ANTIBIOTIC TOLERANCE OF *BURKHOLDERIA*: DISTINGUISHING BETWEEN CLASSICAL RESISTANCE AND PERSISTENCE IN A MACROPHAGE INFECTION MODEL

CRYSTAL COLLINS AND KARA MOSOVSKY*

DEPARTMENT OF BIOLOGICAL SCIENCES, MORAVIAN COLLEGE, BETHLEHEM, PA USA

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ABSTRACT

Burkholderia pseudomallei is a Gram–negative bacillus and facultative intracellular pathogen. It causes the disease melioidosis, which is a potentially fatal human disease found throughout the world but particularly in Southeast Asia and Northern Australia. B. pseudomallei is inherently antibiotic resistant and therefore new therapies are needed to combat this pathogen. Previous studies with the related organism *Burkholderia thailandensis* have shown that the antibiotic ceftazidime does not eliminate all bacteria in an in vitro macrophage model, and the remaining bacteria could still pose a health threat to a potential host. Due to their survival in the presence of antibiotics, we hypothesized that the remaining bacteria were one of two types of antibiotic tolerant cells: classically antibiotic resistant cells or persister cells. To test our hypothesis we isolated the bacteria that had survived ceftazidime treatment in the macrophage infection model and performed additional in vitro experiments to show that the surviving bacteria are neither antibiotic resistant nor persister cells. Instead, they are still susceptible to high doses (200 μg/ml) of the antibiotic over a period of 48 hours ($p<0.001$). We believe the bacteria survive exposure to the antibiotic during the macrophage infection because of their ability to move between intracellular and extracellular compartments, thus avoiding the antibiotic and its deadly effects. Our results provide evidence to suggest that intracellular pathogens, through movement between intracellular and extracellular compartments, may be protected from the effects of antibiotics in similar macrophage infection models.

CORRESPONDING AUTHOR

Kara Mosovsky
mosovskyk@moravian.edu

KEYWORDS

- Persister Cells
- Antibiotic Resistance
- *Burkholderia*
- Macrophages
- Intracellular Bacteria

INTRODUCTION

Burkholderia pseudomallei is a facultative intracellular pathogen and the etiological agent of melioidosis (1, 13, 14, 19). This disease is endemic in regions such as Southeast Asia and Northern Australia and causes potentially fatal infections that can present as pneumonia or septicemia (16). B. pseudomallei is inherently highly antibiotic resistant and due to the swift progression and deadly nature of the disease it causes, is considered to be a bioterrorism threat (17). There is no current vaccine to prevent this

deadly disease, and most of the treatments for melioidosis involve several weeks of intravenous antibiotics, often with the antibiotic ceftazidime (13, 19). However, even with antibiotic treatment, mortality rates still range from 20–50%, and as many as 10% of individuals experience recurrent infections, usually due to relapse of the original infection (17). The high rate of relapse infections would suggest that complete elimination of the pathogen is important for a patient's full recovery. Considering the challenges of treating melioidosis, it is essential to develop new therapies, which could potentially decrease the burden of disease.

A series of previous studies have investigated one alternative treatment option in a macrophage infection model using both the virulent B. pseudomallei and also the closely related and avirulent organism *Burkholderia thailandensis*. These studies show the ability of the antibiotic ceftazidime to interact with the macrophage activator, interferon–gamma (IFN–γ), to synergistically reduce the bacterial burden both inside the infected macrophages (intracellular compartment) and outside of the infected macrophages (extracellular compartment) (10, 12). What was surprising from this study was that the infected macrophages treated with antibiotic alone still harbored a significant amount of extracellular bacteria, even when the antibiotic concentration was approximately five times the minimum inhibitory concentration (MIC) (10). Since current melioidosis treatment relies almost exclusively on antibiotics alone, this result was disconcerting and warranted further investigation. Such remaining bacteria may still pose a significant threat in a host and could explain why patients with melioidosis often develop recurrent infections due to relapse of the original infection. From these previous studies we developed a new research question regarding these surviving extracellular bacteria: are the remaining extracellular bacteria still sensitive to the antibiotic or have they become antibiotic tolerant? This question was the focal point of our study.

