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Enhanced Phagocytic Response of Macrophages to Bacteria by Physical Impact Caused by Bacterial Motility or Centrifugation

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The mechanism of enhanced phagocytic and chemiluminescent responses of macrophages caused by bacterial motility (T. Tomita, E. Blumenstock, and S. Kanegasaki, Infect. Immun. 32:1242, 1981) was studied. Both responses increased up to a certain level with an increased number of motile bacteria, such as *Salmonella typhimurium*, *Escherichia coli*, or *Pseudomonas aeruginosa*, added. In contrast, only a slight increase was observed with the motility (*mot*) mutants of these bacteria, even when 4,000 bacteria per single macrophage were added. If nonmotile bacteria were centrifuged together with a monolayer culture of macrophages, the number of bacteria ingested per macrophage increased dramatically. This phenomenon was not observed in the presence of cytochalasin B or at a low temperature, and about half of the associated bacteria were killed within 30 min of prolonged incubation, indicating that the bacteria were not simply embedded on the macrophage surface. An observed biphasic increase of ingestion with an increase in centrifugal force suggested the existence of a threshold velocity for efficient phagocytosis. The minimum centrifugal force required for maximal response was determined under the conditions in which equalized collision frequency between bacteria and macrophages was maintained when different centrifugal forces were employed. From the value obtained ($5 \times g$), the required rate of movement was calculated as approximately 2.5 μm/s, supposing that the bacterium is spherical and has a 1-μm radius. This value is much lower than the velocity of movement of motile bacteria (20 to 50 μm/s). The results indicate that physical impact caused by bacterial motility is enough to induce a high response of macrophages.

By using several motility (*mot*) mutants of *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa* which lack motility yet possess flagella, we showed in a previous paper (14) that mouse peritoneal macrophages emit chemiluminescence much more efficiently when they are exposed to motile bacteria than when they are exposed to nonmotile bacteria. Chemiluminescence emission is believed to be an indication of the generation of active oxygen species and one of the earliest responses of phagocytes upon exposure to stimuli. We further showed that phagocytosis of motility mutants was much lower than that of their parent or revertant strains. These differences were observed whether or not the bacteria were opsonized and regardless of the source and manner of induction of macrophages. The phenomenon was also observed with polymorphonuclear cells (unpublished observation).

Among several explanations of this phenomenon (14), two may be important: a different frequency of collision and a difference in physical impact to phagocytes between motile and nonmotile bacteria. The results of our preliminary experiments suggested that higher collision alone was not enough to explain the phenomenon, since a similar level of chemiluminescent response obtained with several motile bacteria per macrophage was never evoked with thousands of nonmotile bacteria per macrophage. We therefore tested the other possibility by giving physical force to bacteria to collide with a monolayer of macrophages.

In this paper, we show that the phagocytic response of macrophages to nonmotile bacteria is markedly enhanced by centrifugal force and that there exists a threshold impact force for efficient phagocytosis.

MATERIALS AND METHODS

Bacteria. The bacterial strains used are listed in Table 1. Motility mutants and their parent and revertant strains of *S. typhimurium*, *E. coli* and *P. aerugino-*

TABLE 1. Bacterial strains

Strains	Characteristics
S. typhimurium	
LT2.....	Wild type
SJ442	*motA* mutant of LT2
TT2.....	Mot$^+$ revertant of SJ442
E. coli	
W3110.....	F$^-$ derivative of K-12
TH282.....	*mot5008*::Tn*5* derivative of W3110
TT5.....	Mot$^+$ revertant of TH282
P. aeruginosa	
PAO2003 ...	*argH32 str39 rec2* FP$^-$
MT519	*mot* mutant of PAO2003

sa have been described previously (14). Bacteria were grown in L-broth without glucose (9).

Opsonization of bacteria was performed with homologous normal serum obtained from C3H/He mice (this institute) (14). Under the conditions employed, no agglutination of bacteria was observed.

Macrophages. Resident peritoneal cells were obtained from 8- to 10-week-old female C3H/He mice (this institute) by washing the peritoneal cavity with Eagle minimum essential medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4) (HEPES-MEM). The peritoneal cells (3×10^6) were incubated at 37°C for 2 h in a sterilized plastic liquid scintillation vial or in a plastic dish (35-mm diameter; Corning Glass Works, Corning, N.Y.). After removal of nonadherent cells, adherent cells were kept at 37°C for several hours before use.

