CONCISE COMMUNICATION

Demonstration of a Cell-Mediated Immune Response in Melioidosis

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Melioidosis is a bacterial infection caused by *Burkholderia pseudomallei*. The aim of this study was to determine whether a cell-mediated adaptive immune response against *B. pseudomallei* developed in patients who had recovered from melioidosis. Lymphocyte proliferation assays were done on peripheral blood mononuclear cells from patients ($n = 13$) and control subjects ($n = 10$) to determine the lymphocyte response to *B. pseudomallei* antigens. Production of interferon-γ and interleukin-10 was also determined. Activation of T cell subsets was assessed by fluorescence-activated cell sorter analysis, using antibodies to CD4, CD8, and CD69 antigens. Lymphocyte proliferation and interferon-γ production in response to *B. pseudomallei* antigens were significantly higher ($P < .001$ for both) in patients than in control subjects. There was also an increase in the percentage of activated $CD4^+$ ($P < .004$) and activated $CD8^+$ T cells ($P < .035$) in cell cultures from patients. The development of such a cell-mediated immune response in patients may be essential for their survival.

Melioidosis is a bacterial infection caused by *Burkholderia pseudomallei*. It occurs primarily in tropical and subtropical regions [1]. *B. pseudomallei* is a gram-negative saprophyte, which is widely distributed in soil and water in regions where it is endemic. Infection occurs in humans and animals, and it is acquired by inoculation, inhalation, and possibly ingestion. There is a wide spectrum of clinical presentations resulting from this infection.

B. pseudomallei has been classified as a facultative intracellular pathogen [2–4], which has important implications for the immune system. In intracellular bacterial infections caused by organisms such as *Salmonella* and *Mycobacterium* species, the central role of the T cell in adaptive immunity against the intracellular pathogens has been demonstrated [5]. However, to our knowledge, there is no direct evidence in the literature to suggest the development of an adaptive immune response in *B. pseudomallei* infection. This study was done to determine whether patients surviving melioidosis develop a cell-mediated adaptive immune response.

Subjects and Methods

Subjects. The 13 patients selected for this study had a clinical presentation consistent with melioidosis and a diagnosis confirmed by isolation of *B. pseudomallei* from blood or tissues (table 1). The mean age of the patients was 58 years (range, 31–74 years). Eight of the patients were men, and 5 were women. The cellular assays were done with blood samples from these patients, all of whom recovered from melioidosis and had no clinical evidence of the disease at the time when the assays were done. The mean time after diagnosis of melioidosis in patients was 23 months. The 10 control subjects were from the same geographic region. They had no clinical history of melioidosis and were serologically negative for antibody to *B. pseudomallei*. They included 5 men and 5 women, with a mean age of 51 years (range, 38–62 years).

Lymphocyte proliferation assays. Proliferation assays were done to determine lymphocyte responses to a cocktail of *B. pseudomallei* antigens prepared from strain NCTC13179 by sonication, using a breaking buffer containing leupeptin (0.2 mg/L), pepstatin (0.2 mg/L), and Kunitz DNAse (50 U). Each proliferation assay included samples from up to 3 patients and 2 control subjects. Peripheral blood mononuclear cells (PBMC) separated from heparinized blood were cultured in 96-well plates (10^5 PBMC/well) in RPMI 1640 medium (Life Technologies) supplemented with pooled human serum (10% vol/vol) and antibiotics. Triplicate wells of cells were stimulated with either 1 μg/mL *B. pseudomallei* antigens or 15 μg/mL purified protein derivative (PPD; CSL Biosciences). Cul-

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Informed consent was obtained from study participants. The study followed the National Health and Medical Research Council guidelines (National Statement on Ethical Conduct in Research Involving Humans) and was approved by the Townsville District Health Service Institutional Ethics Committee.

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Table 1. Demographic and clinical details for 13 study patients who recovered from melioidosis.

