Rapid Communication

Adaptive immunity in melioidosis: a possible role for T cells in determining outcome of infection with \textit{Burkholderia pseudomallei}

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Abstract

Melioidosis is a potentially fatal disease caused by the bacterium \textit{Burkholderia pseudomallei}. Individuals with subclinical melioidosis have no apparent clinical signs or symptoms, and are identified only by positive serology. The present study is the first to investigate cell-mediated immune (CMI) responses following in vitro stimulation with \textit{B. pseudomallei} antigens in peripheral blood mononuclear cells (PBMC), collected under field conditions in Papua New Guinea (PNG) from individuals with exposure to \textit{B. pseudomallei} (\(n=13\)). While five had a clinical history of melioidosis (C\(^+\)), the remaining individuals (\(n=8\)) were seropositive, yet healthy with no clinical history of melioidosis (S\(^+\)/C\(^0\)/C\(^0\)). Proliferation and IFN-\(\gamma\) production were significantly greater in lymphocyte cultures from S\(^+\)/C\(^0\)/C\(^0\) individuals compared to C\(^+\) individuals (\(P<0.001\) and \(P<0.05\), respectively). These findings demonstrate that compared to C\(^+\) patients, individuals with subclinical melioidosis have a stronger CMI response to \textit{B. pseudomallei} antigens in vitro. Such a response may be essential for protection against disease progression.

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Keywords: Cell-mediated immunity; Serology; Melioidosis; \textit{Burkholderia pseudomallei}; Papua New Guinea

Introduction

The facultative intracellular bacterium, \textit{Burkholderia pseudomallei}, causes melioidosis, the potentially fatal disease of humans and animals. In humans, infection with \textit{B. pseudomallei} can range from acute fulminating septicemia to a subclinical form, identified only by seroconversion. The present study is the first to investigate cell-mediated immune (CMI) responses following in vitro stimulation with \textit{B. pseudomallei} antigens in peripheral blood mononuclear cells (PBMC), collected under field conditions in Papua New Guinea (PNG) from individuals with exposure to \textit{B. pseudomallei} (\(n=13\)). While five had a clinical history of melioidosis (C\(^+\)), the remaining individuals (\(n=8\)) were seropositive, yet healthy with no clinical history of melioidosis (S\(^+\)/C\(^0\)/C\(^0\)). Proliferation and IFN-\(\gamma\) production were significantly greater in lymphocyte cultures from S\(^+\)/C\(^0\)/C\(^0\) individuals compared to C\(^+\) individuals (\(P<0.001\) and \(P<0.05\), respectively). These findings demonstrate that compared to C\(^+\) patients, individuals with subclinical melioidosis have a stronger CMI response to \textit{B. pseudomallei} antigens in vitro. Such a response may be essential for protection against disease progression.

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Innate immune responses in the initial stages of infection are essential for an efficient immune response against pathogens. It has been shown that defects in phagocytic cell function [14] and the ability of the pathogen to resist killing within phagocytes [15] could lead to persistence of B. pseudomallei. In intracellular bacterial infections, efficient interaction between infected host cells and antigen-specific T cells is required for pathogen elimination [16]. Little is known about the role of adaptive cell-mediated immune (CMI) responses in melioidosis. However, as B. pseudomallei is an intracellular pathogen [17–19], such responses are believed to be essential, particularly since antibodies specific to the organism have little effect in the development of protection [20]. Latent or subclinical forms of melioidosis occur due to the ability of B. pseudomallei to persist in the host for extensive periods before reactivation of infection many months or years later [6,12,21]. Individuals with latent melioidosis have no apparent clinical signs or symptoms, and are identified only by positive serology. Therefore, exposure to, or infection with, B. pseudomallei does not necessarily result in development of clinical melioidosis. Although a recent study demonstrated the development of an adaptive CMI response in culture-confirmed melioidosis patients [22], and a case report demonstrated high CMI in an individual who may have been exposed to B. pseudomallei [23], no previous studies have investigated the CMI responses in a group of individuals with subclinical melioidosis. In the study reported here, CMI responses to B. pseudomallei antigens were compared among a group of seropositive individuals with subclinical infection. In the study reported here, CMI responses to B. pseudomallei antigens were compared among a group of seropositive individuals, with and without a history of culture-confirmed melioidosis. An understanding of the mechanisms that enable seropositive individuals with subclinical infection to control and/or eradicate B. pseudomallei may provide clues for the development of vaccines or immunotherapy. Knowledge of such mechanisms would also have important implications for individuals with subclinical melioidosis who require immunosuppressive treatment for unrelated conditions.

