Yersinia pestis Grows Within Phagolysosomes in Mouse Peritoneal Macrophages

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Transmission electron microscopy was used to determine the intracellular localization of *Yersinia pestis* growing within cultured resident peritoneal macrophages of mice. Monolayers fixed immediately, or as long as 4.5 h, after infection contained yersiniae closely surrounded by a membrane. The vesicles containing the yersiniae were determined to be phagolysosomes by labeling secondary lysosomes of macrophages with electron-dense particles of thorium dioxide. The presence of the label within vesicles containing yersiniae indicates that this pathogen grows within the lysosomal compartment of its target host cell.

Yersinia pestis, the causative agent of plague, is a facultative intracellular parasite of the mammalian reticuloendothelial system (10). Data are scarce concerning the intracellular location of this pathogen. Using electron microscopy, Finegold observed ultrastructurally intact bacilli within phagocytic vesicles in alveolar macrophages of rhesus monkeys that were infected with *Y. pestis* KIM-10 via aerosols (14). The closely related *Yersinia* species, *Y. pseudotuberculosis*, was observed by light microscopy to reside within vesicles in infected HeLa cells (25) but was found free in the cytoplasm of liver and spleen cells from mice dying of experimental pseudotuberculosis (L. A. Dreyfus and R. R. Brubaker, unpublished data cited in reference 4).

In an accompanying paper (23), we report the kinetics for growth of *Y. pestis* in cultured resident mouse peritoneal macrophages. The yersiniae had been grown at 26°C, a temperature that might be found in the gut of a flea. *Y. pestis* grown at this temperature expresses capsular antigen only weakly (15) and fails to show requirements for calcium (16, 27), nucleotides (26), or for the several nutrients (3) that are needed for growth at 37°C. This situation models initial events in infection by *Y. pestis*.

In this paper, we present an ultrastructural characterization of this system. Our goal was to determine the intracellular site in the host macrophage where *Y. pestis* can grow. We found that intracellularly growing yersiniae resided within phagolysosomes; they possess a mechanism for utilizing an environment in which *Escherichia coli* is killed and degraded. We discuss some implications of this finding for studies of the low-calcium-response virulence determinant of *Y. pestis*.

MATERIALS AND METHODS

Bacteria, bacteriophage, and their culture. Organisms and their culture are described in an accompanying paper (23). Bacteria were grown in either 3% NZ amine A (Sheffield Humko Chemical Co., Lyndhurst, N.J.) liquid medium (13) (see Fig. 1) or heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 0.2% D-xylose and 1 mM MgCl₂ (2) (see Fig. 2). Similar ultrastructural findings and kinetics of intracellular growth were obtained with bacteria grown in either medium.

Culture of macrophages. Macrophages were collected and cultured as described in an accompanying paper (23). For electron microscopy, a nominal 2×10^6 macrophages were cultured in 35-mm Nunclon dishes (Nunc/Intermed, distributed by GIBCO Laboratories, Grand Island, N.Y.).

Infection of macrophages. Bacteria were washed once with phosphate-saline buffer (solution "a" of Dulbecco phosphate-buffered saline [12]) and suspended in this buffer at an absorbance at 620 nm of 0.25 or 0.5 (ca. 3.5×10^7 or 7.0×10^7 cells per ml) unless otherwise indicated. Macrophage monolayers were washed twice with Hanks balanced salt solution (HBSS; GIBCO), overlaid with 0.1 ml of bacterial suspension at room temperature, and incubated for 15 min with occasional gentle rocking. Then, they were washed three times with HBSS, overlaid with 2.0 ml of HBSS plus 5% fetal bovine serum (GIBCO) that was heat inactivated (56°C, 30 min), and incubated. This suspending medium does not support growth of *Y. pestis* (unpublished data). A direct count made for macrophages exposed to ca. 3.5×10^7 bacteria grown in heart infusion medium and stained with acridine orange showed that 70% of the macrophages were infected, and on the average, six bacteria were associated with each infected macrophage.