Patient, sex	Age, years	Time since diagnosis[a]	Organ(s) involved	Site(s) of isolation[b]	Antibody titer (IHA)	Maximum SI, mean	Maximum cpm, mean
1, F	74	8	Lungs	Sputum	1:80	254.6	6593
2, F	67	10	Lungs, spleen, skin (S)	Blood, sputum, skin swab	1:640	207.0	4741
3, M	31	9	Lungs	Sputum	>1:5120	146.0	5945
4, F	54	9	Lungs, skin	Skin swab	NS	67.7	2467
5, F	54	8	Skin	Skin swab	1:320	50.9	1197
6, M	33	10	Lungs	Sputum	1:1280	42.7	1001
7, M	64	34	Lungs	Pleural fluid	1:40	41.2	1568
8, F	54	54	Lungs, bone, skin (S)	Blood, skin swab	NS	40.7	833
9, M	58	47	Testis	Scrotal swab	NS	30.5	914
10, M	77	23	Skin	Skin swab	1:1280	28.2	654
11, M	54	5	Skin	Ulcer swab	NS	27.3	813
12, M	61	34	Prostate (S)	Blood, urine	1:640	24.1	555
13, M	68	45	Lungs, prostate	Sputum, urine	NS	13.6	278

NOTE. IHA, indirect hemagglutination; NS, not significant (antibody titer <1:40); (S), septicemic (designation required both clinical features of septicemia and isolation of the organism from blood); SI, stimulation index.

[a] Indicates the period (in months) between first diagnosis and time when the cellular assays were done. None of the patients had clinical evidence of melioidosis at the time the assays were performed.

[b] Skin swabs were obtained from abscesses that either were surgically drained or discharged spontaneously.

ture plates were incubated at 37°C in an atmosphere of 5% CO_2 (vol/vol) in air. Proliferation of cells was determined at 24-h intervals on days 4–7 of culture (4 time points) by measuring [^{3}H]thymidine incorporation (Amersham-Pharmacia Biotech; 1.25 μCi/mL for 4 h). Results were expressed as counts per minute or as stimulation index (SI), the proportion of counts per minute in stimulated cultures compared with that in unstimulated cultures. The maximum SI or counts per minute out of the 4 time points in individual proliferation assays was compared between patients and control subjects.

Determination of cytokine levels by ELISA. Cell culture supernatants collected on days 2 and 6 of culture from parallel experiments were assayed for interferon (IFN)–γ and interleukin (IL)–10 by use of capture ELISA techniques. Paired anti–IFN-γ (clones NIB42 and 4S–B3; PharMingen) or anti–IL-10 (clones JES3-19F1 and JES3-12G8; PharMingen) antibodies were used for the ELISAs, according to the manufacturer's instruction.

Fluorescent-activated cell sorter (FACS) analysis of T cell subsets. Lymphocytes cultured in the presence of *B. pseudomallei* antigens for 2 or 6 days were phenotyped, using isotype-matched controls or the following combinations of monoclonal antibodies (1 μg each; PharMingen): anti-CD3 (Cy-Chrome, HIT3a), anti-CD45 (fluorescein isothiocyanate [FITC], HI30), anti-CD14 (phycoerythrin [PE], M5E2); anti-CD4 (FITC, PRAT4), anti-CD8 (PE, HIT8a), or anti-CD69 (Cy-Chr, FN50). Cells were analyzed by use of a FACS analyzer with CellQuest software (Becton Dickinson). The increase in $CD4^+CD69^+$ and $CD8^+CD69^+$ expression between stimulated and unstimulated cells was considered to reflect activation of $CD4^+$ and $CD8^+$ cells after in vitro stimulation.

Antibody titer. A semiquantitative indirect hemagglutination assay (IHA) was used to determine serum antibody titers to *B. pseudomallei* antigens. Those with a positive reaction (titer >1:40) were confirmed by ELISA for *B. pseudomallei*–specific IgM and IgG [6].

Statistical analysis. Maximal lymphocyte proliferation between 4 and 7 days of culture (maximum SI and counts per minute from 4 time points) for each individual in the patient and control groups and the production of cytokines were analyzed by univariate analysis of variance, using SPSS statistical software (version 8). Dependent variables were tested for normality with a Q-Q plot and were transformed when necessary. Differences in $CD4^+CD69^+$ and $CD8^+CD69^+$ expression in cultured cells from patients and from control subjects were assessed by Student's t test after arc sign transformation of data. Correlation between sets of data was assessed by Pearson's correlation and was considered to be significant if the probability of a type I error was <.05% ($P < .05$). Mean values in the text are expressed as mean ± SEM.