Materials and methods

Study participants

A group of 13 individuals (nine males, four females), currently involved in a prospective study into melioidosis from Balimo, Western Province, PNG (S 8°; E 143°) were selected to participate in the present investigation. The mean age of the individuals was 22 years (range 11–51 years). The cohort represents two groups, the details of which are shown in Table 1. The first group was composed of individuals (n = 5) who were symptomatic and culture positive for melioidosis (C+). Diagnosis was confirmed by isolation of B. pseudomallei from clinical samples. The mean time since diagnosis of melioidosis in the culture positive group was 69 months (range 53–85 months). The second group of individuals (n = 8) was related to the melioidosis patients and was sharing all epidemiological factors at the time the cluster of clinical melioidosis cases was recognized [13]. Subjects in the second group were serology positive, but did not present with any signs or symptoms of infection (S+/C−).

The assays to determine CMI were performed with blood samples from these 13 B. pseudomallei-exposed individuals, none of whom had clinical evidence of melioidosis at the time the assays were performed. Peripheral blood was collected under field conditions in Balimo, PNG, and flown to Townsville, Australia, where it was processed within 12 h of collection. A control group consisted of six subjects who had no clinical history of melioidosis and were serologically negative for antibody to B. pseudomallei (S−/C−), They included three men and three women with a mean age of 40 years (range 25–53 years). The study protocol was

Table 1

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NCM—no clinical melioidosis, asymptomatic.

* Antibody status is considered positive if >1:40 in IHA assay. The antibody status at the time of the outbreak (1998) and the time of CMI assay (2003) is given.
approved by the PNG Medical Research Advisory Committee (MRAC 0302) and informed consent of the subjects was obtained.

**Antibody titers**

An indirect hemagglutination (IHA) test was used to determine melioidosis sero-reactivity according to the method of Ashdown [24]. Briefly, antigen was prepared from pooled supernatants of heat-killed cultures of five *B. pseudomallei* strains. Serum specimens were incubated at 56°C for 30 min and then adsorbed with saline-washed non-sensitized sheep erythrocytes at room temperature (RT) for 15 min before testing. Two-fold dilutions of test serum were performed in isotonic saline (1:5–1:5120), and each dilution was incubated with sensitized ovine erythrocytes for 2 h at RT. The highest dilution at which hemagglutination occurred was recorded as the endpoint titer. An IHA titer of ≥1:40 was considered evidence of recent or remote exposure to *B. pseudomallei*. Positive and negative reference sera were included with each run.

**Proliferation assays**

Proliferation assays were performed to determine lymphocyte responses to a *B. pseudomallei* lysate (BpLy1). BpLy1 is a cocktail of antigens prepared from strain NCTC 13179 by sonication, using a method described previously [22]. PMBC separated from heparinized blood were cultured in 96-well plates [10^6 peripheral blood mononuclear cells (PBMC)/ml] in RPMI 1640 medium (Invitrogen, Australia) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES buffer (25 mM) and 10% pooled human serum. Triplicate wells were stimulated with BpLy1 (1 μg/ml), tetanus toxoid (Ttox; 0.5 U/ml) or phytohemagglutinin (PHA; 10 μg/ml). Culture plates were incubated in 5% CO2 at 37°C. Proliferation of cells was determined at 24-h intervals on days 4–7 of culture (four time points) by measuring [3H]-thymidine incorporation (Amersham-Pharmacia Biotech; 1.25 μCi/ml for 4 h). Results are expressed as log_{10} stimulation index (SI), the proportion of counts per minute in stimulated cultures compared with that in unstimulated cultures. The log_{10} maximum SI recorded at any one of the four time points was compared between seronegative healthy controls (S^-/C^-), seropositive/culture negative (S^+/C^-) and culture positive (C^+) individuals.

