Pathogenicity of High-Dose Enteral Inoculation of *Burkholderia pseudomallei* to Mice

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Abstract. Melioidosis is a frequently lethal tropical infection caused by the environmental saprophyte *Burkholderia pseudomallei*. Although transcutaneous inoculation and inhalation are considered the primary routes of infection, suggestive clinical evidence implicates ingestion as a possible alternative route. We show that in BALB/c and C57BL/6 mice, direct gastric inoculation of high doses of *B. pseudomallei* causes systemic infection that may be lethal or cause chronic disseminated infection. Mice may shed bacteria in the stool for weeks after infection, and high titers of *B. pseudomallei*-specific IgG are detectable. This report of enteric murine melioidosis supports further consideration of this route of infection.

INTRODUCTION

Melioidosis is a serious bacterial infection occurring primarily in southeast Asia and northern Australia. The causative agent is *Burkholderia pseudomallei*, a gram-negative soil saprophyte and potential biothreat agent.¹ Melioidosis occurs after contact with a contaminated environment and therefore is potentially preventable. A major stumbling block to the development of protective strategies is the lack of an evidence base defining routes of acquisition. The prevailing assumption is that most disease occurs as a result of transcutaneous inoculation or inhalation.¹ The role of ingestion as a route of infection remains undefined.² Suggestive but inconclusive evidence is provided by reports of clusters of melioidosis cases in which the strain of *B. pseudomallei* isolated from a common water source was a genetic match for the strain causing disease.^{3–5} Experimental models of melioidosis have focused predominantly on non-enteral routes of infection, all of which have reproducibly resulted in disease.⁶ Experimental enteric infection has received relatively little consideration since the description of melioidosis in 1913 by Whitmore.^{7–10} We developed a model of enteric melioidosis in mice to shed light on ingestion as a route of infection in humans.

MATERIALS AND METHODS

Bacteria. *Burkholderia pseudomallei* 1026b, a clinical isolate obtained from a bacteremic patient from Thailand, was grown in Luria-Bertani broth shaking in air at 37°C, washed twice, resuspended in phosphate-buffered saline (PBS) containing 20% glycerol, and frozen at –80°C. On the day of experiments, the freezer stock was thawed and diluted in PBS to the specific concentration, which was confirmed by quantitative culture on Ashdown agar plates.

Animal model. Six to ten-week old, female, specific pathogen-free BALB/c and C57BL/6 mice were obtained from Charles River Laboratoriwes, Inc. (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME), and maintained in a biosafety level 3 animal facility. Food was withheld from

the mice for three hours preceding inoculation. Inoculation was performed by direct gastric delivery of 200 µL of bacteria using a 22-gauge, 1.5-inch gavage needle. Mice that had inadvertent nasal or respiratory delivery of bacteria or mice that became moribund or died within two hours of infection, suggesting a complicated procedure, were killed and excluded from the experiments.

Thereafter, mice were monitored daily. Ill animals that had ruffled fur, eye crusting, hunched posture, and lack of resistance to handling were deemed terminal and killed (spontaneous death was not required as an endpoint). In the initial experiment, BALB/c and C57BL/6 mice were inoculated with 1×10^3 , 1×10^6 , or 1×10^8 colony-forming units (CFU) ($n = 3$, 5, and 3 and $n = 5$, 5, and 5, respectively). In the second experiment, BALB/c and C57BL/6 mice were inoculated with 1×10^6 , 1×10^8 CFU, or 7×10^8 CFU ($n = 4$, 5, and 4 and $n = 5$, 5, and 7, respectively).

For bacterial culture, the left lung, median hepatic lobe, spleen, brain, and mesenteric lymph nodes were removed in a sterile fashion and homogenized in 1 mL of Dulbecco's PBS. Aliquots (100–200 µL) of homogenized tissue were plated in duplicate on Ashdown agar.¹¹ Stool samples were obtained from mice prior to killing and were homogenized and cultured in a similar manner. *Burkholderia pseudomallei* colonies were counted after 2–4 days of incubation at 37°C. The lower limit of detection was 5–10 CFU/mL. A positive culture was defined as detectable colonies consistent with *B. pseudomallei* morphotypes on both of the duplicate plates. If colony morphology was atypical or there were mixed morphologies, identification of *B. pseudomallei* was confirmed by using a monoclonal antibody-based latex agglutination test.¹² For histologic analysis, organs were fixed in 4% paraformaldehyde before processing. Sections were stained with hematoxylin and eosin, and with Giemsa or Brown and Brenn stains. Slides were reviewed by a veterinary pathologist (HDL). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington.

