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Interaction of Virulent and Avirulent *Listeria monocytogenes* with Cultured Mouse Peritoneal Macrophages

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The interaction between smooth and rough *Listeria monocytogenes* and mouse peritoneal macrophages in culture was investigated. Initially, antibiotics were deleted from the culture medium, and no attempt other than the removal of unphagocytized bacteria by extensive washings was made to control extracellular growth. Under these conditions the monolayers were rapidly destroyed within an 8-h period, and this was associated with increases in the intracellular population of both strains. Extracellular viability counts revealed that washings failed to reduce the bacteria in the medium to less than 10% of the original inoculum. Continuous phagocytosis of *Listeria* which grew logarithmically in the maintenance media appears to account for the observed changes in the number of intracellular bacteria. The data also indicate that it is primarily the free bacteria in the culture medium which are responsible for the cytotoxic effects. In other experiments streptomycin-penicillin solutions were added to the maintenance media after an initial period of phagocytosis. In the presence of antibiotics, the total number of macrophages per field remained relatively constant, and no morphological alterations in the leukocyte cultures were observable. Extensive intracellular multiplication of either strain was not evident in fixed and stained cover slips. Viable intracellular counts reveal that after 24 h there is almost total killing of the rough variant, whereas the smooth strain tended towards complete survival.

Virulence of *Listeria monocytogenes* appears to be dependent upon successful parasitization of the cells of the mononuclear phagocyte system. Evidence in support of this hypothesis has come from studies on the fate of the microorganisms within cultured macrophages. The tissue culture systems which have been employed, however, differed with respect to the source of the leukocyte and whether antibiotics were incorporated into the tissue culture medium to control for extracellular growth of bacteria. Mackaness (14) reported survival and growth of virulent *Listeria* in mouse peritoneal macrophages cultured in the absence of antimicrobial drugs. Extracellular growth was minimized by infecting with a highly dispersed suspension of organisms, and plaque development in the macrophage monolayer was attributed to the actions of those bacteria capable of multiplying within cells. Njoku-Obi and Osebold (15) reported that normal sheep peritoneal macrophages cultivated in Kapral-type culture chambers with normal serum were highly susceptible to the destructive effects of *Listeria* infection, whereas immune cells suspended in immune serum resisted such effects. The workers did not attempt to restrict extracellular growth. Routine viability determinations indicated that continual phagocytosis of bacteria which proliferated freely in the medium were largely responsible for the cytotoxic manifestations. In their studies on the interaction of *Listeria* with guinea pig macrophages in culture, Armstrong and Sword (2) controlled extracellular bacterial growth by incorporating streptomycin into the tissue culture medium. Although the bacterial population increased during the 72-h experimental period, the investigators did not observe an overwhelming cytotoxic effect in stained cover slips. Recently, Cline (5) investigated the phagocytosis and killing of *L. monocytogenes* by human monocytes cultured in medium containing penicillin. Few bacteria survived the 24-h incubation period and there was no mention of injury to the leukocytes.

The present investigation describes the interaction of smooth virulent *Listeria* and a nonpathogenic rough variant with mouse peritoneal macrophages cultured in the presence and absence of antibiotics. This work attempts to further equate virulence of *Listeria* strains with

behavior in tissue culture and serves to reemphasize the difficulties encountered in interpreting data when no attempt is made to control extracellular growth of microorganisms with a relatively short generation time.

MATERIALS AND METHODS

Animals. Swiss albino female mice weighing 20 to 25 g and reared in animal facilities at the University of Massachusetts were used as the source of peritoneal cells for all experiments.

Bacteria. Smooth *L. monocytogenes* strain A4413 was originally isolated at the Communicable Disease Center, Atlanta, Ga., from a child with a fatal infection. When injected into 25-g Swiss mice by the intraperitoneal route, 4.8×10^3 cells of this strain were required to kill 50% of the mice (LD_{50}) within 10 days. Mouse virulence remained constant throughout the study period. Strain 9037-7 (mouse intraperitoneal $LD_{50} > 10^9$) is a rough isolate derived from a smooth virulent strain.

