INFECTION AND IMMUNITY, Sept. 1991, p. 3291–3296
0019-9567/91/093291-06$02.00/0

Vol. 59, No. 9

Growth of *Francisella* spp. in Rodent Macrophages

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Received 5 April 1991/Accepted 30 May 1991

We examined the nature of the interactions between the facultative intracellular pathogens *Francisella tularensis* and *F. novicida* and rodent macrophages. Growth of *F. tularensis* LVS was observed in macrophage monolayers from mice, guinea pigs, or rats. In contrast, *F. novicida* grew in macrophages from mice and guinea pigs but not in macrophages from rats. Transmission electron microscopy studies indicated that both *Francisella* species survive within macrophage phagosomes that are unfused with lysosomes.

Francisella tularensis is a gram-negative, facultative intracellular bacterium that is the causative agent of tularemia in humans. A related organism, *Francisella novicida*, has recently been shown to cause disease in certain types of individuals, most of whom exhibit diminished immune function (12, 28). Little is known, however, about the interaction between these organisms and their host cell, the macrophage. Previous work has been limited to studies of the interaction of *F. tularensis* within macrophages isolated from rabbits (21, 25, 26) and guinea pigs (14, 23, 26). However, the results from most of these studies failed to demonstrate intracellular growth. Moreover, no attempts were made to determine the intracellular compartment in which *Francisella* spp. survive and multiply. In addition, *F. novicida* has never been studied with respect to its interaction with macrophages. In this study, we have systematically examined several parameters of the interaction between these two *Francisella* species and macrophages. Using two *Francisella* species of different virulence, namely, *F. novicida* and the live vaccine strain (LVS) of *F. tularensis*, we examined the intracellular growth of *Francisella* spp. within macrophages from mice, rats, and guinea pigs. Furthermore, we attempted to determine the location of intracellular *Francisella* organisms and provide evidence that both *Francisella* species survive within phagosomal vesicles that have not fused with lysosomes.

Growth of *Francisella* spp. in tissue culture media. One of the difficulties inherent in evaluating the intracellular growth of bacteria is the control of extracellular proliferation. In systems using *Listeria* (22), *Yersinia* (15), and *Salmonella* (8) spp., this is usually accomplished by adding gentamicin to the tissue culture medium. Preliminary attempts to employ gentamicin in our experiments with *F. novicida* indicated that this organism was in a quiescent metabolic state in the tissue culture medium used in our experiments (Dulbecco modified Eagle medium containing 10% defined fetal bovine serum [Hyclone] [DMEM]). Therefore, the growth of *F. tularensis* and *F. novicida* was measured in DMEM and compared with that in tryptic soy broth supplemented with 0.1% cysteine (TSB-C). Wells of a 96-well microtiter plate were inoculated with *Francisella* spp. in either medium, and the bacterial growth was measured over a period of several days by plating serial dilutions of the well cultures on cystine-heart agar containing 5% defibrinated horse blood (CHA-H). *F. novicida* proliferated exponentially in TSB-C; although the growth of *F. tularensis* exhibited a considerably longer lag phase, it, too, grew under the same conditions (Fig. 1). In contrast, after an initial increase in bacterial numbers in DMEM, no subsequent proliferation of either organism was observed. This arrest of growth was not simply due to exhaustion of nutrients in the medium, because the growth of *F. tularensis* LVS in DMEM in which *F. tularensis* LVS had been cultured for 3 days and then sterilized was identical to that observed in fresh medium (data not shown). Although it is not yet possible to account for the modest initial growth of *Francisella* spp. in DMEM, it is clear that DMEM is not a favorable environment for *Francisella* growth. The relative inability of *Francisella* spp. to grow extracellularly in macrophage cultures thus facilitated the development of an in vitro assay for intracellular growth without the complicating parameters of sustained extracellular bacterial growth or the presence of antibiotics in the medium.

