:1	0.010 ± 0.001	99.3 ± 11.7

[a] Resident peritoneal macrophages from C57BL/6J mice were exposed to *S. typhimurium* strain St36 (Fla$^-$) or strain St38 (Fla$^+$) which had been radiolabeled by [^{3}H]leucine incorporation.
[b] MOI, Multiplicity of infection.
[c] Differences in uptake by the two strains were not statistically significant by either method of quantitation.
[d] As determined by Lowry protein assay.

colonization by both bacterial strains was transitory (Fig. 1A), a phenomenon also observed after oral feeding of *Salmonella typhi* to volunteers (7). Our findings support the observations of Tannock et al. (17) who showed that salmonellae do not have to express flagellar or H antigens to colonize the intestinal mucosa; they suggested instead that bacterial survival within the mucosa may depend on the presence of smooth lipopolysaccharide. Hohmann et al. (6) found that *S. typhimurium* immobilization with anti-H antiserum did not reduce intestinal colonization or affect the ability of the bacteria to become systemic. Therefore, flagella are not required for salmonellae to establish intestinal infection or disseminate after oral inoculation.

Both the flagellated and nonflagellated strains were eventually found in the spleens (Fig. 1B) of orally infected animals. Nonetheless, only the flagellated salmonellae were ever consistently detected in primary (arbitrarily defined as between days 0 and 4 of inoculation) blood cultures (Fig. 1C). This observation may reflect the volume of heart blood obtained for blood cultures (200 μl or ca. 10% of total blood volume). Such a sample size may have been too small to reproducibly contain bacteria if only a small number of nonflagellated salmonellae actually became systemic at any one time. Once the nonflagellated bacteria reached the RES, they apparently underwent several divisions (as assessed by kinetics of viable salmonellae counts in spleens of orally inoculated mice) and were readily detectable in the spleen. These nonflagellated organisms were eventually cleared by the innate or acquired immune mechanisms of the host or by both mechanisms, without evidence of morbidity or mortality. By contrast, once the flagellated organisms reached the RES, net growth was exponential, secondary bacteremia was readily detectable by positive blood cultures, and the mice eventually succumbed to murine typhoid. The patterns of pathogenesis for flagellated and nonflagellated *S. typhimurium* support the hypothesis of Hohmann that for an *S. typhimurium* strain to produce pathological murine typhoid in a mouse after oral challenge, the bacteria must not only be able to survive and divide within intestinal lymphoid tissues but also to resist host immune defenses. We concluded from the findings in this oral infection system that flagellated salmonellae were more virulent than nonflagellated salmonellae because of differential net bacterial growth once the organisms reached the RES and not because of differences in degree of intestinal colonization.

To verify this division rate hypothesis, we infected mice i.v. to specifically monitor net multiplication of salmonellae in splenic tissue. Blood clearance experiments indicated that both flagellated and nonflagellated salmonellae were cleared, i.e., taken up by the RES, at the same rates. However, the flagellated bacteria, both motile and nonmotile, were found to grow exponentially in the spleens and livers, whereas the numbers of the nonflagellated strain only increased slightly in the RES. Administration of silica followed by injection of the flagellated and nonflagellated strains of salmonellae showed that the nonflagellated strain could be rendered virulent and hence did not have multiple defects.

In vitro studies with isolated macrophages were then performed to investigate possible mechanisms involved in the differential survival and growth of nonflagellated and flagellated salmonellae in RES tissues. The equally efficient phagocytosis of radiolabeled flagellated and nonflagellated salmonellae confirmed the blood clearance studies. Tomita and Kanegasaki (19) demonstrated in vitro that a Mot$^-$ strain of *S. typhimurium* was not phagocytized as well as a Mot$^+$ strain unless it was centrifuged onto macrophages. In the in vitro assay system we used to study the uptake of radiolabeled salmonellae, the volume of medium which overlaid the macrophages was minimal and may have mimicked the close association between bacteria and macrophage created by centrifugation in the study by Tomita and Kanegasaki.

The results of the in vitro macrophage experiments also indicated that flagellated *S. typhimurium* can survive longer in *Ity^s* macrophages than can nonflagellated salmonellae (Fig. 5). These in vitro data correlate with the in vivo findings, although, unlike the in vivo situation, a net increase in the number of cell-associated bacteria was not observed in vitro. One explanation for the absence of a net increase in numbers of St38 during the limited infection period is that the in vitro assay (8) was designed for a 24-h infection period with the highly mouse-virulent *S. typhimurium* strain TML. The TML strain appears more virulent than St38, as assessed by its more rapid net growth in spleens of *Ity^s* mice (unpublished data).

Several mechanisms may be proposed to explain how flagellated salmonellae survive longer in macrophages than do nonflagellated salmonellae. (i) Flagellated salmonellae may not trigger the full cascade of early microbicidal events associated with the respiratory burst (18). Chemiluminescence studies could be used to detect any differences in initial responses of macrophages to flagellated versus nonflagellated salmonellae. (ii) Flagellated and nonflagellated salmonellae may be sequestered in different compartments of the macrophage, and flagellated bacteria may consequently be shielded from the microbicidal actions of the macrophages better than are nonflagellated bacteria. Electron-microscopic studies could be used to address these issues. (iii) Flagellated and nonflagellated salmonellae may trigger the respiratory burst equivalently and may be localized in the same sites in the macrophages, but the presence of flagella may act as a cloak to partially protect salmonellae from the microbicidal enzymes, pH, and other defenses of the macrophage. In vitro kinetic studies with macrophage phagolysosomes and flagellated and nonflagellated salmonellae could be used to investigate this possibility.

More information on the contribution of flagella to the virulence of *S. typhimurium* for mice can also be obtained by testing the fate of the various isogenic strains used in this study in *Salmonella*-susceptible strains of mice other than *Ity^s*. Such strains include C3H/HeJ (15, 20), F1 male (CBA/N × DBA/2) (13), C57L/J and DBA/2J (A. D. O'Brien, B. A. Taylor, and D. L. Rosenstreich, J. Immunol., in press), and C3Heb/FeJ (5, 12) mice. The genetic defects in these strains of mice affect different host cells and host resistance mechanisms than does *Ity^s*. Therefore, such experiments should reveal whether flagella are required for *Salmonella* survival during other phases of murine typhoid and whether anti-

flagellar antibody is important in expression of acquired resistance to murine typhoid.

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