There are two main types of antibiotic tolerant bacteria. Classical antibiotic resistance is a permanent and heritable tolerance to antibiotics, while persistence is a temporary and non–heritable tolerance (8). Persister cells are thought to be present as a small subpopulation in any large bacterial population and are characterized as slow or non–growing, dormant, bacterial cells that are temporarily tolerant to antibiotics due to their lack of replication (3–5, 8, 9). Additionally, when the antibiotic is removed and persister cells resume replication, most of the progeny of persister cells are susceptible to the antibiotic, a trait that helps distinguish them from classically resistant bacteria (7, 8). While the rate of persister cell formation is typically less than 1% for many pathogens, studies on *Burkholderia* persisters have shown that cultures of *B. pseudomallei* and *B. thailandensis* can harbor around ten times higher frequencies of persister cells compared to other bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (2, 6, 11, 18). We therefore had reason to hypothesize that persister cells may compose some or all of the remaining population of bacteria that had survived antibiotic treatment

during the macrophage infection. The aim of the current study was to determine if the remaining extracellular bacteria were sensitive to antibiotics, antibiotic resistant, or persister cells. By further characterizing the surviving extracellular bacteria, we would have the opportunity to gain insights about the treatment of macrophages in our in vitro model and help inform future treatment options against melioidosis.

MATERIALS AND METHODS

BIOCHEMICALS

Ceftazidime hydrate was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine IFN-γ was purchased from PeproTech (Rocky Hill, NJ). Kanamycin Monosulfate was purchased from MP Biomedicals, LLC (Santa Ana, CA), and Luria Bertani (LB) broth was purchased from ThermoFisher Scientific (Waltham, MA).

BACTERIA

Burkholderia thailandensis E264 was purchased from American Type Culture Collection (Manassas, VA) and used for all experiments. A single isolated colony was inoculated into sterile LB broth and incubated on an ambient air, shaking incubator at 200 rpm for 16 hours at 37°C. Overnight cultures were aliquoted and stored at -80°C in 0.5 ml volumes as a 15% glycerol stock solution. A new vial of *B. thailandensis* was thawed and resuspended immediately prior to use in the macrophage infection assay.

CELL LINES

The murine macrophage cell line, RAW 264.7, was purchased from American Type Culture Collection (Manassas, VA) and used for all macrophage infections. Macrophages were maintained in complete medium consisting of 1x Minimum Essential Medium (MEM; ThermoFisher Scientific) supplemented to a final concentration of 10% EquaFetal Bovine Serum (Atlas Biologicals, Fort Collins, CO), 1x MEM non-essential amino acids, 0.5x MEM essential amino acids, 0.075% sodium bicarbonate (Sigma-Aldrich), and 2 mM L-Glutamine solution. In order to maintain sterility of the cells, passages of the macrophages were maintained in complete MEM supplemented to a final dilution of 100 µg/ml of penicillin-streptomycin solution. All macrophage infections were performed in antibiotic-free complete MEM (cMEM). Macrophages were maintained in a 37°C incubator supplemented with 5% C_O2 and 80% relative humidity.

MACROPHAGE INFECTION ASSAY

The macrophage infection was conducted as previously described (10, 12). Briefly, a hemocytometer was used to appropriately count and dilute the RAW cells to a density of 200,000 cells per well in 0.5 ml of cMEM. After the cells were added to a 24-well plate, they were incubated at 37°C with 5% CO_2 and 80% humidity overnight

for adherence. After this incubation period, the cells were observed under an inverted microscope to determine the general health of the cells. The cells in each well were washed one time, slowly and carefully, with 1 ml of phosphate buffered saline (PBS). A bacteria dilution of *B. thailandensis* in cMEM was then applied to the cells at a multiplicity of infection of 5, and incubated for 1 hour. After the first hour of incubation, the cells were again slowly washed one time with 2 ml of PBS and then 1 ml of a 350 μg/ml kanamycin solution was added into each of the wells and incubated for 1 hour to remove extracellular bacteria. After this final incubation period, the cells were washed two times with 2 ml of PBS to remove the killed extracellular bacteria. Then 0.5 ml of a 10 μg/ml solution of ceftazidime, was added into each of the wells and incubated for 18–24 hours.