Intracellular growth of *F. tularensis* LVS and *F. novicida* in macrophages. To evaluate the intracellular growth of *Francisella* spp. mouse macrophages were plated in 96-well microtiter plates at a density of approximately 2×10^5 macrophages per well. Macrophages were either isolated from the peritoneal cavity by peritoneal lavage or cultured from bone marrow cells in polypropylene bottles in medium containing 10% L-929 cell conditioned medium as a source of macrophage colony-stimulating factor. In either case, the monolayers were washed to remove nonadherent cells. The wells were inoculated with *Francisella* spp. diluted in DMEM, and the plates were spun at $600 \times g$ to enhance the association of bacteria with the macrophages. After incubation for 1 h to allow for phagocytosis, the monolayers were washed three times and incubated in DMEM. At various times, the monolayers were lysed with 0.1% deoxycholate and the bacteria were enumerated as described above. In either resident peritoneal macrophages (Fig. 2A) or bone marrow-derived macrophages (Fig. 2B), both *F. tularensis* and *F. novicida* grew at an exponential rate for 24 to 48 h. Thereafter, the number of *Francisella* organisms remained relatively constant for the remainder of the assay period. Light microscopic examination of the viable infected cultures revealed the presence of intracellular inclusions by their Brownian motion (data not shown), a phenomenon similar to that observed in cells infected with *Chlamydia* spp. The course of *Francisella* replication was also associated with a progressive cytopathic effect, as evidenced by the appearance of clusters of rounded cells and an overall degenerate appearance of the macrophages (data not

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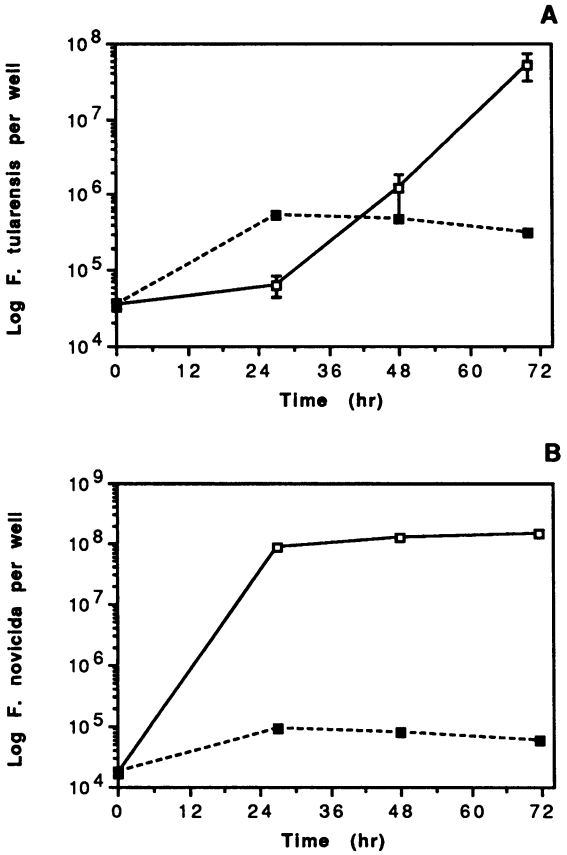

FIG. 1. Growth of *F. tularensis* LVS (A) or *F. novicida* (B) in bacteriological growth medium (TSB-C) or in tissue culture medium (DMEM). Bacteria were grown overnight in TSB-C and then diluted approximately 1:10,000 in either TSB-C (□) or DMEM (■). Then 150 μl was inoculated into each well of a 96-well microtiter plate. After incubation, 100 μl of the culture was removed for bacterial enumeration. Results are expressed as the mean ± 1 standard deviation $\log_{10}$ *Francisella* CFU per well.

