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Brucella abortus L7/L12 Recombinant Protein Induces Strong Th1 Response in Acute Brucellosis Patients

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ABSTRACT

Background: Because of high morbidity of the brucellosis in humans and the potential use of the microorganism as an agent of biologic warfare, protection of effective vaccines and specific diagnostic reagents become necessary to eradicate brucellosis. **Objective:** In this study we aimed to investigate the cytokine responses and changes in peripheral blood lymphocyte subgroups of acute brucellosis patients in response to L7/L12 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) recombinant proteins derived from *Brucella abortus*. **Methods:** levels of IFN-γ, IL-4 and IL-10 secreted from PBMCs of 25 acute brucellosis patients and 15 healthy controls, stimulated with Phytohemagglutinin (PHA), L7/L12 or GAPDH were measured by ELISA. Furthermore alterations in lymphocyte subgroups in response to these *Brucella* antigens were determined by flow cytometry. **Results:** Extracellular IFN-γ levels were found to be elevated after stimulation with L7/L12 in patients with acute brucellosis, whereas no significant changes were found in IL-4 and IL-10 levels. Similar data was also obtained with GAPDH, but the stimulation of IFN-γ production was not observed in all patients and was not as strong as that observed for L7/L12. Moreover, when the distribution of lymphocytes subgroups ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$, $CD3^+CD69^+$ and $CD3^+CD152^+$) was evaluated, it was found that the stimulation with L7/L12 and GAPDH only led to an increase in the percentage of $CD3^+CD69^+$ lymphocytes. **Conclusion:** These data indicate that *Brucella abortus* L7/L12 or GAPDH induce a Th1 type immune response in acute brucellosis patients. Additionally, these recombinant proteins, especially L7/L12, may be used in new vaccine preparations and diagnostic tests.

Keywords: Brucellosis, Cytokine, Recombinant Proteins, T Lymphocytes

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INTRODUCTION

Brucella spp. are facultative intracellular Gram-negative intracellular bacteria that have the ability to survive and multiply within the host phagocytic cells; and therefore, lead to a chronic infection in animals and humans (1). The immune strategies by which *Brucella* evades intracellular killing mechanisms within phagocytic cells are not completely understood. Because of the economic loss due to animal brusellosis, high morbidity of the disease in humans, and the potential use of the microorganism as an agent of biologic warfare, preparation of effective vaccines and specific diagnostic reagents become necessary to eradicate brucellosis (1).

Bacteria, such as *Brucella spp.*, that survive within macrophages, are very sensitive to Th1 immune response that activates the macrophage killing properties mediated by IFN-γ and generates cytotoxic $CD8^+$ T cells (2,3). Therefore, candidate antigens for vaccines should greatly enhance Th1 biased immune response characterized by strong $CD4^+$ and $CD8^+$ T cell immune priming. Recombinant proteins derived from bacterial antigens are attractive candidates for the preparation of efficient, protective and therapeutic vaccines. A wide range of novel *Brucella* polypeptides were produced, characterised, and tested in animal models (3). The L7/L12 ribosomal protein and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which also exist in *Brucella melitensis* and other *Brucella* species (4-6), were identified as an immunodominant antigen from *B. abortus* (6,7). The recombinant L7/L12 protein and plasmid encoding L7/L12 gene can elicit strong cell-mediated immunity and promote protection against *Brucella* infection in mice (8-10). Also, it was reported that mice vaccinated with rGAPDH were able to induce cell-mediated immune response (6). However, very little is known about the human immune response to these proteins. Since there are some differences in terms of host immune responses to brucellosis in different host species (11), investigating immune responses to these proteins in humans is of great importantance. This approach will provide a significant contribution to the understanding of the immunopathogenesis of brucellosis and to the development of new diagnostic and vaccination strategies. In this study, peripheral blood mononuclear cells obtained from 25 acute brucellosis patients and 15 healthy controls were stimulated with Phytohemagglutinin (PHA) and recombinant *Brucella abortus* L7/L12 and GAPDH proteins. Furthermore, the levels of IFN-γ, IL-4, IL-10 secreted from these cells and alterations in lymphocyte subgroups in response to these antigens were measured by ELISA and flow cytometry, respectively.

