

Polymorphonuclear neutrophil response to *Burkholderia pseudomallei* in patients with diabetes mellitus

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Abstract

Background and objectives: *Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially fatal disease endemic in Bangladesh. Diabetes mellitus (DM) is a risk factor for increased susceptibility to *B. pseudomallei* infection. A few studies have been conducted to identify the underlying immunological mechanism responsible for increased susceptibility of individuals with diabetes mellitus to *B. pseudomallei* infection. The present study investigated the polymorphonuclear neutrophil (PMN) response to *B. pseudomallei* in terms of phagocytosis and early respiratory burst in individuals with diabetes mellitus.

Materials and Methods: A total of 5 cases of DM and 5 age and sex matched non-diabetic healthy individuals were enrolled in the study to determine the early respiratory burst and phagocytic ability of PMN to *B. pseudomallei*. The effect of *B. pseudomallei* on phagocytic ability and early respiratory burst of PMN was determined by phagocytic assay and nitroblue tetrazolium (NBT) test respectively. The response of PMN treated with *B. pseudomallei* was compared with that of *Escherichia coli*.

Results: There was no significant ($p>0.05$) difference in phagocytosis of *B. pseudomallei* by PMN between diabetic and non-diabetic cases (21.8 ± 4.64 percent vs 29.25 ± 5.5 percent). But in both diabetic and non-diabetic cases, significantly ($p<0.05$ and $p<0.01$) reduced rate of phagocytosis of *B. pseudomallei* by PMN was observed compared to *E. coli* (21.8 ± 4.64 vs 65 ± 5.36 ; 29.25 ± 5.5 vs 71.25 ± 5.59). Similar results were obtained in terms of phagocytic index. Mean percentage of formazan positive PMN from diabetic cases was not significantly different ($p>0.05$) from non-diabetic healthy cases when cells were treated with *B. pseudomallei* or *E. coli*. In both diabetic and healthy individuals, mean percentage of formazan positive PMN treated by *B. pseudomallei* was not significantly different from that by *E. coli*.

Conclusion: The observations revealed that *B. pseudomallei* was equally capable of inhibiting the phagocytic ability of PMN from both diabetic and non-diabetic individuals. This anti-phagocytic property might play an important role in the pathogenesis of melioidosis.

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Introduction

Burkholderia pseudomallei, a motile gram-negative facultative intracellular bacterium, is the causative agent of melioidosis which ranges from

asymptomatic infection, to localized or disseminated abscess to fatal septicemia [1]. The global burden of melioidosis is 165,000 human cases per year, of which 89,000 (54%) die. The

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bacterium is intrinsically resistant to a wide range of antibiotics and more than 70% of cases die due to treatment with ineffective antibiotics [2-3]. Bangladesh is an endemic country for melioidosis [4]. In Bangladesh, so far, 35 culture-confirmed melioidosis cases were identified from 2001 to 2016; however, true extent of the disease is unknown because of unfamiliarity of the organism to physicians and microbiologists of the country [4]. Human gets infection mainly via traumatic inoculation. After entry into the host, the organism enters into macrophage and may cause latent infection in immunocompetent host and reactivate in immunosuppressed condition [3, 5]. Understanding the pathogenesis of *B. pseudomallei* and the role of host immune response are essential to realize the course of the disease.

Diabetes mellitus is the most common risk factor for melioidosis, and is a co-morbid condition in more than 50% of all melioidosis cases [3]. The risk of diabetic people getting melioidosis is exceedingly higher than the rest of the population [6-7]. Diabetic individuals with poor glycemic control have defects in immune responses against infections [8]. However, the effect of DM on immunopathogenesis of *B. pseudomallei* infection is not clear yet. In BALB/c mice, the virulence of *B. pseudomallei* isolates from DM patients is significantly lower than that of isolates from patients without any risk factor; suggesting immunopathological changes due to diabetes increases the susceptibility to otherwise innocuous strain of *B. pseudomallei* [9].

