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Cytokine Response in Patients with Acute Brucellosis

Alwaleed A Alaidan[1], Iram Saba[2], Ayodele A Alaiya[3], Abdullah A Al-Hokail[4], Tarik A Al-Azragi[5] and Mohamed G Elfaki[6,7]*

[1]*Department of Infection and Immunity, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia*

[2]*Department of Genetics, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia*

[3]*Proteomics, Stem Cell and Tissue Re-engineering Program, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia*

[4]*Department of Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia*

[5]*Department of Internal Medicine, College of Medicine, King Khalid University, Abha, Saudi Arabia*

[6]*National Biotechnology Centre, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia*

[7]*Alfaisal University College of Medicine, Riyadh, Saudi Arabia*

***Corresponding Author:** Mohamed G Elfaki, National Biotechnology Centre, King Faisal Specialist Hospital and Research Centre and Alfaisal University College of Medicine, Riyadh, Saudi Arabia.

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Abstract

Background: Human brucellosis is a contagious infectious disease caused by animal pathogens belonging to genus *Brucella*. The disease constitutes a major public health threat and a major zoonotic disease worldwide. In humans, the disease is primarily caused by *B. melitensis* and exhibited multiple clinical manifestations that are likely attributed to alterations in immune function with consequent immunosuppression.

Objectives: To identify and correlate the expressed cytokines in patients with acute brucellosis.

Methods: Blood specimens were collected from 20 patients with acute brucellosis for the diagnosis and isolation of peripheral blood mononuclear cells (PBMC). PBMCs were spiked *in vitro* with various *Brucella* peptides for the expression and quantification of provoked cytokines using cytokine antibody arrays and ELISA methods.

Results: Our data showed an enhanced expression of both inflammatory and immunogenic cytokines. Representative inflammatory cytokines that were detected include RANTES, MIF/GIF, DER6, complement components (C5; C5a) and the interleukins (IL)-13, and IL-23. The immunogenic cytokines that were detected, on the other hand, include TNF-α, IFN-γ, IL-12 cytokines. Quantitation of the latter cytokines showed 1.5 to 1.8- fold increase when PBMCs were stimulated with whole *Brucella* peptides for 6 days. No cytokine was detected in PBMCs stimulated with single peptide.

Conclusions: The dominant expression of inflammatory cytokines correlated well with the acute presentation of brucellosis. These data are useful in our understanding of the pathophysiology of human brucellosis where the provoked cytokines and chemokines are likely responsible to mediate the inflammatory process seen in our enrolled patients.

Keywords: *Brucellosis; Cytokines; Antibody Arrays*

Introduction

Human brucellosis due to *Brucella melitensis* and/or *B. abortus* is an infectious zoonotic disease characterized by undulant fever, arthritis, spondylitis, endocarditis, osteomyelitis, and hepatosplenic abscesses or brain abscess in rare events [1-3]. In animals, however,

brucellosis is characterized by spontaneous abortion at late gestation, undulant fever, and infertility [4]. The bacterium is small Gram-negative coccobacilli that has the propensity to localize inside monocytes and macrophages (Mφ) [5] of the liver, spleen, bone marrow, uterus, heart, and brain. The replication of brucellae inside cells of the reticuloendothelial system (spleen, liver, and bone marrow) triggers multiple alterations in the cellular pathways that may be responsible for the exhibited protean clinical manifestations with consequent immunosuppression [6,7].

Despite the public health importance of brucellosis, little is known about the immunological control of *B. melitensis* infection in humans. However, in the murine model of infection [8], CD4+ T lymphocytes have been demonstrated to exert their protective effect by production of IFN-γ that activates rodent Mφ to halt the replication of intracellular brucellae [9]. The effector molecules in killing brucellae by IFN-γ–activated Mφ were identified to the production of reactive oxygen intermediates but not to nitric oxide [10]. Regulation of IFN-γ production in this process is mediated by the cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-12 [11] which were both secreted by activated Mφ. In contrast, the counter regulator of IFN-γ production, IL-10, has been demonstrated to exacerbate infection due to *B. abortus* [12]. In humans, however, the cytokine TGF-β has been demonstrated to underlie the depressed function of T cell responses in patients with chronic brucellosis [7]. Further, the lacks of IFN-γ in brucellosis patients [7] coupled by failure of anti-brucella drugs [13] have led to the persistence of brucellae with subsequent tissue retention and disease relapse when the host was immunocompromised. No similar data were reported for patients with acute brucellosis. This study was undertaken to identify the provoked mediators that accentuates the inflammatory process seen in patients with acute brucellosis.