Antibody detection. Heat-killed *B. pseudomallei* 1026b (1×10^6 CFU in 100 µL of PBS) was added to 96-well plates and stored at 4°C overnight. The plates were washed and blocked with 5% skim milk in PBS at 37°C for two hours. After repeat washing, two-fold serial dilutions of serum in PBS from each infected mouse (in duplicate) and from two uninfected mice were added for two hours. The range of dilutions was

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1:32–1:65,536. The plates were washed, goat anti-mouse IgG conjugated to biotin (SouthernBiotech, Birmingham, AL) diluted 1:10,000 in PBS and 1% bovine serum albumin was added, and incubated for two hours. Streptavidin–horseradish peroxidase diluted 1:200 was added for 20 minutes, washing was repeated, and color development was obtained by adding peroxidase substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reaction was quenched with 1 M phosphoric acid and optical densities were determined at an absorbance of 405 nm after subtracting values at 570 nm for correction. A positive antibody titer was defined as the maximal dilution of tested serum that had twice the mean optical density of uninfected serum at the same dilution.

RESULTS

In the initial assessment of pathogenicity from enteral inoculation of *B. pseudomallei*, all BALB/c and C57BL/6 mice administered 1×10^3 or 1×10^6 CFU survived for six weeks. One C57BL/6 mouse in the 10^6 CFU group developed paresis of a hind leg after one week. Of the mice inoculated with

1×10^8 CFU, two of three BALB/c mice and three of five C57BL/6 mice died within five days. The experiment was censored at six weeks. The surviving BALB/c mouse appeared hunched and ill; splenomegaly and a large splenic abscess were observed on a limited necropsy sample.

To confirm these findings, the experiment was repeated. In addition to doses of 1×10^6 and 1×10^8 CFU, a higher dose of 7×10^8 CFU was chosen to determine whether greater lethality could be provoked ($n = 4, 5$, and 4 and $n = 5, 5$, and 7 for BALB/c and C57BL/6 mice, respectively). One of the BALB/c mice that received 1×10^8 CFU was killed because of an isolated inability to lift its head. The combined survival data from the pilot and confirmatory experiments showing partial lethality at the higher inoculating doses are shown in Figure 1. Survival in the two strains of mice did not differ when analyzed by the log rank test.

Lung, liver, spleen, mesenteric lymph node, and, because of the neurologic manifestations observed, brain were harvested and stool samples were obtained from mice in the 1×10^8 and 7×10^8 CFU groups surviving to six weeks. Because abscesses were observed grossly in two BALB/c spleens, particular

FIGURE 1. Combined survival data from pilot and confirmatory experiments showing partial lethality at the higher inoculating doses of *Burkholderia pseudomallei*. A–D, BALB/c and C57BL/6 mice inoculated with 1×10^3 , 1×10^6 , 1×10^8 , or 7×10^8 colony-forming units (CFU) of *B. pseudomallei* by gavage and observed for survival. The 1×10^6 and 1×10^8 CFU data represent two independent experiments combined. BALB/c: $n = 3, 9, 8$, and 4 , respectively. C57BL/6: $n = 5, 10, 10$, and 7 , respectively. There was no difference in survival between mouse strains at any dose by the log rank test. E and F, Spleen section photographed with a $4\times$ objective (E) and liver section photographed with a $20\times$ objective (F) stained with hematoxylin and eosin from a BALB/c mouse inoculated with 1×10^6 CFU six weeks earlier. Multiple, large, confluent abscesses with dark blue necrotic cores efface the normal architecture of the spleen (E). In the liver (F), a focal area of hepatocellular necrosis contains clusters of neutrophilic and mononuclear inflammatory cells. The hypercellular appearance of surrounding sinusoids is caused by increased numbers of inflammatory cells. This figure appears in color at www.ajtmh.org.

