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The Relation of the Cytokines and the CD Markers to the Antibody Titers in Patients with Brucellosis

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Abstract: Human Brucellosis is one of the highly reported zoonotic diseases in the world. In Saudi Arabia, it is a highly reported zoonotic disease. Human immune response to Brucellosis is controversial. The relation of antibody titers to cytokines and CD markers of cells subpopulations was seen important to reveal the immunopathological changes at different stages of Brucellosis. The level of the cytokines, IL-1β, IL-2, IL-4, IL-8, IL-12, INF-γ and TNF-α and the CD markers, CD4$^+$, CD8$^+$, CD19$^+$, NK/CD18$^+$, MHC-II and $\gamma\delta$ TCR were monitored 32 patients with serum titres 1/80, 1/160, 1/320 and 1/640 (8 samples/each patient). The level of the cytokines IL-2, IL-4, IL-12, INF-γ and TNF-α were decreased significantly in all serum titres. However, the CD markers CD8$^+$, NK/CD18$^+$ and $\gamma\delta$ TCR as well as MHC-II increased significantly in the patients with 1/320 titer, Whereas CD19$^+$ cells decreased significantly. The significant decrease of IL-2, IL-4, IL-12, INF-γ and TNF-α even in the titres 1/80 and 1/160 implies that organism could inflicts major defects on the resistance to human Brucellosis. However, increased expression of CD markers, CD8$^+$, NK/CD18$^+$ and $\gamma\delta$ TCR could refer to the nonspecific polyclonal activation during acute Brucellosis. The dichotomy of the Th1/Th2 type cytokines activity was not observed.

Key words: Brucellosis, IL-2, IFN-γ, CD8$^+$, cytokines, *Brucella melitensis*

INTRODUCTION

Human Brucellosis is a worldwide zoonotic disease with half million new cases annually (Pappas *et al.*, 2006). The annual prevalence rate of the disease could exceed ten cases per 100,000 (Franco *et al.*, 2007). In Saudi Arabia, a national survey stated that the prevalence rate in human Brucellosis reached 40 cases per 100,000 (Memish, 2001).

Human Brucellosis is associated with both humoral and cellular immunity. Although antibody responses play certain role in resistance to brucellosis, cell-mediated- immunity (CMI) appears to be the principal mechanism of recovery. In human Brucellosis, IgG is the highest isotype in patients with acute and chronic Brucellosisand in patients without relapse (Araj *et al.*, 1986; Doganay and Bilgehan, 2003) IgM and IgA, however persist in high level during acute stage of the disease (Araj *et al.*, 1986). At the early stage of the infection IgM appears first followed by the domination of IgG. Treatment could reduces the level of the antibodies, but they persist for months or years in the absence of active infection (Ariza *et al.*, 1992).

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Cytokines orchestrate the pathogenesis of Brucellosis through the manipulation of Th1/Th2 phenotypes (Zhan and Cheers, 1995, 1998; Demirdag *et al.*, 2003; Zhan *et al.*, 1996). Th1-type cytokines, mainly interferon-γ (IFN-γ), dominates the acute stage of untreated patients (Zhan and Cheers, 1998, 1993), whereas Th2-type cytokines, mainly interlukin-10 (IL-10), prevail in the chronic stage of the disease (Dornand *et al.*, 2002; Giambartolomei *et al.*, 2002; Fernandes and Baldwin, 1995).

