NOTES

An In Vitro Model System Used To Study Adherence and Invasion of Francisella tularensis Live Vaccine Strain in Nonphagocytic Cells

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In observing Francisella tularensis interactions with nonphagocytic cell lines in vitro, we noted significant adherence, invasion, and intracellular growth of the bacteria within these cells. Francisella tularensis live vaccine strain invasion of nonprofessional phagocytic cells is inhibited by cytochalasin D and nocodazole, suggesting that both the actin and microtubule cytoskeletons are important for invasion.

Francisella tularensis is a highly virulent intracellular bacterial pathogen that causes tularemia in a wide variety of hosts (46). While infection is primarily transmitted through the bite of an infected arthropod or by contact with infected animal material, the capacity of this organism to cause pneumonia infection at a very low dose led to its weaponization by several nations and classification by the CDC as a category A select agent (47). While many laboratories have studied the interaction of F. tularensis with phagocytes, little is known about the interactions of this bacterium with nonphagocytic cells. However, in murine experiments, F. tularensis LVS has been observed within hepatocytes (11, 30), alveolar type II cells (14), and, potentially, early hepatic lesions (39). Other work has found that F. tularensis is protected from gentamicin in human umbilical vein endothelial cells (16) and HepG2 cells (38). In this work, we aimed to quantify and visualize the invasion of several types of nonphagocytic cells by F. tularensis LVS to begin to characterize the cellular mechanisms by which these interactions occur in vitro.

One research group has recently observed structures resembling type IV pili on the surface of LVS (20). In order to ascertain whether F. tularensis LVS is adherent to cells, cell association assays were performed with HeP-2 cells, human bronchial epithelial (HBE) cells, and A549 tissue culture cells. F. tularensis LVS was grown in modified Mueller-Hinton broth (3) supplemented with 150 mM NaCl to an optical density at 600 nm of ~0.3 to 0.5, added to minimal essential medium with 10% fetal bovine serum at a multiplicity of infection of 0.3 to 0.5, and centrifuged at 600 × g to facilitate interactions with cells. After 1 h, the cells were washed with phosphate-buffered saline (PBS), and the monolayer was solubilized with PBS containing 1% saponin before being plated. This treatment did not affect the viability of LVS when plated on modified Mueller-Hinton agar with 0.5% sheep blood (data not shown). Adherence levels of F. tularensis LVS were 0.9% ± 0.04% for HeP-2 cells, 0.5% ± 0.01% for HBE cells, and 0.5% ± 0.08% for A549 cells. The mean CFU recovered per well for each cell type was statistically significantly different from the level of recovery of bacteria that were added to wells without tissue culture cells and treated with gentamicin (P < 0.001).

To more fully characterize F. tularensis adherence, we performed bacterial adherence assays and examined the interactions between LVS and cells using confocal microscopy. Bacteria, labeled with Fluorescein isothiocyanate (FITC) (Molecular Probes) and detected by Alexa 488-conjugated goat anti-rabbit immunoglobulin G (IgG) (Invitrogen) binding, were routinely observed attached to the surfaces of HeP-2, HBE, or A549 cells (Fig. 1). These bacteria appeared to bind specifically to the surfaces of the cells; very few organisms bound to the glass coverslip. These observations provide complementing evidence that the quantitative adherence assay is measuring bacterial attachment to tissue culture cells and not to the tissue culture plate. Additionally, the numbers of bacteria that were attached to the tissue culture cells were consistent with results obtained using the quantitative adherence assay (0.5% to 0.9% adherence is equivalent to 0.5 to 0.9 bacteria per cell).

While examining the 1-h adherence assays, it became apparent that some bacteria were located within the tissue culture cells. Preliminary observations did not detect visible actin re-arrangements as a part of the host cell interactions, in contrast to the interactions that occur with bacterial pathogens such as Salmonella and Shigella spp. and enteropathogenic Escherichia coli. In order to quantitatively assess the level at which F. tularensis is able to invade tissue culture cells, we adapted a gentamicin protection assay that is used to study Francisella bacterium-macrophage interactions and that has been used by us and others to study Salmonella invasion (22, 25, 29, 38). After allowing bacteria to interact with the cell monolayers for 4 h, treating them with 10 μg/ml gentamicin for 1 h, and then washing and lysing them as described above, ~5 × 10^3 to ~5 × 10^4 CFU were consistently recovered from each of the three cell types, representing 0.05% to 0.1% of the inoculum. As a control, we confirmed that treatment of F. tularensis LVS with gentamicin in the absence of eukaryotic cells sterilized the well.
to the limit of detection (<20 CFU/ml). F. tularensis entry (i.e., gentamicin protection) steadily increased in each of the cell types up to 4 h postinoculation (data not shown). Time points beyond 4 h were not useful since intracellular growth of the internalized bacteria obscured the data (8). Due to these results, we used 4-hour incubation times in our standard assays.

