Relapsed Melioidosis Model in C57BL/6 Mice

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Background: *Burkholderia pseudomallei are the causative agents of melioidosis, a disease that has a high relapse rate in endemic areas. The mechanism of relapse is unclear.*
Objective: *This study aimed to establish relapsed melioidosis in C57BL/6 mice by induction with B. pseudomallei.*
Material and Method: *Low doses of B. pseudomallei H777 and its biofilm defective mutant (M10) were intra-gastric fed to C57BL/6 mice. All the infected mice had suppressed immune status by intra-peritoneal injection of hydrocortisone at 2.5 mg per mouse at day 60 post-infection. Inflammatory response to the infection was investigated by histo-pathological studies and monitoring bacterial counts in the blood and organs.*
Results: *All the infected mice were found to have a high infiltration of mononuclear cells at day 60 post-infection. The results showed high bacterial counts in the blood in both strains post-suppressed immune status after two days. The biofilm mutant and wild type strains produced relapse in C57BL/6 mice but the latter was responsible for significantly more severe inflammation than the biofilm mutant.*
Conclusion: *Low immune status may cause relapsed melioidosis in hosts with chronic inflammation.*

Keywords: *Burkholderia pseudomallei, Melioidosis, Relapse*

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B. pseudomallei are a gram negative bipolar staining bacilli. They are environmental saprophytes and can cause a community-acquired bacteremia, named melioidosis, which can grow inside host cells and is not destroyed by nitric oxide from phagocytic cells. Clinical presentation includes a variety of classifications ranging from acute sepsis to sub-acute sepsis, and chronic inflammation. Some patients may not show any symptoms. Ceftazidime is the first drug of choice for melioidosis treatment but not all patients are always cured[1]. Relapse is reported at an unacceptable rate of 4 to 23%[2-4]. Previous studies explained that relapse was due to the production of glycocalyx by bacteria by the formation of micro-colonies in damaged tissues and survival in phagocytic host cells called biofilm[5]. Two of these studies found biofilm production from *B. pseudomallei* was resistant to all antimicrobials[6,7]. However, the mechanism of relapse is still unclear.

The aim of this study was to establish relapsed infection in C57BL/6 mice by induction with low doses of *B. pseudomallei*. The model was subjected to histo-pathological studies and monitored bacterial counts in blood and organs between *B. pseudomallei* wild type and biofilm mutant. The model sought to explain the relationship between relapse and biofilm.

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Material and Method

Bacterial strains

B. pseudomallei H777 was isolated from the blood of a patient admitted to Srinagarind Hospital in 2001. *B. pseudomallei* M10 is a biofilm defective mutant of *B. pseudomallei* H777[8]. The mutant strain was constructed by using the transposon Tn5-OT182[8]. The LD50 of H777 and M10 were 183 and 202 CFU[8].

B. pseudomallei H777 was cultured on Ashdown medium agar at 37°C overnight. *B. pseudomallei* M10 was cultured on LB medium agar supplemented with 10 µg/ml tetracycline. A single

colony of each bacteria was initially grown in Trypticase Soy Broth (TSB) to mid logarithmic growth phase and then sub-cultured into 2% TSB. Bacterial culture was incubated at 37°C in a 200 rpm shaker-incubator for 2 hours and diluted in pyrogen-free saline (PFS) to the desired concentration. The turbidity of bacterial culture was diluted to appropriate infective doses, which were confirmed by plating bacterial suspension on nutrient agar for bacterial count after 30 to 48 hours of incubation at 37°C and expressed as colony forming units (CFU)/ml. All solutions used were sterile and all procedures described were carried out in a bio-safety cabinet.

C57BL/6 mice experimentally infected with B. pseudomallei

C57BL/6 mice aged four to six weeks, each weighing about 20 g, and free of specific pathogens were obtained from the National Laboratory Animal Center (NLAC). The animals (10/group) were infected with 200 CFU of *B. pseudomallei* H777 (group 1) and *B. pseudomallei* M10 (group 2). Mice injected with pyrogen-free saline (group 3) were used as a control. All animal experiments were performed in accordance with the guidelines of NLAC. The infections were done by intra-gastric feeding a total volume of 200 µL containing 200 CFU of bacteria. The animals were observed and their bacterial loads in the blood on days 60 and random organs (such as spleen, lung, and liver) were determined. The histology and pathology of inflammatory responses were investigated. Lung samples were collected 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 cut, de-waxed, and rehydrated through xylene and alcohol and then washed in running tap water for 10 minutes. Hematoxylin and eosin (H&E) was performed. Microscopic tissue sections were analyzed by a licensed pathologist. Uninfected mice were used as a control. The bacteremia and bacterial loads in organs were determined by culture on nutrient agars (Oxoid, Basingstoke, Hants, UK) for 24 to 48 hours and incubated at 37°C. The bacterial colonies that had the typical appearance of *B. pseudomallei* were confirmed by biochemical testing and latex agglutination[9,10].