Light microscopic observations and quantitative plating. Replicate cultures in 16-mm tissue culture wells were maintained alongside the 35-mm dishes and used as previously described (23) to characterize events taking place in samples to be used for electron microscopy.

Labeling of lysosomes with thorium dioxide. We labeled secondary lysosomes with electron-dense thorium dioxide particles, using Thoria Sol (Polysciences, Warrington, Pa.). The presence of these particles in vesicles containing *Y. pestis* or *E. coli* K-12 was used as evidence that lysosomes had fused with phagosomes containing these bacteria (1, 17). The labeling protocol was essentially that developed by Armstrong and Hart (1). This procedure resulted in deposition of electron-dense material only in secondary lysosomes and did not impair the microbicidal capability of the macrophages as judged by their ability to degrade *E. coli* K-12 (see below). Since thorotrast (17) is no longer available, we used Thoria Sol, which is supplied as an aqueous 2% colloidal suspension of 30-nm-diameter particles of thorium dioxide, sufficiently homogeneous in size to be useful as a size standard (data from the manufacturer). Particles of this size should be taken up by pinocytosis rather than phagocytosis (22) and accordingly can be used for direct labeling of secondary lysosomes. The colloid was destabilized and

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formed a macroscopic precipitate when the commercial preparation was diluted directly into "high-serum" medium; this was prevented by first diluting the (autoclaved) colloid 6.4-fold with sterile water. To this was added heat-inactivated fetal bovine serum and 10-fold concentrated stocks of medium 199 and NaHCO_3 (in that order) to give 0.18% Thoria Sol particles, 29% fetal bovine serum, and 71% medium 199 with HBSS.

Preparation of samples for electron microscopy. The macrophages were incubated in three changes of HBSS (a 5-min incubation followed by two 10-min incubations, each in fresh HBSS; 3 to 4 ml per 35-mm dish) to remove fetal bovine serum. The HBSS was replaced with 3 ml of freshly made 2.5% glutaraldehyde (Polysciences) in 0.1 M cacodylic acid plus 1 mM CaCl_2 (pH 7.4) (titrated with NaOH), and the dishes were placed at room temperature for 1 h. To complete the prefixation, the dishes were placed at 4°C overnight (ca. 18 h). The dishes were placed on ice, and the glutaraldehyde was removed by five changes (12 min each; 3 ml per dish) of ice-cold cacodylate buffer: 0.2 M cacodylic acid plus 1 mM CaCl_2 (pH 7.4) (NaOH). The macrophages, still on ice, were then fixed for 2 h with ice-cold 1% OsO_4 (Polysciences) in cacodylate buffer. The preparations were dehydrated through a series of ice-cold ethanol solutions: 50, 70, 80, and 95%, two changes of each (1 min followed by 3 min; 3 ml per dish) with occasional gentle swirling. The dishes containing 95% ethanol were allowed to come to room temperature, and the macrophages were incubated in four 10-min, 3-ml changes of 100% ethanol at room temperature. Then, ca. 3 ml of propylene oxide was added to each dish with swirling to float the polylysine film with its attached macrophages from the tissue culture dish (21). This was immediately transferred to ca. 1 ml of propylene oxide by means of a wooden applicator stick and subjected to two additional changes of propylene oxide (5 min each, ca. 2 ml). The samples were infiltrated in a 3-mm-deep layer of resin mixture containing 60% Poly/Bed 812 (Polysciences) and 40% propylene oxide in capped vials overnight and then in uncapped vials for ca. 3 h. Poly/Bed 812 was prepared according to the directions of the manufacturer. Infiltration was then continued for 3 to 6 h more in a fresh change of full-strength resin mixture, with occasional swirling. The monolayers were transferred to fresh resin in BEEM capsules and embedded for 48 h at 60°C. Silver-gold sections were stained for 5 min with 2% uranyl acetate in 25% ethanol and 5 min with lead citrate (20) and examined with a Philips 301 electron microscope operated at 80 KV, with 25- and 200- μm objective and condenser apertures, respectively.