During early stage of the infection, tumor necrosis factor-α (TNF-α) synthesis is crucial step in initiating the Th1-type responses. TNF-α contributes decisively in the induction of interlukin-12 (IL-12), mediates Th1-type responses, which considered vital for enabling the macrophages to eradicate the persisted infection (Zhan and Cheers, 1995, 1998; Zhan *et al.*, 1996; Demirdag *et al.*, 2003). Therefore, establishment of the infection at the early stages was seen greatly dependent on maintaining the persistence of the organism in the infected macrophages (Fernandes and Baldwin, 1995; Jiang and Baldwin, 1993) by interfering with the apoptosis of the infected macrophages (Zhan *et al.*, 1996, 1998). A consequence to the diminished Th1-type responses, the Th2-type cytokine, IL-10, dominates to enhances the Brucella intracellular survival (Dornand *et al.*, 2002; Giambartolomei *et al.*, 2002). Neutralization of IL-10 with anti-IL-10 monoclonal antibodies resulted in an increase in the IFN-γ production, which enhanced the efficient control of the intracellular Brucella by peritoneal macrophages (Fernandes and Baldwin, 1995). Furthermore, addition of IFN-γ or IL-2 to the macrophages culture enhanced the macrophages capability in clearing the intracellular bacteria (Jiang and Baldwin, 1993).

The Brucella antigens contribute decisively to the regulation of Th1/Th2 dichotomy and immunization with soluble Brucella proteins were seen potent in the stimulation of the IL-4-producing CD4$^+$ T-cells, whereas challenge with live organism stimulated the IFN-γ-inducing CD4$^+$ T-cells (Zhan *et al.*, 1993, 1995). In the other approach, the Brucella species cytoplasmic proteins were seen potent in enhancing the IL-2 and IFN-γ production in the peripheral mononuclear blood cells (PMBC) of the infected patients. Brucella lipopolysaccharides (LPS) was shown to act as potent IL-10 inducer (Kariminia *et al.*, 2002).

The role of CD4$^+$ and CD8$^+$ and $\gamma\delta$ cells in resistance to Brucellosis remained controversial. Yingst and Hoover (2003) reviewed most of the studies on the role of the cells subpopulations in Brucellosis, extensively.

The protective immunity in human Brucellosis appears to be essential for the development of effective human vaccine. However, the nature of immune responses to Brucellosis in human is not fully elucidated. Hence, the main objective of this study was to monitor the changes in the level of cytokines and CD markers in relevance to the antibody titers. This was seen crucial approach for exploiting the immunopathological changes at different stages of the Brucellosis.

MATERIALS AND METHODS

Patients
The Brucellosis patients of this study were classified into two groups:

- **Subacute infection:** This group include all patients with 1/80 and 1/160 serum titres. Their history was taken as an important criterion in excluding their relapse from previous infection. The patients expressed mild symptoms and had transient illness.

- **Acute infection:** Patients that their serum titres 1/320 and above were considered in acute stage of the disease. In addition to their serum titres, their typical sever symptoms and history were considered important criteria in defining their status. Patients of this group were hospitalized and subjected to the typical treatment.

Their infection was confirmed on the basis of typical Brucellosis clinical signs, epidemiological data and laboratory diagnosis. The major clinical signs were fever, splenomegaly, lymphadenopthy, myalgia. Epidemiologically, most of the patients were either as shepherd for the camel or sheep flocks or animal owners.

The serum samples were collected only from those with no medical history of previous Brucellosis and were taken before the antibiotic treatment commence.

Serum Samples

A total of 40 serum samples were collected from local hospitals at three provinces of Saudi Arabia, Al-Ahsaa and Dammam hospitals at the Eastern province, Riyadh Public Hospital at the Central province and Aseer and Najran Hospitals at the Southern province.

The 32 serum samples out of 40 were collected from patients with Brucellosis. The samples were grouped as 8 serum samples from patients of each of the following titres 1/80, 1/160, 1/320 and 1/640. The Brucellosis was diagnosed using tube agglutination test (Biomedical Product Co., Riyadh, Saudi Arabia) and further confirmation with commercial ELISA kit that detects anti-Brucella IgM (Biomedical Product Co., Riyadh, Saudi Arabia). The major antigens in both tests were for *B. abortus* and *B. melitensis.*

The last 8 serum samples were collected from normal donors and were considered as control. Infection of the donors was ruled out on basis of negative tube agglutination test and no Brucellosis in their medical history and any other recent medical complications or infections.