Relapse model

Chronic melioidosis infection models were established with suppressed immune systems by intra-peritoneal injections of hydrocortisone at 2.5 mg per mouse at day 60 post-infection. The animals were observed and their bacterial loads in the blood on days 2 and 4 post-immune suppressed and random organs were determined. The histology and pathology of inflammatory responses were investigated.

Determination of bacterial load in blood and organs of infected animals

Blood was collected from each animal via retro-orbital puncture with a sterile heparinized capillary tube. The bacteremia and bacterial loads in organs were determined by culture in nutrient agars (Oxoid, Basingstoke, Hants, UK) for 24 to 48 hrs and incubated at 37°C. Individual blood samples were spread on nutrient agar plates for colony counts and expressed as CFU/ml. All groups randomly sacrificed two mice for bacterial loads in organs at days 2 and 4 post-immune suppressed, and when the infected animals became moribund, they were euthanized to collect their livers, lungs and spleens. Each organ was homogenized and cultured to determine the bacterial loads. The colonies with a typical appearance of *B. pseudomallei* were biochemically tested and immunologically identified by the latex agglutination test[9,10].

Results

C57BL/6 mice experimentally infected with B. pseudomallei

B. pseudomallei in blood and organs

All the infected mice were positive in blood culture on day 2 post-infection. On day, 60 post-infection, all infected mice gave high bacterial counts in spleens, lungs, and livers but not in the blood. The histology and pathology revealed highly dense mononuclear cell infiltration and granulomas (Fig. 1). More severe chronic inflammation was found in the H777 group than in the M10 group.

Relapse model

Chronic melioidosis mice were positive in blood culture on day 2 post-immune suppressed. The bacterial count shown in Table 1 was found to be highly significant in the H777 group when compared to the M10 group. Splenomegaly was seen in day 4 post-immune suppressed in both the H777 and M10 groups.

Discussion

Relapse is high in melioidosis cases. This study reported the establishment of a relapsed mouse model that provided a much-needed tool to investigate the pathogenesis and host immune response to