MATERIALS AND METHODS

Study Population. Patients were grouped as acute brucellosis according to duration of the disease (<12 months). The patient group included fifteen females and ten males (n = 25) (mean age: 46.7 ± 12.6 years) with clinical and laboratory diagnosis of acute brucellosis. The healthy control group consisted of eight females and seven males (n = 15) (mean age: 31.3 ± 9.6 years) with no history of *Brucella* infection. Brucellosis was diagnosed in these patients on the basis of clinical, serological and bacteriological examinations. The diagnostic criteria were based on isolation of *Brucella spp.* from blood culture (BACTEC 9050, Becton-Dickinson Diagnostic Instrument System, Sparks, USA)

and/or a single *Brucella* titre of 1/160 by a standard tube agglutination test or Coombs test and, confirmed by a 2-mercaptoethanol (2-ME) test titre of ≥ 1/160, in association with compatible clinical findings. The controls had no brucellosis history, and no clinical and serological findings related to brucellosis or with any other disease. The study was approved by the Ethical Committee of Uludag University, Bursa, Turkey, and all subjects gave written informed consent.

***Brucella Abortus* Recombinant Proteins.** Both recombinant *Brucella abortus* proteins, L7/L12 and GAPDH, were expressed in *E. coli* harboring the pMAL-c2 vector (New England Biolabs, Beverly, Mass.) and purified by the amylose resin column (New England Biolabs) as described previously (6,8).

Mononuclear Cell Culture. Heparinized peripheral venous blood samples were taken from all patients before the onset of treatment. Peripheral blood mononuclear cells (PBMC) from acute brucellosis patients (study group) and healthy subjects (control group) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation and cultured in RPMI-1640 medium (Sigma, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Israel), 10 mM HEPES buffer (Sigma), 2 mM L-glutamine (Sigma), 100 U of penicillin/ml and 100 µg of streptomycin/ml (Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. The cells (5 x 10^5/well) were stimulated in a 48-well plate with 3 µg/ml phytohemagglutinin (PHA) (Sigma), 5 µg/ml of L7/L12 and/or 5 µg/ml of GAPDH. The complete culture medium (cRPMI) was used as a negative (unstimulated) control. The supernatants were collected from the cells that were stimulated with PHA after 2 days and from the cells that were stimulated with L7/L12 and GAPDH at the end of the sixth day. These supernatants were then kept at – 86°C until used. The remaining cells were resuspended in cRPMI and immediately analyzed by flow cytometry for evaluating lymphocyte subgroups.

Detection of Cytokines. The supernatants that were stored at –86°C were used for evaluating extracellular cytokines. Cytokines (IFN-γ, IL-4, IL-10) were detected using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (BIOSOURCE, CA, U.S.A.) with paired cytokine specific monoclonal antibodies according to manufacturer's directions. The minimum detectable concentrations of IFN-γ, IL-4 and IL-10 were 4, 2 and 1 pg/ml, respectively.

Analysis of Lymphocyte Subpopulations by Flow Cytometry. Cells were washed at 300 x g for 10 min at 4°C and stained with monoclonal antibody pairs (Beckman Coulter, Miami, FL, USA and Immunotech, Marseille, France): CD3-FITC/CD4-PE, CD3-FITC/CD8-PE, CD4-FITC/CD25-PE, CD3-FITC/CD69-PE, CD3-FITC/CD152 (CTLA4)-PE for 15 minutes at room temperature in the dark. All antibodies were FITC or PE-labeled mouse IgG1. Lymphocyte subpopulations were evaluated by flow cytometer (Epics XL.MCL, Beckman/Coulter, Miami, FL, USA). Each lymphocyte subpopulation count was expressed as a percentage of the total number of lymphocytes.