Results

Antibody titers at the time of the study ranged from 1:5 to 1:5120 (table 1). However, there was no correlation ($P > .05$) between maximal antibody titer at the time of hospitalization (data not shown) or investigation (table 1) and cell proliferation or cytokine production. There also was no correlation between the time since diagnosis of melioidosis and proliferation of lymphocytes in response to *B. pseudomallei* antigens, and there were no differences between the parameters assessed for the patients who initially presented with or without septicemia.

The maximum SIs and the maximum counts per minute observed in the lymphocyte proliferation assays in response to *B. pseudomallei* antigens were compared for patients and control subjects (table 1). There was a significantly higher proliferation of lymphocytes in cell cultures derived from patients (mean SI, 75.0 ± 21.4; mean cpm, 2120 ± 604) than in those derived from control subjects (mean SI, 11.7 ± 2.1; mean cpm, 311 ± 76), as assessed by both SI ($P < .001$; figure 1) and counts per minute ($P < .001$). Although high levels of proliferation were observed in patients (mean SI, 152.7 ± 42.6) and in control subjects (mean SI, 155.6 ± 26.3) in response to PPD stimulation, the difference between the groups was not significant ($P > .05$).

IFN-γ production in response to *B. pseudomallei* antigens was significantly higher on days 2 ($P < .001$) and 6 ($P < .001$)

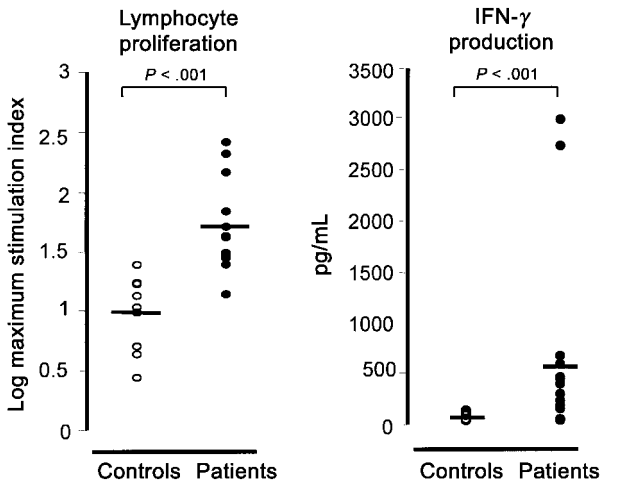

Figure 1. Proliferation of lymphocytes and interferon (IFN)–γ levels in peripheral blood mononuclear cell (PBMC) culture supernatants derived from PBMC from patients and healthy control subjects in response to *Burkholderia pseudomallei* antigens. *Left graph,* The maximal proliferative responses of lymphocytes from patients who had recovered from *B. pseudomallei* infection (●; $n = 13$) after stimulation with *B. pseudomallei* antigen were significantly greater ($P < .001$) than those of lymphocytes from healthy control subjects (○; $n = 10$). *Right graph,* Culture supernatants from PBMC from healthy control subjects (○; $n = 10$), with no evidence of previous exposure to *B. pseudomallei,* produced minimal levels of IFN-γ in response to *B. pseudomallei* antigens, compared with those from patients (●; $n = 13$). Mean values are shown *(horizontal lines)*.

(figure 1) in cell cultures derived from patients than in those derived from control subjects. There was a positive correlation between IFN-γ production and maximal proliferation of lymphocytes obtained from patients after *B. pseudomallei* antigen stimulation at days 2 ($r = 0.69$; $P < .001$) and 6 ($r = 0.69$; $P < .001$). Such a positive correlation was not observed in control subjects. There was no significant difference in IFN-γ production between cell cultures derived from patients (2183 ± 958 pg/mL) and control subjects (1436 ± 1971 pg/mL) after stimulation with PPD. A positive correlation was observed between the amount of IFN-γ production and maximal proliferation of lymphocytes in cell cultures derived both from patients ($r = 0.65$; $P < .001$) and from control subjects ($r = 0.69$; $P < .001$) in response to PPD at day 6. Levels of IL-10 in cell culture supernatants of patients and control subjects were not significantly different at day 6 of culture after stimulation with *B. pseudomallei* antigens. However, high IL-10 levels were detected in cell culture supernatants derived from both patients (704 ± 163 pg/mL) and control subjects (768 ± 134 pg/mL) after stimulation with *B. pseudomallei* antigens. There was no correlation between IL-10 production and cell proliferation or with IFN-γ production.