Collected samples were transported immediately in ice box to the laboratory and kept at -20°C. The laboratory diagnosis was performed at each of the given hospitals where the samples were collected.

Whole Blood Samples

The whole blood samples were used to examine the changes in the CD markers of the PMBCs. They were collected from patients of different antibody titers and controls. However, it was only possible to collect five blood samples from patients with serum titre 1/320 and controls. The lack of enough blood samples from patients of other serum titres was mainly due to the difficulties in reaching the patients before the start of the antibiotic treatment.

Determination of Cytokines Level Using ELISA Test

The level of human cytokines, Interleukin-1β (IL-1β), IL-2, IL-4, IL-8, IL-12, INF-γ and TNF-α was determined using anti-human cytokines ELISA kits (R and D, USA). The determination of the cytokines level was carried out according to the manufacturer's directions.

Flowcytometry for the Determination of the CD Markers

The flow cytometry was performed at the Regional Laboratory and Blood Bank, Dammam, Eastern Province. Flow cytometry analysis to identify the percentage of CD4$^+$, CD8$^+$, NK/CD18$^+$, MHC-II, CD19$^+$ and $\gamma\delta$ TCR was performed using FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The CD markers were analyzed using Becton Dickinson Simultest IMK-Lymphocyte kit.

To each of the six tubes, 20 μL of each of the following reagents were added, anti-CD45$^+$/CD14$^+$, control reagent, anti-CD3$^+$/CD19$^{+,}$ anti-CD3$^+$/CD4$^+$, anti- CD3$^+$/CD8$^+$, anti- CD3$^+$/CD16$^+$+CD56$^+$ was added, respectively. Then 100 μL of whole blood that contains approximately 3.5-9.4×10^4 cells was added to each tube. The cells were mixed thoroughly at low speed for 3 sec and then incubated in the dark for 15-30 min at room temperature. After lysing with 2 mL of 1x lysing buffer and incubation for 10-12 min tubes were centrifuged and the cells pellet washed and 0.5 mL of 1% paraformaldehyde

was added to each tube. Finally, the labelled cells suspensions were analyzed by the flow cytometer Becton Dickinson for two-colour analysis with SimulSET program guides.

Statistical Analysis

The t-test and analysis of variance were performed using the SPSS software (SPSS Inc.).

RESULTS

The Level of Cytokines at Different Titers

The analysis of variance (ANOVA) revealed significant (p>0.05) decrease in the cytokines level of IL-2 (p>0.0008), IL-4 (p>0.0009), IL-12 (p>0.0001), IFN-γ (p>0.0005) and TNF-α (p>0.0001) with all titres. The IL-1β and IL-8 levels however, expressed no significant differences with all titres. The mean cytokines levels (pg mL^{-1}) of different serum titres are shown Table 1.

The Percentage of Different CD Markers

Flowcytometry of different CD markers from patients with serum titre 1/320 indicated significance increase in their percentage (Fig. 1). The t-test analysis indicated significant (p>0.05)

Table 1: The mean cytokines level (pg mL^{-1}) in relation to the antibody titres