Statistical Analysis. Data were expressed as mean ± SEM. A Wilcoxon Signed Rank Test was used for evaluating lymphocyte subpopulations and extracellular cytokines. Comparison between groups was made using the Mann-Whitney U Test. The fold changes (FC) in the levels of cytokines secreted from stimulated (S) *versus* unstimulated (US) PBMCs were calculated with the following formula; FC = S – US / US. Afterwards, the data were compared statistically using the *SPSS 13.0* statistical *software* (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of L7/L12 and GAPDH on Cytokine Secretion. The peripheral blood mononuclear cells obtained from patients with acute brucellosis and from healthy subjects were stimulated with PHA, L7/L12 and GAPDH prior to the measurement of extracellular cytokines (IFN-γ, IL-4, IL-10). Interestingly, the spontaneous production of IFN-γ was significantly lower in unstimulated cells of the patients in comparison to those of the healthy controls (Table 1). Both L7/L12 and GAPDH seemed to increase extracellular IFN-γ production in brucellosis patients ($p<0.05$) compared to unstimulated cells (Table1). However, only the stimulation of PBMCs with L7/L12 induced IFN-γ production in each patient with 6.66 $\pm$ 2.02 fold increase on average, whereas IFN-γ production after stimulation was not altered in healthy controls (Figure 1). On the other hand GAPDH did not lead to a significant difference in INF-γ production between patients and healthy control individuals (1.47 $\pm$ 0.8 and 1.44 $\pm$ 1.78 fold increases, respectively) (Figure 1).

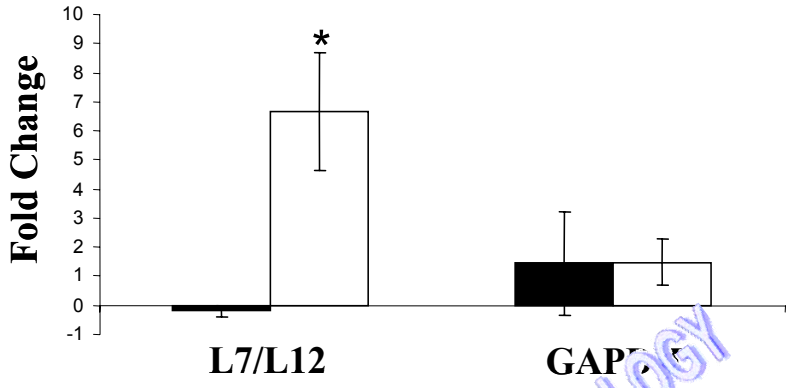

Figure 1. Fold changes in IFN-γ levels after stimulation of PBMCs with L7/L12 and GAPDH in healthy controls (black bars) and Brucellosis patients (open bars). (Healthy controls *vs*. patients * $p<0.001$)

Interleukin-4 levels measured in the supernatants of PBMCs from patients with brucellosis in response to L7/L12 or GAPDH were below the detection limit (Table 1). Statistically significant increases were found when the IL-10 levels within the supernatants of the cells stimulated with PHA, L7/L12 or GAPDH were compared with those of unstimulated cells in patients and healthy controls (Table 1). However, there was no significant difference in IL-10 production between cells from patients or healthy control individuals, following stimulation with either L7/L12 or GAPDH (Table 1).

Effect of L7/L12 and GAPDH on Lymphocyte Subpopulation. In our study, no statistically significant differences were found when the percentages of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$ and $CD3^+CD152^+$ lymphocytes within the PBMCs stimulated with L7/L12 or GAPDH were compared with those in unstimulated cells both from patients and healthy subjects (Table 2).

Table 1. Comparison of IFN-γ, IL-4 and IL-10 levels secreted from PBMCs stimulated with L7/L12 and GAPDH in healthy controls and brucellosis patients.

