Interactions between Bordetella bronchiseptica and its Host

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Bordetella bronchiseptica is a common mammalian respiratory pathogen and is the closely related progenitor of the human pathogen *Bordetella pertussis*, the organism responsible for whooping cough. Mice are a natural host of *B. bronchiseptica*, making them an appropriate model for the study of many aspects of the bacterium's biology and its interactions with host cells. In this study the types of host cells present in the lungs early in the infection and the effects of bacterial virulence factors on phagocytic cells were determined. In addition, the wild type *B. bronchiseptica* strain RB50 was transformed with the plasmid CC2, which encodes yellow fluorescent protein, for future observation of direct bacterial-host interactions.

Introduction

Bordetella bronchiseptica, the pathogenic agent responsible for kennel cough in dogs and rhinitis in pigs, is a gram-negative coccobacillus closely related to the human pathogen *B. pertussis,* which is responsible for whooping cough. These organisms share several virulence factors, such as adenylate cyclase toxin and the attachment protein filamentous hemagglutinin. These virulence factors are transcriptionally regulated by the Bvg AS signal transduction system **(Salyers 2002)**. This system senses environmental conditions and mediates the transition between infectious (Bvg +) and non-infectious (Bvg -) phases of the organism **(Yuk 1998, 945)**. In the case of *B. bronchiseptica,* this system also regulates the expression of a type III secretion system (TTSS) **(Yuk 1998, 945)**. The TTSS forms a needle-like protein structure on the surface of many pathogenic microorganisms and it is used to introduce toxins or other proteins directly into the cytoplasm of host cells. It has previously been documented that this secretion system induces caspase-1 independent necrosis of epithelial cells and macrophages in culture, suggesting it is very important for the pathogenicity of the organism **(Yuk 1998, 945)**. Our group determined which host cells are present in the lungs early in the infection and studied what type of cell death mechanism TTSS induces on phagocytic cells. In addition, the wild type strain RB50 of *B. bronchiseptica* **(Cotter 1994, 3381)** was also transfected with the plasmid CC2, which encodes yellow fluorescent protein (YFP). To validate the ability of the transfected bacteria to colonize the respiratory track of its natural host, the mouse, we compared the bacterial population of YFP labeled bacteria found in the lungs to that of unlabeled RB50. Since it appears that the presence of YFP does not alter the ability of the bacteria to infect the respiratory tract of mice, we believe that this system can be used to visually examine interactions between the bacteria and the early inflammatory cells.

Materials and Methods

Host Cell Population. Three C57BL/6 mice, obtained from Jackson Laboratories (Bar Harbor, ME), were sedated with isofluorane (Abbott Laboratories) and inoculated intranasally with $5.0x10^5$ colony forming units (CFU) of RB50 diluted into a volume of 50uL of phosphate-buffered saline (PBS). The host cells present in the lungs on day 3 post-inoculation were determined from lung homogenates. The lungs were homogenized in 10ml of DMEM plus 10% Fetal Calf Serum (FCS) through a Cellector™ screen (Bellco Glass, Inc.). 5□g of DNAse were added to the lung homogenate to decrease the level of cell clumping. 5ml of the homogenate were added to 6ml of the Histopaque® 1119 gradient (Sigma) and centrifuged at 3000rpm for 30min. The visible layer of cells close to the top of the gradient was removed. 300_l of this cell suspension were added to the CytoSpin® glass slide apparatus, centrifuged for 30min at 300rpm and Giemsa stained. The different leukocyte types were counted by microscopy to a total of 300 cells per slide.

Neutrophil Isolation. Neutrophils were isolated from C57BL/6 mice blood in a 1:1 mixture of Histopaque® 1077 and 1119 gradients (Sigma). The mixture was centrifuged at 3000rpm for 30min. The visible layer of cells close to the top of gradient was removed. Cells were centrifuged at 13000rpm for 1min to replace the gradient with DMEM/F12 medium without phenol red plus 10% FCS.