Materials and Methods

Study population

Twenty patients with acute brucellosis were recruited from 2 hospitals in Riyadh region namely, King Khalid University Hospitals (KKUH), and the King Faisal Specialist Hospital and Research Centre (KFSHRC). All patients were studied for their occupational history, clinical presentation, and laboratory findings. Subsequently, 12 milliliters (12 mL) of blood was collected from each patient after verbal consent approval as stated by KFSHRC Ethics Committee. The collected blood was used for microbial culture, serum extraction, and PBMCs isolation. The diagnosis of brucellosis was based on clinical symptoms, microbiological culture, and PCR assays as previously described [14,15].

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected heparinized blood by differential centrifugation [16] over Ficoll-Hypaque gradient (Pharmacia Fine chemicals, Piscataway, N.J.). Cells at the interface were collected by aspiration, washed 3x with PBS containing 5 mM EDTA, and resuspended in complete RPMI-1640 medium (GIBCO, Grand Island, N.Y.) containing HEPES, glutamine, streptomycin, penicillin, and 10% heat-inactivated human AB serum. The viability of the cells was found to be > 95% by trypan blue exclusion dye. For future studies, PBMCs were cryopreserved in liquid nitrogen at 2 x 10^6 cells/ml in 90% fetal bovine serum (Sigma-Aldrich, St Louis, MO, U.S.A.) containing 10% DMSO.

In Vitro stimulation of PBMCs with *Brucella* proteomes

To determine the immunologic potential of *Brucella* peptides in the induction of cytokines, sick PBMCs from patients diagnosed with brucellosis were co-cultured with whole *Brucella* peptides as well as with selected peptides in some experiments as previously described [7]. PBMCs were cultured in 6-well plates in complete RPMI-1640 medium supplemented with 10% fetal bovine serum. The cells were subsequently stimulated with whole *Brucella* peptides, PHA, selected *Brucella* peptides and incubated for 6 days at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Supernatants were collected and tested for cytokines induction and production by antibody arrays and ELISA as described by the manufacturer (R and D Systems, Inc., Minneapolis, MN, U.S.A).

Cytokine antibody arrays

Array membranes with 96 spots (12 x 8) of adsorbed monoclonal antibodies against various human cytokines (Proteome Profiler™ human cytokine array kit) were purchased from R and D Systems, Inc., Minneapolis, MN, U. S. A. Membranes were incubated with diluted

serum samples (1:5 dilution) or with supernatants from PBMCs spiked with *Brucella* peptides. Subsequently, membranes were processed for the detection of cytokines spot using chemiluminescence imaging system as recommended by the manufacturer.

Statistical analysis

Quantitative data for cytokines level was analyzed by Student's *t*-test for significance at $p \leq 0.05$ using the Statistical Analysis System soft wares (SAS Institute, Cary, NC, U.S.A.).

Results

Diagnostic profile of patients with brucellosis

Most patients suspected with brucellosis were presented with headache, high fever, back pain, myalgia, and joints problem. In one patient there was evidence of hallucination suspected with neurobrucellosis. The diagnosis was confirmed by culture of blood specimens and PCR assay. Microbiological examination of blood specimens showed the growth of pale white pinpoint coccobacilli colonies suggestive of *Brucella* cells. However, genomic DNA isolated from putative colonies or from *Brucella*-positive sera showed an amplification of a 223-bp target sequence of a gene encoding for the 31- kDa *Brucella abortus* antigen as previously reported by our group (data not shown).