Collection of macrophages. Mouse peritoneal macrophages were harvested, quantitated, and processed by modification of the procedure of Cohn and Benson (6). Mice were injected with Scherer's Maintenance Medium, containing 5 units of heparin per ml of solution, and rapidly killed with ether. Each animal's abdomen was gently massaged and, after reflection of the skin, the peritoneal washings were removed with a 5-ml syringe and a 22-gauge 1.5-inch (3.81-cm) needle and pooled in 15-ml, sterile, siliconized, conical, centrifuge tubes. The cells were then sedimented at $165 \times g$ for 10 min, the supernatant fluid was decanted, and the pellet was suspended in the tissue culture medium which consisted of 80% (vol/vol) Scherer's Medium containing 0.2 mM glutamine plus 20% heat-inactivated fetal calf serum. Leukocyte counts were performed in a hemacytometer, and the cells were diluted to give a concentration of about 10^6 cells per ml.

Cultivation of macrophages. One milliliter of cells suspended in complete medium were dispensed in Leighton tubes containing flying cover slips (9 by 22 mm). The tubes were gassed with a 5% CO_2-air mixture, closed with rubber stoppers, and incubated at 37 C for 1 h to allow for attachment of the cells to the cover slips. The medium was then withdrawn, and the cell layer was washed twice with Scherer's Medium to remove nonadhering cells. The wash fluid was removed, and the cells on the cover slips were overlaid with 1 ml of fresh culture medium.

Infection of macrophage cultures. A tryptose (Difco) broth culture was prepared by inoculating from a thawed, frozen tryptose-agar slant and incubating for 18 h at 37 C. The culture was centrifuged at $3,000 \times g$ for 10 min, and the pellet was washed twice in phosphate-buffered saline. The bacteria were suspended in tissue culture medium, and the cell concentration was adjusted to insure an infectivity ratio of approximately 1 after addition of 1 ml of the suspension to the washed monolayer. After a 2-h period of phagocytosis, the infecting medium was removed by aspiration, and the cells were washed four to five times by vigorous shaking in 5 ml of Hanks solution to remove the free *Listeria*. To evaluate the effectiveness of this procedure, bacterial plate counts were performed on each of the individual wash fluids. Fresh culture medium was added to the cell layers, and the tubes were gassed, plugged, and reincubated at 37 C. In experiments where restriction of extracellular growth of bacilli was required, the medium was supplemented with 50 μg of streptomycin and 50 units of penicillin per ml.

Enumeration of cultures. At 2-h intervals after infection, cover slips were removed from control and infected tubes, fixed in methanol, stained with May-Grunwald Giemsa, and mounted in balsam for direct microscopic counting. The bacterial content and the proportion of infected cells were determined by observing 50 different oil immersion fields. The percentage of destruction of macrophages was obtained by counting the total number of cells per 100 fields in the infected cultures and comparing these with controls. The tubes were washed to remove the antibiotics, and the number of viable intracellular bacteria was determined by plate counts of distilled water lysates of macrophages fractured by shaking for 1 h at room temperature with no. 10 glass beads. Extracellular growth of bacilli was followed by culturing dilutions of the maintenance medium.

RESULTS

Initial experiments were performed to determine whether intracellular events could be separated from extracellular interactions of *Listeria* with macrophages if no attempt was made to restrict growth of the bacteria in the culture medium. After the allotted 2-h period of phagocytosis, the monolayers were vigorously washed and incubated again with culture medium devoid of antibiotics. Duplicate cover slips were removed immediately after washing and at 2-h intervals to determine the fate of intracellular bacteria. The data are expressed as the number of intracellular bacteria per 100 infected macrophages; the number of intracellular bacteria per 100 macrophages of the total population, and the percentage of infected macrophages. These parameters fix the initial level of infection with some precision and describe the dynamics of the host-parasite relationship. At an infectivity ratio of 1.2 *Listeria* per macrophage, it appears that the microorganisms rapidly increase within the cells until a maximum intracellular population of approximately seven bacteria per macrophage is realized (Fig. 1). Parallel experiments performed on the rough variant 9037-7 show that, at an input multiplicity of 1.45 bacteria per macrophage, the results were similar to those obtained

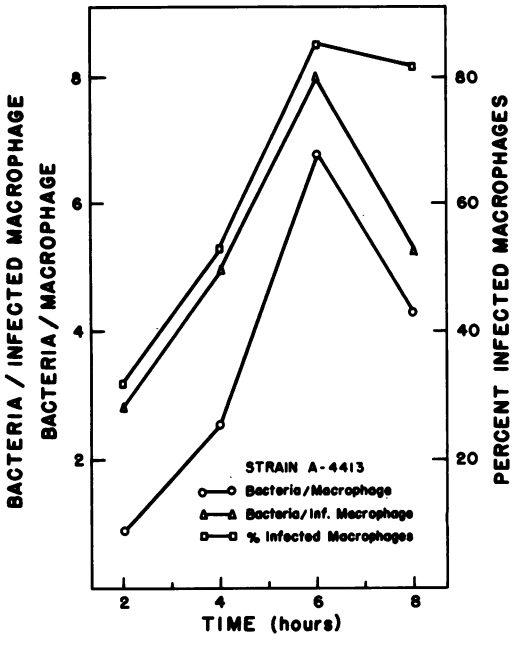

FIG. 1. *Microscopic counts of intracellular smooth Listeria in macrophage culture.*