Detection of Cell Death Mechanisms. To determine the cell death mechanism induced by RB50 on host phagocytic cells, 10^6 C57BL/6 mouse neutrophils and 10^6 cells of the RAW 264.7 mouse macrophage cell line **(Ralph 1977, 950)** were infected with heat-killed RB50, live RB50 or the type III secretion system mutant WD3 (only RAW 264.7) **(Yuk 1998, 945)** at a multiplicity of infection (MOI) of 30 for 2hr at 37°C. To determine the cell death mechanism induced, the cells were centrifuged at 5000rpm for 1min and washed once with PBS. They were then resuspended in 100_l of Annexin-V binding buffer (10mM HEPES pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$, and 0.1% BSA). 0.5_l of 40ng/ml Annexin-V-FITC solution, a kind gift of Dr. Margaret S. Halleck, and 1_l of 1.0mg/ml propidium iodide (PI) solution were added to the cell suspension and incubated for 5min at room temperature. After the incubation, 500_l of binding buffer were added. The samples were kept on ice, protected from light and a total of 20000 cells per sample were screened by FACScan™ flow cytometry (Becton Dickinsion).

Bacteria and Plasmid. The wild type strain of *B. bronchiseptica* RB50 **(Cotter 1994, 3381)** was transformed by bacterial mating with the E. coli strain SM10 carrying the plasmid CC2 encoding YFP (fig.1, 2), a gift from Dr. Peggy Cotter. Proper insertion of the plasmid into the bacterial chromosome was confirmed with polymerase chain reaction (PCR) using the primers CWSSHR3 and CWBpGR3, also gifts from Dr. Peggy Cotter, under standard conditions. Bacterial fluorescence was also confirmed via fluorescent microscopy. Successfully transformed bacteria were maintained on Bordet Gengou (BG) agar (Difco) with 7.5% sheep blood, 20_g/ml of streptomycin and 60_g/ml of gentamycin. Bacteria were grown in Stainer-Scholte broth with 60_g/ml of gentamycin prior to mouse inoculation.

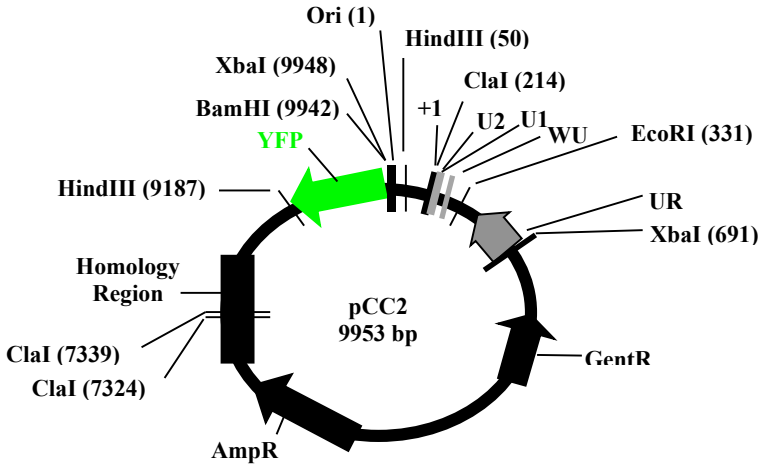

Figure 1.

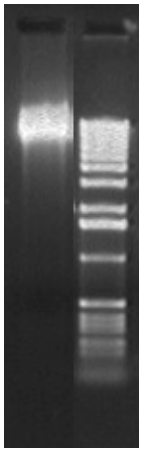

Figure 2.

Figure 1. Genetic map of the plasmid CC2 that encodes yellow fluorescent protein.
Figure 2. 1% agarose gel with purified plasmid CC2 (lane 1) and a 10 kbp standard DNA ladder. The plasmid is 9953 base pairs long.

