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Brief communication (Original)

Elevated intracellular levels of iron in host cells promotes *Burkholderia pseudomallei* infection

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Background: *Burkholderia pseudomallei* is the causative agent of melioidosis. The disease is endemic in northeast Thailand. Several studies have reported the effect of iron-overloaded environment in promoting bacterial infection. However, little is known about the effect of host cell iron elevation on melioidosis.
Objective: We investigated the impact of increased host cell iron levels on *B. pseudomallei* infection.
Methods: HeLa and A549 cell monolayers were supplemented with ferrous sulfate ($FeSO_4$) and ascorbic acid to increase intracellular iron levels. The iron elevated host cells were infected with *B. pseudomallei* and examined for plaque formation, cell invasion, intracellular survival and multinucleated giant cell (MNGC) formation and compared to bacteria infecting control cells.
Results: The ability of *B. pseudomallei* to form plaques in iron supplemented HeLa cells and to invade iron supplemented A549 cells was significantly higher. Furthermore, the intracellular survival of *B. pseudomallei* and the ability to induce MNGC formation in iron-supplemented host cells was greater than in infected control cells.
Conclusion: Elevation of iron levels in host cells promotes *B. pseudomallei* infection.

Keywords*: Burkholderia pseudomallei,* intracellular survival, invasion, iron elevation, virulence

Iron is an essential element of all living cells. Infected mammalian host cells require iron to generate an effective immune response [1] whilst the pathogen requires iron to support growth [2]. In host cells, several mechanisms operate to withhold iron from potential pathogens. One of the most effective is to decrease availability of iron by production of transferrin [3], lactoferrin [4] and hepcidin [5]. They compete with host cells for the available iron. Bacteria can produce siderophores, high affinity iron-binding compounds that take up iron from host cells by superior binding strength. Many diseases, such as hemochromatosis and thalassemia, predispose to iron overload. This has detrimental effects on many organ systems including the heart, liver and lungs, and may promote infection [6]. Several studies have reported the effect of an iron-overloaded environment on bacterial infection such as elevated levels of iron in Caco-2 human intestinal cells promoted invasion by *Salmonella enterica* serovar Enteritidis and increased cytokine/chemokine mRNA expression [7]. Infection of rabbits with *Pseudomonas aeruginosa* after injection of iron compounds resulted in rapid bacterial growth and death from an otherwise non-lethal dose [8].

Burkholderia pseudomallei is a Gram-negative intracellular bacterium and causative agent of melioidosis in humans and animals. South-east Asia, especially northeastern Thailand and northern Australia are endemic areas [9]. *B. pseudomallei* can infect many organs, most commonly the lungs. As iron is an essential element for growth of bacteria, maintaining free iron concentrations at extremely low levels (less than 10^{-18} M) is a first line of host defense against invading pathogens [10]. In order to acquire iron from the host, *B. pseudomallei* produces a siderophore (malleobactin) under the control of ferric uptake regulator (*fur*) gene [11]. Using DNA microarrays, it was shown that genes encoding siderophore-mediated iron transport and heme receptors were induced when the organism was grown in media with reduced iron content [12]. Wanachiwanawin [13] showed that iron-overloaded

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E-beta thalassemic patients had more frequent episodes of infection with *B. pseudomallei*.

The objective of this study was to examine the impact of host cell iron status on *B. pseudomallei* infection. Monolayers of tissue culture cells with elevated iron levels were infected with *B. pseudomallei* and several bacterial virulence phenotypes including plaque formation, cell invasion, intracellular survival and multinucleated giant cell (MNGC) formation were compared to bacteria infecting control cells.

Materials and methods

Bacteria, cell cultures and treatment

B. pseudomalllei K96243 was cultured in Luria-Bertani medium (LB) or trypticase soy broth (TSB) (Difco®, Dickinson, France). The culture was grown at 37°C. Human epithelium (HeLa) and human respiratory epithelial (A549) cell lines were obtained from the American Type Culture Collection and cultured in DMEM or RPMI 1640 medium (Gibco Laboratories, New York, USA) supplemented with 10% FBS (HyClone, Utah, USA) at 37°C in 5% CO_2.

