BURKHOLDERIA PSEUDOMALLEI BIOFILM PLAYS A KEY ROLE IN CHRONIC INFLAMMATION IN C57BL/6 MICE

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Abstract. *Burkholderia pseudomallei* is a causative agent of melioidosis. Clinical signs of melioidosis vary from acute septicemia to chronic inflammation or subclinical infection. This study investigated the role of *B. pseudomallei* biofilm in chronic inflammation in lungs of infected C57BL/6 mice. Low doses of *B. pseudomallei* H777 and its biofilm defective M10 mutant were fed intra-gastrically to C57BL/6 mice and inflammatory responses were investigated by histopathological techniques. Two hundred colony forming units (CFUs) of *B. pseudomallei* H777 induced chronic inflammatory responses in mice on day 20 post-infection, with discrete interstitial infiltration by mononuclear inflammatory cells. On day 40 post-infection, there were marked thickening of alveolar septa and congested capillaries, which increased in severity by day 60. On the other hand, mice infected with *B. pseudomallei* M10 showed less mononuclear infiltration. The results indicate that *B. pseudomallei* defective in biofilm production gave rise to less severe pathology, resulting a higher rate of survival in infected mice; and pulmonary melioidosis could be developed in C57BL/6 mice by intra-gastric feeding makes it a possible animal model of chronic human melioidosis.

Keywords: *Burkholderia pseudomallei*, chronic inflammation, inflammatory cells, intra-gastric feeding

INTRODUCTION

Burkholderia pseudomallei is a causative agent of melioidosis, a disease endemic in Southeast Asia and northern Australia (Limmathurotsakul *et al*, 2011). In northeastern Thailand, melioidosis is most commonly reported in Ubon Ratchathani Province (Limmathurotsakul *et al*, 2010). Infection is thought to be acquired through a wound in the skin, ingestion or inhalation of aerosolized *B. pseudomallei* (Cheng and Currie, 2005). Clinical manifestations can vary from acute or latent infection to chronic localized pathogenic symptoms. Melioidosis is commonly manifested in patients with compromised immunity, such as those with diabetes

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and/or thalassemia (Leelarasamee, 2004).

Currently, there is no vaccine for humans to counter the disease. Untreated septicemic illness has a high (80-90%) mortality rate and death occurs within 24-48 hours after the initial onset of symptoms (Chaowagul, 2000). The recommendation for antibiotic treatment is high dose intravenous ceftazidime or carbapenem administered for 10-14 days (Chaowagul, 2000; Dance, 2002). Early treatment with ceftazidime can decrease mortality by about 50% but the rate of relapse is still high (Inglis *et al*, 2006). Despite reports of chronic melioidosis, the pathogenesis mechanism is still unclear (Conejero *et al*, 2011), and in part this may be due to a lack of suitable models.

B. pseudomallei-infected C7BL/6 mice tend to develop chronic infection as they are more resistant to infection than BALB/c mice when infected with the same dose (Leakey *et al*, 1998). Moreover, chronically-infected C57BL/6 mice model does not mimic chronic infection in humans when bacteria are given via an intra-nasal route (Leakey *et al*, 1998; Hoppe *et al*, 1999). However, a recent study demonstrated chronic inflammation in C57BL/6 mice that mimicked chronic human melioidosis by means of intra-nasal route with low dose [100 colony forming units (CFU)] of *B. pseudomallei* (Conejero *et al*, 2011). However, the study required technically challenging intra-nasal bacterial inoculations of anesthetized mice, which had undesirable side-effects.

Hence, this study developed a C57BL/6 mice model of chronic inflammation melioidosis by direct delivery of *B. pseudomallei* by intra-gastric feeding. This technique did not need anesthetization and preparing bacteria in agar bead to produce granuloma inflammation. Using this animal model, role of *B. pseudomallei* biofilm associated with chronic inflammation in melioidosis was evaluated using wild type and biofilm-defective bacteria.