RESULTS

Conditions for morphological studies. At high multiplicities of infection (MOI), the macrophages apparently were saturated with respect to their ability to support bacterial uptake and growth. An MOI of 100, 500, or 3,000 resulted in the same number of yersiniae per well after infection and washing of the macrophages (1×10^6 to 2×10^6 CFU per well), and no growth occurred during 10 h of incubation (data not shown). This problem was eliminated by lowering the MOI to 35.

It was important to confirm that the dynamics of intracellular growth after infection at this MOI were similar to those found previously with lower MOI; accordingly, we compared the growth of *Y. pestis* KIM after infection at MOI of 3.5 and 35. The 10-fold difference in MOI simply resulted in a 10-fold difference in bacteria initially present in the system, with no difference in the apparent generation time (1.5 h).

***Y. pestis* growing in macrophages is surrounded by a membrane.** With the electron microscope, we saw that each intracellular bacterium was surrounded by a membrane that was closely apposed to the bacterial wall (Fig. 1). The membrane appeared to have discontinuities (e.g., Fig. 1A, arrows), but these may have arisen from oblique sections through foldings; the membrane often was observed to have a wavy contour, and a faint continuation of the membrane profile sometimes could be seen within regions of apparent discontinuity. An observation of potential significance was that the *Y. pestis*-containing vacuoles often contained small vesicles, some of which appeared to be closely associated with the bacterial outer membrane, perhaps blebbing from it (Fig. 1B and C).

Intracellular *Y. pestis* resides within phagolysosomes. To determine whether the yersinia-containing vacuoles were phagolysosomes, we labeled secondary lysosomes of macrophages with thorium dioxide and examined *Y. pestis*-containing vacuoles for the presence of the electron-dense label.

To test the degradative capabilities of these macrophages, we exposed them for 1 h to *E. coli* K-12, well known to be readily killed by macrophages. In all specimens observed, *E. coli* cells within macrophages were in various stages of degradation within vacuoles also containing thorium dioxide particles. This shows that these macrophages had the ability to degranulate and degrade *E. coli*. However, macrophages cultured the same way permitted *Y. pestis* to retain ultrastructural integrity (Fig. 2) despite degranulation; intracellular *Y. pestis* resides within the lysosomal compartment of the cell. Figure 2B shows a specimen in which lysosome-phagosome fusion was taking place at the time of fixation.

DISCUSSION

We have begun to characterize the environment in which *Y. pestis* grows in its host target cell, the resident macrophage. In plague, monocytes and macrophages presumably represent a privileged environment (10, 23), available immediately upon injection of the bacteria by flea bite. In our study, we have modeled initial events in infection by exposing cultured resident peritoneal macrophages to yersiniae grown at 26°C, a temperature that might be found in a flea and at which *Y. pestis* is not heavily encapsulated and does not show several nutritional requirements seen at 37°C. Our major finding is that this facultative intracellular parasite grows within phagolysosomes.

One outcome of our preliminary investigations may deserve pursuit in future experiments: our initial experiments with high MOI resulted in a fixed number of bacteria per well (ca. 10^6) and a failure of that number to increase upon continued incubation. Significantly higher bacterial yields resulted consistently when the initial infection was made with lower MOI, suggesting that yersiniae present at high density exerted a direct toxic effect as opposed to initial saturation of the system with respect to the supply of nutrients for bacterial growth. Electron microscopic observations of monolayers infected at high MOI showed that ultrastructurally intact yersiniae were intracellular and enclosed within a vesicle (data not shown), so the apparent toxicity did not block growth by preventing uptake into the cell. It is likely that the macrophage actively contributes to the maintenance of a proper environment for development of *Y. pestis* and that this process is disrupted at high MOI.