The impact of diabetes in *B. pseudomallei* infection clearly delineates that the host immune response dictates the outcome of *B. pseudomallei* infection. In addition to acquired immune response, innate immune responses might play a critical role for the initial control of infection. Polymorphonuclear neutrophil (PMN), a cell of innate immunity, is considered the first line of defense against invading microorganisms. *In vivo* findings suggest that depletion of PMN from mice resulted in an acute and rapidly lethal *B. pseudomallei* infection, indicating the importance of PMN in early clearance [10]. Mice lacking NADPH oxidase, an important enzyme in the generation of the neutrophil respiratory burst, were more susceptible to *B. pseudomallei* disease [11].

Diabetes mellitus, the primary predisposing condition for melioidosis, is associated with impaired chemotaxis, phagocytosis, oxidative burst, and killing activity of neutrophils [12]. Increased incidence of melioidosis is also noted in neutropenic patients [13], as well as patients with chronic granulomatous disease [14]. Interestingly, treatment of melioidosis with the neutrophil-differentiating cytokine granulocyte colony-stimulating factor (G-CSF) showed mixed results [15]. It has reported to reduce the mortality of melioidosis patients in Australia [15], but is only associated with prolonged survival in Thai patients [16]. Thus, all these *in vivo* reports suggest the important role of neutrophils in controlling *B. pseudomallei* infection, but how *B. pseudomallei* affects neutrophil is not clear yet.

In this study, we aimed to observe PMN responses to *B. pseudomallei* in Bangladeshi people with diabetes mellitus. This would help us to understand the role of innate immunity in the pathogenesis of *B. pseudomallei*.

Materials and methods

Study population and sample collection: Cases of diabetes mellitus of different duration and age and sex matched apparently healthy non-diabetic individuals were enrolled in the study. All diabetic cases were on different oral hypoglycemic agents. Cases with chronic disease, known hematological disorders, acute infection, leucocytosis and fever in last one month were excluded. About 2-3 ml of blood was collected from each individual with aseptic precautions in a sterile heparin tube and kept in ice until used. All assays to measure the PMN response to *B. pseudomallei* and *E. coli* were done within 2-3 hrs of blood collection. Informed consent was obtained from all study population prior to collection of blood sample. The study was approved by the Ethic Review Board of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM).

Bacterial strains and cell preparation: *B. pseudomallei* strain (CS6887) isolated from a Bangladeshi melioidosis patient and *Escherichia coli* ATCC 25922 strain were used to measure the early respiratory burst and phagocytic function of PMN. The response of PMN to *B. pseudomallei* (CS6887) was compared to that of *E. coli*. The bacteria were

stored in trypticase soya broth (TSB, HiMedia Laboratories Pvt. Ltd., India) with 15% glycerol at -20°C until used. A single colony of *B. pseudomallei* or *E. coli* was suspended separately in 5 ml TSB in two tubes and incubated aerobically overnight at 37°C to obtain a cell suspension of 3×10^8 colony forming units per ml (CFU/ml).

Phagocytic assay: Phagocytic assay was performed as previously described [17]. Briefly, 20 μl of growth of either *B. pseudomallei* or *E. coli* in TSB (6×10^6 cells) was added to 500 μl of whole blood sample in two tubes and mixed thoroughly by gentle shaking. The tubes were incubated aerobically at 37°C for 30 minutes. After 30 minutes, the mixture was mixed with gentle shaking. Then, duplicate smears were made on two glass slides from each tube and air dried. Before making smear, the glass slides were soaked in xylene overnight and then washed with absolute ethanol and air dried for minimizing the clumping of neutrophils [18]. One slide was stained with Leishman stain and another one with 0.5% safranin stain. Smears were examined under the oil immersion lens and 200 neutrophils were counted (Figure-1a, 1b). The percentage of PMN with phagocytosed bacteria was calculated by: $\{(\text{Number of PMN with phagocytosed bacteria} \div \text{Total PMN counted}) \times 100\}$. The phagocytic index per neutrophil was estimated by the formula: $\{\text{Total number of intracellular bacteria} \div \text{Total PMN with phagocytosed bacteria counted}\}$ [17].