On days 2 and 6, the percentage of activated $CD4^+$ ($CD4^+CD69^+$) and $CD8^+$ ($CD8^+CD69^+$) T cell populations after *B. pseudomallei* antigen stimulation was higher in cultured cells derived from patients than in those derived from control subjects. At day 6, there was a significant difference ($P < .004$) between the percentages of activated $CD4^+$ T cells in patient and in control samples (2.09% ± 0.52% vs. 0.26% ± 0.12%, respectively). A similar trend was observed between activated $CD8^+$ T cells from patient and control samples (1.70% ± 0.34% vs. 0.80% ± 0.15%, respectively; $P < .035$).

Discussion

The data presented here demonstrate that lymphocytes from patients who had recovered from melioidosis (compared with those from control subjects) proliferate in vitro in response to *B. pseudomallei* antigens, produce significant amounts of IFN-γ, and may involve both activated $CD4^+$ and $CD8^+$ T cells. To our knowledge, this is the first direct demonstration of the possible role of lymphocytes in the development of a cell-mediated adaptive immune response in melioidosis. A positive IHA test result (titer >1:40) is considered to be serologic evidence of *B. pseudomallei* infection [6], although it is not indicative of active disease. In this study, a wide range of antibody titers in the patient group was observed. This is consistent with another study that found that levels of IgG were not predictive of disease outcome [7], which suggests a need for cell-mediated responses in the development of protective immunity.

Assessment of lymphocyte proliferation and production of IFN-γ in PBMC cultures after in vitro exposure to bacterial antigens provides a measure of antigen-specific lymphocyte responses to recall antigenic challenge. The significantly higher proliferation of lymphocytes and the high levels of IFN-γ in culture supernatants from patients (figure 1) are indicative of the recognition of *B. pseudomallei* antigens by memory T cells. The patients and control subjects participating in this study had comparable cell-mediated immune responses to the antigen PPD, as assessed by lymphocyte proliferation and IFN-γ production. A crude preparation of *B. pseudomallei* antigens was used in this study, to include all possible combinations of antigens to which the host might be exposed during the disease process.

IL-10 is produced late after endotoxin challenge, predominantly by monocyte/macrophages and T cells, which supports the view that it is a natural defense mechanism against excessive inflammatory responses after infection [8]. It is also a potent inhibitor of IFN-γ and IL-12. High levels of IL-10 in lymphocyte cultures are known to suppress cell proliferation and production of IFN-γ [9]. In this study, there was no significant difference in the amount of IL-10 in cultures from the 2 groups. Therefore, the inhibitory effects of IL-10 in cell cultures from both groups may have been similar. Although clinical studies have demonstrated an association between elevated levels of IL-10 and other proinflammatory mediators and increased

mortality [10–13], the precise role of IL-10 in suppression of immune responses in melioidosis is unclear.

CD69, a protein expressed early on the surface of stimulated T cells, is used as a marker of activation and correlates with the antigen-specific proliferative response of lymphocytes [14, 15]. In this study, CD69 expression was assessed 2 and 6 days after stimulation, because we believed that detection of a limited number of *B. pseudomallei*–specific T cells in cultures at a very early stage would be technically difficult. Increased CD69 expression can also be attributed, in part, to the presence of high levels of IFN-γ. In this study, the expression of CD69 in both T cell subsets was higher in patients than in control subjects, indicating a possible role for both $CD4^+$ and $CD8^+$ T cells in *B. pseudomallei*–specific immune responses.

The significantly higher cell proliferation, IFN-γ production, and activation of T cell subsets observed in the patient group, compared with control subjects, after in vitro challenge with *B. pseudomallei* antigens may be explained by antigen-specific memory cell responses. It is tempting to speculate that the patients included in this investigation survived the disease because of the development of a protective adaptive immune response to *B. pseudomallei*. The current study provides valuable information that should influence the design of future studies, with larger groups of patients, correlating T cell function and changes in cytokine levels with outcome. Adoptive transfer experiments in animal models are required before definitive conclusions can be reached on the development of a protective immune response to *B. pseudomallei*.

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