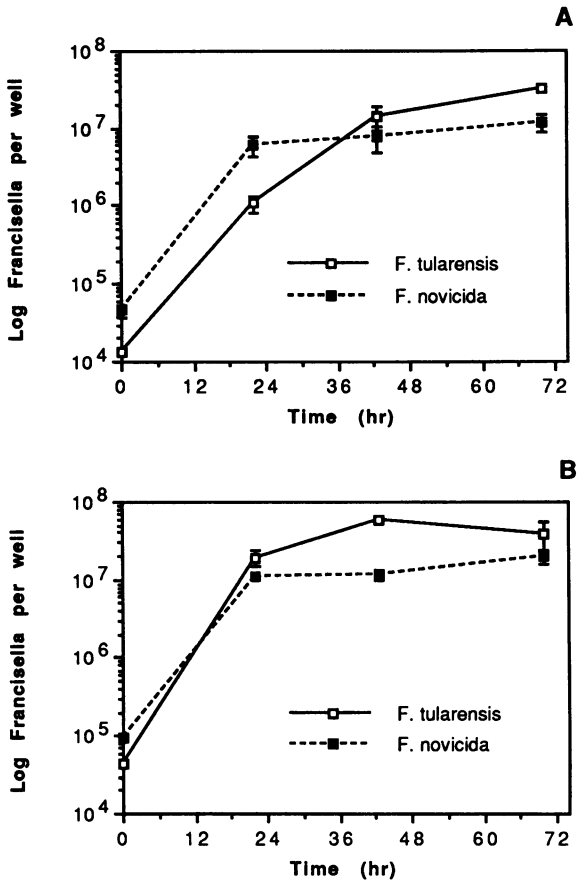

FIG. 2. Growth of *F. tularensis* LVS and *F. novicida* in mouse resident peritoneal macrophages (A) or mouse bone marrow-derived macrophages (B). Macrophage monolayers containing approximately 2×10^5 macrophages were allowed to phagocytize *Francisella* bacteria for 1 h, after which uningested bacteria were washed away. The cells were incubated and lysed at the indicated times by the addition of an equal volume of 0.2% sodium deoxycholate. Bacteria were enumerated by plating serial dilutions on CHA-H, and the results are expressed as the mean ± 1 standard deviation $\log_{10}$ *Francisella* CFU per microtiter well.

shown). It is likely, therefore, that the cessation of bacterial proliferation is due to the destruction of the favorable growth environment within macrophages and not the exhaustion of chemical nutrients in the medium.

A separate experiment was performed to evaluate the possibility that *Francisella* spp. grew in the extracellular environment under the influence of growth-promoting factors secreted by macrophages. Two-chamber wells were formed in 24-well microtiter plates by inserting a 0.1-μm polycarbonate membrane filter chamber (Transwell; Costar Nucleopore, Toronto, Canada) into each well. Macrophages were cultured in DMEM at a density of 10^6 cells per well in the lower chamber. *F. tularensis* LVS and *F. novicida* were diluted in DMEM and inoculated into the upper chamber, and bacterial growth was monitored for the next 3 days. Control wells contained DMEM alone in the lower well. The growth rate of *Francisella* spp. was essentially the same in the presence or absence of macrophages in the lower chamber (data not shown), indicating that macrophages do not simply support the extracellular growth of *Francisella* spp. by the secretion of a soluble substance.

Intracellular growth of *Francisella* spp. in macrophages from different species of rodents. Previous studies have indicated that rodent species exhibit different levels of resistance to infection with *F. novicida* (18) and various strains of *F. tularensis* (7). Therefore, it was of interest to determine whether these differences were related to the ability of these organisms to proliferate inside macrophages. Resident peritoneal macrophages were harvested from mice, rats, or guinea pigs and infected in vitro with either *F. tularensis* LVS or *F. novicida* as described above. *F. tularensis* proliferated at a similar rate in macrophages harvested from the three species of rodents (Fig. 3). Likewise, *F. novicida* grew equally well in either mouse or guinea pig macrophages. However, *F. novicida* was unable to proliferate in macrophages isolated from rats, except for a slight increase in bacterial numbers early in the course of infection, such that the difference between bacterial growth in rat macrophages and that in mouse or guinea pig macrophages was greater than 100-fold by 24 h after the infection.

Localization of *Francisella* bacteria in the intracellular environment. To identify the intracellular compartment in which *Francisella* spp. grow, macrophages adherent to Thermanox coverslips were infected and processed for