Mouse Experiments with RB50-YFP. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). 20 mice were sedated with isoflurane (Abbott Laboratories) and intranasally inoculated with $5.0x10^5$ colony forming units (CFU) of RB50-YFP diluted into a volume of 50uL of phosphate-buffered saline (PBS). 4 mice per time point were sacrificed on days 1,3,7,14, and 28 to determine bacterial CFU from the lungs. The organs were homogenized in 1 ml of PBS, diluted and plated on BG agar with 7.5% sheep blood and 20_g/ml of streptomycin for 48hr at 37°C for CFU counts. The data obtained were compared to previously recorded data of RB50 in C57BL/6 mice **(Kirimanjeswara 2003, 1719)**.

Results

Host Cell Population. The host immune system responds quickly to the presence of the pathogens in the respiratory track. To determine which cell types respond to the infection by day 3 post-inoculation we prepared slides with lung homogenates. At this time point, the vast majority of the cells were macrophages (65%), followed by neutrophils (20%) and lymphocytes (15%), (fig. 3).

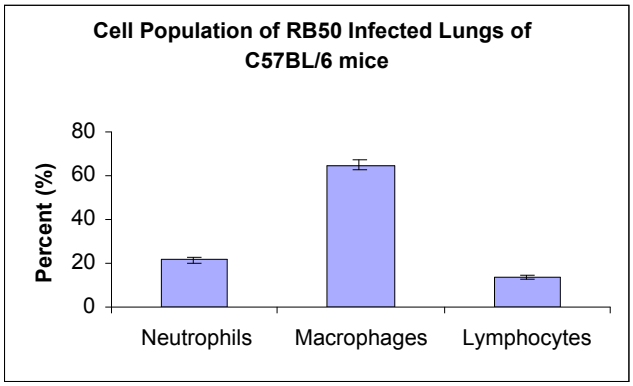

Figure 3. Host immune cells present in the lungs of RB50 infected C57BL/6 mice on day 3 post-infection. Data is expressed in average ± standard error.

Cell death mechanism. Since previous experiments have indicated that *B. bronchiseptica* induces a vigorous cellular response during infection, the interactions of *B. bronchiseptica* with immune cell populations were examined. FITC-labeled Annexin-V was used to detect apoptosis and PI was used to detect necrosis of neutrophils and macrophages induced by RB50 infection in-vitro. First, of the RB50 infected neutrophils screened, 30% underwent apoptosis and 32% underwent necrosis. These results are significantly higher than those observed for heat-killed bacteria infected neutrophils: 14% apoptosis, 3% necrosis (fig.4). Second, in the case of the RB50 infected RAW 264.7 macrophages, 43% were observed to undergo necrosis while only 5% underwent apoptosis. The level of necrosis induced by RB50 is considerably higher than the level of apoptosis or necrosis induced by heat-killed bacteria (3% and 2% respectively) or WD3 (4% and 3% respectively) (fig.5).

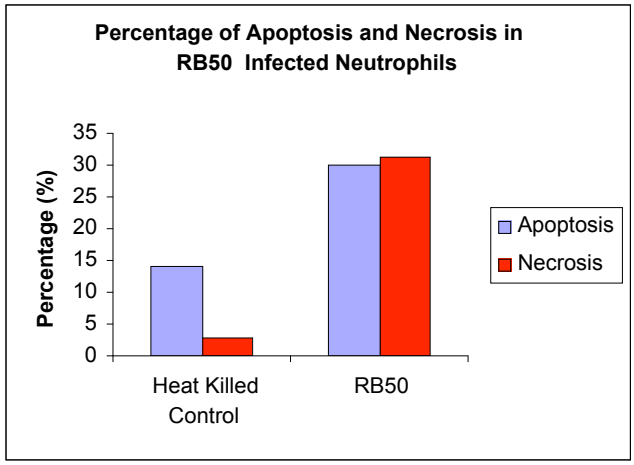

Figure 4.