MATERIALS AND METHODS

Bacterial strains

B. pseudomallei H777 was isolated from blood of a patient admitted to Srinagarind Hospital, Khon Kaen, Thailand in 2001 (Taweechaisupapong *et al*, 2005). *B. pseudomallei* M10 is a biofilm-defective mutant of *B. pseudomallei* H777 constructed using transposon Tn5-OT182 inactivation and tetracycline selection (Taweechaisupapong *et al*, 2005). LD_{50} (50% lethal dose concentration) of H777 and M10 is 183 and 202 CFU (Taweechaisupapong *et al*, 2005). *B. pseudomallei* H777 was cultured on Ashdown medium agar (Hardy Diagnostics, Santa Maria, CA) at 37°C overnight. *B. pseudomallei* M10 was similarly cultured on LB medium agar (Oxoid, Basingstoke, Hants, UK) supplemented with 10 μg/ml tetracycline. A single colony of each bacterium initially was grown in trypticase soy broth (TSB) (Oxoid, Basingstoke, Hants, UK) to mid-logarithmic growth phase and then sub-cultured into 2% TSB. Bacterial culture then was incubated at 37°C with shaking for 2 hours and diluted in pyrogen-free saline (PFS) (HyClone, Logan, UI) to appropriate inoculation doses determined by plating bacterial suspension on nutrient agar (Oxoid) and bacterial counting after 30-48 hours of incubation at 37°C (expressed as CFU)/ml). All solutions used were sterile and all procedures described were carried out in a bio-safety cabinet.

B. pseudomallei experimental infection of C57BL/6 mice

Male pathogen-free C57BL/6 mice, 6 to 8 weeks of age and weighing about 20 g, obtained from the National Laboratory

Animal Center (NLAC), Thailand were infected intra-gastrically with 200 μl of 200 CFU *B. pseudomallei* H777 (group 1, 15 animals) and *B. pseudomallei* M10 (group 2, 15 animals). Mice injected with PBS (group 3, 15 animals) were used as control.

The study was carried out in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the Northeast Laboratory Animal Center, Khon Kaen University. The animal-use protocol was approved by the Animal Ethics Committee, Northeast Laboratory Animal Center, Khon Kaen University (AEKKU-NELAC 16/2557).

Histopathology examination

Lung samples were collected from infected mice randomly chosen from each group in neutral sodium salt-buffered formalin on days 20, 40, 60, and 80 post-infection. Samples were fixed for 1 month, embedded in paraffin wax and 3 μm sections were sliced, de-waxed and rehydrated with xylene and alcohol, respectively and washed in running tap water for 10 minutes. Tissue slices were stained with hematoxylin and eosin (H&E) and examined under a light microscope by a licensed pathologist.

Determination of bacterial load in blood and organs of infected mice

Blood (300 μl) was collected from each animal via retro-orbital puncture using a sterile heparinized capillary tube. On days 20, 40, 60, and 80 post-infection the liver, lung and spleen were removed from two mice chosen randomly from each group and each organ individually homogenized and cultured on nutrient agar for 24-48 hours at 37°C. Bacterial colonies with a typical appearance of *B. pseudomallei* were biochemically tested and immunologically identified by latex agglutination test (Wuthiekanun *et al*, 1996; Sirisinha *et al*, 1998). Individual blood samples were spread on nutrient agar plates and cultured as described above. Colony counts are expressed as CFU/ml. Blood cultures also were determined on day 2 post-*B. pseudomallei* feeding to confirm infection.

Statistical analysis

Statistical analysis was performed using Exact Probability test to compare survival rate among groups. Average survival days are calculated using *t*-test and Mann-Whitney *U* test. Data are considered statistically significant when *p*-value < 0.05. Analysis of data was performed using SPSS version 21 (IBM, Armonk, NY).

RESULTS

***B. pseudomallei* intra-gastrically infected C57BL/6 mice have positive bacterial load in organs but not in blood**

Chronic inflammation melioidosis animal model was successfully developed by feeding with a low dose (200 CFU) of *B. pseudomallei* as lung lesions were observed to be infiltrated by lymphocytes and epithelioid macrophages on day 20 (Fig 1). Mononuclear cell infiltration was found in lung tissues and a number of infected mice started to die from day 30. Some mice exhibited dense mononuclear cell infiltration and positive *B. pseudomallei* cultures were obtained from the lung and spleen. Severe inflammation was found on day 60 as evidenced by highly dense mononuclear cell infiltration and granulomas. Although inoculated mice were still asymptomatic through to day 80. The control group showed normal alveoli and intact terminal bronchioles through to day 80.

