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The Profile of Cytokines and IgG Subclasses in *BALB/c* Mice after Immunization with *Brucella* Ribosomal Gene

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Abstract: This study was evaluated the ability of DNA vaccine encoding L7/L12 protein of *Brucella* sp. to induce cellular and humoral immune responses in *BALB/c* mice and the profile of cytokines and IgG sub classes were determined. Intra muscular vaccination of mice using L7/L12 gene. Three vaccinations at 3 week intervals were performed. Cytokines and IgG subclasses were analyzed 3 week after the last DNA vaccination. Splenic lymphocytes from L7/L12pCDNA3-vaccinated mice produced high levels of IFNγ (3100 pg mL^{-1}) and low levels of IL-5 (300 pg mL^{-1}), 3 weeks post-vaccination. The L7/L12pCDNA3 immunizations elicited high IgG2a isotype response in mice immunized. This antigen also induced IgG1 titers which were slightly lower than the IgG2a titers. Immunological analysis shows the appropriate immune response in *BALB/c* mice model after vaccination with L7/L12 gene. The high level of IFNγ and low level of IL-5 in combination with high IgG2a/ IgG1 ratio show the activation of Th1 cell response. The lower bacterial cfu from vaccinated mice in comparison with control groups show the efficiency of L7/L12 DNA vaccination in mice model.

Key words: *Brucella abortus*, L7/L12 gene, DNA vaccination

INTRODUCTION

Brucella abortus, a gram-negative bacterium, is pathogenic for cattle, causing abortion in pregnant females due to colonization of the placenta and sterility in males and it causes a debilitating fever (undulant fever) in humans (Aleixo *et al*., 1999; Smith and Ficht, 1990). Brucellosis still constitutes an important health problem in many developing countries and in some developed areas of the world (Boschiroli *et al*., 2001). Vaccination is an essential element in the control of brucellosis. The vaccinal *B. abortus* strain 19 [S19] has been used successfully for eradication of brucellosis in cattle (Jinkyung Ko and Splitte, 2003).

However, due to its virulence for humans and its possible abortion in pregnant animals (Adone and Ciuchini, 2001), alternative vaccinal approaches are needed.

DNA vaccines are promising delivery systems for many infectious diseases which provide prolonged antigen expression, leading to amplification of immune response and induce memory responses (Gurunathan *et al*., 2000a; Klinman *et al*., 1998). Moreover, endogenous expression of antigen from DNA introduced into host cells leads to processing and presenting the peptide with the major histocompatibility complex class I hence induces cytotoxic T-lymphocyte (Corr *et al*., 1996; Ulmer *et al*., 1993).

Moreover, DNA vaccines themselves possess their own adjuvant activity because of the presence of unmethylated CpG motifs in particular base contents (Mor, 1998; Sato *et al*., 1996). This type of vaccine is capable of eliciting strong cell-mediated immunity that is required for control of infection by many intracellular agents including *Brucella* sp. (Gurunathan *et al*., 2000b; Lai and Bennett, 1998; Prud'homme *et al*., 2001; Golding *et al*., 2001; Oliveira and Splitter, 1995; Smith and Ficht, 1990).

It was previously reported that DNA vaccine encoding the L7/L12 ribosomal protein from *B. abortus* has induced an appropriate immune response with some level of protection in mice model but they did not report cytokines profile and IgG sub classes (Kurar and Splitter, 1997). In the other study was designed to evaluate the

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immunogenicity and the protective efficacy of a divalent fusion DNA vaccine encoding both the *Brucella abortus* L7/L12 protein but with pcDNA3.1 vector (Luo *et al*., 2006).

The present study shows that intramuscular (i.m.) immunization of mice with L7/L12 gene generates a strong specific humoral and Th1 responses with high level of IFN-γ and IgG2a with pcDNA3 vector.

MATERIALS AND METHODS

This study was conducted at the Tarbiat Modares University, Medical Faculty, Department of Microbiology from October 2002 to January 2004.