	IFN–γ (pg/ml)		IL-4 (pg/ml)		IL-10 (pg/ml)	
	Patients	Controls	Patients	Controls	Patients	Controls
US	58.3±28.4	637.4±274.6	U	U	292.1±65.3	276.9±31.4
PHA	5273.8±869.8*	4618.3±1236.2***	71.7±7.8	96.9±8.3	579.3±31.8 **	585.1±44.5**
L7/L12	776.9±200.1**	418.4 ±113.7	U	U	638.6±48.5*	552.8±72.3*
GAPDH	274.0±84.5*	568.0±314.8	U	U	718.8±72.6*	696.9±70.8*

PBMCs (5 x 10^5/ml) from healthy controls and brucellosis patients were incubated in the absence (US) or presence of PHA (2µg/ml) for 2 days, followed by incubation with L7/L12 (5µg/ml) or GAPDH (5µg/ml) for 6 days, harvesting the culture supernatants and assaying for IFN-γ, IL-4 and IL-10 levels by ELISA (Unstimulated *vs* stimulated * $p<0.05$; ** $p<0.01$; *** $p<0.001$).
U: Undetectable

In brucellosis patient group, the percentage of $CD3^+CD69^+$ lymphocytes were significantly higher in cells stimulated with L7/L12 or GAPDH than those of unstimulated cells ($p<0.01$). The percentages of $CD3^+CD69^+$ lymphocytes in the PBMCs of healthy controls did not significantly change after stimulation with L7/L12 or GAPDH (Table 2). However, there were no statistically significant differences in all lymphocyte subpopulations between the healthy and patient groups.

DISCUSSION

The effective protective immunity against intracellular bacteria depends on acquired cellular immunity and the activation of macrophages by cytokines, particularly IFN-γ, which are secreted by T lymphocytes (12). Although there are limited observations in relation with immune responses in human brucellosis, some studies demonstrated that *B. abortus* induces the secretion of the Th1 type cytokines, especially IFN-γ, by $CD4^+$ and $CD8^+$ T lymphocytes (13-16). However, it was also shown that treatment of macrophages with optimal concentrations of IFN-γ still allows some intracellular *Brucella* to survive (17,18). In addition, *Brucella* disrupts major histocompatibility complex (MHC) class II expression and antigen presentation, and therefore, can escape from $CD4^+$T cell-mediated cellular immune response (18,19). In previous animal studies, it was well documented that the GAPDH and L7/L12 induced production of IFN-γ and therefore led to very strong specific Th1 type T cell responses (3,6,8,9). However, it is unclear whether these recombinant protein antigens would have the same effect in human cells.

Table 2. Peripheral blood lymphocyte subpopulations in PBMCs stimulated with PHA, L7/L12 and GAPDH in healthy controls and brucellosis patients.

	US	PHA	L7/L12	GAPDH
$CD3^+$ T cells				
Patients	90.1±4.7	69.2±20.9	89.6±7.1	89.9±9.5
Controls	89.5±4.3	71.8±10.8	87.8±7.2	91.8±3.6
$CD3^+CD4^+$ T cells				
Patients	53.7±0.5	19.5±3.7	57.2±3.0	47.4±5.4
Controls	61.4±2.5	38.4±4.0	59.3±3.2	63.3±4.5
$CD3^+CD8^+$ T cells				
Patients	21.6±3.7	10.2±2.5	22.9±2.6	24.4±4.3
Controls	24.1±1.6	18.7±1.2	26.1±3.8	23.3±1.7
$CD4^+CD25^+$ T cells				
Patients	3.1±0.4	17.0±3.3	3.6±0.6	3.1±0.4
Controls	6.3±0.6	31.3±6.2	12.9±5.3	10.6±4.4
$CD3^+CD69^+$ T cells				
Patients	1.5±0.3	19.0±5.2*	3.4±0.6*	3.4±0.6*
Controls	4.7±1.2	49.6±4.5*	2.6±0.7	6.3±4.1
$CD3^+CD152^+$ T cells				
Patients	0.5±0.5	0.8±0.2	7.8±7.8	0.5±0.4
Controls	2.0±0.6	4.0±1.8	1.6±0.5	1.7±1.5

PBMCs (5 x 10^5/ml) from healthy controls and brucellosis patients were incubated in the absence (US) or presence of PHA (2µg/ml) for 2 days, followed by incubation with L7/L12 (5µg/ml) or GAPDH (5µg/ml) for 6 days, and harvesting the culture supernatants for the analysis of lymphocyte subpopulations by flow cytometry (Unstimulated *vs.* stimulated * $p<0.01$).