The survival rate of infected group 1 is significantly lower than that of control group 3 ($p = 0.021$), whereas the survival

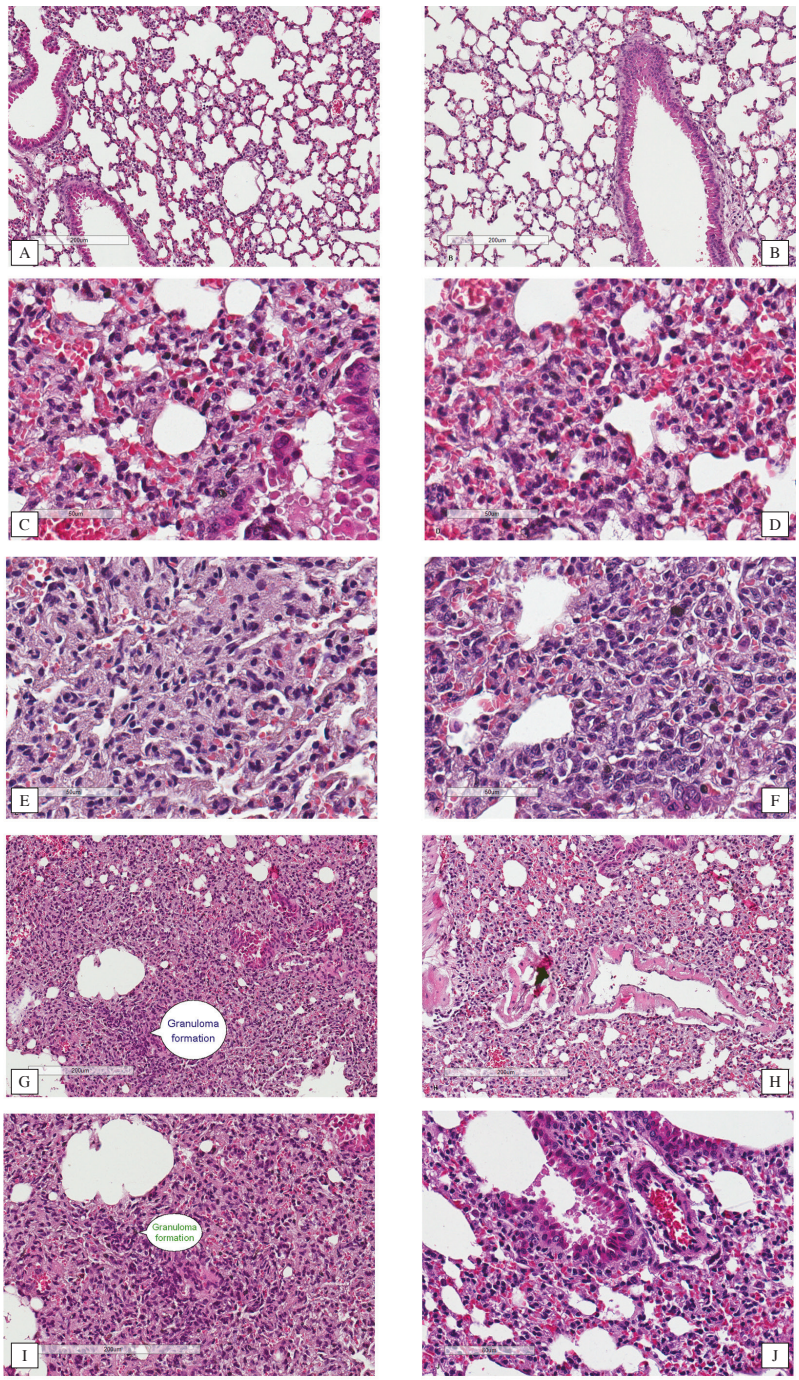

Fig 1–Hematoxylin and eosin stained sections of C57BL/6 mouse lung tissue following intra-gastric inoculation with 200 CFU *Burkholderia pseudomallei* H777 (C, E, G, I and J) or M10 (biofilm defective mutant) strain (D, F and H). Mice were sacrificed and lungs removed, sectioned, and stained on day 20 (A, C, and D), day 40 (E and F), day 60 (B, G, H, and I), and day 80 (J) post-infection. A and B, uninfected control. Magnification of each panel is indicated: A,B,H,G,I = 200 μm; C,D,E,F = 50 μm; J = 80 μm.