TESTING FOR ANTIBIOTIC RESISTANCE

After 24 hours of infection and treatments, the remaining extracellular bacteria were carefully resuspended in their wells and 100μl was directly plated onto agar with varying concentrations of ceftazidime. The agar plates contained either no antibiotic, 10 μg/ml of ceftazidime, or 200 μg/ml of ceftazidime (which equates to ~100x minimal inhibitory concentration (MIC) of 1.75 μg/ml) (20). Plates were incubated in an ambient air incubator for 24–36 hours at 37°C and then inspected for growth.

TESTING FOR PERSISTER CELL FORMATION

After the 18-hour treatment period of the macrophage infection assay, macrophages were inspected for apparent signs of infection and consistency between wells. Remaining extracellular bacteria were resuspended slowly and carefully, and transferred to 2 ml tubes, combining two identical wells into 1 tube due to low numbers of remaining bacteria. Tubes of bacteria were centrifuged at 12000 xg for five minutes, the supernatant was carefully removed, and a 1 ml wash with complete cMEM was added to each tube. After a second spin, the pellets were reconstituted in 0.5 ml cMEM and the entire tube contents were plated into separate wells of a new 24-well plate. Ceftazidime was re-added to a final concentration of 200 μg/ml and returned to the incubator at 37°C with 5% CO_2 and 80% humidity. Subjecting bacteria to very high doses of antibiotics is one way to identify persister cells (7). Serial dilutions of the surviving bacteria were plated at various time points post-antibiotic treatment, and bacterial density was calculated as colony forming units per milliliter (CFU/ml) after incubation of the agar plates.

STATISTICAL ANALYSES

Prism software version 7.01 (GraphPad, La Jolla, CA) was used to plot the mean and standard error of the mean (SEM) for different treatment groups. One-way analysis of variance (ANOVA) and Tukey's post test was used to compare differences between three or more treatment groups.

RESULTS

TESTING FOR CLASSICAL RESISTANCE

Previously, we found that treatment of *B. thailandensis*–infected macrophages failed to kill all extracellular bacteria even after 18–24 hours of exposure to ceftazidime above the MIC. To determine if these remaining bacteria had developed classical resistance to ceftazidime during the treatment, we first recapitulated the standard in vitro infection with appropriate treatments. We then harvested the remaining survivors from the treatment wells and plated them on antibiotic–infused agar. After incubation, bacteria were only detected on the antibiotic–free control plates, with the greatest density of bacteria coming from the untreated control wells of the macrophage infection (Table 1).

Ceftazidime treatment of infected macrophages yielded many surviving bacteria on the antibiotic–free control plates, and the combination treatment of ceftazidime and IFN–γ yielded no detectable surviving bacteria. No extracellular bacteria grew on ceftazidime–infused agar, regardless of the concentration and regardless of the treatment the infected macrophages had received. With these results in mind, we next asked whether or not the bacteria that were surviving 18–24 hrs of ceftazidime treatment during the macrophage infection were persister cells.

Macrophage Treatment Groups	No Ceftazidime	Ceftazidime-infused agar plates (10 μg/ml)	(200 μg/ml)
Control	++	–	–
Ceftazidime	+	–	–
Ceftazidime + IFN-γ	–	–	–

[a]no visible growth = –, minimal growth = +, abundant growth = ++

Table 1: Remaining bacteria fail to grow on antibiotic-infused agar. RAW macrophages were infected with *B. thailandensis*, and treated for 18 hours with ceftazidime alone (10 μg/ml) or the combination of ceftazidime (10 μg/ml) and IFN-γ (10 ng/ml). Surviving extracellular bacteria were then plated onto agar plates without antibiotic, or with 10 μg/ml or 200 μg/ml ceftazidime. This table represents similar data from two independent experiments.

TESTING FOR PERSISTER CELLS

To determine if the remaining extracellular *B. thailandensis* were persister cells, we again harvested the bacteria from the end of the macrophage infection to determine their sensitivity. After 18 hours of ceftazidime treatment (10 μg/ml) of the infected macrophages, the remaining extracellular bacteria were separated from the macrophages, processed, and re-subjected to a higher concentration of ceftazidime to identify persister cells. At different time points well contents were serially diluted and plated onto LB agar. As shown in (Figure 1), the bacteria that had previously survived antibiotic treatment in the presence of the macrophages were now killed by high doses of ceftazidime (200 μg/ml) over the 48 hour time frame, with most of the killing occurring by 24 hours. From time point 0 to 3 hours, there was no statistical difference in how many bacteria were killed by the antibiotic. There was however, statistically significant killing at the time point for 24 hours ($p<0.01$). By 48 hours, we were unable to detect any viable *B. thailandensis* cells ($p<0.001$), indicating that all bacteria had been killed.