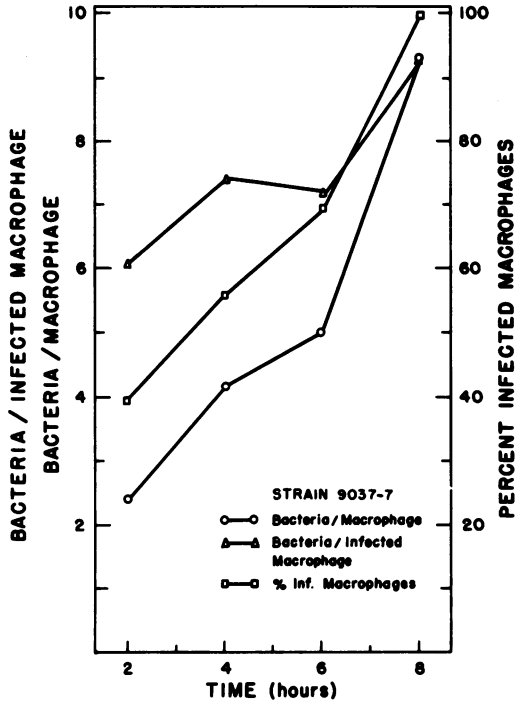

FIG. 2. *Microscopic counts of intracellular rough Listeria in macrophage culture.*

for the virulent isolate (Fig. 2). The phagocytic index was somewhat greater as was the percentage of bacteria-containing cells. Although the rapid increase in the average number of bacteria infected macrophage would seem to indicate intracellular multiplication, subsequent experiments reveal this to be predominantly the result of continuous phagocytosis of bacteria from the medium.

Counts of the number of leukocytes per field were taken to estimate the extent to which bacteria were being released from lysed cells. Both strains initiate rapid cytolysis, and only a small number of morphologically intact macrophages were seen attached to the cover slip after the first 8 h of infection (Fig. 3). Many of the *Listeria* freed by continuously lysing cells would be taken up by neighboring macrophages but, as the number of leukocytes decline with time, more cells escape phagocytosis and multiply freely in the culture fluid. Viable counts of the culture medium revealed that the extracellular population was never reduced to less than 10% of the original inoculum (Fig. 4). These cells were found to multiply in the culture medium with a mean generation time of 45 min and, at the point of maximum phagocyte destruction, the number of bacteria in the culture fluid exceeded 10 million/ml. Based on calculated intracellular generation times of 4.8 and 5.1 h for *Listeria*

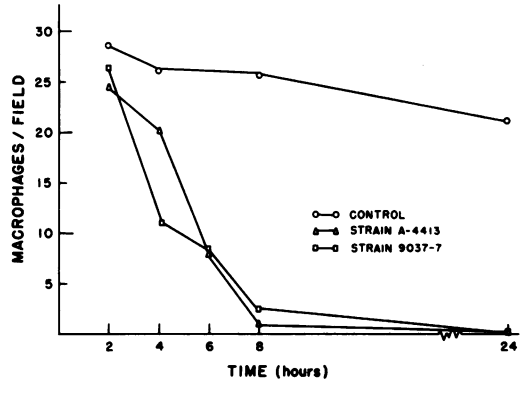

FIG. 3. *Kinetics of macrophage destruction after infection with smooth and rough Listeria.*

growing in vivo in mouse spleen and liver, respectively (14), it appears that intracellular multiplication of the microorganisms contributes little to the cytotoxic manifestations. Extrapolation of these figures to the mouse macrophage system indicated that massive phagocyte destruction had occurred before the bacilli were afforded an opportunity for a single division.