Bacterial strains and media: *B. abortus* 544 (virulent strain) and *B. abortus* strain 19 (S19) were obtained from Pasteur Institute of Iran. *Brucella* cells were grown on 2YT agar (10 g of yeast extract, 10 g of tryptone and 5 g of NaCl L^{-1}). For vaccination or challenge, the colonies were suspended and washed two times in a sterile Phosphate-buffered Saline (PBS) and the No. of bacteria was measured by determining the cfu on 2YT agar plates.

Extraction of chromosomal DNA: Chromosomal DNA prepared according to standard CTAB/NaCl method (20). Quality and quantity of purified genomic DNA was assayed by 0.8% agarose gel electrophoresis in 1xTBE buffer and spectrophotometrically (260/280 nm), respectively.

Plasmid preparation: The *Brucella* L7/L12 gene which isolate and characterized previously (Abtahi *et al*., 2004) (flanked by *Bam*HI/*Xho*I sites) were ligated into the multiple-cloning site of the mammalian expression vector pcDNA3 (*in vitro* gen), giving pcDNA-L7/L12. *Escherichia coli* DH5α cells (Stratagene, La Jolla, Calif.) were transformed with the recombinant plasmid and grown in Luria-Bertani broth containing ampicillin (100 μg mL^{-1}). Plasmid DNA for *in vitro* transfection or mouse immunization was extracted from a 16 h culture and purified using the Endo-Free Plasmid Giga kit (Qiagen, Chatsworth, Calif.). The concentration and purity of the plasmid were determined by measuring the optical density ratio A_{260}/A_{280}. Plasmid DNA was adjusted to a final concentration of 1 mg mL^{-1} in PBS and stored at -80°C till further use.

DNA vaccination and challenge of *BALB/c* mice: Female *BALB/c* mice 6 weeks old were obtained from the Razi Vaccine and Serum Research Institute of Iran randomly distributed into three groups so that each group consisted of 16 mice. The mice were injected through intramuscular (i.m.) route in the tibialis anterior muscles with 100 μg of pcDNA-L7/L12, or pcDNA3 (in 50 μL sterile saline) and PBS as negative controls each group was inoculated three times at 3-week intervals. The immune response (four mice per group) was analyzed 3 weeks after the last DNA immunization. Positive control mice (n = 8) received an intraperitoneal (i.p.) injection with 5×10^4 cfu of *B. abortus* vaccine strain S19 in 100 μL of sterile PBS 4 weeks before the challenge. For the challenge inoculum, mice received an i.p. injection with approximately 5×10^4 cfu of *B. abortus* 544 in 100 μL of sterile PBS, 4 weeks after the last vaccination. Colonies were counted and expressed as $\log_{10}$ cfu per spleen.

Determination of isotype-specific immunoglobulin by ELISA: Expression and purification of the recombinant L7/L12 was described previously (Abtahi *et al*., 2004).

Serum samples were analyzed for specific anti L7/L12 antibodies. Briefly, standard ELISA plates (MaxiSorp; Nunc A/S, Denmark) were coated overnight at 4°C with recombinant protein L7/L12 (50 μL well) at a final concentration of 2.5 μg mL^{-1} in PBS (pH 7.4). After three wash cycles with PBS, the plates were saturated for 2 h at RT with 150 μL of blocking buffer (PBS with 1% BSA). Plates were washed with PBS containing 0.1% Tween 20. Serially twofold-diluted individual serum samples (1:100) were added to the plates and incubated for 1 h at RT. The serum from nonimmunized mice was used as a negative control. After five washing cycles, plates were incubated with goat biotinylated anti-mouse immunoglobulin G1 (IgG1) or IgG2a (1:1000 diluted, Amersham) for 1 h at RT. After washing, streptavidin-horseradish peroxidase (1:1000 diluted in PBS, Amersham) was added and incubated for 1 h at RT. After excessive washing TMB (3,3',5,5'-tetramethyl benzidine) in citrate-phosphate buffer (0.05 M Na_2HPO_4, 0.025 M citric acid, pH 5.0) and 2 mM H_2O_2 were added to monitor the peroxidase activity. The reaction was stopped after 20 min by addition of 2 M H_2SO_4. The optical density was measured at 450 and 630 nm on a Bio Kinetics Reader EL-340 (Biotek Instruments, Winooski, Vt.). The end point titer was defined as the highest dilution of serum giving an optical reading of two times the reading of the negative control.

