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Original article

Smooth *Brucella* strains invade and replicate in human lung epithelial cells without inducing cell death

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Abstract

Inhalation is a common route for *Brucella* infection. We investigated whether *Brucella* species can invade and replicate within alveolar (A549) and bronchial (Calu-6 and 16HBE14o-) human epithelial cells. The number of adherent and intracellular bacteria was higher for rough strains (*Brucella canis* and *Brucella abortus* RB51) than for smooth strains (*B. abortus* 2308 and *Brucella suis* 1330). Only smooth strains exhibited efficient intracellular replication (1.5–3.5 log increase at 24 h p.i.). A *B. abortus* mutant with defective expression of the type IV secretion system did not replicate. *B. abortus* internalization was inhibited by specific inhibitors of microfilaments, microtubules and PI3-kinase activity. As assessed with fluorescent probes, *B. abortus* infection did not affect the viability of A549 and 16HBE14o- cells, but increased the percentage of injured cells (both strains) and dead cells (RB51) in Calu-6 cultures. LDH levels were increased in supernatants of Calu-6 and 16HBE14o- cells infected with *B. abortus* RB51, and to a lower extent in Calu-6 infected with *B. abortus* 2308. No apoptosis was detected by TUNEL upon infection with smooth or rough *B. abortus*. This study shows that smooth brucellae can infect and replicate in human respiratory epithelial cells inducing minimal or null cytotoxicity.

Keywords: *Brucella*; Respiratory epithelium; Invasion; Replication

1. Introduction

Brucellosis is a worldwide distributed zoonotic disease caused by *Brucella* species, which can be transmitted from animals to humans by several ways. Inhalation of infected aerosols is one of the most common routes for human infection. Outbreaks of human brucellosis due to airborne transmission have occurred in abattoirs [1], laboratories [2], and rural settings [3]. Notably, brucellosis is considered the commonest laboratory-acquired infection, and aerosols have been implicated in most of these cases [4]. The fact that *Brucella* can be easily aerosolized and easily transmitted through inhalation greatly contributes to its consideration as a potential biological weapon [5]. *Brucella* species are currently listed within category B in the bioterrorism agent lists from CDC and NIAID.

In spite of the importance of the airways for *Brucella* entry to the organism, the respiratory route of infection has been explored in only a few animal studies evaluating vaccines [6] or the kinetics of systemic spread after inhalation [7]. However, the interaction of *Brucella* with the pulmonary tissues has not been studied, and it remains unknown how the bacterium reaches the systemic circulation from the airways. It can be speculated that the bacterium infects some pulmonary cells, either transiently or persistently, before gaining access to the systemic circulation. Notably, in mice infected with aerosolized *Brucella abortus*, lung CFU counts increase steadily until week 4 p.i. and remain high at least until week 8 p.i. [8]. Therefore, *B. abortus* seems to be able to replicate and persist within the lung, at least in mice.

Several pathogens usually acquired through inhalation can invade epithelial respiratory cells, including *Mycobacterium*

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tuberculosis [9], *Francisella tularensis* [10], *Burkholderia cepacia* [11], and *Bacillus anthracis* [12], and most are also capable of intracellular replication in such cells. Epithelial infection may result in the induction of a proinflammatory response and/or cell death by necrosis or apoptosis. *M. tuberculosis* and *B. anthracis* induce the death of alveolar epithelial cells, probably allowing these bacteria to disrupt the epithelial layer and gain access to blood vessels [12–14]. Moreover, it has been shown that *M. tuberculosis* released from lysed alveolar epithelial cells can infect adjacent cells in the monolayer [15].

While it is well established that *Brucella* invades and replicates within phagocytic and nonphagocytic cells [16,17], no studies have been performed on the interaction of these bacteria with lung epithelial cells.