To confirm the results of the quantitative invasion assay, we examined F. tularensis interactions with tissue culture cell lines by using confocal microscopy. Coverslips were fixed with 3.7% paraformaldehyde for 15 min, permeabilized with 0.2% Tween 20 in PBS, and labeled as described above. Cellular actin was visualized with rhodamine phalloidin according to the manufacturer's instructions (Invitrogen). A primary goal of these experiments was to confirm that the organisms were located physically within the host cells. As shown in Fig. 2A, C, and E, bacteria are clearly associated with the tissue culture cells, with virtually no organisms adhering to the glass slide. Using ImageJ software (http://rsb.info.nih.gov/ij/), we could demonstrate that the cell-associated bacteria were intracellular because vertical digital slices (Fig. 2B, D, and F) of the areas indicated in Fig. 2A, C, and E demonstrate that the bacteria are surrounded by actin-associated membranes. Virtually the same results were obtained for each of the tissue culture cell lines tested (Fig. 2).

In order to preliminarily determine what cellular mechanisms might be required for the internalization of F. tularensis LVS, HEP-2 tissue culture cells were treated with either 2 μg/ml cytochalasin D to inhibit actin filament polymerization or 10 μg/ml nocodazole to inhibit microtubule polymerization from 30 min prior to infection, and 1-h invasion assays were performed. The treatment of HEP-2 cells with cytochalasin D almost completely abrogated the entry of bacteria into HEP-2

FIG. 1. Confocal microscopy of F. tularensis LVS adherence to A549 (A), HEP-2 (B), and HBE cells (C). Images were taken 1 h postinfection and are z projections of stacks of images. F. tularensis LVS (white) was detected with rabbit Francisella tularensis antiserum and goat anti-rabbit IgG conjugated to Alexa 488; F-actin was stained with rhodamine phalloidin (gray). The white arrows in each panel indicate some of the adherent bacteria present on each cell type. Bars, 10 μm.

FIG. 2. Confocal and transmission electron microscopy of F. tularensis entry into A549 cells (A, B), HEP-2 cells (C, D), and HBE nonphagocytic tissue culture cells (E, F, G). (A, C, and E) Z-projections of stacks of images of a 4-h invasion assay showing bacteria (green) that are located beneath the membrane of the infected nonphagocytic cells. White lines indicate areas showing that bacteria are surrounded by actin-associated membranes. (B, D, and F) Selected slices through the z projections stacks along the indicated lines. Bacteria are immunolabeled green with primary rabbit Francisella tularensis antiserum and secondary goat anti-rabbit IgG conjugated to Alexa 488. Cellular F-actin is stained red with rhodamine phalloidin. (G) Transmission electron microscopy of HBE cells infected with the virulent F. tularensis strain 1547-57 for 1 h. Arrows indicate the internalized bacteria. Experiments performed with LVS detected similar internalization events (data not shown). Bars, 10 μm (A, C, E), 5 μm (B, D, F), and 500 nm (G).
cells (2.4% ± 3.1% of wild-type entry). The treatment of cells with nocodazole reduced the invasion of HEp-2 cells by LVS to 26.9% ± 12.6% of that of untreated cells. *Salmonella enterica* serovar Typhimurium was included as a control for the efficacy of cytoskeletal disruption since *Salmonella* invasion requires actin polymerization (17, 19, 21, 26). As expected, serovar Typhimurium invasion was reduced to 1.4% ± 0.1% by treatment with cytochalasin D, but nocodazole treatment did not significantly reduce the invasion of HEp-2 cells by *Salmonella*. These data indicate that both the actin and microtubule cytoskeletons are important for the invasion of HEp-2 cells by LVS. We extended our characterization of *F. tularensis* entry into epithelial cells by incubating virulent *F. tularensis* subsp. *holarctica* strain 1547-57 or *F. tularensis* LVS with HBE cells and examining the interactions by transmission electron microscopy. As shown in Fig. 2G, two organisms were observed inside of an HBE cell, apparently having just entered the cells. It is unclear whether the intracellular organisms were confined within a vacuolar membrane or whether they had escaped into the cell cytoplasm. Virtually the same results were obtained when infecting HBE cells with *F. tularensis* LVS (data not shown).