In our study the levels of extracellular IFN-γ produced from the peripheral blood mononuclear cells (PBMCs) of the patients with acute brucellosis following stimulation with L7/L12 and GAPDH was increased in comparison to IFN-γ levels spontaneously secreted from the unstimulated cells, whereas there was no statistically significant difference between IFN-γ levels produced by stimulated and unstimulated cells in healthy subjects. These data demonstrate that IFN-γ responses to L7/L12 and GAPDH are antigen-specific. Rafiei et al. (20) reported that the heat-inactivated *Brucella melitensis* Rev-1 induced production of IFN-γ, in both patients and healthy controls compared to the cultures without antigen stimulation. However, it was also mentioned that production of IFN-γ was much higher in acute brucellosis when compared to the chronic one and the healthy control group. In contrast with our data, the reason for the production of IFN-γ in response to the heat-inactivated *Brucella melitensis* Rev-1 in healthy subjects may be due to the antigenic complexity of this strain and/or because of using whole blood stimulation, which could activate the cells of innate immune system, such as NK cells or macrophages. Another interesting finding of our study was that L7/L12 induced strong Th1 response (approximately 6-7 folds) almost in every patient while GAPDH did not lead to significantly increased Th1 response in some of the patients. This data suggests that L7/L12 is a reliable candidate protein which can be used for the preparation of efficient *Brucella* vaccines. IFN-γ production capacity of PBMCs in response to

recombinant antigenic proteins such as purified protein derivative (PPD), culture filtrate protein (CFP)-10 and early secreted antigenic target (ESAT)-6 is used in the diagnosis of tuberculosis (21). Likewise, an immunodiagnostic method may be developed for the diagnosis of brucellosis by measuring IFN-γ production following stimulation by *Brucella* recombinant proteins, especially L7/L12.

Our study also demonstrated that PHA led to an elevation in IFN-γ levels in both patients and healthy controls, and this increase in cytokine production was higher in patients when compared with the controls. Similarly, Akbulut et al. (22) have demonstrated increased percentage of IFN-γ-producing T lymphocytes in acute brucellosis patients in comparison to the control group. In addition, diminished production of Th1 cytokines, IFN-γ and IL-2, was found to be associated with T-cell anergy to *Brucella* antigens and disease chronicity (20,23). Also, proliferative responses of $CD4^+$ T lymphocytes to PHA in chronic brucellosis patients has been found to be significantly low (24). The level of Th1 cytokines, especially IFN-γ, seems to be related to the clinical status of the disease and defective T lymphocyte proliferation, and IFN-γ production in response to the mitogens may be suggestive of a tendency to chronicity.

We also observed that the IFN-γ secreted from the unstimulated cells of the acute brucellosis patients was prominently lower than that of the healthy subjects. These data were in correlation with that of Rafiei et al. (20) which suggest that the low IFN-γ production by unstimulated cells is due to the production of Th2 or regulatory cytokines such as IL-10. However, in our study, no difference was observed between patients and healthy subjects when the levels of IL-10 produced by unstimulated cells were evaluated.

Interleukin-4, which is produced by Th2 cells, is an anti-inflammatory cytokine that prevents the Th1 cell response (25). It also prevents macrophage activity by decreasing IFN-γ production from $CD4^+$ T cells and can block T cell proliferation by decreasing IL-2 receptor expression (26). Rafiei et al. (20) found no difference in production of IL-4 in response to heat inactivated *B. melitensis* Rev-1. In another study using a murine model, Rosinha et al. (6) investigated humoral and cellular immune responses to *Brucella* rGAPDH and rL7/L12, and showed that *in vitro* stimulation of splenocytes from primed mice was able to produce IFN-γ and TNF-α but not IL-4. In our study, IL-4 levels synthesized by PBMCs from controls and patients with brucellosis in response to L7/L12 and GAPDH were below the detection limit. Only, PHA led to significant increases in both controls and patients. Therefore, it is worth measuring the levels of other Th2 cytokines such as IL-5 and/or IL-13 in further experiments instead of IL-4.