B. pseudomallei growth in iron-restricted and supplemented medium

The effect of iron on *B. pseudomallei* growth was investigated by adding 0.5 mg/mL deferoxamine (DFO) (Sigma Chemical Co., Missouri, USA) in LB medium to chelate the available iron in the medium. Then, the DFO-treated LB medium was supplemented with ferrous sulfate ($FeSO_4$) (Sigma Chemical Co.) at concentrations of 10, 100 and 1,000 µg/mL plus 0.1 mg/mL ascorbic acid (Sigma Chemical Co.) before *B. pseudomallei* inoculation and incubation at 37°C. At various time points, the cultured broth was sampled for enumeration of *B. pseudomallei* by plating the serial dilutions of bacterial culture on trypticase soy agar (TSA) (Difco®).

Development of host cell elevated iron level and measurement of intracellular ferritin

HeLa and A549 cell monolayers were grown to confluence overnight in culture medium supplemented with 10% (v/v) FBS without antibiotics in 24-well tissue culture plates (Costar, Massachusetts, USA). The cell monolayers were washed once with 10 mg/mL EDTA in 1 × phosphated-buffered saline (PBS) to remove excess iron. Fresh medium containing various $FeSO_4$ concentrations to increase iron levels in A549 and HeLa cells plus 0.1 mg/mL ascorbic acid were added. Then, the iron treated host cells were incubated overnight at 37°C in 5% CO_2. Ascorbic acid had been reported to enhance iron available by reducing ferric complexes to the ferrous form [14]. Cell viability was examined by staining with 0.4% trypan blue dye (Gibco Laboratories) and counting viable cells under a light microscope (Olympus®, Tokyo, Japan). Intracellular ferritin was measured to determine the iron status. In brief, iron treated host cells were lysed with 0.1% Triton X-100 (Sigma-Chemical Co.) and the intracellular ferritin in the lysates was measured by a Spectro ferritin kit (Ramco Laboratoies, Texas, USA) according to the manufacturer's instructions. Three independent measurements were performed from triplicate wells.

Plaque, invasion, intracellular multiplication and survival assays

A plaque assay was performed in HeLa cell monolayer supplemented with iron. After infection with *B. pseudomallei* K96243 at multiplicity of infection (MOI) of 10 for two hours, cells were overlaid with 0.5% agarose containing DMEM medium, 10% FBS, 4.5 mg/mL of D-glucose to sustain cell viability, and 250 µg/mL of kanamycin to kill extracellular bacteria. To enhance visualization of the plaques, a second agarose overlay containing 0.01% neutral red was added, and the plaques were observed four hours later. Plaque-forming efficiency was measured as previously described [15]. An invasion assay was performed in the presence and absence of iron. Briefly, the A549 monolayer was treated to increase iron level and infected with *B. pseudomallei* K96243 at MOI of 50 for two hours. At three hours post-infection, the viable intracellular bacteria were released using 0.1% Triton X-100 and enumerated by plating serial dilutions on TSA. Bacterial colony forming units (CFU) were counted after 36-48 hours of incubation at 37°C. For the intracellular multiplication and survival assay, iron-treated A549 cells were infected as described above for the invasion experiment, except with the MOI of 10. At various intervals (2, 4, 6, 8, and 12 hours), cell monolayers were washed and lysed to estimate CFU counts. Monolayer integrity and viability was verified at the end of all assays.

MNGC formation assay

The assay was performed as described previously [16] with some modifications. Briefly, the iron

supplemented A549 cell monolayer was inoculated with *B. pseudomallei*. At various time points after exposure, the infected cells were stained with Giemsa (Merck, Darmstadt, Germany). The percentage of MNGC reflected the number of nuclei within multinucleated cells/total number of nuclei counted × 100. MNGC was defined as a cell with three or more nuclei per cell.

Statistical analysis

A statistical software package (Graphpad® Prism4) was used for data analysis and graphics. All tests for significance were performed using the Student's *t*-test. A P-value of less than 0.05 was considered statistically significant.

Results

Effect of iron on B. pseudomallei growth

To demonstrate the iron requirement for *B. pseudomallei* growth, an iron-chelating agent, DFO was added to *B. pseudomallei* cultures. It was found that *B. pseudomallei* could not grow in 0.5 mg/mL DFO-treated LB medium. When 10 and 100 μg/mL $FeSO_4$ was added to DFO-treated LB medium, the CFU counts of *B. pseudomallei* increased significantly (data not shown), indicating that iron is essential for *B. pseudomallei* growth. In addition, this finding may explain the report that occurrence of *B. pseudomallei* in acidic bore water in Northern Australia was strongly associated with high iron levels [17]. However, when $FeSO_4$ concentrations were increased to 1,000 μg/mL, the survival of *B. pseudomallei* decreased suggesting that excessive amount of iron may have toxic effects on *B. pseudomallei*.