To determine whether the invasion of tissue culture cells by *F. tularensis* LVS was followed by significant bacterial replication, samples were examined by confocal microscopy at 8 h and 24 h postinfection. At 8 h postinfection, groups of dividing bacteria were observed within tissue culture cells (data not shown). At 24 h postinfection, significant bacterial growth was observed in each of the three tissue culture cell types (Fig. 3). In the majority of instances, the bacterial growth was clumped together as microcolonies within the cytoplasmic space of the cell. At 24 h, these microcolonies typically surrounded the nucleus of the cell, which was apparent by the cellular space that lacked significant bacterial growth. Occasionally, the intracellular bacteria displayed unusual immunostaining patterns, although this was not consistently observed (Fig. 3B). In some instances, lysed eukaryotic cells with visible gaps in their cortical actin staining were observed with bacteria apparently exiting the cell (data not shown).

To quantify the growth within the cells, intracellular growth curve experiments were performed with HEp-2, HBE, and A549 cells. Wells were infected and treated as for the invasion assays described above, but after 1 h, gentamicin was removed and fresh medium was added. Cells were lysed with PBS and 1% saponin at appropriate time points. Viable-cell counts of well lysates showed that the bacteria began to multiply at 5 h postinfection and continued to grow at a steady rate up to the 25-h time point. Within this 20-h time period, the bacterial load increased ~1,000-fold in each of the three tissue culture cell types (growth differences between cell lines were not statistically significant). The rate of growth was comparable to that of the organisms in optimized modified Mueller-Hinton broth and was similar to that observed in macrophage studies. After 24 h in tissue culture, some cells could be seen detached from the surface, although the cell monolayer remained largely intact. These data demonstrate that *F. tularensis* LVS is capa-
ble of invading and replicating within nonphagocytic tissue culture cells, indicating that entry into and growth within nonphagocytic cells during Francisella infection may contribute to pathogenesis and disease progression.

In this work, we have presented an initial characterization of the ability of F. tularensis LVS to adhere to and invade nonphagocytic cells. It is well established that the virulence of F. tularensis depends upon the ability to grow within host cells. These bacteria can enter macrophages, from various hosts, via CR3 receptor (7, 9, 10), mannose receptor (4, 44), or scavenger receptor A (36) and replicate (1, 2, 15, 18, 32, 34, 42, 45). Studies of interactions between host macrophages and F. tularensis have identified genes (i.e., mglA, igLA, igLB, igLC, igLD, pdpA to -D, and acpA) that are involved in modifying the macrophage intracellular environment to permit intracellular replication (2, 5, 6, 27, 28, 33, 35, 40–43). The molecular details of these modifications are not well understood, but the Francisella intracellular compartment is clearly different from a typical phagolysosome (31, 42, 43).

The work described here contributes to the emerging picture of F. tularensis pathogenesis and is consistent with the findings of others (11, 14). Entry into nonphagocytic cells is typically an active process for the microorganism since the cells typically lack innate uptake mechanisms for large particles. Our observations that F. tularensis efficiently adheres to, enters, and replicates within nonphagocytic hepatocytes and alveolar type II epithelial cell lines provide additional evidence that the interactions of F. tularensis with nonphagocytic cells may play an important role in its virulence strategy. Work is under way in our group to identify the factors involved in entry and to characterize the mechanism of action.

Another aspect of this work is that the development of this tissue culture model will allow comparisons of the intracellular growth mechanisms of F. tularensis within phagocytic and nonphagocytic cells. While it is likely that growth mechanisms in phagocytic and nonphagocytic cells will overlap significantly, it is also possible that F. tularensis will interact uniquely with each cell type due to differences in entry and/or differences in the signals received from different cells. Identification and characterization of the virulence factors required in each intracellular environment would further elucidate the unique requirements for survival of intracellular bacterial pathogens like F. tularensis in host cells.

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