Thioglycolate-induced macrophages were obtained from C3H/He mice injected intraperitoneally with 2 ml of thioglycolate medium 4 days before harvest.

Measurement of chemiluminescence. Luminol-dependent chemiluminescence was assayed at 37°C in a temperature-stabilized liquid scintillation spectrometer (Beckman LS 233) in the out-of-coincident mode, as described previously (14).

Phagocytosis test of monolayer macrophages. The phagocytosis test of monolayer macrophages was done as described previously (14). Monolayers of macrophages in a plastic dish were incubated with various numbers of bacteria. After incubation at 37°C for various periods, the monolayers of cells were vigorously washed twice with 10 ml of phosphate-buffered saline containing cytochalasin B (1 μg/ml). The monolayers were dried, fixed with methanol, and stained with Giemsa stain. The percentage of macrophages ingesting bacteria and the mean number of ingested bacteria per cell were determined under a microscope by counting the number of bacteria per cell for at least 200 macrophages. The ingested bacteria were observed in a limited area of macrophage.

Phagocytosis test of suspension culture of macrophages. Thioglycolate-induced macrophages (5×10^6 cells) were mixed with either opsonized or nonopsonized bacteria (1.5×10^8 or 5×10^8 colony-forming units, respectively) in 1 ml of suspension in a siliconized test tube. The mixture was incubated at 37°C for 20 min under continuous agitation provided by shaking the test tube in an oscillating shaker moving at 120 strokes per min. After the addition of cytochalasin B (5 μg), the macrophages were washed twice by centrifugation at $110 \times g$ for 5 min to remove free bacteria. Washed cells were smeared on glass slides with a Cytospinner (Clay Adams), and the slides were fixed and stained with Giemsa stain. Quantitation of ingestion was done as described above.

Phagocytosis in mouse peritoneal cavity. One milliliter of bacterial suspension containing 5×10^8 bacteria was injected into the peritoneal cavities of C3H/He mice. After 15 min, peritoneal cells were collected by washing the peritoneal cavities with ice-cold HEPES-MEM containing cytochalasin B (5 μg/ml). The cells were washed twice by centrifugation at $110 \times g$ for 5 min with fresh medium. Washed cells were smeared on glass slides with a Cytospinner. After drying, the cells were fixed with methanol and stained with Giemsa stain. Quantitation of phagocytosis was done as described above.

Phagocytosis experiments under centrifugation. To a monolayer of macrophages in a plastic dish (35-mm diameter), 2 ml of a bacterial suspension in HEPES-MEM was added. The dish was fixed on the bucket of a swing rotor (7-cm radius) and centrifuged at 37°C unless otherwise stated. After centrifugation, the macrophages were washed vigorously with phosphate-buffered saline containing cytochalasin B (1 μg/ml), fixed, and stained. Quantitation of phagocytosis was done as described above.

Intracellular killing of nonmotile *S. typhimurium*. Monolayers of macrophages and nonopsonized *S. typhimurium* SJ442 *motA* (6×10^7 cells) were centrifuged at $20 \times g$ for 15 min, as described above. The monolayers were washed twice with 10 ml of HEPES-MEM to remove free bacteria, and prewarmed medium (2 ml) was added to the dishes. After incubation at 37°C for 30 min, the medium was removed and 1 ml of ice-cold phosphate-buffered saline was added. Macrophages were lysed by scraping with silicon rubber (14), and the lysates were plated on L-broth agar plates. Lysates of macrophages without prolonged incubation were also plated as zero time controls.

Measurement of bacterial motility. The measurement of bacterial motility was done under a microscope with a Petroff-Hauser counting chamber (20 μm in depth; Erma Optical Co. Ltd. Tokyo, Japan) at 37°C. The rate of bacterial motility was calculated from the time required for the bacteria to pass through 250 μm (5 to 12 s in the case of *S. typhimurium*). The rates of at least 50 bacteria were measured.