rate of infected group is not different from group 3 ($p = 0.462$) (Table 1).

Although all infected mice had positive blood culture on day 2 post-infection, blood cultures on days 20, 40, 60, and 80 exhibited negative results (data not shown). However, *B. pseudomallei* H777 was found in the lung of infected group 1 on day 20 and in the spleen on day 40 through to day 80, while in infected group 2 *B. pseudomallei* M10 became present in the lung and spleen on day 40 onwards (Table 2).

Biofilm plays a role in chronic inflammation in *B. pseudomallei*-infected mice

Histopathological studies of lungs from C57BL/6 mice at day 20 post-infection revealed a discrete interstitial infiltration by mononuclear cells with marked thickening of alveolar septa causing congested alveolar capillaries (Fig 1). Alveoli decreased in size on day 40. Severe inflammation was seen on day 60 post-infection. In addition, there was marked intense parenchymal infiltration by epitheliod histiocytes and small to large lymphocytes causing destruction of parenchymal tissues. *B. pseudomallei* strains H777 and M10 revealed similar pathology but M10 showed significantly less severe inflammation than H777 (Fig 1). *B. pseudomallei* strain M10 presented minimal foci of mild inflammation response, predominantly by presence of mononuclear cells, with an absence of neutrophils. Control mice showed signs of restoration of lung tissue dominated by weak cellular infiltration on day 20 post-infection and throughout the experiment.

DISCUSSION

This study successfully established a C57BL/6 mouse model with chronic *B. pseudomallei* infection in the lungs via intra-gastric inoculation, employing a ball-tip stainless steel 22 gauge gavage needle used to prevent any damage to the esophagus, passing gently through the mouth and pharynx into the esophagus, directly depositing the bacterial suspension into the stomach (Goodyear *et al*, 2012).

As the oral route was used for infection, the influence of gastric acid was a concern since it is known that exposure to pH < 3 for 15 minutes is lethal to bacteria and there reduced susceptibility to infection by ingested bacterial pathogens (Tennant *et al*, 2008). Nonetheless, the oral route of infection can be an animal model of human melioidosis especially in regions where *B. pseudomallei* is found in soil and water such as parts of northeastern Thailand. A low (200 CFU, approximately 1 LD_{50} amount) bacterial dose was used to enable colonization of the mice by a chronic challenge (Taweechaisupapong *et al*, 2005). Histopathological studies using two strains of *B. pseudomallei*, H777 and biofilm-deficient M10 mutant, showed less pathology of the lung generated by the latter mutant bacteria.

Intranasal challenge with low doses of *B. pseudomallei* induces chronic inflammation and mice begin to die from day 10 post-infection (Conejero *et al*, 2011). The median survival time is 58 days and 20% of mice exhibit no clinical signs of illness 3 months post-challenge when the experiment was terminated. In our study an intra-gastric administration by oral gavage induced chronic inflammation in mice by day 20 post-infection and mice started to die from day 30. The dense inflammatory infiltration in mice lungs mimicked that of chronic human melioidosis (Wong *et al*, 1995; Limmathurotsakul and Peacock, 2011).

Table 1
Number of surviving C57BL/6 mice during the period of experiment.