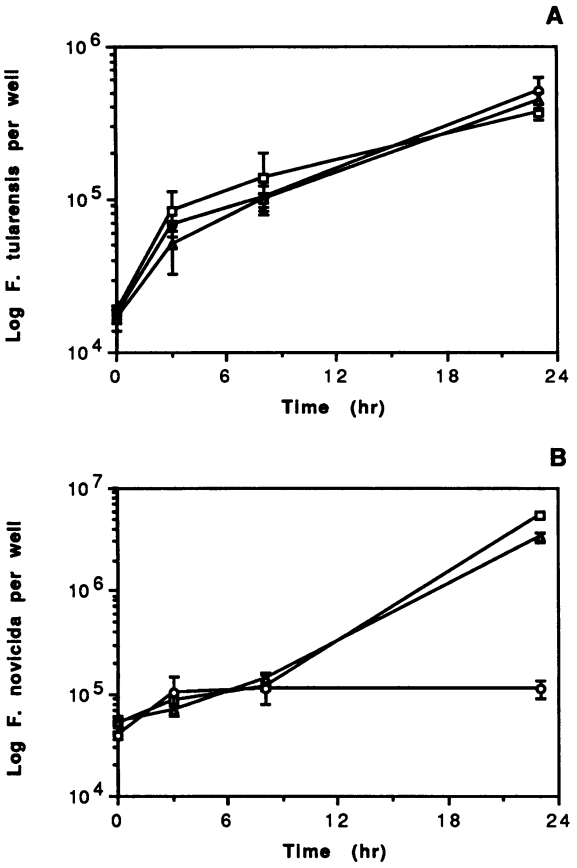

FIG. 3. Growth of *F. tularensis* LVS (A) and *F. novicida* (B) in resident peritoneal macrophages from mice (□), rats (○), or guinea pigs (△). The experimental procedure was as described in the legend to Fig. 2, and the results are expressed as the mean ± 1 standard deviation $\log_{10}$ *Francisella* CFU per microtiter well.

transmission electron microscopy 4 to 7 h later. *Francisella* bacteria appeared as round or elliptical electron-dense bodies located inside membrane-bounded phagocytic vesicles within the macrophages (Fig. 4). The vesicles contained one or more bacteria, and frequently the bacterial cells were surrounded by a clear space. Phagocytic vesicles containing bacteria also frequently contained membranous or amorphous bodies. Small clear vesicles (lysosomes) were commonly associated with the phagocytic vesicles.

To determine the intracellular location of ingested bacteria, macrophages were labeled before infection with colloidal thorium (24). In macrophages so treated, the electron-opaque thorium appeared as clusters of granular material (Fig. 5A, B, and E). Frequently, small clusters of granular material occurred adjacent to phagocytic vesicles containing *Francisella* spp. In macrophages infected with *F. novicida*, the proportion of phagocytic vesicles containing bacteria together with thorium was 13.2% (17 of 129 vesicles in nine macrophages). The proportion of vesicles containing both thorium particles and *F. tularensis* LVS was 22.5% (16 of 71 vesicles in five macrophages). In the control preparation in which macrophages were infected with nonpathogenic *Escherichia coli* K-12 (data not shown), 77.9% of the phagocytic vesicles contained thorium (60 of 77 phagosomes in five macrophages). The differences between *Francisella* spp. and *E. coli* in the percentage of fused phagolysosomes were tested for significance by using a Kruskall-Wallis nonparametric test on arcsine-transformed data. Both *Francisella* species were significantly different from the control (*E. coli*) organism ($0.005 < P < 0.01$).

In a separate experiment, macrophages infected with *F. novicida* were processed for acid phosphatase cytochemistry (10). Cells so treated typically had an electron-dense reaction product deposited over vesicular structures within the macrophage (Fig. 5C and D). In general, the reaction product was associated with vesicles that were distinct from bacterium-containing vesicles. Interestingly, when the reaction product was associated with bacterium-containing vesicles, it was most commonly deposited over a small vesicular inclusion within the phagocytic vacuole. Although this vesicle has the appearance of a lysosome, its intraphagosomal localization is unusual and its biological significance is as yet undetermined.

We have described a macrophage culture system in which *F. tularensis* LVS and *F. novicida* proliferate in different populations of macrophages, including resident peritoneal macrophages and macrophages derived in vitro from bone marrow progenitor cells. Although *Francisella* spp. grew to a small extent in tissue culture medium alone, the data indicate that the bacterial growth observed in macrophage monolayers is mainly due to intracellular replication. First, in this system, non-cell-associated bacteria were removed at the zero time point by extensive washing of the monolayers with medium, thus minimizing the ratio of extracellular to intracellular bacteria at the outset of infection. Second, the growth in tissue culture medium alone was not greater than 10-fold, whereas the growth in macrophage cultures exceeded 100- to 1,000-fold.

It is not known whether *Francisella* spp. are able to proliferate to a small extent extracellularly in vitro once they are released (by lysis) from the favorable growth environment of the macrophage. Tissue culture medium containing serum evidently contains a growth inhibitor or lacks the nutrients that are essential for sustained *Francisella* growth. Extracellular replication of *Francisella* spp. after release might therefore represent the division of bacteria that have already begun to proliferate intracellularly. In either case, *Francisella* spp. must depend upon the intracellular environment of the macrophage for extracellular growth, however limited that might be.