**IFN-γ production**

IFN-γ production was determined using QuantiFERON-TB assay (Cellestis) with modifications to the manufacturer’s instructions. Briefly, heparinized whole blood (1 ml) was added to 24-well culture plates and wells were stimulated with BpLy1 (1 μg/ml), Ttox (0.5 U/ml) or PHA (10 μg/ml). Plates were then incubated for 24 h in 5% CO2 at 37°C. Following incubation, contents of each well were centrifuged (10 min; 500 × g) and plasma removed and stored at −70°C until further analysis. IFN-γ levels were measured in samples from individual wells as per the manufacturer’s instructions. Briefly, plasma samples (50 μl) were added in duplicate to 96-well plates coated with anti-human IFN-γ monoclonal antibody. Conjugate (murine monoclonal anti-human IFN-γ-horseradish peroxidase; 50 μl) was subsequently added and plates were incubated for 1 h at RT. After washing, chromogen (3,3',5,5' -tetramethylbenzidine) was added and color development was stopped (0.5 M H2SO4) after incubation for 30 min at RT. Absorbance of each well was determined with a microtiter plate reader (Multiskan EX355 v1.0, Labsystems, Finland) at 450 nm, with a reference wavelength of 650 nm. Recombinant human IFN-γ standards were included to construct a standard curve. IFN-γ levels of unstimulated wells were subtracted from stimulated wells and results are expressed as international units per milliliter (IU/ml), relative to the standard preparations.

**Statistical analysis**

Maximal lymphocyte proliferation between days 4 and 7 of culture (maximum SI from four time points) for each individual in the three groups and the production of IFN-γ was analyzed by univariate analysis of variance (ANOVA), using SPSS statistical software (version 8). Dependent variables were tested for normality with a Q-Q plot and were transformed where necessary. Correlation between sets of data was assessed by Pearson’s correlation and was considered significant if the probability of a type I error was <0.05% (P < 0.05). Mean values in the text are expressed as mean ± SEM.

**Results and discussion**

The clinical spectrum of melioidosis is wide, ranging from asymptomatic infection to acute fulminant septicemia [1,25,26]. *B. pseudomallei* can remain latent in the host for long periods of time and the disease may recrudesce clinically when conditions become favorable for the bacterium to multiply. In endemic areas, a significant proportion of apparently healthy individuals have antibodies to *B. pseudomallei* [2,27–29]. These antibodies may result from the transition from asymptomatic infection to acute fulminant septicemia. In endemic areas, a significant proportion of apparently healthy individuals have antibodies to *B. pseudomallei* [2,27–29]. These antibodies may result from the transition from asymptomatic infection to acute fulminant septicemia. In endemic areas, a significant proportion of apparently healthy individuals have antibodies to *B. pseudomallei* [2,27–29]. These antibodies may result from the transition from asymptomatic infection to acute fulminant septicemia.
present infection with *B. pseudomallei*. A wide range of antibody titers was observed in the seropositive individuals included in this study although no correlation was found between antibody levels and disease outcome (Table 1). This is consistent with previous studies [20,22]. It should be noted that while the initial IHA titer was negative for individual no. 5, diagnosis of melioidosis was based on isolation of *B. pseudomallei* from a groin abscess. Therefore, this individual was included as a C+ patient in the present study.

The current study was the first to compare CMI responses within *B. pseudomallei*-exposed individuals with disparate outcomes of infection, to *B. pseudomallei* antigens in vitro. We focused attention on a group of individuals in whom *B. pseudomallei* infection produced either clinical or subclinical melioidosis. Since neither group had clinical melioidosis at the time of this study, results are unlikely to have been influenced by possible immunoregulatory effects that may be associated with an ongoing systemic infection. It is possible however, that infection persisted, although both groups would have been subject to this risk, since treatment may not eradicate *B. pseudomallei* [21,29,30].