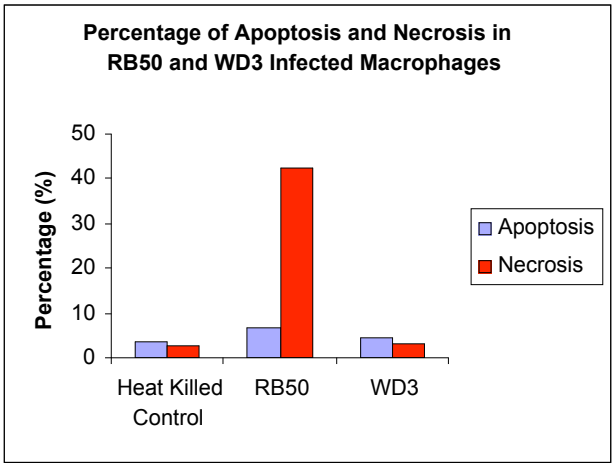

Figure 5.

Figures 4 and 5. The cell death mechanism induced by RB50 infection was analyzed by flow cytometry. Apoptosis was detected with FITC-labeled AnnexinV and necrosis was detected with propidium iodide. A total of 10^6 neutrophils isolated from C57BL/6 mice and macrophages of the RAW 264.7 mouse cell line were infected and 20000 cells were screened with flow cytometry.

pCC2 Insertion and Expression. In order to visually examine the interactions of *B. bronchiseptica* with responding host cells, the pCC2 plasmid, containing the YFP gene was transfected into wild type strain RB50 of *B. bronchiseptica*. After bacterial mating, several transformants were obtained. The transformants were subcultured on BG agar with gentamycin and streptomycin for 48hr and a colony from the subculture was selected for PCR screening to confirm proper insertion of pCC2 into the bacterial chromosome. The plasmid was inserted properly as it can be observed on figure 6. Proper expression of YFP was confirmed by microscopy (fig. 7)

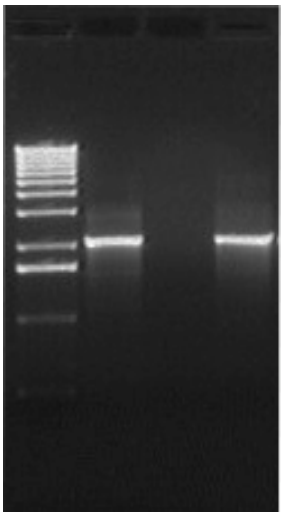

Figure 6.

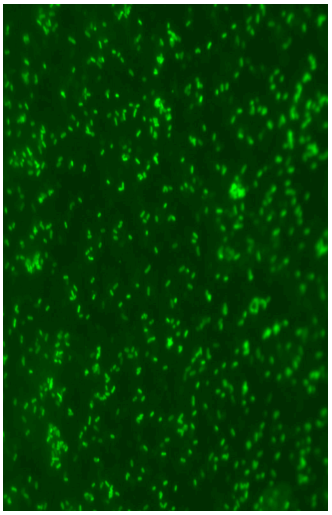

Figure 7.

Figure 6. 1% agarose gel with 10 kbp standard DNA ladder (lane 1), PCR positive control product of integrated plasmid (lane 2), negative control of integrated plasmid (lane 3), and pCC2 integrated plasmid (lane 4). PCR was carried out under standard conditions to confirm proper integration of pCC2 into the bacterial chromosome using the primers CWSSHR3 and CWBpGR3. Integrated plasmid is 2.1 kbp long.

Figure 7. Fluorescent micrograph of *B. bronchiseptica* strain RB50 expressing YFP.

Bacterial CFU. To confirm that the expression of YFP would not alter *B. bronchiseptica* colonization, bacterial numbers in the lungs of mice infected with either the wild type or YFP expressing strains of *B. bronchiseptica* were compared. After 28 days of infection, no significant difference in bacterial population between RB50 and RB50-YFP infected lungs was observed (fig.8).