care was taken to avoid cross-contamination between organs. Ashdown agar and the latex agglutination assay were used to confirm growth of *B. pseudomallei*. Four of six BALB/c mice but only one of six C57BL/6 mice had detectable bacteria in at least one organ or in stool sample (Table 1). No bacteria were recovered from mesenteric lymph nodes but several mice had positive brain cultures. Recoverable bacteria in any organ were only detected when bacteria were shed in the stool. To examine dissemination in the early phase of infection, BALB/c ($n = 4$) and C57BL/6 ($n = 5$) mice were inoculated with 7×10^8 CFU and killed after 24 hours. Lung, liver, spleen, mesenteric lymph node, and stool samples were cultured (Table 1). *Burkholderia pseudomallei* was recovered from three BALB/c mice and from one C57BL/6 mouse.

Histopathologic examination of organs from BALB/c mice six weeks after enteral infection with 1×10^6 – 7×10^8 CFU infection showed a range of inflammatory lesions. Splenic abscesses were characterized histologically by multiple large coalescing foci of densely necrotic neutrophils and parenchymal cells

surrounded by scant histiocytes and a thin fibrous capsule (Figure 1E). Liver sections were characterized by small, widely scattered focal abscesses, prominent Kupffer cells, and an abundance of inflammatory cells within sinusoids (Figure 1F). Extramedullary hematopoietic foci were common within the liver. Mild focal inflammation was present within or near heart valves and focal gliosis was noted within the brain. Lung histopathologic abnormalities were absent. Giemsa and Brown and Brenn stains failed to identify organisms within lesion sites.

To determine whether enteral infection induced an adaptive immune response, serum from surviving mice infected six weeks prior with 1×10^6 – 7×10^8 CFU were tested for IgG to heat-killed *B. pseudomallei* (Table 1). All mice with recoverable bacteria on organ sampling had titers of at least 1:32,768 but titers were variable in culture-negative mice.

DISCUSSION

We show in this exploratory study that at high doses *B. pseudomallei* administered enterally to mice may be rapidly lethal or cause chronic disseminated infection. Infection by this route induces an antibody response. Mice may shed bacteria in the stool for at least six weeks and may demonstrate neurologic involvement. The establishment of this experimental model bolsters efforts to understand the route of infection in human disease.

Melioidosis, which occurs only upon exposure to a contaminated environment, can potentially be prevented if the route of infection is understood. *Burkholderia pseudomallei* may survive for years in water under experimental conditions,¹³ and the evidence implicating ingestion as a possible route of infection in humans underscores the need for experimental animal models.^{3–5} Although others investigators have reported that oral inoculation may be lethal,^{7,9,10} our study has defined the clinical and pathologic features of enteric disease. Inoculating doses of 10^8 CFU may induce lethal disease in BALB/c and C57BL/6 mice. Unlike intravenous and intranasal infection models, we did not observe a survival difference between mouse strains.^{14,15} The enteral dose required for lethality in both strains is substantially higher than the approximately 5×10^2 CFU/lung that is lethal in an inhalation model of murine melioidosis (West TE, unpublished data). How this translates to infectious doses in humans is presently unclear. There are few data that indicate likely concentrations of *B. pseudomallei* in environmental water sources in disease-endemic areas. Additionally, chronic ingestion of the pathogen may conceivably result in different clinical manifestations, and impaired host defenses, as are commonly associated with melioidosis, may dramatically increase susceptibility to infection by this route.

Although *B. pseudomallei* survives poorly in the acidic gastric environment,¹⁶ our data show that invasive infection nonetheless may occur after high-dose enteric infection. Given the negative liver cultures at 24 hours, bacterial spread is conceivably initiated by mesenteric lymphatics rather than the portal venous system. The pattern of distant organ infection suggests hematogenous dissemination. Our observations of neurotropism are not unique because bacteria have been cultured from brain in oral, intranasal, intravenous and intraperitoneal models of *B. pseudomallei* infection.¹⁰ Neurologic infection in humans has been well documented, particularly in Australia.¹ Although intranasal mouse infection models probably cause

TABLE 1
Distribution of recovered *Burkholderia pseudomallei* and serum IgG titers after enteral infection of mice*