In the present study we have investigated whether different *Brucella* species can adhere, invade and replicate within alveolar and bronchial human epithelial cells. We have also addressed the type of internalization involved and the effect of the infection on cell viability.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. abortus 2308, *Brucella suis* 1330 (both smooth virulent strains), *B. abortus* RB51 (rough vaccine strain), a local isolate of *Brucella canis* (rough virulent strain), and a *virB10* polar mutant derived from *B. abortus* 2308 were grown in tryptic soy broth at 37 °C with agitation. For *B. abortus* expressing green fluorescent protein (GFP) the medium was supplemented with 50 μg/ml kanamicin. Bacteria were washed twice with sterile phosphate buffered saline (PBS) and inocula were prepared in sterile PBS on the basis of the OD readings, but the actual concentration was later checked by plating on agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

2.2. Cell culture

The A549 cell line (human type II alveolar epithelial) was grown in DMEM supplemented with 2 mM L-glutamine, 10% FBS (Gibco, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. The human bronchial epithelial cell line Calu-6 was grown in supplemented RPMI 1640 (Gibco). The 16HBE14o- human cell line, established from normal bronchial epithelium by transfection with the SV40 genome defective in the origin of replication (kindly provided by Dr. Dieter Gruenert, University of California, San Francisco), was grown in fibronectin/collagen/BSA-coated flasks in supplemented minimal essential medium. For infection assays, all epithelial cell lines were seeded at 2×10^5 cells/well in 24-well plates and cultured in a 5% CO_2 atmosphere at 37 °C for 24 h in antibiotic-free culture medium.

2.3. Cellular infections

Infections were performed at multiplicities of infection (MOI) of 200 bacteria/cell. After dispensing the bacterial suspension the plates were incubated for 2, 4 or 16 h at 37 °C under 5% CO_2 atmosphere. At the end of this incubation (time 0 p.i.), each well was washed three times with sterile PBS. For quantification of intracellular bacteria, the infected monolayers were incubated in the presence of 100 μg/ml of gentamicin (Sigma, USA) and 50 μg/ml of streptomycin (Sigma) to kill extracellular bacteria [18,19]. At different times after antibiotics addition (2, 24 or 48 h) cells were washed with sterile PBS and lysed with 0.2% Triton X100. Serial dilutions of the lysates were plated on agar to enumerate colony forming units (CFU). The number of adherent bacteria was obtained by subtracting the number of intracellular bacteria from the number measured in the absence of antibiotics, both at 2 h p.i.

2.4. Inhibition of internalization

Infection experiments in the presence of specific inhibitors were carried out to examine whether *B. abortus* 2308 internalization by alveolar epithelial cells depends on actin polymerization (cytochalasin D), microtubules (colchicine), PI3-kinase signalling (LY294002), or bacterial protein synthesis (chloramphenicol). Cytochalasin D (5, 2.5, 1 and 0.5 μg/ml) and LY294002 (50, 25, and 10 nM) were solubilized in dimethyl sulfoxide (DMSO). Colchicine (10, 5 and 1 μM) and chloramphenicol (100 and 50 μg/ml) were solubilized in water. All inhibitors were obtained from Sigma, and the concentrations used were based on similar studies in cultured epithelial cells [9,20]. The individual inhibitors were added to A549 monolayers 1 h prior to the addition of *B. abortus*, and were maintained throughout the infection period. Control wells without inhibitors were infected in parallel. Plates were centrifuged for 10 min at room temperature and incubated for 2 h before replacing the culture medium with medium containing gentamicin and streptomycin. The number of viable intracellular bacteria (CFU) was determined 2 h later as described above. At the concentrations used, neither inhibitors nor DMSO had an effect on epithelial cell viability.

2.5. Confocal microscopy

Cells seeded onto glass coverslips were infected overnight with GFP-labeled *B. abortus*, washed and treated with gentamicin/streptomycin as described above. At different times post-infection the coverslips were fixed with 4% paraformaldehyde and mounted. To quantify infected cells, the coverslips were examined by confocal microscopy (C1 Confocal Microscope, Nikon, USA), using 60× plan oil immersion lens. Pictures were acquired and processed using Photoshop software (Adobe System Inc., USA).