RESULTS

Phagocytic response of macrophages in suspension culture to motile and nonmotile bacteria. The higher phagocytic response observed with a monolayer culture of macrophages exposed to motile bacteria in a previous study (14) was also demonstrated in a suspension culture, in which macrophages and bacteria were continuously agitated in a siliconized tube. As shown in Table 2, the number of phagocytized bacteria was much higher when motile bacteria were used than when nonmotile bacteria were used. A higher phagocytic response to motile bacteria

TABLE 2. Phagocytic response of macrophages to motile and nonmotile *S. typhimurium* in agitating suspension culture[a]

S. typhimurium strain	Opsonization with normal serum	Mean no. of bacteria ingested per cell[b]	% Macrophages ingesting[b]
LT2 (wild type)	+	21.5 ± 1.0	>99
	−	12.9 ± 0.9	98 ± 1
SJ442 (*motA* mutant)	+	1.7 ± 0.4	56 ± 2
	−	0.5 ± 0.1	40 ± 2
TT2 (Mot$^+$ revertant)	+	21.2 ± 1.7	>99
	−	13.2 ± 0.7	99

[a] Thioglycolate-induced macrophages (5×10^6) and either opsonized bacteria (1.5×10^8) or nonopsonized bacteria (5×10^8) were used.

[b] Means of two experiments ± standard errors.

TABLE 3. Ingestion of motile and nonmotile *S. typhimurium* by peritoneal macrophages in situ

S. typhimurium strain	Motility	Mean no. of bacteria ingested per cell[a]	% Macrophages ingesting[a]
LT2 (wild type)	+	15.4 ± 1.7	96 ± 2
SJ442 (*motA* mutant)	−	1.9 ± 0.3	53 ± 4
TT2 (Mot$^+$ revertant)	+	16.1 ± 1.2	97 ± 2

[a] Means of three experiments ± standard errors.

was also observed in mouse peritoneal cavities (Table 3). In this experiment, a certain number of bacteria were injected into the cavity; after 15 min, macrophages were washed out and the number of bacteria ingested was counted. The results indicate that enhanced response of macrophages caused by motility of bacteria is a general phenomenon.

In other experiments, monolayer cultures of macrophages and bacteria were agitated to test whether agitation affects the phagocytic response. Only a slight increase was observed when nonmotile bacteria and monolayer cultures were agitated (data not shown).

Phagocytic and chemiluminescent responses of macrophages to a large number of nonmotile bacteria. If the frequency of collision between macrophages and bacteria determines the level of phagocytic and chemiluminescent responses of macrophages to bacteria, higher responses of macrophages can be expected upon exposure to a larger number of nonmotile bacteria. However, as shown in Fig. 1, only a slight increase in phagocytic and chemiluminescent responses was observed upon exposure to larger numbers of nonmotile *S. typhimurium*, and the level of the responses never reached that obtained with motile bacteria, even when 1,000 times the number of nonmotile bacteria was used. Similar results were obtained with nonmotile mutants of *E. coli* and *P. aeruginosa* (data not shown). These results suggest that a higher frequency of collision caused by Brownian movement or agitation is not enough to induce efficient phagocytic and chemiluminescent responses of the cells.

Requirement of physical impact for efficient response of marcophages to bacteria. Since the higher collision frequency caused by Brownian movement or agitation was not enough to provoke a high level of response induced by motile bacteria, we further examined the effect on phagocytosis of physical impact given by centrifugal force between nonmotile bacteria and monolayer macrophages. As shown in Table 4, if nonmotile mutants of *S. typhimurium*, *E. coli*,