The proliferative response of lymphocytes in cell cultures derived from *B. pseudomallei*-exposed individuals (log\(_{10}\) mean SI, 2.64 ± 0.1; range 2.12–3.48) was significantly higher following stimulation with BpLy1 (*P* < 0.001) compared to lymphocytes derived from control subjects (log\(_{10}\) mean SI, 1.55 ± 0.1; range 1.02–1.76) (data not shown). In contrast, high levels of proliferation in controls (log\(_{10}\) mean SI, 2.48 ± 0.3; range 1.12–1.76) and in *B. pseudomallei*-exposed subjects (log\(_{10}\) mean SI, 2.30 ± 0.2; range 1.00–3.57) were measured in response to stimulation with Tox ( *P* > 0.05; data not shown). These results support the findings of our previous study [22,23].

Proliferative responses to BpLy1 for both C+ (*P* < 0.001; log\(_{10}\) mean SI, 2.38 ± 0.3; range 2.12–2.61) and S+/C− (*P* < 0.001; log\(_{10}\) mean SI, 2.74 ± 0.2; range 2.11–3.48) individuals were significantly higher than responses of S−/C− (log\(_{10}\) mean SI, 1.55 ± 0.13) (Fig. 1a). Lymphocyte proliferative response to *B. pseudomallei* antigens was highest in seropositive individuals with no clinical history of melioidosis (S+/C−) (*P* < 0.001; Fig. 1a). In contrast, comparable proliferation in response to stimulation with the Tox antigen was observed for S−/C− (log\(_{10}\) mean SI, 2.48 ± 0.3), S+/C− (log\(_{10}\) mean SI, 2.32 ± 0.3) and C+ (log\(_{10}\) mean SI, 2.34 ± 0.3) individuals, indicating that their CMI responses were not depressed (Fig. 1a). Similarly, no significant differences were observed in the proliferative response to PHA stimulation between lymphocytes from S−/C− (log\(_{10}\) mean SI, 2.11 ± 0.2), S+/C− (log\(_{10}\) mean SI, 2.54 ± 0.2) and C+ (log\(_{10}\) mean SI, 2.35 ± 0.3) individuals (Fig. 1a).

Whole blood from S+/C− subjects also produced the highest levels of IFN-γ (mean IU/ml, 10.4 ± 3; range 0–37.7) when stimulated with BpLy1 (Fig. 1b), reflecting the proliferative responses to *B. pseudomallei* antigens (Fig. 1a). These levels were significantly higher than for C+ individuals (*P* < 0.05; mean IU/ml, 2.8 ± 1.6; range 0–8.76) but not compared to S−/C− (*P* > 0.05; mean IU/ml, 5.5 ± 2; range 0–9.5). No significant differences in IFN-γ levels in whole blood stimulated with Tox or PHA were demonstrated between S−/C− (Tox, 0.3 ± 0.2 IU/ml; PHA, 0.7 ± 0.5 IU/ml) and C+ (Tox, 0.4 ± 0.2 IU/ml; PHA, 0.7 ± 0.5 IU/ml) individuals (Fig. 1b). However, compared to *B. pseudomallei*-exposed individuals, IFN-γ production in response to Tox ( *P* < 0.05; 4.2 ± 1.6 IU/ml) and PHA ( *P* < 0.05; 4.3 ± 2.4 IU/ml) stimulation was increased in whole blood from S−/C− subjects.

Strong CMI responses demonstrated in individuals with subclinical melioidosis may be essential for protection against progression of *B. pseudomallei* infection. Although lower than S+/C−, individuals with a history of clinical melioidosis (C+) still demonstrated increased lymphocyte proliferation when compared to healthy, seronegative controls (S−/C−). Acquired immunity against pathogens is mediated by antigen-specific memory B and T lymphocytes and triggers a rapid, effective immune response following re-exposure to the microorganism. The extent of in vivo T cell expansion in a primary infection influences the size of memory T cell populations [31]. Both antigen and inflammatory factors are believed to jointly promote effector T cell proliferation, although antigen presentation alone is essential for expansion of the memory T cell compartment [32]. The findings of the current study suggest that although C+ individuals generate *B. pseudomallei*-specific memory T cells, this lymphocyte subset may be reduced or less responsive compared to the *B. pseudomallei*-specific memory T cells of S+/C− individuals. Irrespective of the reasons for alterations in immune responses, individuals who fail to mount an adequate CMI response may succumb to clinical melioidosis.