Analysis of cytokine expression by antibody arrays

Among all peptides tested, only *Brucella* whole cell peptides were found promising to provoke a cellular response in PBMCs culture. None of the surface, cytosol, or mitochondrial fractions of the solubilized proteins was able to induce an immunologic response alone. However, when a high throughput chemiluminescence imaging system was used, a consortium of both inflammatory and immunogenic cytokines was detected in supernatant fluids from PBMCs spiked with whole *Brucella* cell lysate. Among the detected inflammatory cytokines, RANTES, MIF/GIF, DER6, complement components (C5; C5a) and the interleukins (IL)-13, and IL-23 (Figure 1). Concomitantly, an enhanced expression of immunogenic cytokines was evidenced by the detection of TNF-α, IFN-γ, IL-12 cytokines (Figure 2). The concentration of immunogenic cytokines was abundant in PBMCs spiked with *Brucella* whole cell peptides compared to untreated PBMCs (Figure 2). In essence, the blot had maintained the specificity of any overexpressed protein in this high throughput chemiluminescence imaging system.

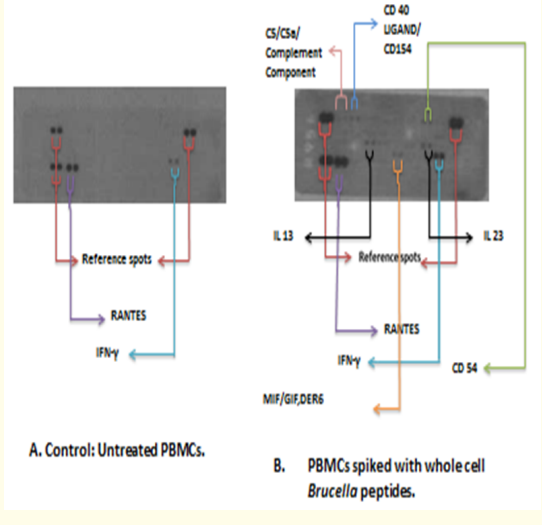
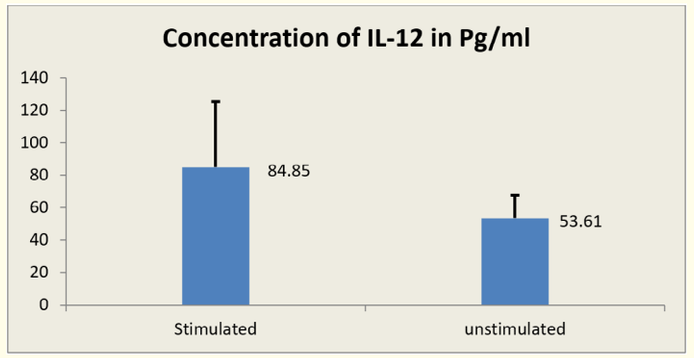
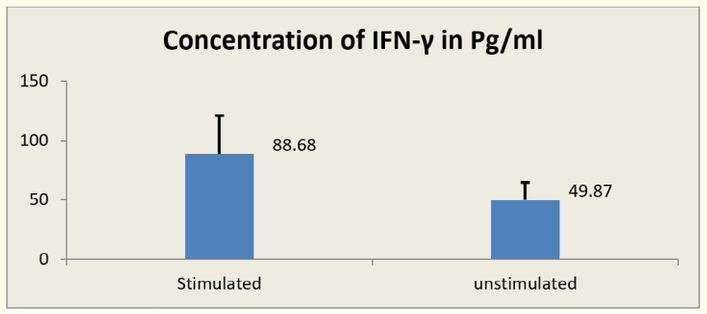


Figure 1: *Cytokines expression by antibody arrays in PBMCs of patients with acute brucellosis. Supernatant fluids from PBMCs spiked with or without whole cell Brucella peptides were tested for multiple cytokines expression using the Proteome ProfilerTM human cytokine array kit. Signals of the recognized cytokines were visualized by using chemiluminescence reagents.*