Eliminating the effects of continuous phago-

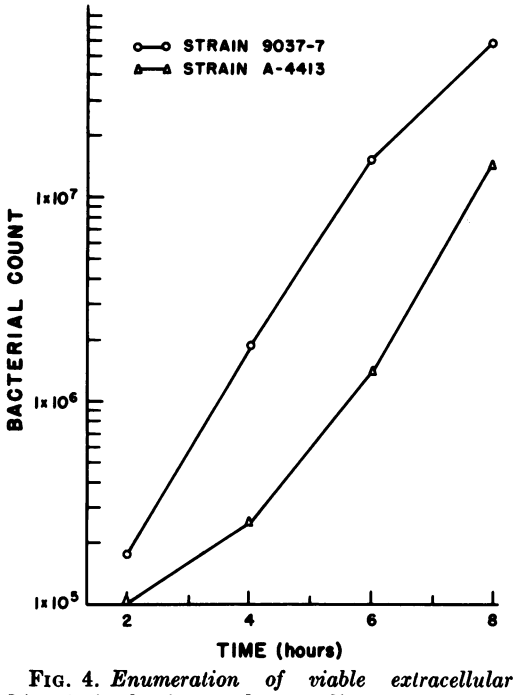

FIG. 4. *Enumeration of viable extracellular Listeria in the tissue culture medium.*

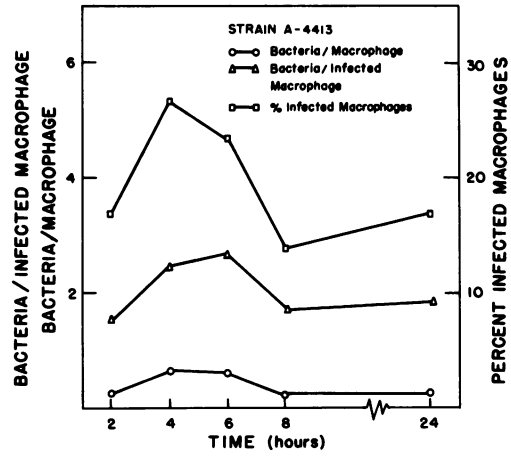

FIG. 5. *Microscopic determinations of the number of smooth Listeria within macrophages cultured in the presence of antibiotics.*

cytosis required that expermiments be performed to insure extracellular killing of bacteria. After the initial period of phagocytosis, tissue culture medium containing antibiotics was added to the washed tubes. The data obtained from microscopic counts of stained cover slips indicate that the total intracellular population of the smooth isolate remained essentially stationary throughout the experimental period (Fig. 5). The increase in the number of bacteria per macrophage that occurs between the 2nd and 4th h after ingestion could represent a brief period of intracellular multiplication or phagocytosis of dead or dying bacteria from the culture fluid. The data obtained for the interaction of strain 9037–7 with macrophages cultured in the presence of antibiotics show a steady decline in the intracellular population of the rough strain (Fig. 6). In addition to being more vulnerable to postphagocytic destruction, the rough isolate is differentiated from the virulent strain by its greater susceptibility to ingestion by macrophages. The number of macrophages per oil immersion field were periodically counted to determine whether bacteria could have been killed by exposure to the antibiotics in the culture medium after their release from leukocytes. Evidence against this possibility was obtained by a failure to observe significant differences between the number of macrophages

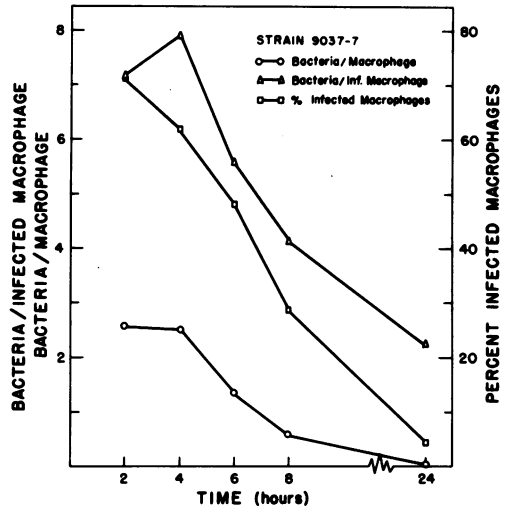

FIG. 6. *Microscopic determinations of the number of rough Listeria within macrophages cultured in the presence of antibiotics.*

TABLE 1. *Counts of the number of macrophages per microscopic field during infection with smooth and rough Listeria*

H after infection	Macrophages per field			
	Expt I		Expt II	
	Smooth	Control	Rough	Control
2	17	18	18	17
8	16	15	19	17
24	14	13	18	18