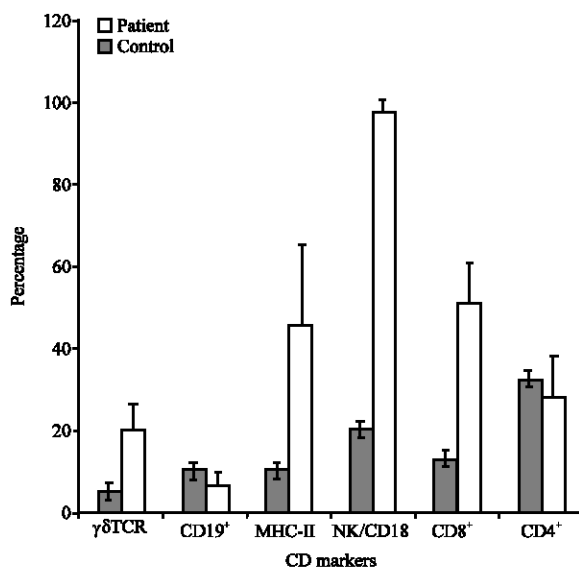| Reciprocal antibody titers	(Cytokines levels (pg mL^{-1})						
	IL-1β	IL-2	IL-4	IL-8	IL-12	IFN-γ	TNF-α
Control	11.2±3.3	130.2±32	74.2±5.9	88.82±0	34.9±3	99.4±13.6	86.6±29.3
80	1.3±3.2	23.2±28	8.9±12.1	66.06±45.5	3.9±4.1	22.5±26.4	8.4±18
160	0.2±0.4	2.1±6.1	10.7±16.1	99.12±102.1	1.7±1.4	13.3±11.8	3.4±9.6
320	10.1±25.1	7.0±11.1	3.0±4.1	572.5±974	5.7±6.5	6.6±7.5	5.5±15.5
640	1.6±1.9	17.9±19.6	5.0±8.3	262.8±347	4.4±4.3	29.3±15.3	5.3±7.2

Values are shown in mean±SE

Fig. 1: Mean percentage of CD markers of PMBCs from patients with 1/320 antibody titre

increase in the CD8^{+} (p>0.0001), NK/CD18^{+} (p>0.009), MHC-II (p>0.001) $\gamma\delta$ TCR (p>0.001) and significant decrease in CD19^{+} (p>0.006). The CD4^{+} cells level failed to indicate significant change (p>0.18).

The flow cytometry of the CD markers of some of the serum titres was performed and the results were appeared to be close to that of the above findings but were not included due to the low numbers of samples for each of the serum titres.

DISCUSSION

Human Brucellosis is dominated by the Th1-type cytokines, IFN-γ and IL-2, during the early stages of the infection, whereas IL-10, a Th2 type cytokine, dominates the chronic stage of the disease which enhances the bacterial manoeuvres for the formidable persistence in macrophages (Fernandes and Baldwin, 1995; Rafiei *et al.*, 2006).

The results indicated significant decrease of the cytokines, IL-2, IL-4, IL-12, IFN-γ and TNF-α with all serum titres. IL-1β and IL-8, however showed no significant differences with that of the controls. Reports on the role of cytokines immunological activities in acute human Brucellosis were controversial. The attempt to monitor the cytokines, IL-1, IL-2, IL-4 and TNF-α during acute Brucellosis failed, but the levels of IL-8, IL-12 and IFN-γ indicated significant rise (Ahmed *et al.*, 1999). On the other hand, the phytohaemagglutinin (PHA)-stimulated lymphocytes of untreated patients failed to express any significant increase in the IL-12 level, whereas IFN-γ production appeared to be defected (Rodriguez-Zapata *et al.*, 1996). Interestingly, the IFN-γ and TNF-α levels in patients with acute Brucellosis were significantly elevated, but the level of the IL-4 failed to present any significance (Demirdag *et al.*, 2003). In contrast to the previous studies, the current findings do not correlate with the studies that claim the dominance of the Th1 cytokines in acute Brucellosis (Zhan and Cheers, 1995, 1998; Demirdag *et al.*, 2003; Zhan *et al.*, 1996). However, the results are in accordance with the reports that advocate the severe depression of IL-2, IFN-γ and TNF-α (Rodriguez-Zapata *et al.*, 1996). In general, the results do not substantiate the domination of the Th1/Th2 responses that was reported previously (Zhan and Cheers, 1995, 1998; Demirdag *et al.*, 2003; Zhan *et al.*, 1996), which correlate with other report (Demirdag *et al.*, 2003).