The mechanisms whereby *Francisella* spp. are able to resist the microbicidal activities and multiply within macrophages await further investigation. Some organisms, notably *Salmonella typhimurium* (5), *Leishmania* spp. (1, 6), and *Mycobacterium lepraemurium* (11), are apparently able to withstand the lysosomal killing mechanisms. Others, such as *Listeria monocytogenes* (27) and *Trypanosoma cruzi* (20), are able to escape from the phagosome and are evidently thus protected from lysosomal attack. A third mechanism of intracellular survival, exhibited by pathogens such as *Mycobacterium tuberculosis* (3, 4), *Legionella pneumophila* (13), *Toxoplasma gondii* (16), and *Chlamydia* spp. (9, 19), is the prevention of fusion of the phagosome with the lysosome. In this study, we report the results of electron microscopic examination of intracellular *Francisella* spp. (Fig. 4 and 5) that indicate that *Francisella* spp. may survive within a membrane-bounded phagocytic vesicle. In addition, there is some evidence suggesting that lysosomes fail to fuse with phagosomes containing *Francisella* bacteria. Although we observed a small percentage (13 to 23%) of bacteria within secondary lysosomes, this proportion is comparable to that found with *M. tuberculosis* (3, 4) and may reflect the fusion

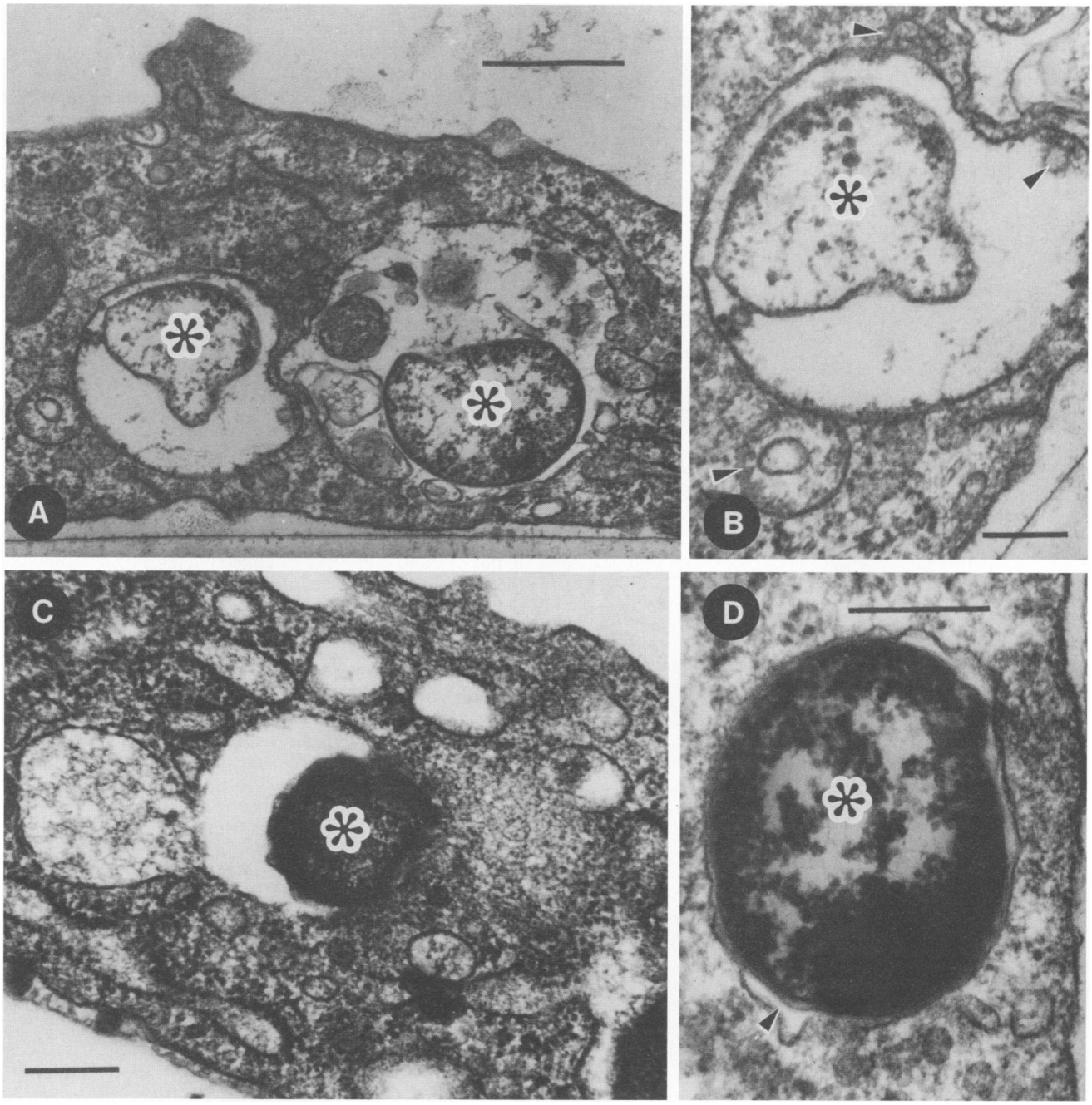

