Original Article

Evaluation of the immunogenicity and the protective efficacy in mice of a DNA vaccine encoding SP41 from *Brucella melitensis*

Ayman Al-Mariri and Abdul Q. Abbady

Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria, Damascus, Syria

Abstract

Introduction: *Brucella melitensis* is a facultative intracellular Gram-negative bacterial pathogen that may enter the host via ingestion or inhalation, or through conjunctiva or skin abrasions. Some *Brucella* spp surface proteins (SPs) play an important role in bacterial adhesion and invasion and thus represent targets for the host immune system. *Brucella* spp surface protein with apparent molecular mass of 41 kDa interacts selectively with HeLa cells.

Methodology: To evaluate the role of SP41 (41 kDa) as a DNA vaccine against *Brucella* spp., pCISP41, a plasmid construct for protein expression in mammalian cells, was established. Exogenous SP41 was detected in pCISP41-transfected Vero cell line by immune blotting using specific polyclonal antibody. The protective role of pCISP41 against *B. melitensis* 16M in mice was evaluated by measuring B and T cell responses in comparison to those achieved with attenuated *B. melitensis* Rev. 1 vaccine.

Results: BALB/c mice injected with pCISP41 were able to develop SP41-specific serum immunoglobulin G (IgG) antibodies. In addition, splenocytes from DNA-SP41-vaccinated mice elicited a T-cell-proliferative response and also induced gamma interferon (IFN-γ) production, but not interleukin-5 (IL-5), suggesting the induction of a T-helper-1-dominated immune response. Vaccination with attenuated *B. melitensis* Rev.1 strain induced better protection levels than DNA vaccination with SP41 against *B. melitensis* 16M in mice.

Conclusion: Such responses play an important role against intracellular infecting agents such as *Brucella* spp. Altogether, our data suggest that SP41 may represent a promising candidate for DNA vaccination against brucellosis, but more investigation to increase its protective efficacy should be done.

Key words: *Brucella*; SP41; DNA vaccine; CMI; vaccine

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Introduction

Brucella melitensis is a facultative intracellular pathogen and one of the etiological agents of brucellosis that can infect humans and animals [1]. The disease brucellosis is a highly common bacterial zoonosis worldwide, and a major cause of human disease and economic loss of cattle [2]. Because of the economic losses to the sheep and goat industry caused by *B. melitensis*, as well as the zoonotic infections caused by these bacterial species, great efforts were made to eradicate ovine brucellosis worldwide [3]. To achieve this objective, a vaccine strain of *B. melitensis*Rev.1 has been used with relatively good results [4]. However, Rev.1 has several drawbacks. For example, it is difficult to differentiate between vaccinated and naturally infected animals, as Rev.1 elicits antibodies against smooth lipopolysaccharide, and it induces abortion in pregnant animals showing a likelihood of changing to a virulent form and also be fully virulent for humans [5]. A vaccine that will be non-infectious to humans but effective in stimulating a broad protective immune response is needed to control brucellosis [6]. The host resistance to *B. melitensis* depends mainly on acquired cell-mediated immunity (CMI) [7]. *In vivo*, Th1 differentiation depends on IL-12, and Th17 differentiation depends on IL-6 and TGF-b [7]. Th17 responses have been shown to contribute to host defense against several extracellular pathogens such as *Klebsiella pneumoniae* [2], as well as against intracellular microorganisms such as *B. abortus* [8]. The development of a Th1 subset of CD4+ lymphocytes secreting gamma interferon (IFN-γ) is important, since it is a crucial cytokine that can upregulate the anti-*Brucella* activity of macrophages [9,10]. The historical basis of DNA vaccines rests on the observation that direct *in vitro* and *in vivo* gene transfer of recombinant DNA by a variety of techniques resulted in the expression of protein [11].

DNA vaccination might provide several important advantages over current vaccines, as follows: (i) DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I restricted CD8+ T-cell responses, which may be advantageous compared with conventional protein-based vaccines, while mitigating some of the safety concerns associated with live vaccines; (ii) DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relative ease [12,13]; (iii) DNA vaccine provides prolonged antigen expression, leading to the amplification of immune response and induces memory responses against infectious agents [14]. This type of vaccine is capable of eliciting the strong CMI that is required for the control of infection by many intracellular agents [15]. This kind of immune response is of paramount importance against *Brucella*spp [16]. Several studies of murine models have been conducted to test the abilities of different proteins of *Brucella* to induce a protective immune response. Recombinant antigens of *Brucella* spp. such as Bacterioferritine (BFR) and the P39 [17], HtrA [18], GroEL [19,20], GroES [21], Cu-Zn superoxide dismutase (SOD) [22,23], YajC [24], L7/L12 [25], outer membrane protein 31 (Omp31) [26], ialB and omp25 [16], BLS-L7/L12 [27], BLSOmp31 [28] and lumazine synthase [29] have been shown to induce humoral and CMI responses in mice. In addition, Omp31, P39, L7/L12, BLSOmp31, lumazine synthase and peptides comprising certain epitopes of Cu-Zn SOD induced some level of protection in a mouse model of infection. Castañeda-Roldán*et al*. showed that a surface protein (SP41) of *Brucella* spp can bind to cellular sialic acid residues of HeLa cells [30]. SP41 is associated with bacterial adherence and invasion of HeLa cells. Adhesions in the form of outer membrane proteins may mediate direct binding of the bacteria to host cells or favor colonization by mediating bacteria-bacteria interactions. Bacterial adherence is then considered an important virulence trait, because it enables bacterial pathogens to deliver toxins efficiently to host tissues, to interact closely with the cell membrane favoring intracellular penetration to overcome peristaltic clearance, and to establish microbial communities in biological niches [31]. Biochemical analysis of SP41 revealed that this protein is the predicted product of the ugpB locus, which showed significant homology to the glycerol-3-phosphate-binding ATP-binding cassette (ABC) transporter protein that is found in several bacterial species. The aim of this study was to evaluate the protective capacity of immunization with plasmid DNA carrying the *B. melitensis* 16M SP41 gene (pCISP41).