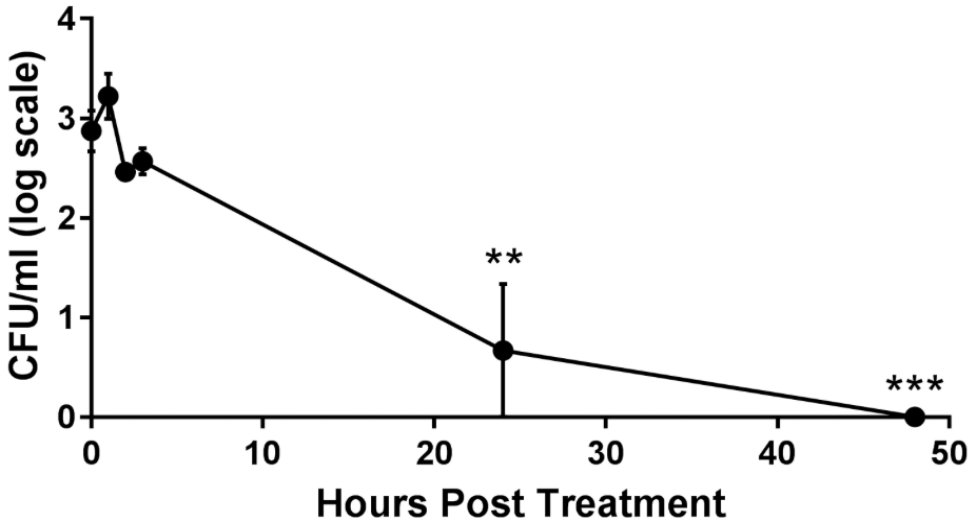

Figure 1: After 18 hour macrophage infection, remaining extracellular bacteria are still sensitive to high doses of antibiotic. RAW cells were infected with *B. thailandensis*. After 18 hours of treatment with 10 μg/ml ceftazidime, the surviving extracellular bacteria were removed, washed, and re-subjected to ceftazidime at 100x the MIC (200 μg/ml) in a macrophage-free system. Bacterial density was determined at time points 0, 1, 2, 3, 24, and 48 hours by plating dilutions of the well contents. Data is representative of three similar and independent experiments that showed similar trends in killing. Means and SEM were plotted for each time point. One-way ANOVA was used to determine statistically significant differences from time point 0, with ** indicating $P < 0.01$, and *** indicating $p < 0.001$.

DISCUSSION

The purpose of these experiments was to determine if the extracellular bacteria that were surviving 18 hours of treatment with ceftazidime in the macrophage infection model were classically resistant or if they were considered to be persister cells. Similar to Titball *et. al.* we plated the bacteria onto antibiotic-infused media to test for antibiotic resistance due to spontaneous mutations (2). In Table 1, we showed a lack of growth on the antibiotic-infused plates, which serves as evidence that the extracellular bacteria were not classically resistant. In classical resistance, the

progeny of the resistant cells would also be resistant, therefore we would expect to see whole colonies of antibiotic-resistant bacteria on the plates infused with antibiotic concentrations above the MIC. Instead, we were only able to see growth on the antibiotic-free control plates. Since we did not plate all of the volume from each of the treated wells of the macrophage infection, there is also a limit of detection to our assay, and we cannot say with certainty that the well contents did not contain any antibiotic resistant bacteria. Regardless, we were able to determine that the majority of the extracellular bacteria are not resistant.

Previous studies found low bacterial burden in the extracellular compartment following macrophage treatment with ceftazidime and IFN-γ (10). Here we were unable to detect any extracellular bacteria from the wells of infected macrophages that had received the combination treatment group, which indicates that this population of bacteria is very small, and provides further evidence that the combination therapy may provide significantly more protection than the antibiotic alone. While Table 1 shows evidence that the remaining bacteria are not classically resistant, from these results alone, we cannot rule out the possibility that some, or many, of the treatment-surviving bacteria are persisters. If persisters existed following treatment in the macrphage infection model, then they would tolerate high doses of antibiotics on the agar plates, but the great majority of their progeny would be susceptible, and therefore, we wouldn't be able to visualize any colonies on the agar plate. Since this theory mirrored what we observed, we hypothesized that some remaining bacteria may be persisters.