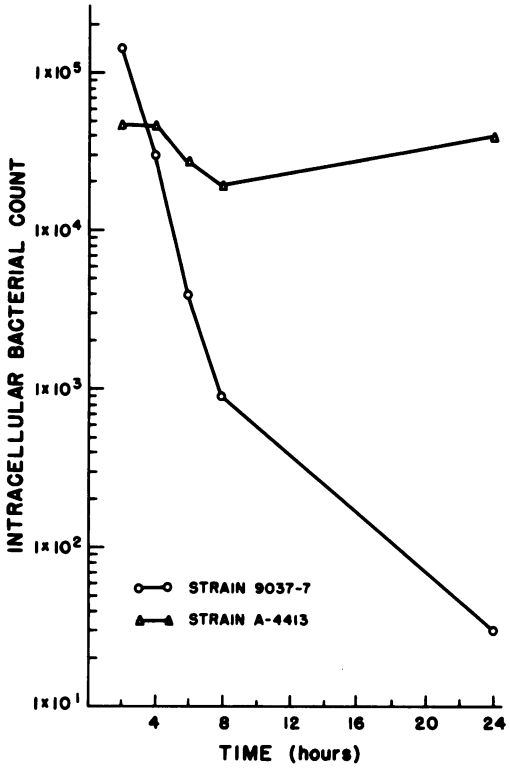

FIG. 7. *Intracellular viability counts of Listeria in macrophages cultivated with antibiotics.*

per field in infected and control cultures (Table 1). After 24 h, infected cells were unaltered and could not be morphologically distinguished from control macrophages. Since this series of experiments did not allow differentiation of living from killed bacteria, intracellular viable counts were simultaneously performed (Fig. 7). The rough strain died linearly with time while the virulent strain tended towards complete survival. During the first 8 h, a minimal decline was observed in the intracellular number of smooth bacteria. However, after 24 h, the count approximated the count obtained immediately after the initial period of phagocytosis.

DISCUSSION

Several investigators have attempted to distinguish intracellular proliferation of bacteria from the accumulation of bacilli which multiplied extracellularly. Mackaness (13) compared extracellular growth of *Staphylococcus aureus* after infection of macrophages in fluid and plasma clot cultures. He noted that the solid medium prevented efficient phagocytosis and allowed logarithmic increments in the extracellular population, whereas in suspension culture the growth potential was inhibited by continuous phagocytosis. The experiments demonstrated that assessment of the fate of intracellular bacteria becomes impossible when macrophages are not inhibited from phagocytizing bacteria multiplying freely in the medium. Kapral and Shayegani (12) restricted extracellular multiplication by washing away the extracellular organisms as rapidly as they multiplied. The procedure was found to be as effective as antibiotics in controlling the number of extracellular bacteria. Other workers have assumed that extracellular growth could be minimized by thorough washing of cover slips immediately after phagocytosis (9). In contrast, Mackaness (13) reported that it was nearly impossible to wash all bacteria from a glass surface after they had settled in the presence of serum protein. Recently, Patterson and Youmans (16) examined the problem of extracellular multiplication of *Mycobacterium tuberculosis*. The workers thoroughly washed the macrophage cultures after phagocytosis and cultured the medium at 24-h intervals to determine the number of extracellular mycobacteria. They concluded that only a small percentage of extracellular bacilli would remain after washing, and those found at 24 h would predominantly represent progeny of bacteria which multiplied intracellularly and were released subsequent to lysis of macrophages. Their assumption that three washes would remove 99.9% of the nonphagocytized bacilli was not verified by a zero time count. The present experiments indicate that after exhaustive washing numerous bacteria remain and multiply in the tissue culture medium. It is largely the progeny of these cells which contribute to the rapid destruction of the macrophages. Bacteria released from dead or dying phagocytes would add to the extracellular pool of *Listeria*, and continuous phagocytosis of these cells would make any attempt at enumeration of intracellular growth virtually impossible. The rapid death of the macrophages is apparently attributable to the activities of extracellular as well as ingested bacteria. Macrophage death was seen to occur in the absence of heavily infected cells. When the extracellular growth of *Listeria* was restricted, bacteria ingested prior to addition of antibiotics were unable to destroy the phagocytes. A high level of parasitism seems to be required for initiation of toxic manifestations. This is in accord with the observation that derangements in the cytoplasm of guinea pig macrophages by *Listeria* occurred only in cases where extensive intracellular multiplication had occurred (2).