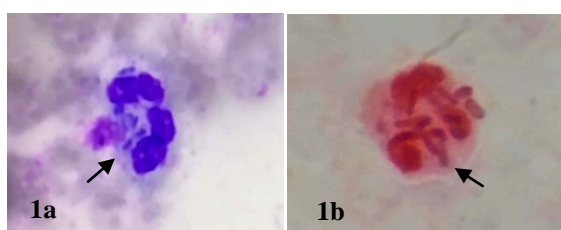


Fig-1. Photomicrographs showing PMN with phagocytosed bacteria (arrow); (a) Leishman stain (b) Safranin stain ($\times 1000$)

Nitro blue tetrazolium (NBT) test: Early respiratory burst of PMN to *B. pseudomallei* was determined by NBT test as previously described [19-20]. Formation of formazan by reduction of NBT dye following antigenic stimulation of PMN indicates occurrence of early respiratory burst in the cell.

Twenty microliter of either live *B. pseudomallei* or *E. coli* in TSB (6×10^6 cells) was added to 500 μl of whole blood sample in two tubes and mixed thoroughly by gentle shaking. *E. coli* was used to compare the response of PMN to *B. pseudomallei* while TSB without any bacteria served as a negative control. The tubes were incubated aerobically at 37°C for 30 minutes. Then 500 μl of 0.2% NBT solution was added to each of the above mentioned tubes and mixed thoroughly by gentle shaking. Solution of 0.2% NBT was prepared by dissolving 2 mg NBT dye (Abcam, UK) with 40 μl absolute ethanol; the dissolved solution was made up to 1 ml by adding 960 μl phosphate buffered saline (PBS). To dissolve completely, the solution was heated at 60°C for 20 minutes. This solution was made freshly for each batch of test. The tubes were again incubated aerobically at 37°C for 25 minutes after adding NBT solution. After 25 minutes, the tubes were mixed with gentle shaking. Then, duplicate smears were made on two glass slides from each tube and air dried. The glass slide was prepared as described above. One slide was stained with Leishman stain and another one with 0.5% safranin stain. Smears were examined under oil immersion lens and 200 neutrophils were counted. Neutrophils with a single, large, dense and deep-blue colored cytoplasmic deposit of formazan (reduced NBT, Figure-2a, 2b) were counted as “positive” cells. The PMN stained blue in Leishman stain while it was reddish in colour by safranin stain (Figure-2a, 2b). The formazan containing monocytes were not taken into account. Safranin stain permitted easier identification of formazan positive PMN due to an excellent contrast between the color of PMN and formazan. The percentage of formazan positive PMN was calculated as: $(\text{Number of formazan positive PMN} \div \text{Total PMN counted}) \times 100$

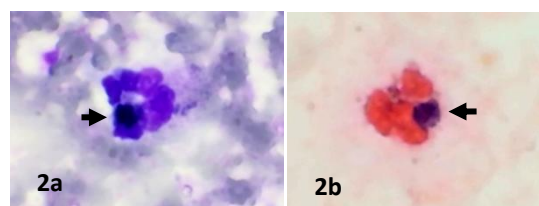


Fig-2. Photomicrographs showing deep-blue formazan deposits (arrow) positive PMN. (a) Leishman stain and (b) Safranin stain ($\times 1000$)

Results

A total of 5 patients with DM and 5 age and sex matched healthy non-diabetic individuals were included in the study. The mean duration of diabetes was 5.2 ± 1.34 years. The mean (\pm SE) postprandial (2 hrs after breakfast) blood glucose level of diabetic cases was 20.4 ± 1.36 mmol/l at the time of blood collection. The age range of the participants was 40-43 years and all were female.