The *Y. pestis* used in this study and *E. coli* K-12 were taken up in similar numbers (data not shown), probably via phagocytosis, whereby they enter the lysosomal environment. However, *E. coli* was rapidly degraded in our experi-

FIG. 1. Transmission electron photomicrographs of *Y. pestis* within cultured peritoneal macrophages of mice. These specimens illustrate the membrane (M) that was always found surrounding intracellular *Y. pestis* (Y). Since these specimens were fixed for electron microscopy late in infection, it was common to observe several bacteria in a vacuole. B and C illustrate the tiny vesicles (V) frequently seen associated with the bacterial outer membrane, sometimes apparently blebbing from it. Bars represent 0.1 μm .

FIG. 2. Transmission electron photomicrographs of *Y. pestis* KIM in thorium dioxide-labeled phagolysosomes within cultured resident peritoneal macrophages of mice. Lysosomes in macrophages were labeled with electron-dense thorium dioxide particles, and the macrophages were then infected with *Y. pestis* KIM (MOI of 35) and incubated for 1 h before fixation for electron microscopy. (A) Label can be seen within the tightly fitting phagolysosome, indicating that degranulation has occurred. (B) Specimen illustrating the fusion of thorium dioxide-laden lysosomes with phagosomes containing yersiniae. Bars represent 0.5 μ m. Labels are *Y. pestis* (Y), phagolysosomal membrane (PL), lysosome (L), polylysine film (F) from the tissue culture dish, to which some thorium dioxide particles have adhered, and label probably associated with a phagolysosome viewed in a "polar-cap" section (+).

ments, whereas the yersiniae thrived. Therefore, we believe that *Y. pestis*, like *Salmonella typhimurium* (9) and *Coxiella burnetii* (7, 8), grows within phagolysosomes; perhaps the phagolysosomal membranes disappear late in the infection of a macrophage, when this cell has become necrotic. Such has been observed by Finegold (14) and Dreyfus and Brubaker (cited in reference 4). Our study may have failed to include the terminal stages of the infection cycle, because moribund macrophages tended to detach from the dishes and may have been removed by the many washings used in sample preparation for electron microscopy.

Our study has some important implications for studies of one of the virulence determinants of *Y. pestis*, known as the Vwa⁺ phenotype (19) or low-calcium response (J. D. Goguen, J. Yother, and S. C. Straley, submitted for publication). This determinant includes the coordinate expression of two properties: (i) the requirement for calcium or a nucleotide for growth at 37°C (6, 16, 26) and (ii) synthesis of two protein antigens called the plague virulence antigens V and W. In vitro, these antigens are synthesized only at 37°C and in the absence of calcium, i.e., in a simulated cytoplasmic environment (5, 24). Accordingly, it has been thought that they mediate events during intracellular growth of yersiniae. The V antigen is a protective antigen and is believed to be important in the pathogenesis of plague (18), but the mechanism for this protection is unknown. The present study suggests that in vivo, *Y. pestis* may be taken into a macrophage along with a small volume of calcium-containing extracellular fluid and may reside within a space that is topologically outside of the cell. If this topology is maintained with respect to the direction of calcium transport, then the virulence antigens may not be expressed by *Y. pestis* during growth within the phagolysosome. Perhaps only just before bursting of the macrophages are the bacteria free in the cytoplasm in which they express V and W. According to this model, the V antigen may mediate extracellular events, such as subsequent encounters with neutrophils or activated macrophages. This could account for the ability of this antigen to be protective against experimental plague in mice. We currently are testing these ideas.

A second point concerns the intracellular growth environment of *Y. pestis*. Recent physiological studies characterizing this low-calcium response were made, using neutral or alkaline pH (11, 26, 27; Goguen et al., submitted). The present study points to the importance of including acidic conditions among those examined, since the phagolysosomal environment in which *Y. pestis* resides during intracellular growth probably is acidic.

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