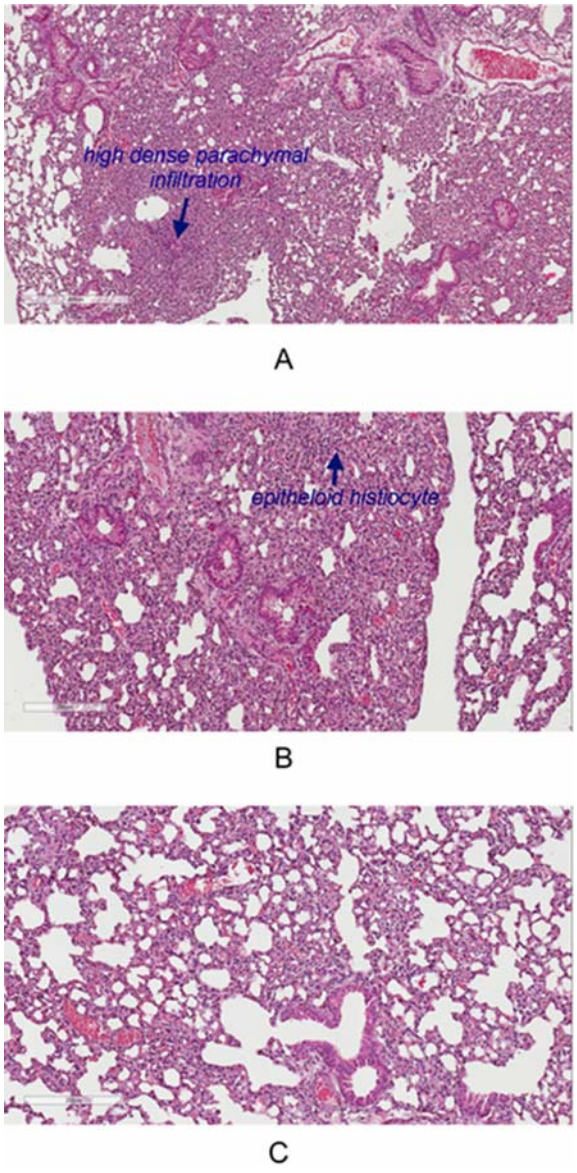

Fig. 1 Hematoxylin and eosin staining of lung tissue in C57BL/mice challenged with B. pseudomallei at day 60 post infection. Mice infected with *B. pseudomallei* strain H777 (A and B) showed high dense parenchymal infiltration and epitheloid histiocyte. Mice infected with *B. pseudomallei* strain M10 showed less mononuclear cells infiltration (C).

relapsed melioidosis and to evaluate new strategies of interventions against the disease. This study was successful in the establishment of C57BL/6 mice with relapsed melioidosis. The route of infection was intra-gastric and the route of immune suppression was intra-peritoneal. Infection intra-gastric was concerned with low pH that may destroy and ingest the bacteria. However, 200 CFU/ml (approximately 1LD50) was appropriated to establish and cause chronic inflammation in C57BL/6 mice (in a previous and unpublished study by the authors). Two strains of *B. pseudomallei*, H777 and M10, were studied. Both strains developed chronic inflammation and high dense infiltration of the lung parenchyma by epitheloid histiocytes and small to large lymphocytes were found on day 60 post-infection. However, the H777 group had inflammatory cells of high dense infiltration. Inflamed mice had suppressed immune status by hydrocortisone. High bacterial counts were observed in the blood on day 2 post-immune suppressed and high bacterial counts in spleens and lungs were observed in randomly sacrificed mice. Moreover, splenomegaly was seen on day 4 post-immune suppressed. The relapsed melioidosis in mice model exhibited a disease profile similar to the first episode of the acute septic melioidosis model. Primary infection with 100 LD50 of *B. pseudomallei* caused acute septic melioidosis, in that the animals developed high TNF-alpha, interleukin-gamma, high bacterial counts in the blood, splenomegaly, and abscess formation in spleens and lungs[11]. This study showed high bacterial count on days 2 and 4 post-immune suppressed. Relapsed mice started to die on day 5 post-immune suppressed in the H777 group. The severity of the disease depended on the virulence of the bacterial strain. The M10 group is a biofilm defective mutant of *B. pseudomallei* H777, and the strain had all the virulence factors like H777 except that it could not form biofilm. Nevertheless, the M10 strain was able to develop chronic inflammation but less severe than that found in H777 strain, as seen in the minimal foci of mild inflammatory response and predominantly mononuclear cells. However, when the M10 strain infected mice were treated with hydrocortisone, positive blood culture on day 2 post-immune suppressed similar to the H777 group was found. It is possible that other virulence factors, such as lipopolysaccharide, flagellin, and capsules, were active and might have played a role in inflammation. A previous study showed *B. pseudomallei* with uncommon LPS types (smooth type B and rough type) may be associated with relapse[12]. This contrasts with the work of Limmathurostsakul et al that found that relapse was associated with biofilm formation of the primary infection isolate, but not associated with LPS type[13]. The study that is the subject of this paper found the relationship between relapse and biofilm formation as a biofilm-defective mutant group that can

Table 1. Average bacterial count in blood from chronic inflammation mice post immune suppressed

Day post-immune suppressed	Mean bacteria count (CFU/ml)	
	H777 group (n = 6)	M10 group (n = 6)
2	358	274
4	472	126

Table 2. Average bacterial count in organs from chronic inflammation mice post immune suppressed

Day post-immune suppressed	Mean bacteria count (CFU/ml)					
	H777 group (n = 2)			M10 group (n = 2)		
	Spleen	Lung	Liver	Spleen	Lung	Liver
2	386	TNTC	458	294	428	TNTC
4	425	TNTC	462	342	284	394

TNTC = Too numerous to count

develop in relapse but in a less severe form than in the wild type.