The data obtained present little evidence for

the occurrence of intracellular multiplication but support the previously cited findings of Mackaness (14) that virulence of *Listeria* reflects an ability to survive in macrophages. He observed that the microorganism was promptly inactivated by mouse peritoneal cells unless virulence was raised by mouse passage or by selection of strains which survived and grew in homologous macrophages in vitro. The ability of the derived strains to grow and produce plaques in cultured macrophages correlated with increased survival rates in the spleen and liver. In the present study no attempt was made to increase virulence of the smooth bacteria. It may be that this microorganism represents a strain of intermediate pathogenicity which exerts little aggressive activity towards macrophages and is in turn resistant to their bactericidal components.

It is evident that assessment of the intracellular fate of *Listeria* must be obtained under conditions of restricted extracellular growth. However, incorporation of antibiotics into the culture fluid may alter the host-parasite relationship and obscure determinations of intracellular multiplication. Several investigators have obtained evidence in support of the idea that the bactericidal capabilities of macrophages are facilitated by their capacity to concentrate antibiotics from the culture medium (3, 4, 7, 8, 10, 12, 16). Armstrong and Sword (2) were able to show a one log increment of *L. monocytogenes* strain 34s within guinea pig macrophages cultured in the presence of 20 μg of streptomycin per ml. Intracellular growth, however, ceased after 24 h and declined slightly during the next 2 days. Interruption of growth may have been coincidental with the intracellular accumulation of bactericidal levels of antibiotic. This is in keeping with the report of Bonventre and Imhoff (3) that streptomycin is steadily concentrated in macrophages over a period of 6 days. Recently, Adam et al. (1) reported a rapid enhancement in the destruction of bacteria by macrophages when phagocytosis is allowed to proceed in the presence of subliminal amounts of streptomycin. In the present experiments, the penetration of small amounts of antibiotics into the macrophages may have been sufficient to prevent bacterial division.

Our inability to detect brief periods of intracellular multiplication might be explained by additions to the medium of penicillin as well as streptomycin. Simon and Sheagren (17) determined the effect of penicillin on the growth of *Listeria* in bovine-gamma globulin (BGG) immune guinea pig peritoneal macrophages cultured with and without BGG antigen. They observed that the number of viable intracellular bacteria counted at 4 h after infection was consistently lower in cultures reincubated with an antibiotic. Their penicillin effect may be questionable, since the control involved its deletion and the problem concerning the extracellular population appears. It is interesting to note that, at infectivity ratios ranging from 5 to 12 bacteria per macrophage, the control monolayers bathed in 1.2 μg of sodium penicillin/ml were badly damaged by 4 h and completely destroyed in 24 h. Their strain of *Listeria* (ATCC 19115) retained guinea pig virulence throughout the study period and was of the same serotype (4b) as strain A4413. Cline (5) studied the interaction of 32P-labeled *L. monocytogenes* strain 4524B with human monocytes cultured in medium containing penicillin (50 units/ml). Radioactivity estimations of disrupted macrophages revealed that by 24 h the number of viable colony-forming units was approximately 10% of that initially ingested by the macrophages. This was also true for a virulent subline of UCLM-1 which was obtained by passage through human macrophages. However, radioautographic evidence was obtained for bacterial multiplication within the cells. Replication occurred early, and after 24 h few intracellular labeled organisms were observed in the radioautographs of the monocytes.

Based on our quantitative assay of viable intracellular *Listeria*, it appears that most of the smooth bacteria initially ingested survived the 24-h experimental period. Visual enumeration, however, revealed that the number of bacteria per cell fluctuated during the first 8 h. It therefore seems reasonable to suggest that the bacteria were in a state of flux and underwent alternating or perhaps continuous cycles of division and cell death. If so, viability counts performed at our selected intervals might have missed those periods of multiplication and would only have shown net effects. The employment of phase-contrast optics for continuous observation of single infected cells might provide useful information regarding this possibility.

The somewhat discrepant reports on the interactions of *L. monocytogenes* with macrophages in culture may be explained in part by differences in the experimental methods. The investigators used different *Listeria* strains, and dissimilar procedures were employed for maintenance of virulence. There was variation in the nature and source of the phagocyte; composition of the culture medium and type and concentration of the antibiotic used to control extracellular growth. In a sense the reports should not be viewed as conflicting, since it is a fundamental principle that virulence can be described only within the confines of a given experimental system, and extrapolation to other systems is fallacious without

experimental evidence. However, if we are to accurately correlate virulence of bacteria with precise behavior in a given macrophage culture, then it must be insured that the findings are adequately corrected for antibacterial effects related to the intracellular accumulation of antibiotics from the culture medium.

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