Establishment of iron-rich host cells

Cell cultures were treated overnight with various concentrations of $FeSO_4$ (10, 100 and 1,000 μg/mL) coupled with 0.1 mg/mL ascorbic acid and the ferritin level was measured to monitor the intracellular iron status. We found that addition of 10, 100, and 1,000 μg/mL $FeSO_4$ plus ascorbic acid in HeLa cells caused ferritin level to increase from 53.25±1.25 ng protein/mL (unsupplemented control) to 116.35±9.45, 136.0±14.5, and 187.0±22.2 ng protein/mL, respectively. Although we noted that increasing $FeSO_4$ amounts also resulted in higher ferritin levels, we also observed a detrimental effect on the viability of cells at the concentrations of 100 and 1,000 μg/mL $FeSO_4$ (less than 80% at these levels). Therefore, a $FeSO_4$ level of 10 μg/mL was chosen to generate an iron-elevated status and yet maintain HeLa cell integrity. For respiratory epithelial A549 cells, addition of 10, 100 and 1,000 μg/mL of $FeSO_4$ plus ascorbic acid elevated the ferritin level from 45.65±3.55 ng protein/mL (unsupplemented control) to 237.65±21.75, 287.25±34.05, and 840.10±10.05 ng protein/mL, respectively. We observed that the viability of A549 cells was reduced dramatically at $FeSO_4$ concentration of 1,000 μg/mL. Therefore, 100 μg/mL $FeSO_4$ with 0.1 mg/mL ascorbic acid was chosen to induce iron loading in A549 cells. Having established a reliable protocol for induction of iron elevated host cells, we then investigated the effect of iron on *B. pseudomallei* infection.

Elevation of host cell iron level enhances virulence phenotypes of B. pseudomallei

To monitor whether the increasing intracellular iron levels can affect dissemination of *B. pseudomallei* from cell to cell, plaque forming efficiency was measured. **Figure 1A** shows that *B. pseudomallei* infection of 10 μg/mL $FeSO_4$-treated HeLa cells results in significantly increased plaque formation when compared to control cells ($p < 0.05$).

An invasion assay was performed to address whether this phenotype resulted from increased bacterial entry into host cells. As shown in **Figure 1B**, the ability of *B. pseudomallei* to invade 100 □g/mL $FeSO_4$-treated A549 cells (1.0±0.2%) was greater than that in the unsupplemented control (0.41±0.07%) ($p < 0.05$). This indicates that increasing host cell iron availability can promote *B. pseudomallei* invasion and that increased plaque formation may at least in part, be due to the increased invasion of *B. pseudomallei* into host cells. This result is consistent with a previous report that elevated enterocyte iron concentration enhanced *Salmonella* invasion [7].

The effect of elevating iron levels on the intracellular growth of *B. pseudomallei* after successful invasion of non-phagocytic cells was also investigated. The MOI of *B. pseudomallei* infections to the iron-treated A549 and control cells were adjusted so the initial number of bacterial entry into both cells (two hours) was not significant different ($p > 0.05$). As shown in **Figure 1C**, the number of intracellular *B. pseudomallei* in iron-treated A549 cells at 4, 6, and 8 hours post-infection were significantly increased when compared to untreated A549 cells.

This finding suggests that respiratory epithelial A549 cells with iron-enriched status promote intracellular multiplication and survival of *B. pseudomallei*.

MNGC formation is a unique characteristic of *B. pseudomallei* infection in tissue cultures. This remarkable feature was observed also in the tissues of melioidosis patients [18]. The exact reason for MNGC formation remains unknown but it is tempting to speculate that these structures are formed to enable *B. pseudomallei* to evade the antibody mediated killing mechanism. **Figure 2A** illustrates that the generation of MNGC by *B. pseudomallei* infected A549 cultures supplemented with iron is significantly greater when compared to control cells from 6 to 12 hours post-infection. Giemsa-stained MNGCs in iron-treated and control cells at 8 hours post-infection are shown in **Figure 2B**.

Discussion

We have developed a tissue culture model with elevated intracellular levels of iron to investigate virulence of *B. pseudomallei*. The first phenotype observed was increased ability of *B. pseudomallei* to form plaques in infected cells. Efficiency of plaque formation determines the whole process of infection including the ability of bacteria to invade, survive, replicate and spread from cell to cell. Further investigation suggested that the increased plaque formation efficiency observed in iron-supplemented host cells infected by *B. pseudomallei* may result not only from the increased invasion efficiency but also intracellular survival and replication of *B. pseudomallei*.