FIG. 4. (A) Transmission electron microscopy preparation of macrophages infected with *F. novicida*. Bacterial cells (*) occur within phagocytic vesicles along with other vesicular and amorphous material. Bar, 0.5 μm. (B) Detail of phagocytic vesicle containing *F. novicida* (*) within a macrophage. Note the small vesicles within the phagocytic vesicle and the small clear vesicles associated with the outer surface of the vesicle (arrows). Bar, 0.25 μm. (C) Phagocytic vesicle in macrophage containing *F. tularensis* LVS (*). Vesicles containing amorphous or electron-lucent material are commonly associated with bacterium-containing vesicles. Bar, 0.25 μm. (D) Detail of a phagocytic vesicle containing *F. tularensis* LVS (*). Note that the periplasmic space and the outer membrane are discernable, as is the membrane limiting the phagocytic vesicle. Bar, 0.25 μm.

of lysosomes with phagosomes containing degenerate bacteria.

One finding of particular interest in this study was the marked difference between *F. tularensis* LVS and *F. novicida* with respect to their relative growth rates within rat macrophages. It has been shown that rats are relatively resistant to death caused by infection with either *F. novicida* (18) or virulent *F. tularensis* (7). *F. tularensis* LVS is capable of growing in the livers and spleens of rats (17) to an extent comparable to that in the livers and spleens of mice (2), an observation consistent with our in vitro results. Thus, there must be other determinants of mortality besides visceral bacterial growth. The growth of *F. novicida* in the tissues of rats has not been reported; it is tempting to speculate that