It was proposed that bacterial persistence requires significant reduction in the biological activities of the pro Th1-type cytokines (Zhan and Cheers, 1995, 1998; Demirdag *et al.*, 2003; Zhan *et al.*, 1996). Therefore, during the acute stage the fate of the infection is dictated by the strength of the Th1 responses. Resistance to the infection will be overridden, if the TNF-α response fades, which affects the Th1 responses through the depression of IL-12 (Zhan and Cheers, 1995; Demirdag *et al.*, 2003; Dornand *et al.*, 2002). Hence, the onset of the antibody production could be an indication on the establishment of the infection rather than a resistant process (Baloglu *et al.*, 2000). Elevation in the antibody responses could be due to the capability of the organism to elicit wide scale of T-independent polyclonal B-cell activation (Golding *et al.*, 2001). The polyclonal activation was seen as consequence of the shaded LPS that initiate Th2-type response (Kariminia *et al.*, 2002). In addition, impairment of antigen processing and decrease in the CD4^{+} and B-cells at the late stages of acute infection clearly refers to the sever assaults on these immune factors (Golding *et al.*, 2001; Moreno-Lafont *et al.*, 2002).

The previous studies failed to draw precise role for IL-4 and IL-8 in acute Brucellosis. In this study, significant decrease of the IL-4 level might refer to the lethal impact on CD4^{+} Th2 cells.

Although the flowcytometry analysis of the PMBCs from infected patients was restricted to the serum titre 1/320, the results indicated significant rise in the CD8^{+} NK/CD18^{+}, MHC-II, $\gamma\delta$ cells with exception of CD19^{+} and CD4^{+}. The significant increase in the CD8^{+}, NK and $\gamma\delta$ cells as well as the MHC-II upregulation was reported elsewhere (Yingst and Hoover, 2003; Moreno-Lafont *et al.*, 2002, 2003; Celik and Akbulut, 2005). However, the proliferative response of CD4^{+} cells in Brucellosis is matter of controversy (Yingst and Hoover, 2003).

The results of the significant decrease in the CD19$^+$ cells is in accordance with the previous report (Celik and Akbulut, 2005) but the significance of this decrease on the immune responses was not elucidated.

Although most of the studies indicated significant rise of CD8$^+$, NK and $\gamma\delta$ T-cells during acute Brucellosis, their role in the resistance to the infection remained controversial. For instance, proliferative response of the above cells subpopulations was seen normal when they were pulsed with PHA and/or Brucella antigens (Moreno-Lafont *et al.*, 2002, 2003). It was speculated that majority of these cells were of low specificity. Hence, it is plausible to assume that proliferation of $\gamma\delta$ T-cells and NK cells could be identical to that of CD8$^+$ cells due to certain non-specific signal(s) (Yingst and Hoover, 2003). Studies on the memory in human Brucellosis are essential to unravel the essence of T-cells specific activation.

In general, it is difficult to conceive that elevated $\gamma\delta$ T-cells, CD8$^+$ and NK cells could play a decisive role in resistant to Brucellosis after the onset of the acute infection. Simultaneous increase in cells subpopulations with decrease in IFN-γ, IL-12, IL-2, IL-4 and TNF-α bolster the fact that the proliferative response of the major cells subpopulations is an immunopathological consequence of Brucellosis.

It can be concluded therefore, that increase in the antibody titres during acute Brucellosis most probably does not reflect a resistance process. The inhibition that was inflicted upon the cytokines, IL-2, IL-12, IFN-γ and TNF-α in the subacute stage (1/80 and 1/160 titres) could substantiate the notion that organism initiates its lethal effects way beyond the antibody response commence.

In view of the current results, the development of vaccine for human Brucellosis should consider the manipulation of antigens that are involved in initiating the early immunopathological responses.

Finally, the results indicated the importance of studying the cytokines and the CD markers in relation to the antibody titres. However, the limited numbers of the patients and absence of certain cytokines like IL-10 and transforming growth factor-β and CD markers of memory cells appear essential for future study to provide further detailed insight on the immunological responses at different stages of the disease.

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