Interleukin-10 is known as a regulatory cytokine that decreases the production of IFN-γ and IL-12. This cytokine has an anti-inflammatory effect both *in vivo* and *in vitro*, and it can suppress protective immune response by preventing cytokine mediated macrophage activation and by inhibiting a Th1 type of cell differentiation (25,27). Previously, it has been shown that IL-10 decreases *in vitro* production of IFN-γ in splenocytes stimulated by *Brucella* antigens and *in vitro* neutralization of endogenous IL-10 in *Brucella* antigen-stimulated cultures of splenocytes from infected mice resulted in decreased bacterial loads (28). These data suggest that IL-10 may be downregulating the immune response to *B. abortus* by affecting both macrophage effector function and the production of IFN-γ. Oliveira et al. (29) measured IL-10 in culture supernatants of splenocytes from MHC Class I and MHC Class II knockout mice stimulated with *B. abortus*, and demonstrated that splenocytes from MHC class I knockout mice lacking $CD8^+$ T cell-

mediated response produced two-fold higher levels of IL-10 than those of the control and the MHC class II knockout mice. According to these data they suggested that $CD8^+$ T cells may inhibit the synthesis of Th2 type cytokines such as IL-10. In our study, an IL-10 level was found to be increased in both unstimulated and stimulated cells from healthy subjects and patients. However, there was no significant difference between all groups studied. Similarly, Rafiei et al. (20) did not find significant differences in IL-10 production in response to mitogen or a specific antigen between brucellosis patients and the healthy controls. Therefore, all these data suggest that the induction of IL-10 production by *Brucella* specific antigens or mitogens in both patients and healthy controls is likely due to non-specific stimulation by *Brucella* components. This non-specific IL-10 production may play a role in the survival of *Brucella* during natural infection and may also reduce the immunogenicity of the proteins used, most likely by inhibiting robust development of a Th1-type response.

In brucellosis $CD4^+$, $CD8^+$ and γδ T lymphocytes produce IFN-γ and increase the bactericidal function of macrophages. Also, $CD8^+$ and γδ T lymphocytes directly kill infected macrophages by mainly using apoptotic mechanisms. In addition, Th1 type antibodies like IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis in mice (14). Although a previous study by Araya et al. (30) showed that both $CD4^+$ or $CD8^+$ T cells are important in controlling infection, more recent studies (19,28,30,32-34) reported that $CD8^+$ T cells are more prominent in immunity to brucellosis. In our study, there were no significant differences in $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$, and $CD3^+CD152^+$ lymphocyte subpopulations in response to L7/L12 and GAPDH both in patients and controls. In an earlier study, brucellosis patients responded to slat extractable antigen from *B. melitensis*, RCM-BM, with increased CD69 expression on T cells (34). Similarly, the percentage of $CD3^+CD69^+$ T lymphocytes increased in response to L7/L12 and GAPDH in acute Brucellosis patients but not in healthy controls in our study. These findings indicate that T lymphocytes are specifically activated in response to peptide antigens in acute Brucellosis.

To summarize, this study demonstrated that recombinant L7/L12 and GAPDH induce a Th1-type of immune response in acute brucellosis patients. However, we should stress that L7/L12 can elicit a more efficient and reliable Th1 type response. Therefore, our study suggests that L7/L12 can be a proper antigen for new *Brucella* vaccines based on the induction of cellular immune response. In addition, strong IFN-γ production induced by L7/L12 could be used as an immunological method for the diagnosis of brucellosis. However, further studies are required to determine a more detailed role of L7/L12 and GAPDH in inducing strong cellular immune responses in brucellosis.

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