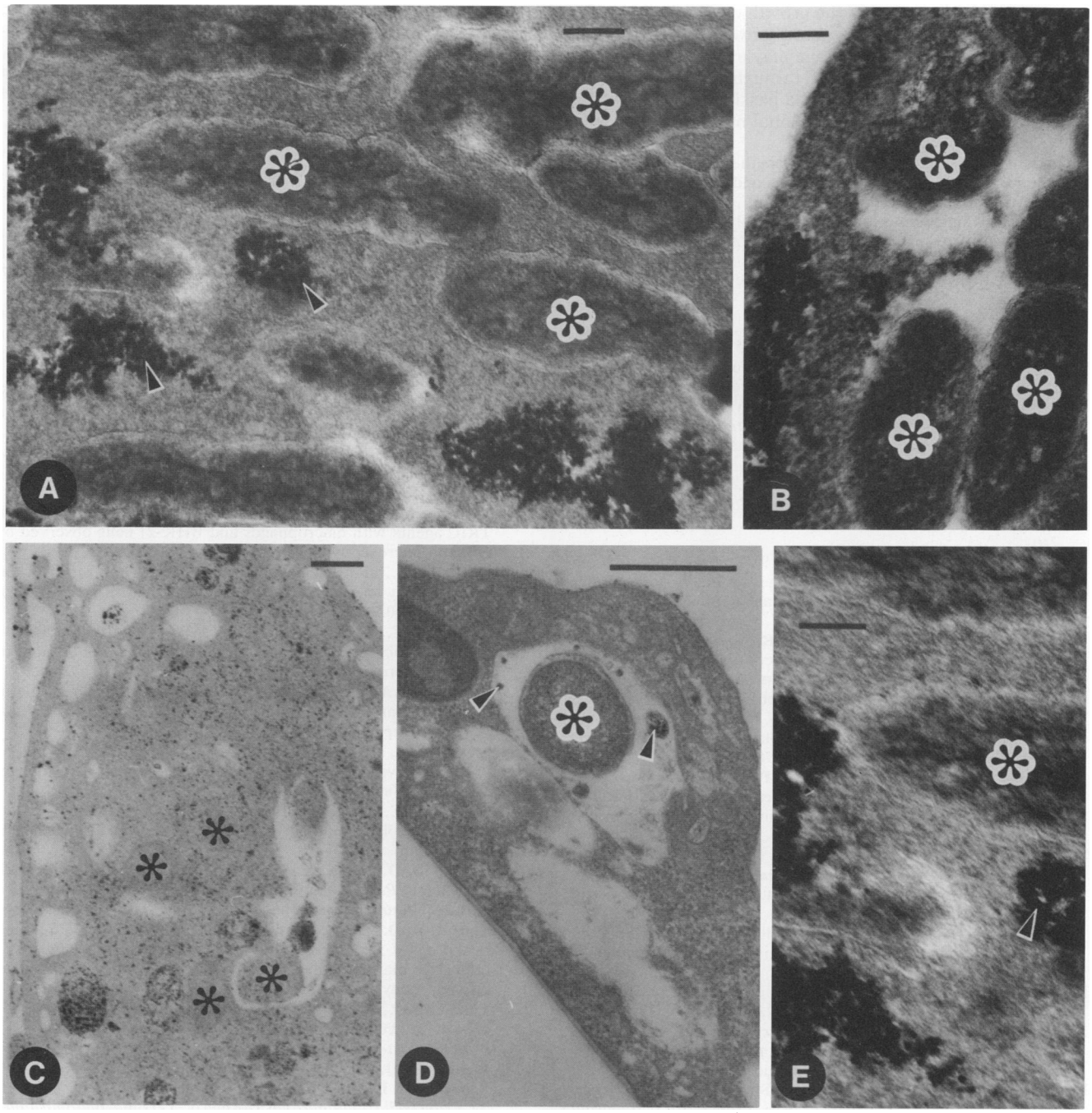

FIG. 5. (A and B) Transmission electron micrograph of a macrophage in which phagolysosomes are labeled with thorium (arrows). The phagocytic vesicles containing *F. novicida* (*) appear not to have fused with lysosomes but have thorium-containing vesicles associated with the outer edge of the phagosomes. Bars, 0.25 μm. (C and D) Sections of cells prepared with the Gomori cytochemical method for localization of acid phosphatases. The reaction product is not deposited over vesicles containing *F. novicida* (*). In some phagocytic vesicles (D), the reaction product is associated with the small vesicular inclusion (arrows). Bars, 1 μm. (E) Transmission electron micrograph of a macrophage preloaded with thorium and subsequently infected with *F. tularensis* LVS. The electron-opaque thorium particles (arrow) occur in clusters associated with the cytoplasmic surface of the phagocytic vesicles containing bacteria (*). Bar, 0.25 μm.

one of the reasons for the relative resistance of rats to infection with *F. novicida* is the inability of this organism to grow within rat macrophages.

Although is not yet apparent why *F. novicida* should be unable to grow within rat macrophages, a number of possibilities may be proposed. First, *F. novicida* may be deficient in a ligand, or rat macrophages may be deficient in a receptor, that is necessary to mediate phagocytosis. Indeed, the growth curve in rat macrophage cultures resembles that in medium in the absence of cells. Second, assuming that *F. novicida* gains entrance to the intracellular environment, it is possible that rat macrophages exert some bactericidal activ-

ity on *F. novicida* such that the growth curve obtained (Fig. 3B) is the net result of *F. novicida* growth and destruction. Alternatively, the rat macrophage may exert a bacteriostatic effect upon *F. novicida*, either through an active growth-inhibitory mechanism or through a passive process, such as depriving the organism of an essential nutrient for growth.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the British Columbia Health Care Research Foundation to F.E.N. and from the Natural Sciences and Engineering Research Council and the Medical Research Council of Canada to R.D.B. L.S.D.A. is supported by the Medical Research Council of Canada.

We thank J. L. Doran for the critical review of this paper.

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