Table-1 shows phagocytosis of *B. pseudomallei* and *E. coli* by PMN in study population. In DM cases, the rate of PMN with phagocytosed *B. pseudomallei* was significantly ($p=0.0102$) low compared to PMN with phagocytosed *E. coli* (21.8 ± 4.64 percent vs 65 ± 5.36 percent). Similar, significantly ($p=0.0004$) low rate of phagocytosis of *B. pseudomallei* by PMN was observed in non-diabetic healthy controls compared to PMN with phagocytosed *E. coli* (29.25 ± 5.5 percent vs 71.25 ± 5.59 percent). There was no significant difference in the rate of phagocytosis of *B. pseudomallei* by PMN between diabetic cases and non-diabetic healthy controls (21.8 ± 4.64 percent vs 29.25 ± 5.5 percent). Similar result was observed for phagocytosis of *E. coli* by PMN in diabetic cases and non-diabetic healthy controls (65 ± 5.36 percent vs 71.25 ± 5.59 percent).

The detail result of phagocytic index of PMN in diabetic cases and healthy controls stimulated with

B. pseudomallei and *E. coli* is shown in Table-2. In DM cases, the phagocytic index of PMN stimulated with *B. pseudomallei* was significantly ($p=0.0180$) low compared to PMN stimulated with *E. coli* (1.45 ± 0.1 vs 2.31 ± 0.15). Similar, significantly ($p=0.0072$) low phagocytic index of PMN stimulated with *B. pseudomallei* was observed in non-diabetic healthy controls compared to PMN stimulated with *E. coli* (1.73 ± 0.16 vs 2.66 ± 0.21). There was no significant difference ($p=0.1733$) in the phagocytic index of PMN between DM cases and non-diabetic healthy controls stimulated with *B. pseudomallei* (1.45 ± 0.1 vs 1.73 ± 0.16). Similarly, no significant ($p=0.2101$) difference was observed in the phagocytic index of PMN between diabetic cases and non-diabetic healthy controls stimulated with *E. coli* (2.31 ± 0.15 vs 2.66 ± 0.21).

Cytoplasmic formazan formation in PMN indicated early respiratory burst following stimulation with *B. pseudomallei* or *E. coli*. The difference of formazan positive PMN from diabetic cases and non-diabetic healthy controls was not significant ($p=0.6453$) when PMNs were stimulated with *B. pseudomallei*. In case of *B. pseudomallei*, mean percentage of formazan positive PMN in diabetic cases was 17.8 ± 2.05 compared to 19.4 ± 2.63 in non-diabetic healthy controls. Likewise, when stimulated with *E. coli*, formazan positive PMN from diabetic cases (13.4 ± 1.7 percent) was also not significantly ($p=0.6361$) different from non-diabetic healthy

Table-1: Rate of phagocytosis of *B. pseudomallei* and *E. coli* by PMN in study population

Study population	Mean % (\pm SE) of PMN with phagocytosed		p value*
	<i>B. pseudomallei</i>	<i>E. coli</i>	
DM (n=5)	21.8 ± 4.64	65 ± 5.36	0.0102
Non-diabetic healthy (n=4 ^a)	29.25 ± 5.5	71.25 ± 5.59	0.0004
p value**	0.331	0.4521	

Note: *paired student's t test; ** unpaired t test; a= one case was omitted due to inconsistent result.

Table-2: Phagocytic index of PMN in study population stimulated with *B. pseudomallei* and *E. coli*

Study population	Phagocytic index (Mean \pm SE) of PMN for		p value*
	<i>B. pseudomallei</i>	<i>E. coli</i>	
DM (n=5)	1.45 ± 0.1	2.31 ± 0.15	0.0180
Non-diabetic healthy (n=5)	1.73 ± 0.16	2.66 ± 0.21	0.0072
p value**	0.1733	0.2101	

Note: *paired student's t test; ** unpaired t test

Table-3: Rate of formazan positive PMN in study population stimulated with *B. pseudomallei* and *E. coli*

Study population	Mean % (\pm SE) of formazan positive PMN treated with		p value*
	<i>B. pseudomallei</i>	<i>E. coli</i>	
DM (n=5)	17.8 \pm 2.05	13.4 \pm 1.7	0.1248
Non-diabetic healthy (n=5)	19.4 \pm 2.63	15.2 \pm 3.26	0.1433
p value **	0.6453	0.6361	

Note: *paired student's t test; ** unpaired t test

individuals (15.2 \pm 3.26 percent). No significant difference ($p=0.1248$ and $p=0.1433$; Table-3) in formazan positive PMNs was observed between *B. pseudomallei* and *E. coli* in both diabetic and non-diabetic healthy cases.