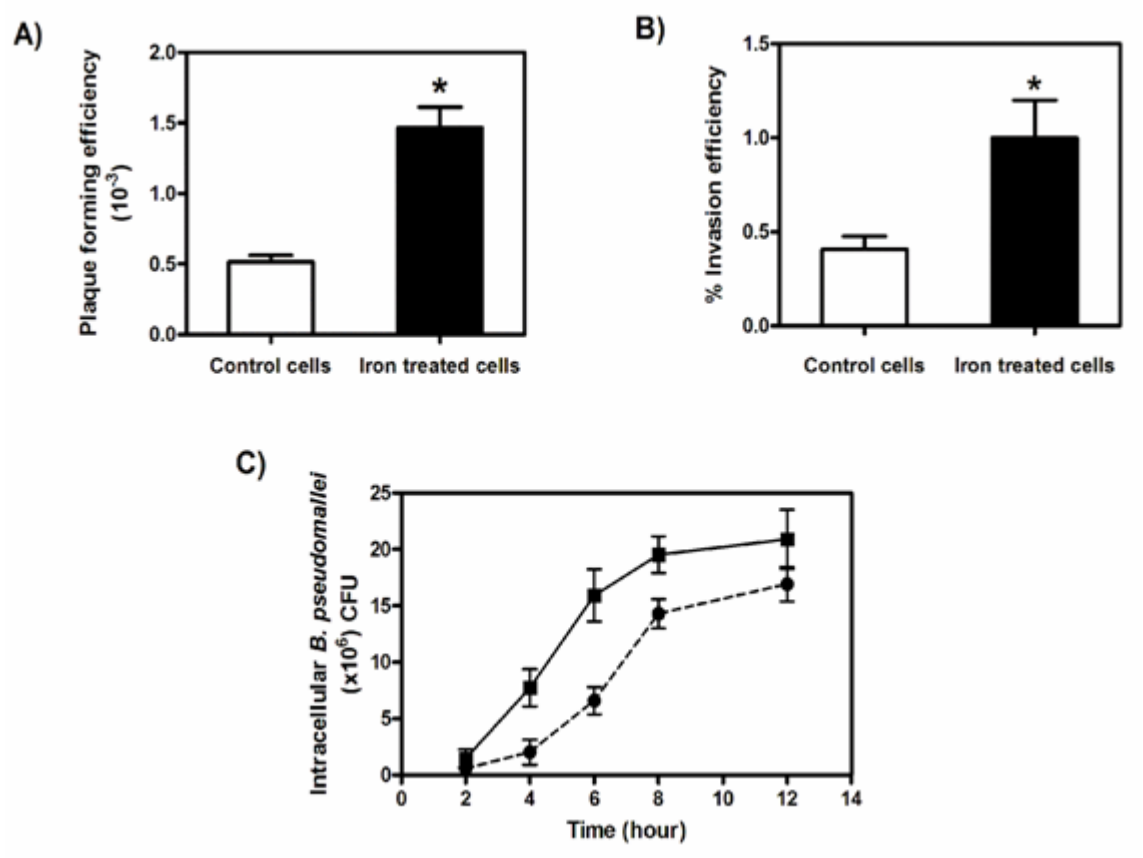

Figure 1. Effect of iron supplementation on the plaque formation, invasion efficiencies and intracellular multiplication of *B. pseudomallei*. **A:** Plaque-forming efficiency of control untreated (□) and $FeSO_4$-treated HeLa cells (■) were determined after infection with *B. pseudomallei*. **B:** Invasion efficiency of *B. pseudomallei* in $FeSO_4$-treated A549 cells (■) compared with control untreated cells (□). Asterisk indicates significant difference ($p < 0.05$, *t*-test). **C:** Intracellular multiplication of *B. pseudomallei* in control untreated (●) and $FeSO_4$-treated (■) A549 cells was evaluated post-infection by CFU count.