Disparate CMI responses in vitro are documented for subclinical and clinical infections with several intracellular bacteria [33–35]. In many ways, *B. pseudomallei* infection parallels the characteristics of tuberculosis, caused by the intracellular bacterium *M. tuberculosis*. One-third of the world population is infected with *M. tuberculosis*, although only 5–10% of non-HIV-infected individuals develop active tuberculosis during their lifetimes [36]. Latent tuberculosis can be identified by a positive tuberculin skin test. Patients with advanced or disseminated tuberculosis are often anergic on skin testing for delayed-type hypersensitivity (DTH) and have poor lymphocyte proliferative responses to antigens of *M. tuberculosis* in vitro [33,34]. In contrast, patients who have successfully controlled the primary *M. tuberculosis* infection have a vigorous DTH reaction and their T cells respond well to antigen [33]. Similarly, studies of *Leishmania panamensis* found a significantly greater in vitro expansion of CD4+ and CD8+ T cells from subclinically infected individuals stimulated with *Leishmania* antigen than those who had experienced recurrent leishmaniasis [35]. In a mouse
model of leishmaniasis, failure to control disease progression was associated with a population of Lyt-2-T cells that can prevent the induction or expression of curative CMI, thus exacerbating disease development [37]. Protective T cells produce IFN-γ and macrophage-activating factor when cultured in vitro with leishmanial antigens, whereas disease-promoting T cells do not [37].

A clinical study of seven cases of acute melioidosis demonstrated a significant decrease in the total number of lymphocytes and helper T cells, as well as a reduction in the T helper/T suppressor subset ratio [38]. Delayed hypersensitivity responses as assessed by reaction to 2,4-dinitrochlorobenzene were also dramatically reduced to 0% from a normal response of 80% [38]. It was demonstrated that treatment with levamisole, a potent immunostimulant, in combination with conventional antibiotic therapy, improved cellular immunity and recovery from melioidosis [38]. A more recent study carried out by our group detected a reduction in total lymphocyte, T cell and NK cell numbers in patients with acute melioidosis [39]. Unlike the previous report by Tanphaichitra and Srimuang [38], a reduction in the ratio of CD4+/CD8+ T cells was not observed [39]. However, the significance of both findings is confounded by the fact that many of the patients were undergoing chemotherapy or corticosteroid therapy, or had other associated risk factors or diseases which may have influenced the T helper/T suppressor ratio at the time of assessment. For this reason, the true effect of human melioidosis on lymphocyte subsets would be difficult to define from these studies. However, with the use of available murine models, further characterization and comparison of B. pseudomallei-specific T cells in subclinical vs. clinical melioidosis is warranted.

In B. pseudomallei infection, IFN-γ is essential for host survival and is produced predominantly by NK cells and CD8+ T cells [40,41]. There are several examples of infectious diseases that have an in vitro defect in antigen-induced IFN-γ production that is limited to the infecting pathogen alone. Cells from patients with lepromatous leprosy, severe cutaneous and visceral leishmaniasis, and tuberculosis produce low or undetectable levels of IFN-γ in response to their respective antigens but typically generate IFN-γ normally after stimulation with mitogens or other microbial antigens [42]. Following treatment, IFN-γ production occurs in
response to the specific antigen to which the patients' T cells originally failed to react. In contrast, T cells from patients with better-controlled manifestations of the same infections, such as tuberculoid leprosy or mucocutaneous leishmaniasis, readily secrete IFN-γ in response to specific microbial antigen [42]. This may account for the differences in IFN-γ levels observed between C+ and S/C− individuals in the current study.

Following in vitro stimulation with B. pseudomallei antigens, the significantly higher lymphocyte proliferation and IFN-γ production observed in asymptomatic, seropositive individuals compared with individuals with a history of clinical melioidosis may reflect differences in their antigen-specific memory T cell populations. This study provides further evidence of differences in immune responses to B. pseudomallei that appear to determine the outcome of natural infection. The results indicate that individuals who fail to mount an adequate CMI response may succumb to infection. Alternately, those who develop strong specific CMI response to B. pseudomallei may not develop clinical disease. Such responses are essential for providing protection against B. pseudomallei infection.

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References