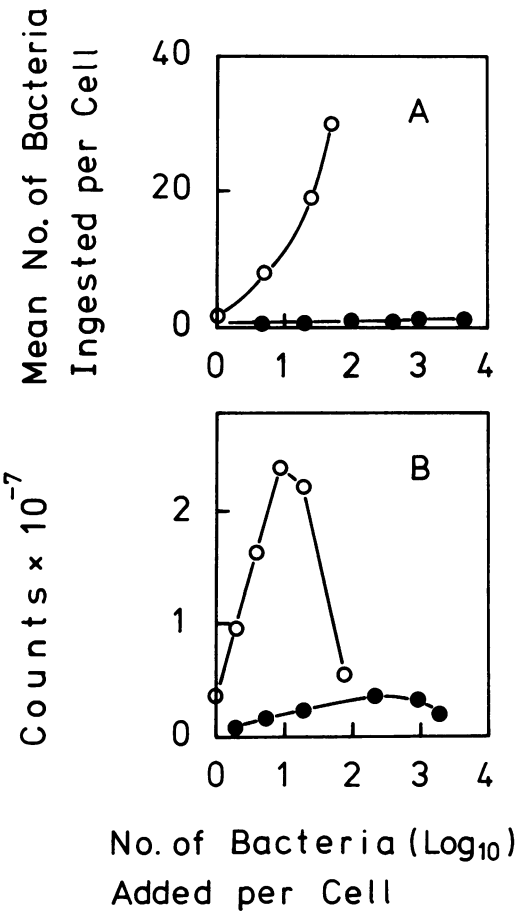

FIG. 1. Phagocytic and chemiluminescent responses of macrophages to motile and nonmotile *S. typhimurium*. Phagocytic (A) and chemiluminescent (B) responses of macrophages (approximately 1.5×10^6 cells) to the indicated numbers of *S. typhimurium* LT2 (○) and SJ442 *motA* (●) were assayed as described in the text. In the phagocytosis test, cells and bacteria were incubated at 37°C for 20 min without agitation.

TABLE 4. Effect of centrifugation on phagocytosis of nonmotile bacteria by macrophages[a]

Bacteria	Centrifugation	Presence of cytochalasin B	Mean no. of bacteria ingested per cell[b]	% Macrophages ingesting[b]
S. typhimurium				
LT2 (parent)	−	−	5.8 ± 0.2	82 ± 4
	−	+	0.1 ± 0.04	9 ± 2
SJ442 (*motA* mutant)	−	−	0.2 ± 0.07	11 ± 2
	+	−	6.9 ± 0.4	83 ± 9
	+	+	0.2 ± 0.04	10 ± 2
E. coli				
W3110 (parent)	−	−	25.6 ± 2.1	99 ± 1
TH282 (*mot* mutant)	−	−	0.6 ± 0.2	13 ± 4
	+	−	27.9 ± 1.1	99 ± 1
	+	+	0.4 ± 0.1	10 ± 3
P. aeruginosa				
PAO2003 (parent)	−	−	2.8 ± 0.2	63 ± 5
MT519 (*mot* mutant)	−	−	0.1 ± 0.05	6 ± 2
	+	−	2.6 ± 0.3	59 ± 5

[a] A monolayer of macrophages (approximately 1.5×10^6 cells), on which 2 ml of bacterial suspension was overlayed, was centrifuged at $50 \times g$ for 15 min at 37°C. The following numbers of nonopsonized bacteria were used: *S. typhimurium*, 2.8×10^8; *E. coli*, 3.6×10^8; or *P. aeruginosa*, 1.7×10^8.

[b] Means of three experiments ± standard errors.

and *P. aeruginosa* were centrifuged together with a monolayer of macrophages, the numbers of mutants ingested (or tightly associated) were similar to the numbers of motile parents ingested without centrifugation. About 50% of these bacteria were killed within 30 min of prolonged incubation (data not shown). In the presence of cytochalasin B (Table 4) or at 4°C (data not shown), this enhanced effect caused by centrifugation or bacterial motility was diminished completely, and the number of ingested bacteria under such conditions declined to those of nonmotile bacteria without centrifugation.

We further tested the effect of different centrifugal forces on the phagocytic response of monolayer macrophages. As shown in Fig. 2, the number of bacteria ingested increased linearly with increments of centrifugal force up to around $10 \times g$, and then the increase became gradual. The biphasic increase of the number of ingested bacteria was demonstrated whether or not the bacteria were opsonized (Fig. 2), and these results imply the existence of a threshold velocity of bacteria above which macrophages respond well to bacteria and engulf them efficiently. Incidentally, the frequency of collision between monolayer macrophages and bacteria is considered to increase linearly with the increase in centrifugal force (see below).

Threshold velocity for induction of maximum response of macrophages. To determine the real threshold velocity, an experiment was designed in which collision frequency was constant for different centrifugal forces. Since collision frequency increases proportionally as the sedimentation velocity (i.e., centrifugal force) of nonmotile bacteria increases, it is necessary to reduce the number of bacteria added in inverse proportion as centrifugal force (g force) increases. The results obtained in such experiments are shown in Fig. 3, which shows that above $5 \times g$, the number of bacteria ingested per cell was constant. By using Stokes law, the velocity of sedimentation of nonmotile bacteria at $5 \times g$ was calculated to be approximately 2.5 μm/s, supposing that the bacterium has a spherical shape and a 1-μm radius (see Discussion).