We decided to identify potential persisters as done previously by treating the bacteria with a high dose of antibiotic and looking for a plateau in the killing over time (4, 9). In such cases, the bulk of the population of bacteria is killed, while the antibiotic tolerant persisters survive. Persisters are identified as the subpopulation of surviving bacteria after the bulk of the population has been eliminated. Graphically, the sensitive majority of the population dies quickly over time, but then the killing curve plateaus because the remaining persisters are tolerant to the antibiotic (4, 9). Previous experiments with *B. pseudomallei* 82 showed that bacteria exposed to cefotaxime at 100x the MIC, without shaking, were killed modestly for 24 hours, after which there was no additional killing throughout the 33 hour experiment (11). In these experiments, there was less than a 10 fold overall decrease in bacteria from time 0 through the 33 hour antibiotic treatment period, showing tolerance of the bacteria to the antibiotic. We expected persister cells in our assay to show a similar trend in tolerance when exposed to 100x the MIC of antibiotic over a period of time, even though we used *B. thailandensis* instead of *B. pseudomallei* and a different cephalosporin antibiotic. If most of the remaining bacteria were indeed persisters, we would have expected to see little killing or at least a plateau in the killing over time. In Figure 1 we show that the remaining extracellular bacteria are still sensitive to high doses of ceftazidime over a 24 hour period, with no detectable bacteria by 48 hours. While we certainly saw significant killing over time, we cannot say definitively that the antibiotic completely sterilized the wells, due to our limit of detection. Based on standardized definitions of the limit of detection (15) we estimate that the number of remaining

bacteria at 48 hours post-treatment is <1 CFU/ml. Overall, our evidence has suggested that the extracellular bacteria that survive the 18–24 hour treatments during the macrophage infection remain sensitive to both low (10 μg/ml) and high doses (200 μg/ml) of the antibiotic ceftazidime. Previous studies have suggested that the transitioning of extracellular *B. thailandensis* to the intracellular space, upon uptake, or transitioning of intracellular bacteria to the extracellular space, upon macrophage lysis, may influence *in vitro* macrophage infection models (10).

We now expand upon that hypothesis, suggesting that the dynamic movement of extracellular bacteria to the intracellular compartment may protect them from the antibiotic. This protected movement between compartments may explain why bacteria are still present in low numbers in the extracellular niche of the macrophage infection model after 18 hours of treatment, and yet are still sensitive to the antibiotic. Perhaps it is the rapid movement between extracellular and intracellular compartments that accounts for their protection from the antibiotic. Future studies will attempt to tease out these nuances of the macrophage infection model through visualization of real-time infections. The interactions between bacteria in the intracellular and extracellular compartments could also be studied through repeated saline washes of the extracellular compartment throughout the 18 hour infection. If there is a dynamic movement of bacteria between compartments, and if the bacteria are not able to re-infect the macrophages once released to the extracellular compartment, we would expect the intracellular numbers to fall more quickly over time upon treatment with the synergistic combination of IFN-γ and ceftazidime.

Even if the bacteria are neither resistant nor persister cells, there might be some difference in susceptibility of the extracellular bacteria that remain at the end of the macrophage infection compared to bacteria from untreated wells of the macrophage infection. Future experiments to compare the rate of killing of the extracellular bacteria that came from antibiotic-treated macrophages to those that came from untreated macrophages, and even to bacteria never exposed to macrophages. These experiments could determine if interactions with the macrophages affords the bacteria any amount of tolerance to the antibiotic. Lower doses of antibiotic could also be used against these groups to compare killing curves.

In conclusion, we have shown evidence that the *B. thailandensis* cells that survive ceftazidime treatment for 18–24 hours in the macrophage infection model are still sensitive to high and low concentrations of ceftazidime, and they are not classically resistant nor are they persister cells. We hypothesize that the ability of the bacteria to move between intracellular and extracellular compartments during the macrophage infection likely accounts for their ability to survive long periods of time in the presence of bactericidal concentrations of ceftazidime. Future studies will continue to investigate various host-pathogen interactions of this macrophage infection model.

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