Number of remaining C57BL/6 mice after two mice from each group (n = 15) were sacrificed on designated day post-infection									
Days post-infection	Infected/injected with								
	PFS			H777			M10		
	Died	Sacrificed	Surviving	Died	Sacrificed	Surviving	Died	Sacrificd	Surviving
0	0	0	15	0	0	15	0	0	15
20	0	2	13	0	2	13	0	2	13
30	0	0	13	1	0	12	0	0	13
40	0	2	11	1	2	9	1	2	10
50	0	0	11	0	0	9	0	0	10
60	0	2	9	2	2	5	0	2	8
70	0	0	9	0	0	5	0	0	8
80	0	2	7	1	2	2	1	2	5
Surviving mice (p-value)		7			2 (0.021)			5 (0.462)	
Average survival days		80			61			74	
SD (p-value)		0			20 (0.033)			15 (0.462)	

PFS, pyrogen-free saline; H777, *Burkholderia pseudomallei* wild type strain; M10, *B. pseudomallei* biofilm-defective mutant strain.

Table 2
Burkholderia pseudomallei load in the spleen and lung from two C57BL/6 mice from each group.

Days post-infection	PFS		H777		M10	
	Lung (CFU/ml)	Spleen (CFU/ml)	Lung (CFU/ml)	Spleen (CFU/ml)	Lung (CFU/ml)	Spleen (CFU/ml)
20	NG	NG	67	NG	NG	NG
40	NG	NG	318	485	309	328
60	NG	NG	> 500	264	362	225
80	NG	NG	378	274	298	354

PFS, pyrogen-free saline; H777, *Burkholderia pseudomallei* wild type strain; M10, *B. pseudomallei* biofilm-defective mutant strain; NG, no growth. Note that the animals were those sacrificed from Table 1.

The role of biofilm formation in chronic inflammation was ascribed to an initiation of host immune response rendering bacteria to establish a biofilm community, which is difficult to eradicate and perpetuates inflammation by promoting further inflammation (Wolcott *et al*, 2008). Moreover, prolonged host inflammatory responses to microbes may also participate in the pathogenesis of chronic infection. Many chronic infections in humans are related to biofilm formation, such as cystic fibrosis, endocarditis, periodontitis, and chronic wound (Parsek and Singh, 2003). The difference between wild type and biofilm-defective mutant bacteria in the induction of inflammation may be due to attachment of the wild type microbe to surfaces of organs and subsequent aggregation in a hydrated polymeric matrix of their own synthesis, thereby forming a biofilm (Parsek and Singh, 2003). These sessile biofilm communities then give rise to non-sessile individuals. Thus, the biofilm-forming bacterial strains are able to turn into planktonic bacteria that rapidly multiply and disperse (Wolcott *et al*, 2008). The formations of these sessile communities are at the root of many persistent and chronic bacterial infections.

The use of *B. pseudomallei* biofilm-deficient M10 mutant resulted in mononuclear infiltration but the density of inflammatory cell infiltration was less than that caused by wild type *B. pseudomallei* H777. Possibly, the loss of biofilm formation resulted in less inflammation as the presence of biofilm results in resistance to immune clearance and chronic inflammation (Calo *et al*, 2011). Moreover, biofilm formation also affects anti-microbial therapy, resulting in resistance to antibiotics by limiting drug diffusion into the biofilm matrix and heterogeneity of bacterial growth within the biofilm (Niels *et al*, 2010). Stimulation of bacteria to produce biofilm results in certain genes to be resistant to anti-microbial agents (Sawasdidoln *et al*, 2010). However, although *B. pseudomallei* M10 was unable to form a biofilm chronic inflammation still occurred in the infected mice. This may be due to presence of other virulence factors, such as lipopolysaccharide, flagellin and capsule (Taweechaisupapong *et al*, 2005).

The role of phagocytic cells in inflammation depends the duration of the infection (Kolaczkowska and Kubes, 2013). In the case of acute melioidosis in BALB/c mice, infiltration of neutrophils and macrophages was observed with some necrosis. Acute inflammation mice model exhibits splenomegaly by day 4 post-infection (Lever *et al*, 2009). Both macrophages and neutrophils play key roles in early resistance to *B. pseudomallei* infection in various animal models (Katrin *et al*, 2006; Easton *et al*, 2007; Wiersinga *et al*, 2008) and histological analysis of tissues from patients with melioidosis demonstrated the presence of mostly neutrophils along with macrophages at the infection sites (Wiersinga *et al*, 2008**)**. However, we found only mononuclear cells at the site of infection in mice. Neutrophils may have been present in early period of inflammation but were not sufficient enough to control infection. This finding is in accord with a study by Conejero *et al* (2011) that did not find any recruitment of neutrophils in chronic murine melioidosis. Chanchamroen *et al* (2009) observed impaired phagocytosis and an inability to delay apoptosis of neutrophils in diabetic melioidosis patients and infection by *B. pseudomallei* with reduced uptake by neutrophils, attributed to the possibility that neutrophils being recruited early to the site of infection but do not persist (Kolaczkowska and Kubes, 2013).