Discussion

In recent time, an increase in melioidosis cases has been observed in Bangladesh. DM is the major risk factor for melioidosis [3]. The aim of this study was to determine neutrophil responses to *B. pseudomallei* in diabetic patients *in vitro*. PMN is the first line host defense against bacterial infection. After invasion of bacteria, PMNs migrate to the infection site, phagocytose and kill the bacteria by oxygen-dependent or oxygen-independent pathway [21].

In the present study, PMN from both diabetic and non-diabetic healthy participants showed significantly lower rate of phagocytosis of *B. pseudomallei* compared to that of *E. coli*. The phagocytic index (number of intracellular bacteria/PMN) was also significantly lower for *B. pseudomallei* compared to *E. coli* in both groups. Earlier, Mulye *et al.* also showed that in comparison to *E. coli*, *B. pseudomallei* was less efficiently phagocytosed by PMNs of both diabetic and healthy subjects [22]. Capsule might be a factor that could have prevented PMN to recognize and internalize the offending bacteria. Such anti-phagocytic property due to the presence of carbohydrate capsule have been described for a number of gram-positive and negative bacteria namely *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Haemophilus influenzae* and *Klebsiella pneumoniae* [23-24]. However, in the present study, although *B. pseudomallei* and *E. coli* both were capsulated, *B. pseudomallei* was significantly less phagocytosed

than *E. coli* by PMN from both study groups. This result suggests that *B. pseudomallei* might have additional virulence factor(s) that is more effectively preventing phagocytosis by PMN. So, further studies are required to investigate anti-phagocytic property of *B. pseudomallei* in more detail.

However, in the present study, PMN showed no significant differences in the rate of phagocytosis and phagocytic index for both *B. pseudomallei* and *E. coli* between diabetic and non-diabetic participants. Similar observation was reported earlier which showed no difference in the rate of phagocytosis of *B. pseudomallei* by PMN of diabetic and non-diabetic cases [25]. However, the study reported reduced rate of phagocytosis in diabetic cases with very poor glycemic (HbA1c>8.5%) control [25]. Another study group found reduced PMN uptake of virulent K1/K2 *Klebsiella pneumoniae* in diabetic patients compared with healthy subjects [26].

In oxygen dependent killing mechanism, NADPH-oxidase enzyme complex present in PMN is activated, transfers electron to oxygen molecule and forms superoxide. This superoxide kills bacteria. In this study, NBT dye was used to detect superoxide formation. NBT dye is reduced to insoluble deep-blue or purple colored deposit called formazan by transfer of electron by NADPH-oxidase enzyme complex. Therefore, formazan formation indicates superoxide formation in terms of early respiratory burst within PMN [21].

In the present study, the early respiratory burst in PMN following stimulation with either *B. pseudomallei* or *E. coli* was not significantly reduced in diabetic than non-diabetic population. Though there was no significant reduction of early respiratory burst in PMN from both diabetic and non-diabetic cases for *B. pseudomallei* and *E. coli*, we did not assess the actual killing of the ingested

bacteria. It is important to know the actual killing capability of *B. pseudomallei* by PMN from both diabetic and non-diabetic cases because *B. pseudomallei* can quickly escape from the endosome/phagosome of host cells and persists within the cytoplasm of those cells. Therefore, *B. pseudomallei* can spread from cell to cell avoiding the host extracellular environment [27]. This might play an important role to in the pathogenesis of melioidosis among diabetics as their other immune parameters are compromised [28]. Also, it is needed to see if there is any alteration of oxygen independent killing of *B. pseudomallei* by PMN of diabetic patients.

In the present study, the rate of phagocytosis, phagocytic index and early respiratory burst of PMN for both *B. pseudomallei* and *E. coli* were though less in diabetic than that of non-diabetic cases, these were not significant. This could be due to low number of cases tested or because of short duration (around 5 years) of diabetes in our cases. Therefore, further study with large number of cases is needed to see the response of PMN to *B. pseudomallei* in diabetics.

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