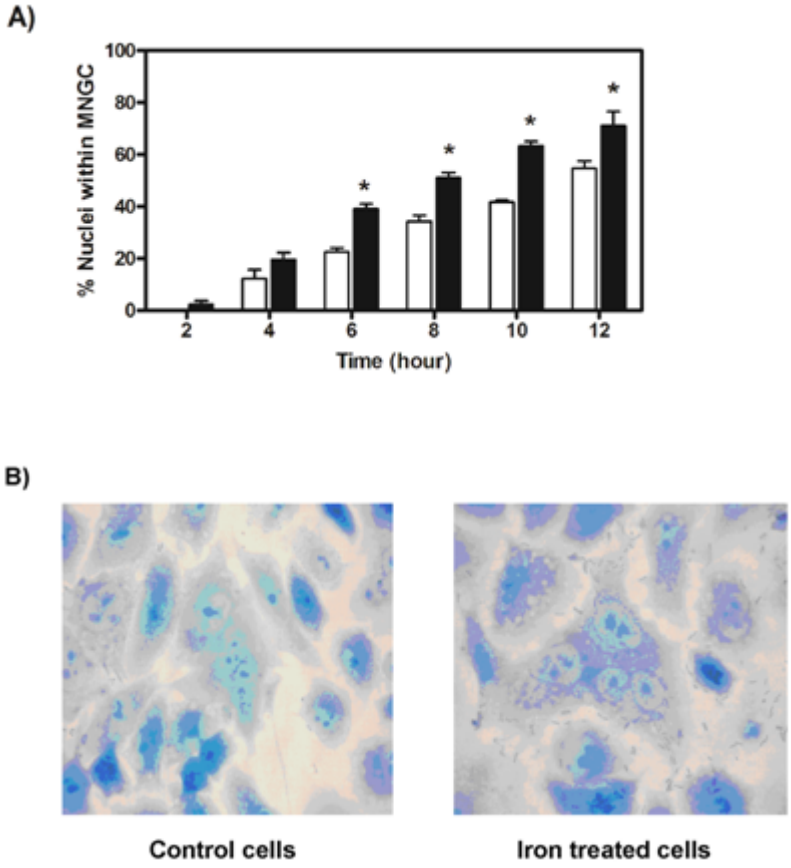

Figure 2. Effect of host cell iron supplementation on *B. pseudomallei* induced MNGC formation. **A:** Percentage of MNGC formation in control untreated (□) and $FeSO_4$-treated (■) A549 cells after *B. pseudomallei* infection. Asterisks indicated significant difference ($p < 0.05$, *t*-test). **B:** Giemsa staining of *B. pseudomallei*-induced MNGC at 8 hours post-infection of $FeSO_4$-treated A549 and control untreated cells. Bar, 10 µm.

The finding that the intracellular growth of *B. pseudomallei* was increased in iron-supplemented cells led us to hypothesize that the higher efficiency of *B. pseudomallei*-induced MNGC formation in A549 cells may be the result of increased survival and replication of intracellular *B. pseudomallei*. It had been reported that facultative intracellular bacteria including *M. tuberculosis*, *Salmonella enteritis* serovar Typhi, and *Chlamydia pneumoniae* are dangerous when they are growing within iron-rich host cells [19]. Furthermore, elevated iron stores are associated with HIV disease severity and mortality [20]. Chronic transfusion results in accumulation of excess iron leading to an increase in infection [21]. Injection of iron into mice and rats markedly increases the virulence of several pathogens [22]. Elevated iron concentrations in the lung can be used for microbial metabolism, resulting in more virulent and persistent infections [8]. As the most commonly infected organs found in melioidosis are the lungs, it is possible that patients with iron overload may be at increased risk of *B. pseudomallei* infection.

The majority of patients with clinical melioidosis have underlying diseases. In northeastern Thailand, the prevalence of a thalassemia trait, homozygous hemoglobin E or hemoglobin E trait is approximately 40% of the population [23]. It has been documented that thalassemic patients have an increased risk of *B. pseudomallei* infection [13, 23]. Gaining control over iron homeostasis may be important in reducing *B. pseudomallei* infection or disease severity. This suggestion is supported by a recent report that a combination of antibiotics and iron chelator could successfully block biofilm formation and improve treatment outcome of *P. aeruginosa* infection [24].

Conclusion

By treating both human cervical epithelial HeLa and human respiratory epithelial A549 cells with FeSO4 coupled with ascorbic acid, we generated an elevation of the iron level, which was monitored by measuring intracellular ferritin induction. This iron rich state enhanced the invasion efficiency, intracellular survival and replication of *B. pseudomallei* compared with infected control cells suggesting that elevated host cell iron levels promotes *B. pseudomallei* infection.

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