Hydrocortisone-treated mice were found to be susceptible to chronic melioidosis inflammation and developed acute septic melioidosis within 2 days post-immune suppressed. However, immuno-compromised individuals were more likely to develop severe acute septicemia and relapse. The H777 group showed more severe chronic inflammation and higher bacterial counts in the blood after immune-suppressed when compared to the M10 group. This model showed that a biofilm defective mutant strain can develop chronic inflammation and when immune status was suppressed, the mice suffered relapsed infection. However, infection was less severe and had a significantly lower number of bacteria in the blood on days 2 and 4 post-immune suppressed (Table 1). Moreover, relapsed mice in the M10 group were able to survive more than 7 days post-immune suppressed. Biofilm formation may have an important role in relapse as seen in the lower bacterial counts in the blood when compared to the wild type group. Moreover, the study found dead relapsed mice in the wild type group within 7 days.

Conclusion

This study established a relapsed melioidosis in mice model. The route of infection was intra-gastric. Chronically inflamed mice had suppressed immune status by hydrocortisone treatment. Relapsed mice revealed acute septic inflammation like the first episode of infection as bacteremia and splenomegaly.

What is already known on this topic?

Relapsed melioidosis was established in C57BL/6 mice by intra-gastric infection. Relapsed mice revealed acute septic inflammation like the first episode of acute melioidosis. Relapsed model presented high bacteremia and splenomegaly. Severe inflammation mice were observed in *B. pseudomallei* H777 infected group.

What this study adds?

This is the first study for intra-gastric infection to relapsed melioidosis in C57BL/6 mice. Intra-gastric fed to C57BL/6 mice are convenience, no need equipments, and special skill.

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Potential conflicts of interest

None.

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แบบจำลองการเกิดการกลับเป็นซ้ำในโรคเมลลิออยโดสีสในหนู C57BL/6 mice

ภาวนา พนมเขต, ปาริชาติ วงศ์เสนา, สุรศักดิ์ แว่นรัมย์, สุรศักดิ์ วงศ์รัตนชีวิน, ธนัชพร บาตรโพธิ์

ภูมิหลัง: *Burkholderia pseudomallei เป็นสาเหตุของโรคเมลิออยโดสีสมีรายงานอัตราการเกิดการกลับเป็นซ้ำในพื้นที่ระบาดในอัตราสูง กลไกการเกิดการกลับเป็นซ้ำยังไม่ชัดเจน*

วัตถุประสงค์: *สร้างแบบจำลองการเกิดการกลับเป็นซ้ำในหนู C57BL/6 mice โดยการเหนี่ยวนำด้วยเชื้อ B. pseudomallei*

วัสดุและวิธีการ: *เชื้อ B. pseudomallei สายพันธุ์ H777 และเชื้อ H777 กลายพันธุ์ไม่สามารถสร้างไบโอฟิลม์สายพันธุ์ M10 ระดับต่ำ ถูกปล้อนให้หนู C57BL/6 mice และหนูทุกตัวถูกฉีดยากดภูมิด้วย hydrocortisone ขนาด 2.5 มิลลิกรัม ต่อ หนู 1 ตัว ผ่านทางหน้าท้องในวันที่ 60 หลังจากหนูติดเชื้อ การอักเสบจากการติดเชื้อถูกศึกษาด้วยการย้อมทางจุลพยาธิวิทยา และติดตามจำนวนแบคทีเรียในเลือดและอวัยวะภายใน*

ผลการศึกษา: *หนูทุกตัวที่ติดเชื้อพบการมาของ mononuclear cells ในวันที่ 60 หลังการติดเชื้อ เมื่อถูกกดภูมิ 2 วัน พบแบคทีเรียในเลือดจำนวนมาก ทั้งสองสายพันธุ์ สายพันธุ์ที่ไม่สร้างไบโอฟิลม์และสายพันธุ์ดั้งเดิมสามารถทำให้เกิดการกลับเป็นซ้ำได้แต่สายพันธุ์ดั้งเดิมสามารถทำให้เกิดการอักเสบได้รุนแรงกว่าสายพันธุ์ที่ไม่สร้างไบโอฟิลม์อย่างมีนัยสำคัญ*

สรุป: *ในภาวะที่ผู้ป่วยติดเชื้อ B. pseudomallei แบบเรื้อรังเมื่อภูมิคุ้มกันต่ำสามารถเกิดการกลับเป็นซ้ำของโรคเมลิออยโดสีสได้*