Lymphocyte proliferation assay: Proliferation was determined by measuring the level of incorporation of [^{3}H] thymidine into the DNA of actively dividing cells. The spleenocytes were removed and homogenized with RPMI 1640 medium (Gibco, BRL) and centrifuged for 10 min at 1,200x g and the cell pellets were washed two times with RPMI 1640 supplemented with 5% Fetal Bovine Serum (FBS) (Sigma). Erythrocytes were lysed with Gey's solution, by incubating the mixture for 10 min at 4°C. The

lymphocytes were washed two times with RPMI 1640 containing 5% FBS by centrifugation. Spleenocytes resuspended in complete medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin [100 U mL^{-1}], streptomycin [100 μg mL^{-1}], gentamicin [50 μg mL^{-1}] and 5×10^{5} M 2-mercaptoethanol) were cultured at 2×10^{-5} cells per well in 96-well flat-bottom microwell plates. Cultures in quadruplicate were stimulated with 2.5 μg of L7/L12. Concanavalin A (ConA) (Sigma, St. Louis, Mo.) at a concentration of 0.5 μg mL^{-1} and RPMI were used as positive and unstimulated controls, respectively. After 72 h at 37°C in a humidified 5% CO_2 incubator, cells were pulsed with 0.5 μCi of [^{3}H] thymidine/well (ICN) for 18 h. The cells were harvested onto glass filter strips. Tritiated-thymidine incorporation was counted by liquid scintillation spectroscopy with a Beta plate counter (WALLAC Oy, Turku, Finland). The mean No. of Counts Per Minute (CPM) and standard error of the mean for each quadruplicate set of cells were determined.

Measurement of cytokines in supernatants: The release of IFNγ and interleukin-5 (IL-5) in murine splenocyte culture supernatants were measured after 72 h of incubation with antigen as described for the lymphocyte proliferation assay. IFNγ and IL-5 were assayed according to the manufacturer's instructions with using specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (Pharmingen, San Diego, Calif.).

Statistical analysis: The data were analyzed by Student's t-test. p-value of <0.05 was considered to be significant.

RESULTS

Vaccination with L12/L7 confers protection against *B. abortus* 544 challenged *BALB/c* mice: To determine the protection potency of immunization with the candidate DNA vaccines against *B. abortus* 544 infection, *BALB/c* mice immunized three times with either pcDNA3L7/L12 or pcDNA3. They were infected 4 weeks later with *B. abortus* 544. Four weeks after challenge, mice were killed and cfu in the spleens were quantitated. The mice which were immunized by pcDNA3L7/L12 showed significant level of protection at 4 weeks after challenge (Table 1). There is no significant difference between the number of the bacteria isolated from the spleens of the nonvaccinated and pcDNA3L7/L12 or pcDNA3 vaccinated animals (Table 1). In contrast, at a vaccination-to-challenge interval of 4 weeks, mice vaccinated with strain S19 demonstrated an efficient protection.

The humoral response to L7/L12 induced by DNA vaccination: Sera collected 3 weeks after the last vaccination were assayed for the presence of L7/L12 specific antibodies by indirect ELISA using the relevant purified recombinant proteins. The pcDNA3L7/L12 immunizations induced antibody IgG2a responses to L7/L12 that were 2.35±0.15. This antigen also induced IgG1 titers which were lower than the IgG2a titers (1.4±0.15). Immunization with pcDNA and PBS did not induce any production of anti-L7/L12 antibodies (1.375±0.15).

T-cell proliferative response by splenocytes from DNA-vaccinated mice: To further investigate the cellular immune response induced by the plasmid vectors, we analyzed the proliferative T-cell response.

T-cells were isolated and purified from spleens of the mice immunized. As shown in Fig. 1, L7/L12 DNA vaccination resulted in specific T-cell proliferation in response to either the related recombinant antigen. This specific induced proliferative response was also found at 3 weeks post vaccination. In contrast, immunization with pcDNA3 or PBS did not have any effects on the level of T-cell proliferative response (Fig. 1). The ConA mitogen as positive control was able to induce T-cell proliferation in all cases (data not shown).

