Persistence of *Burkholderia multivorans* within the Pulmonary Macrophage in the Murine Lung

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Members of the *Burkholderia cepacia* complex (BCC) are opportunistic pathogens in patients with cystic fibrosis (CF) (8). In Canada, the outcome of infection with members of the BCC appears to be species dependent (16). The worst prognoses are associated with *Burkholderia cenocepacia* infections, which have the highest rates of transmissibility and mortality. *Burkholderia multivorans* infections are also common among CF patients, but these infections are associated with lower rates of transmissibility and mortality. The mechanisms underlying the pathogenic differences in these two species are poorly understood (7). Many potential virulence factors for BCC clinical isolates have been identified and evaluated, including a type III secretion system, the cable pilus, flagellin, and ornibactin (9, 13, 15, 18, 19, 21). However, these determinants are typically studied in epidemic CF isolates and therefore almost always represent a virulence determinant for *B. cenocepacia*. A cross-species comparison of BCC virulence in a pulmonary model has not been done previously.

We used a model of pulmonary infection that shows clear differences between infection with *B. cenocepacia* and infection with *B. multivorans*. In this intranasal infection model of BALB/c mice, *B. cenocepacia* strain C6433 promoted a more vigorous host response and was rapidly cleared from the lung, whereas *B. multivorans* strain C5568 established a persistent infection in pulmonary macrophages. *B. multivorans* C5568 and *B. cenocepacia* C6433 were recovered from patients with CF and are part of the Canadian *B. cepacia* Research and Referral Repository. They were stored at −70°C in Mueller-Hinton broth. Animal studies were approved by the University of British Columbia Animal Care Committee (UBC-ACC no. A98-0138). Intranasal infection experiments were conducted with healthy female mice as previously described (5). All results are expressed below as means ± standard errors of the means. All data were analyzed by the Mann-Whitney test for nonparametric, unpaired values. *P* values of <0.05 were considered statistically significant.

*B. multivorans* C5568 persisted in the lungs of BALB/c mice, while *B. cenocepacia* C6433 was cleared (Fig. 1A). Mice infected with *B. multivorans* sustained a pulmonary infection for 4 days. In contrast, *B. cenocepacia* was cleared quickly from the lungs of infected mice.

The general health of animals was assessed daily by using a three-point system based upon weight, food consumption, and general appearance. Animals were observed for their responses to infection and were considered unwell when two of the following three observations were made: >7% weight loss, <3 g of food consumed daily, or appearance of systemic illness (ruffled coats, huddled position, lethargy). *B. cenocepacia* C6433 infection caused more weight loss than *B. multivorans* C5568 infection (Fig. 1B). The mean weight loss in animals infected with *B. cenocepacia* was significantly greater (*P* = 0.004) than the mean weight loss in animals infected with *B. multivorans* on days 1 through 3 of the experiment. A higher proportion of *B. cenocepacia*-infected animals than of animals given *B. multivorans* displayed other signs of systemic illness on days 1 and 2 postinfection. On day 1, 62.5% of the mice (30 of 48 mice) challenged with *B. cenocepacia* but only 27.6% of the mice (13 of 47 mice) challenged with *B. multivorans* appeared to be ill. On day 2 postinfection, 60.6% of the mice (20 of 33 mice) challenged with *B. cenocepacia* appeared to be ill, compared with 32.0% of the mice (8 of 25 mice) infected with *B. multivorans*.

Analyses of pulmonary cells and cytokines were carried out with groups of three mice per time point. Bronchoalveolar lavage (BAL) was performed on euthanized mice in situ as previously described (4). Cells were counted with a hemocytometer; 100-μl aliquots were deposited onto glass slides by using a cytocentrifuge and were stained with Hemacolor (EM Diagnostic Systems, Gibbstown, N.J.) for differential counting. *B. cenocepacia* C6433-infected mice displayed greater pulmonary host responses than mice infected with *B. multivorans* C5568 (Fig. 2). The number of neutrophils in the lungs of infected mice peaked on day 1 of infection for both the *B. multivorans*- and *B. cenocepacia*-challenged groups (Fig. 2B). The maximal number of neutrophils was significantly greater in the BAL fluid of mice infected with *B. cenocepacia* than in the BAL fluid of mice infected with *B. multivorans* (*P* = 0.0173). The number of neutrophils in the lungs of both...
groups diminished quickly thereafter, and there was no difference between B. multivorans- and B. cenocepacia-challenged mice in the recovery phase. The number of macrophages recovered from the BAL fluid of infected mice increased on day 1, peaked on day 2 of infection, and was sustained at day 4. There was no difference in the numbers of macrophages recovered from the BAL fluids of mice infected with the two strains (data not shown).

BAL fluid was frozen at −70°C until an enzyme-linked immunosorbent assay analysis for mouse interleukin-1β (IL-1β), macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor alpha (R&D Systems, Minneapolis, Minn.) was performed. All three cytokines exhibited peak recoverable levels for the lavage at 3 h postchallenge, and there was a quick decline in the detectable levels thereafter (Fig. 2A). The level of IL-1β in B. cenocepacia-challenged mice was significantly higher (P < 0.0001) than the level detected in B. multivorans-challenged mice 3 h postinfection. The levels of MIP-2 and tumor necrosis factor alpha in B. cenocepacia-challenged mice did not differ significantly from those in B. multivorans-challenged mice on any day of infection (data not shown).