To determine whether the velocity of movement of motile bacteria is greater than this value, we measured the velocity of motile *S. typhimurium*, *E. coli*, and *P. aeruginosa* organisms under a microscope. The value was within the range of 20 to 50 μm/s for all bacteria, indicating that the movement of motile bacteria is much faster than the threshold velocity for inducing maximal response of macrophages.

DISCUSSION

In this paper, we showed that the phagocytic response of macrophages to nonmotile bacteria was markedly enhanced by centrifugal force, which is considered to cause physical impact between bacteria and macrophages. Furthermore, we determined the minimal centrifugal force, above which nonmotile bacteria were phagocytized by macrophages as efficiently as motile, parent bacteria. By using Stokes law (8) and supposing that the shape of the bacterium is

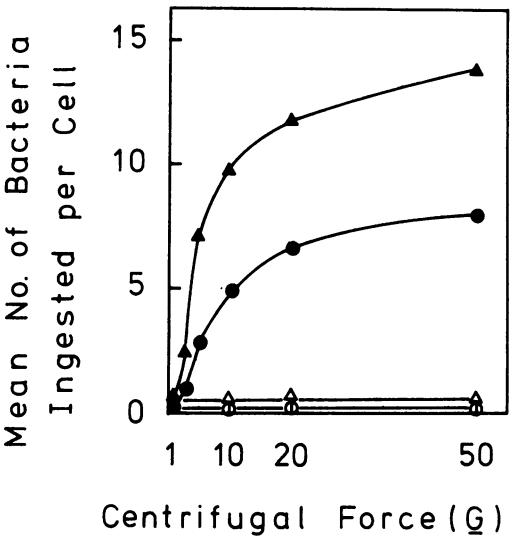

FIG. 2. Effect of different centrifugal forces on phagocytosis of nonmotile *S. typhimurium* by monolayer macrophages. A monolayer of macrophages (approximately 1.5×10^6 cells) and 2 ml of bacterial suspension in a plastic dish (diameter, 35 mm) were centrifuged at the indicated forces at 37°C for 15 min. Opsonized (3×10^7) (triangles) or nonopsonized (1.6×10^8) (circles) *S. typhimurium* SJ442 was used. The experiments were performed in the presence (open symbols) or absence (closed symbols) of cytochalasin B (5 μg/ml).

spherical, the sedimentation velocity (v) of a nonmotile bacterium can be evaluated by the following equation: $v = 2r^2 (\rho - \rho_0) F/9\eta$, where r is the radius of the bacterium, ρ is the density of the bacterium, ρ_0 is the density of water at 37°C, η is the viscosity of water at 37°C, and F is the centrifugal force. The following dimensions were employed: ρ, 1.15 g/cm^3 (12, 13); ρ_0, 0.994 g/cm^3 (3); and η, 0.00694 poise (3). If the radius of the bacterium is 1 μm, the sedimentation velocity of nonmotile bacteria at $5 \times g$ is calculated to be 2.5 μm/s. The velocity of bacterial movement observed with motile bacteria was much higher than the threshold velocity, indicating that the physical impact caused by bacterial motility is enough to enhance the phagocytic response of macrophages.

Ingestion of particles such as zymosan was also enhanced by centrifugation, and there exists a threshold force for efficient phagocytosis (unpublished observation). For zymosan, the threshold force was under $2 \times g$, and significant phagocytosis was observed even without centrifugation (i.e., $1 \times g$). Centrifugation or agitation has also been employed empirically by other investigators to get efficient phagocytosis of bacteria, yeasts, or erythrocytes. For example, Wood and Smith (15) showed that centrifugation at 2,000 rpm for 5 min of a mixture of leukocytes and nonopsonized, nonmotile bacteria, such as *Klebsilla, Pneumococcus*, or *Staphylococcus* species, results in marked phagocytosis of these bacteria, in contrast to a simple mixing of the two, although these investigators considered that a sandwich of bacteria between phagocytes was the cause of the enhancement of phagocytosis. Kielian and Cohn (6) used centrifugation for phagocytosis of serum-opsonized, heat-killed yeasts. Ehlenberger and Nussenzweig (4) used centrifugation as a contact-inducing agent to stimulate phagocytosis of antibody-coated sheep erythrocytes. As shown in this paper, physical impact brought about by centrifugation or motility enhances phagocytosis, probably overcoming a physical barrier such as