Table 1: Protection efficacy against *B. abortus* 544 in *BALB/c* mice induced by vaccination with pcDNA3-L7/L12 compared to live strain *B. abortus* S19 or pcDNA3 alone

Experimental group in spleen	*Brucellae*±SD	Mean log_{10}
PBS	4.9±0.9	(9×10^4)
pcDNA	4.9±0.017	(9×10^4)
pvDNA3-L7/L12	4.5±0.07	(3×10^4)*
B. abortus	2.9±0.08	(800)**

The data represent the mean cfu (log_{10}) of three different experiment±SD. *Significantly different from PBS-treated mice (p<0.05). **Significantly different from PBS-treated mice (p<0.001)

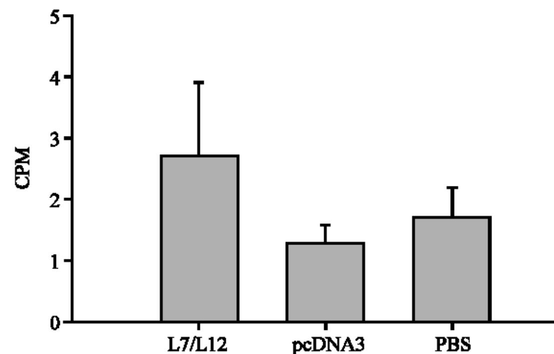

Fig. 1: Lymphocyte proliferation assay of splenocytes derived from *BALB/c* mice immunized with pcDNA-L7/L12, pcDNA3 and PBS three weeks post immunization

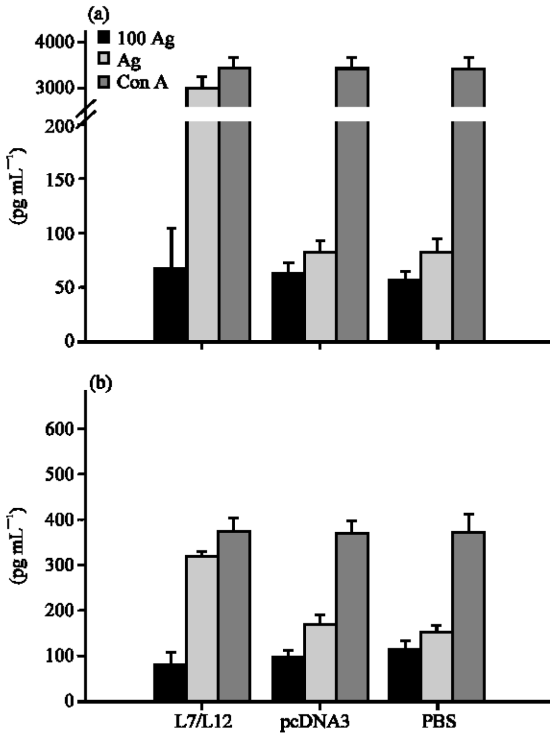

Fig. 2: Quantitative ELISA analysis of IFNγ (a) and IL-5 (b) secretion by murine lymphocytes isolated after DNA immunization (pcDNA3 or pcDNA3L7/L12) and subsequent stimulation in culture using recombinant L7/L12 antigen

Cytokines profile of vaccinated *BALB/c* mice: Cytokine production from splenocytes of four vaccinated mice per group, 3 weeks after the last vaccination is shown in Fig. 2. Splenic lymphocytes from pcDNA3L7/L12-vaccinated mice released up to 3100 pg of IFNγ mL^{-1}, 3 weeks post vaccination. By contrast, little if any IL-5 (300 pg mL^{-1}) was detected. Mice vaccinated with pcDNA3 produced neither IFNγ nor IL-5 upon restimulation with the antigen. The media did not induce IFNγ or IL-5 production, whereas ConA induced a large quantity of IFNγ (Fig. 2a) and minimal amount of IL-5 (Fig. 2b). Taken together, these results indicated that there was a significant Th1 biased response after the immunization with pcDNA3L7/L12.