Lung tissue was removed on day 4 of infection and used for immunofluorescent localization of bacteria. Positive staining was observed only in B. multivorans-infected lungs, in accordance with the number of CFU. Lungs and tracheae were excised in toto from euthanized mice, fixed in acetone-methanol, dehydrated, and embedded in paraffin. The antibodies used in this study were rabbit polyclonal sera generated in our laboratory against B. multivorans FC147, rat anti-mouse Mac-3 monoclonal antibody (BD Biosciences), Alexa 488-conjugated goat anti-rabbit antibody, and Alexa 594-conjugated goat anti-rat antibody (Molecular Probes, Eugene, Oreg.). All antibodies were diluted 1/500 in blocking buffer (phosphate-buffered saline [PBS] containing 0.5% [vol/vol] Tween 20 and 5% [vol/vol] normal goat serum). Histological sections were blocked in PBS containing 1% normal goat serum and 0.5% Tween 20 (Sigma). Samples were incubated with primary and secondary antibodies, in sequence, for 1 h at room temperature. Some sections were counterstained with Mayer’s hematoxylin (Sigma Diagnostics, St. Louis, Mo.). A persistent B. multivorans infection was localized primarily with Mac-3-positive macrophages in the lungs on day 4 of infection. Lung sections were either immunostained for bacteria prior to Mayer’s hematoxylin counterstaining or immunostained for both bacteria and Mac-3 antigen (Fig. 3). Sections immunostained with the B. multivorans-specific polyclonal antibody showed that 39.0% of the
FIG. 3. Indirect immunofluorescent localization of persistent *B. multivorans* C5568. BALB/c mice were intranasally challenged with \( \sim 1.0 \times 10^7 \) CFU of *B. multivorans* strain C5568, and lung tissue was processed for immunohistological analysis on day 4 of the infection. Sections were either immunostained for bacteria prior to Mayer’s hematoxylin counterstaining (A to D) or double immunostained for both bacteria and the Mac-3 antigen without counterstaining (E to H). A positive bacterial signal (green) was localized primarily in lymphoid aggregates (A and B) or with mononuclear cells in the airspace (C and D). Double immunostaining showed that the positive bacterial signal (green) frequently colocalized with Mac-3-positive cells (red) in these areas (lymphoid aggregates [E and F] and macrophages [G and H]), suggesting that macrophages were the primary host cells interacting with *B. multivorans* strain C5568 at this point in infection.
bacteria localized to lymphoid aggregates and that 7.3% of these bacteria were organized as bronchus-associated lymphoid tissue (Fig. 3A and B); 56.1% of the bacteria localized with isolated macrophages (Fig. 3C and D), and 4.9% localized with bronchiolar epithelium (data not shown). Similar patterns of bacterium-host association were observed in the sections subjected to double immunostaining. In these sections, 76.5% of the bacteria localized to areas that were also positive for the Mac-3 antigen; 28.8% of these double-positive loci were cells within lymphoid aggregates (Fig. 3E and F), and 51.9% were
alveolar macrophages (Fig. 3G and H). Also, 5.8% of the double-positive cells appeared to be in bronchiolar epithelia, whereas 13.5% of the double-positive cells occurred in areas where there was collapse or excessive erythrocyte penetration.

Day 4. *B. multivorans*-infected lung samples were diced to obtain 1-mm cubes, processed, and stained as previously described (20), except for an initial 1.5-h fixation with 1% glutaraldehyde and the exclusion of tannic acid. Ultrathin sections were viewed with a Hitachi H7600 transmission electron microscope. Electron-dense bacteria were observed in membrane-bound vacuoles of macrophages in the alveolar space (Fig. 4A to D) of *B. multivorans*-challenged mice. Bacteria were distinguished from cytoplasmic granules by morphology and by the distinct presence of both bacterial outer membranes and vacuolar membranes. Macrophages in *B. multivorans*-challenged lung samples showed an increased presence of cytoplasmic vacuoles, regardless of whether they contained bacteria; some samples had phagolysosomes containing degradated material (Fig. 4E). No bacteria were observed in conducting airways or lymphoid aggregates by transmission electron microscopy. Macrophages from PBS-challenged lungs appeared to be slightly smaller than macrophages from infected lungs, and they had few cytoplasmic vacuoles and no phagolysosomes (Fig. 4F). No bacteriomorphic, electron-dense, membrane-bound structures were observed in macrophages or any other cell type in PBS-challenged lungs.

Here we describe a pulmonary model of BCC infection in an immunocompetent murine host, which differentiates the cell type in PBS-challenged lungs. Both *B. multivorans*-challenged lung samples showed an increased presence of cytoplasmic vacuoles, regardless of whether they contained bacteria; some samples had phagolysosomes containing degraded material (Fig. 4E). No bacteria were observed in conducting airways or lymphoid aggregates by transmission electron microscopy. Macrophages from PBS-challenged lungs appeared to be slightly smaller than macrophages from infected lungs, and they had few cytoplasmic vacuoles and no phagolysosomes (Fig. 4F). No bacteriomorphic, electron-dense, membrane-bound structures were observed in macrophages or any other cell type in PBS-challenged lungs.

We established a model of pulmonary infection in immunocompetent mice which discriminates *B. multivorans* strains from *B. cenocepacia* strains on the basis of persistence and host response. We propose that *B. multivorans* C5568 is capable of persisting in the host by establishing an intracellular infection in macrophages and that *B. cenocepacia* C6433 is cleared rapidly from the host because it is not able to gain such a foothold and in the process induces a more vigorous and damaging host response than strain C5568. This model system also has the capacity to evaluate BCC virulence determinant mutants. In future experiments with these two strains and various pulmonary host cell populations we will examine the molecular relationship between pathogen and host. Such studies should provide further information about the precise mechanisms of intracellular survival and may instruct novel therapeutic approaches.

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