A study using an acute melioidosis BALB/c mouse model infected with 100 doses of 50 LD_{50} amounts of *B. pseudomallei* induced splenomegaly with abscesses and all infected mice died 7-14 days post-infection (Panomket *et al*, 2009). Typically, BALB/c mice are very susceptible and have been used regularly to study acute melioidosis (Hoppe *et al*, 1999). On the other hand, C57BL/6 mice show increased resistance to *B. pseudomallei* infection even though they are unable to clear the infection (Barnes *et al*, 2001). Thus this study chose C57BL/6 mice to develop an animal model of human chronic melioidosis.

Splenomegaly and massive destruction of lymphoid tissue are common features of chronic melioidosis in animals (Conejero *et al*, 2011) but no splenomegaly was observed in any infected C57BL/6 mice in this study. However, *B. pseudomallei* was found in lung and spleen cultures. Conejero *et al* (2011) reported that a murine model of intra-nasally infection by *B. pseudomallei* shares features observed in human chronic melioidosis. Histological characteristics of this murine model revealed development of granulomas, caseous necrosis, multi-nucleated giant cells, and fibrosis. A novel model of chronic *B. pseudomallei* infection in rats using agar beads showed granulomatous pathology (van Schaik *et al*, 2008). In our study, epitheliod-like cells and showing granuloma formation were found in mice with chronic infection of *B. pseudomallei*. This granuloma formation was similar to those in C57BL/6 mice infected intranasally with a low dose of *B. pseudomallei* (Conejero *et al*, 2011) and in other infections such as tuberculosis, chronic broncho-pulmonary *Pseudomonas aeruginosa* and schistosomiasis (van Schaik *et al*, 2008; Conejero *et al*, 2011; Aubry, 2012). Multinucleated giant cells are also present in autopsy samples from humans who died from melioidosis (Conejero *et al*, 2011).

B. pseudomallei is most commonly found in soil, especially at a depth of 25-45 cm. However, bacteria can move to the surface with rising water table during the wet season. High incidence of melioidosis was found during the rainy season (Cheng and Currie, 2005). *B. pseudomallei* usually enters the host body via cuts and sores in the skin or via inhalation of dust or droplets. However, in endemic regions, there is a close association between melioidosis and rainfall (Parameswaran *et al*, 2012). Ingestion of *B. pseudomallei* may also occur. Intranasal inoculation of *B. pseudomallei* 1026b resulted in gastrointestinal (GI) tract as the primary site of bacterial persistence during the chronic infection phase (Goodyear *et al*, 2012). Moreover, *B. pseudomallei* preferentially colonizes the stomach following oral inoculation, and chronically colonized GI tract likely serves as a reservoir for dissemination of infection to extra-intestinal sites (Goodyear *et al*, 2012).

In summary, this study presents an unusual mode of *B. pseudomallei* infection via the intra-gastric route and resulting in melioidosis-induced pneumonia and the development of granuloma formation. These results confirm the recent report of two melioidosis patients resulting from drinking *B. pseudomallei* contaminated water (Limmathurotsakul *et al*, 2014). The development of a murine model of human chronic melioidosis via intra-gastric inoculation is easier and does not require anesthetization. Application of this model allowed identification of significantly different chronic inflammation pathologies caused by wild type and biofilm-deficient *B. pseudomallei* strains. The findings of the study should prove useful in future investigations of the role of biofilm in relapse infection.

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Declaration of conflicts of interests

The authors declare that there are no conflicts of interests with respect to the research, authorship, and/or publication of this article.

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