Protection against *B. abortus* 544 challenge in immunized mice: To determine whether the immunization with the candidate DNA vaccines induced protection against *B. abortus* 544 infection or not, the mice immunized three times with pcDNA3L7/L12 was infected 4 weeks later with *B. abortus* 544. Four weeks after challenge, mice were killed and cfu in the spleens were quantitated. The mice which were immunized by pcDNA3L7/L12 showed significant level of protection at 4 weeks after challenge (Table 1). No significant difference in the number of the bacteria isolated from the spleens of the nonvaccinated and pcDNA3-vaccinated animals was observed (Table 1).

DISCUSSION

Several reports have been shown that DNA vaccination stimulated long-lived humoral and cellular immune responses *in vivo* against viruses, bacteria, or parasites in a variety of animal models (Donnelly *et al*., 1997; Krishnan, 2000; Ulmer *et al*., 1996). DNA vaccine provides prolonged antigen expression, leading to amplification of immune response and it induces memory responses against infectious agents (Gregoriadis, 1998; Talwar *et al*., 1999). Moreover, endogenous expression of antigen from DNA introduced into host cells leads to processed peptides presented with the major histocompatibility complex class I and it can induce cytotoxic T lymphocytes (Donnelly *et al*., 1997; Gurunathan *et al*., 2000a). This type of vaccine is capable of eliciting strong CMI that is required for control of infection by many intracellular agents, like *Brucella* (Golding *et al*., 2001; Kaufmann *et al*., 1995; Splitter *et al*., 1996).

Kurar and Splitter (1997) created DNA vaccines with using pcDNA3 and p6 vectors containing the gene for ribosomal protein L7/L12. Their studies revealed that the constructs could induce both specific antibodies and T-cell-mediated immune responses accompanied by a significant level of protection compared to the controls. But in the study they did not detect cytokines profiles and IgG subclasses.

In this study, demonstrate that intramuscular (i.m.) immunization of mice with pcDNA-L7/L12 generates a strong specific humoral and T-cell responses with tendency of Th1 like cytokines. Three weeks after the last vaccination we found high titers of specific IgG2a in mice immunized with plasmid pcDNA-L7/L12. The IgG2a / IgG1 ratio was approximately, greater than 1. Since, the isotype profile of antibody response is a reflection of the T-helper-cell types (Jones and Winter, 1992; Mosmann and Sad, 1996), these results suggest that i.m. pcDNA-L7/L12 DNA vaccination can induce Th1 responses. In fact, it was IgG2a isotype of the antibody in the infected animals that helped in clearance of pathogens (Mallick *et al*., 2007). Mice vaccinated i.m. with pcDNA3 did not produce detectable antibodies against L7/L12 antigen. This is also in agreement with the earlier observation that recombinant L7/L12 protein could serve as serological diagnostic antigen in ELISA (Mor, 1998).

The induction of T-cell immune responses after DNA immunization was then evaluated by measuring T-cell-proliferative and cytokine responses after *in vitro* stimulation of splenic cells with purified recombinant L7/L12 protein. L7/L12 induced a high T-cell-proliferative response with production of significant levels of IFNγ but no IL-5. Even a single dose of DNA immunization (L7/L12) was able to elicit a detectable immune response. These results are in agreement with those obtained with the CpG adjuvanted recombinant proteins; because of induce a strong Th1 type immune response (Gurunathan *et al*., 2000b).

It is well documented that cellular immune response plays a major role in the establishment of a protective response against *Brucella* (Jiang and Baldwin, 1993; Stevens *et al*., 1992). Therefore designing of a preventive vaccine against brucellosis must be based on its capacity to generate a strong Th1-type immune response, with high levels of IFNγ and IgG2a involved in the immune process. Previously reports of i.m. pcDNA-L7/L12 administration have demonstrated that inoculation with this vector elicits cellular immune response, as characterized by high especially in increasing the levels of IFNγ production by T cells and high production of IgG2a in immunized animals (Oñate *et al*., 2003).

In conclusion, this study has shown that i.m. inoculation with pcDNA-L7/L12 elicits a strong cellular protective immune response, stimulating Th cells. The IFNγ-induced expression of immunoglobulin of the IgG2a isotype is widely recognized as a characteristic of a Th1-type immune response. Th cells from immunized mice produced high levels of IFNγ and IgG2a, which have been reported to be essential for protective immunity against *Brucella*.

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