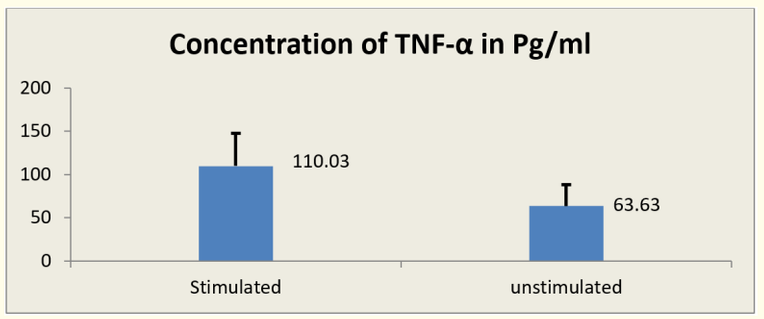


Figure 2: *Concentration of IL-12, IFN-γ, and TNF-α in patients PBMCs stimulated with whole brucella peptides for 6 days. PBMCs-stimulated with selected Brucella peptides produced no cytokines.*

Discussion

In this study, the cytokine profile was investigated in patients with acute brucellosis in an effort to delineate their role in the clinical manifestations presented in patients with acute brucellosis due *B. melitensis*. Our dot blot results confirmed the detection of defense proteins elicited by sick PBMCs from patients with acute brucellosis. The specificity of dot blot proteomes by chemiluminescence imaging system coupled with the quantitation of these cytokines at the translation level had sufficed us for not pursuing cytokines mRNA expression at the transcriptional level. However, these defense proteins are potential mediators for both inflammation and immunity to *Brucella* infection [5,17].

Although most of the immunologic processes to *Brucella* infection were mediated by mononuclear cells [17], the data collected from an *in vitro* culture of sick PBMCs spiked with *Brucella* antigens supported the induction of cell-mediated immune responses. The detection of immune cytokines, inflammatory cytokines, chemokines, and complement molecules by dot blot method clearly supported our hypothesis that these *Brucella*-sick PBMCs were already primed *in vivo* with *Brucella* antigens to mount a cellular response *in vitro*. The resultant products were known to mediate cellular immunity via the IFN-γ pathway [18,19] as well as inflammation through the release of complement factors, chemokines and inflammatory cytokines [10,20]. The chemokines are known to attract immune cells to the site of infection and thus, they represent the initial host response to *B. melitensis* infection [5]. Further, the detection of IL-12, IFN-γ, and TNF-α by PBMCs-stimulated with *Brucella* antigens clearly reflects the role of these immunogenic cytokines in the clearance of *B. melitensis* infection at later stage of infection. None of these cytokines were detected in *Brucella*-positive sera in our studied group (data not shown). Therefore, the existence of these two processes (inflammatory and immunogenic cytokines) clearly reflects the turnover of protein molecules involved in pathogenesis and immunity to *Brucella* infection [21]. This notion was supported by the abundance of cellular proteomes as previously

described [22]. The involvement of these cellular proteomes in the cell signaling pathways awaits further study. However, at the translational level these inflammatory mediators are likely responsible for the expression of arthritis, spondylitis, osteomyelitis, and undulant fever that were commonly presented in patients with brucellosis. Therefore, for future therapy of brucellosis, inclusion of cytokines and their antagonists as therapeutic agents is necessary to alleviate the inflammatory process in conjunction with current anti-brucella drugs [13].

Conclusions

The array of cytokines and chemokines secreted by PBMCs is pivotal for the selection of candidate proteomes that trigger cell-mediated immunity as well as important targets for future drug discovery against human brucellosis.

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Competing Interest

The authors declare that they have neither competing nor conflict of interests.

Author's Contribution

AAA wrote and reviewed the manuscript. IS performed the laboratory assays and reviewed the manuscript. AAA isolated the proteomes and reviewed the manuscript. AAH and TAA were our clinicians who recruited and treated the enrolled patients and reviewed the manuscript. MGE designed the study, run the PBMCs culture and had wrote, reviewed, edited and submitted the manuscript.

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