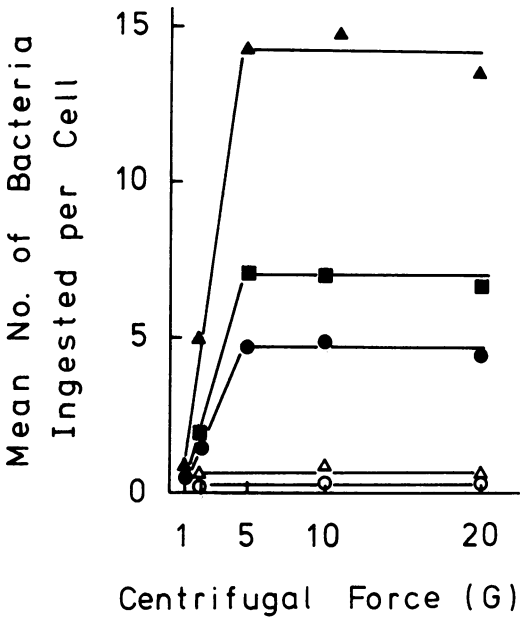

FIG. 3. Centrifugal force required for efficient phagocytosis of nonmotile *S. typhimurium*. Monolayer macrophages (approximately 1.5×10^6 cells) and 2 ml of bacterial suspension in a plastic dish (diameter, 35 mm) were centrifuged at the indicated forces at 37°C for 15 min. To equalize collision frequency at different centrifugal forces, the following numbers of bacteria were employed, respectively, for the experiments at 1, 2, 5, 10, and $20 \times g$: opsonized bacteria (triangles), 1.2×10^9, 0.6×10^9, 0.24×10^9, 0.12×10^9, and 0.06×10^9; nonopsonized bacteria (circles), 3×10^9, 1.5×10^9, 0.6×10^9, 0.3×10^9, and 0.15×10^9; nonopsonized bacteria (squares), 6.0×10^9, 3.0×10^9, 1.2×10^9, 0.6×10^9, and 0.3×10^9. Quantitation of phagocytosis was done as described in the text. Open symbols, cytochalasin B added (5 μg/ml); closed symbols, no addition.

electrostatic repulsion due to a negative charge on the surface of the phagocytes and bacteria. Physical impact may also play an important role in phagocytosis in vivo.

In the experiment shown in Fig. 1, the generation of chemiluminescence drastically decreased with an increased number of motile bacteria added above about 10 bacteria per cell. In contrast, phagocytosis still increased up to around 100 bacteria per cell. The results suggest that too much close contact of bacteria injured the cell membrane of macrophages, even when macrophages still retained phagocytic activity. This may be caused by production of active oxygen from the macrophage itself. Such susceptibility was also reported by McGee and Myrvik (10), who showed that activated but not normal alveolar macrophages were injured upon phagocytosis of zymosan. As reported by us (14) and others (11), activated macrophages produce much more active oxygen than normal (resident) or induced macrophages. McGee and Myrvik (10) also showed that the addition of catalase, cytochrome *c*, and ascorbate had some protective effect, which indicates the involvement of active oxygen for cell membrane injury.

Physical impact due to motility not only enhances phagocytosis by professional phagocytes but also may play some role in the bacterial association and invasion of tissue and cells. As shown by Kihlström and Edebo (7), killed *S. typhimurium* interacts less efficiently with HeLa cells than does living bacteria. Guentzel and Bery (5) showed that motility or flagella or both contribute to the adsorption of *Vibrio cholerae* to intestinal mucosa. Craven and Montie (2) also reported that motility (and/or chemotaxis) seems to be associated with the virulence of some *P. aeruginosa* strains in a burned mouse model. In the case of *Salmonella* species, however, it is not certain whether motility plays some role in infection, although motile *Salmonella* organisms were definitely ingested much more efficiently than were nonmotile bacteria. If macrophages are not activated, they cannot kill the virulent bacteria efficiently (1), even if they engulf them rapidly. Thus, such bacteria may be delivered to other places, such as the mesenteric lymph node, the spleen, and the liver.

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