		Positive cultures		IgG titer
Mice		24 hours	6 weeks	
BALB/c				
Inoculum = 7×10^8 CFU				
Mouse 1	LN, stool	–	–	
Mouse 2	None	–	–	
Mouse 3	Spleen, stool	–	–	
Mouse 4	Lung, spleen, LN, stool	–	–	
Mouse 5	–	Brain, stool	$\geq 1:65,536$	
Mouse 6	–	Stool	$\geq 1:65,536$	
Mouse 7	–	None	$< 1:32$	
Inoculum = 1×10^8 CFU				
Mouse 1	–	None	$< 1:32$	
Mouse 2	–	Liver, spleen, stool	$\geq 1:65,536$	
Mouse 3	–	Spleen, brain, stool	$\geq 1:65,536$	
Inoculum = 1×10^6 CFU				
Mouse 1	–	–	1:256	
Mouse 2	–	–	1:256	
Mouse 3	–	–	$\geq 1:65,536$	
C57BL/6				
Inoculum = 7×10^8 CFU				
Mouse 1	Lung, stool	–	–	
Mouse 2	None	–	–	
Mouse 3	None	–	–	
Mouse 4	None	–	–	
Mouse 5	None	–	–	
Mouse 6	–	None	$< 1:32$	
Mouse 7	–	None	1:16,384	
Mouse 8	–	None	1:256	
Mouse 9	–	None	$< 1:32$	
Inoculum = 1×10^8 CFU				
Mouse 1	–	None	1:2,048	
Mouse 2	–	Brain, stool	1:32,768	
Inoculum = 1×10^6 CFU				
Mouse 1	–	–	$< 1:32$	
Mouse 2	–	–	$< 1:32$	
Mouse 3	–	–	1:32,768	
Mouse 4	–	–	1:4,096	
Mouse 5	–	–	1:1,024	

*Specimens sampled at 24 hours were lung, liver, spleen, mesenteric lymph node (LN), and stool. Specimens sampled at 6 weeks were lung, liver, spleen, mesenteric LN, brain, and stool. *B. pseudomallei*-specific IgG titers were measured 6 weeks after infection. CFU = colony-forming units; – = not measured; none = negative culture for all specimens sampled.

direct infection of the brain by the olfactory nerve,¹⁷ our results suggest that *B. pseudomallei* acquired enterally can cross the blood-brain barrier.

Our stool culture data indicate that *B. pseudomallei* survives for up to six weeks within the lumen of the gut. All mice with detectable infection in any organ had positive stool cultures; thus, recoverable fecal bacteria may be a marker for invasive infection. Likewise, in humans with melioidosis, *B. pseudomallei* has been isolated from rectal swabs, always in association with bacterial growth from other sites.¹⁸ The shedding of bacteria in stool, particularly on a chronic basis, raises important questions about mechanisms of environmental contamination in disease-endemic areas.

Enteric melioidosis induces an adaptive immune response in BALB/c and C57BL/6 mice. Not surprisingly, the *B. pseudomallei*-specific IgG titer was uniformly higher in the presence of persistent infection. Some mice had no measurable antibodies or detectable bacteria at six weeks, supporting the hypothesis that even at high inoculating doses, infection was not established in these animals. Robust antibody titers were measured in several mice that did not have bacteria recovered from the organs or stool sampled. These mice may have had foci of persistent infection in other organs that were not sampled, or may have developed but then cleared infection prior to sampling. A similar phenomenon after ingestion of *B. pseudomallei* might explain the seropositivity observed in otherwise healthy humans.¹⁹

Potential limitations to our model include the use of a thawed glycerol bacterial stock, which may have altered the interaction of the pathogen with the mucosal immune system. We have used the same stock extensively in an aerosol model of murine melioidosis and found our results to be reproducible and largely consistent with the published literature (West TE, unpublished data). Although we excluded cases of obvious aspiration during inoculation, we cannot exclude microaspiration after gastric delivery of bacteria. However, only two of nine mice had recoverable bacteria in the lung 24 hours after enteral infection, suggesting that this finding was not likely to be a dominant phenomenon.

In summary, this animal model provides supportive evidence that melioidosis may be initiated by ingestion of *B. pseudomallei*. However, high inoculating doses were required to demonstrate virulence in bolus infection of immunocompetent mice, and the parallels to human melioidosis remain uncertain. Additional investigations of environmental contamination, of experimental enteric disease models, including chronic ingestion and immunocompromised hosts, and of human melioidosis are required.

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