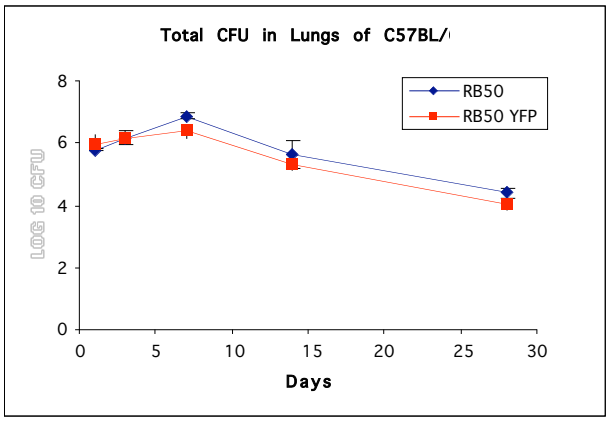

Figure 8. Infection time course of C57BL/6 mice with RB50-YFP compared to wild type RB50. Data is expressed in average ± standard error.

Discussion and Conclusions

In this study, we determined which host cells respond to the *Bordetella* infection on day 3 post-infection from the observation of leukocytes isolated from infected lung homogenates. With this technique we found that the majority of the cells present at this stage of the infection are macrophages followed by neutrophils and lymphocytes. This technique will help us determine which cells are present throughout the course of infection.

Since macrophages and neutrophils are the main host immune cells that interact directly with the bacteria, we explored what were the effects of this interaction on these cell types. It has been reported previously that the expression of a type III secretion system by *Bordetella bronchiseptica* induces caspase-1 independent necrosis on epithelial cells and macrophages in-vitro **(Stockbauer 2003, 123)**. With the use of Annexin-V-FITC to detect apoptosis and propidium iodide to detect necrosis through flow cytometry, we confirmed these findings in-vitro with the RAW 264.7 mouse macrophage cell line and purified neutrophils form C57BL/6 mice. We compared the cell death mechanisms induced by the wild type *B. bronchiseptica* strain RB50 and the type III secretion system mutant WD3 on RAW 264.7. We found that a significantly higher amount of necrotic cell death was induced by RB50 compared to the level of apoptosis. This level of necrotic cell death was also significantly higher than the level of necrosis induced by WD3. In the case of neutrophils, very similar levels of apoptosis and necrosis induced by RB50 were observed, however neutrophils undergo phagocytosis-mediated apoptosis **(Kobayashi 2002, 6901)**. This could explain the relatively high level of apoptosis induced by the heat-killed bacteria (~15%) and the high level of apoptosis observed in RB50-infected neutrophils. In order to confirm this hypothesis, further experiments should be carried out. Experiments should also be done to determine what cell death mechanism WD3 induces on neutrophils.

The expression of yellow fluorescent protein by *B. bronchiseptica* is a very useful technique that will allow us to visualize many of the interactions that occur between RB50 and its host during the course of infection. This technique would also be useful to better understand other pathogens. In our study, we successfully transformed the organism with the plasmid CC2, which encodes yellow fluorescent protein (YFP). We showed, with the use of standard PCR techniques, that this plasmid integrated efficiently into the *Bordetella* chromosome. However, proper insertion of the plasmid into the bacterial chromosome does not guarantee normal colonization of the organism. To address this question we carried out a 28 days mouse infection time course and compared the total CFU of bacteria on days 1,3,7,14, and 28 with previously recorded data **(Kirimanjeswara 2003, 1719)**. No significant difference between RB50-YFP and RB50 bacterial numbers was observed. This suggests that the integrated plasmid does not affect the ability of the organism to colonize the lower respiratory track of mice. However, in order to compare the two strains with higher accuracy, both time courses must be carried out side-by-side.

In conclusion, our group determined that most of the host cells that respond to the infection on day 3 post-inoculation are macrophages. We determined that the bacterium induced mostly necrosis on neutrophils and macrophages mediated by the type III secretion system, although further studies should be carried out to confirm these results. We also genetically

modified the wild type strain RB50 of *Bordetella bronchiseptica* with the plasmid pCC2 to induce the expression of yellow fluorescent protein. This technique would enable us to observe direct interactions between the bacteria and the host immune system during the course of infection.

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