Methodology

Animals

Seventy female BALB/c mice (7 to 8 weeks old, purchased from Charles River Laboratories, L'Arbresle, France) were randomly distributed into four experimental groups. The mice were kept in conventional animal facilities and received water and food.

Bacterial strains and growth conditions

B. melitensis 16M and vaccinated strain *B. melitensis* Rev.1 were obtained from the University of Namur (Belgium). *Brucella* was grown under optimal conditions in 2YT (peptone, 16 g/l; yeast extract, 10 g/l; NaCl, 5 g/l and distilled water to 1 l) (Difco, USA) overnight at 37°C and 5% CO_2 to ensure sufficient cell density; or in 2YT-Agar (agar 13 g/l), for 72 h at 37°C and 5% CO_2 [17]. All experiments with live *Brucella* spp were performed in biosafety level 3 facilities. *Escherichia coli* strain TOP10 was used to prepare the plasmid constructs. The *E. coli* TOP10 cultures were routinely grown at 37°C in Luria-Bertani broth (LB) or agar and were supplemented, when required, with 100 µg/ml ampicillin. For infection experiments, *B. melitensis*16M were grown for 48 hours in 2YT agar. The bacteria were suspended in a sterile phosphate-buffered saline (PBS). *B. melitensis*16M abundance in PBS was monitored by recording the optical density (OD) at 590 nm. The exact doses were assessed retrospectively by viable counts on 2YT agar plate.

Production of recombinant SP41 protein

Briefly, the respective gene SP41 was cloned into a pET-15b expression vector and the resulting plasmid was introduced in *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA); then the positive clones were selected. The recombinant proteins were expressed successfully in transformed bacteria by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium, and then purified with a Ni2-HiTrap chelating 5-ml prepacked column (Amersham Pharmacia Biotech, Freiburg, Germany) using imidazole as the elution reagent, according to the manufacturer's protocol. The lysates of transformed cells and the purified protein were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays. The purified protein was then stored at -70°C until

used for enzyme-linked immunosorbent assay (ELISA) or for *in vitro* stimulation of splenocytes.

Polyclonal antibodies against SP41

A New Zealand white female rabbit was injected intradermally with 100 µg of rSP41 in Freund's adjuvant (complete for the initial injection; incomplete for subsequent intramuscular injections) diluted 1:1 with sterile saline. Three inoculations were performed at two-week intervals. Antisera were then collected 10 days after the last injection. Working dilutions of 1:500 of antisera were used in this work.

Subcloning of SP41 gene in mammalian expression plasmid pCI

Oligonucleotide primers were designed to amplify the whole sequence of SP41 gene from *B. melitensis* bv. 1 str. 16M based on the published corresponding genomic sequences (GeneBank accession no. NC_003318). SP41 gene is located in the *B. melitensis* bv. 1 str. 16M chromosome II in a complementary position from 659855 bp to 661156 bp. A large gene fragment containing a full-length open reading frame of SP41 gene was first amplified by PCR from the genomic DNA of *B. melitensis*16M using (forward) 5′-TAAAGATCCAGAGGAGCTTT-3′ and (reverse) 5′-CATTGTCTCGATATGATGGA-3′. The first PCR product was used as a template in a second PCR run to amplify the exact SP41 gene length by its original start and stop codons. For this aim, primers were designed according to the SP41 gene nucleotide sequence and contained one artificial restriction site at each end: (forward) 5′-ATACA**CTCGAG**ATGTTCACCCGTCTGATCACG-3′ and (reverse) 5′-CGAGA**TCTAGA**ATTATTGAGCTGCGGCGATTG-3′, where the underlined nucleotides indicate the position of XhoI and XbaI restriction enzymes (Fermentas GMBH, Opelstraße, Germany), respectively. The amplified SP41 gene fragment was subcloned into the mammalian expression vector pCI downstream of the cytomegalovirus promoter (Promega, Boston, USA). PCR was performed in a 10 µl volume containing 1 ng DNA template, 5 pmol of each primer, 10 mM deoxyribonucleoside triphosphate, 10× PCR buffer, and 5 U AmpliTaq polymerase (Qiagen, Valencia, USA). PCR amplification was conducted with a DNA thermal cycler under the following conditions: denaturation at 95°C for 3 minutes, annealing at 55°C for 45 seconds and extension at 72°C for one minute (35 cycles). The amplified products were purified with the Quiaex II gel extraction kit (Qiagen) and digested with XhoI and XbaI restriction endonucleases. After digestion, the PCR product was purified again by the same procedure and ligated to the predigested pCI vector. This construction was used to transform *E. coli* TOP10, and a single recombinant clone was selected. Plasmid DNA was extracted with the Miniprep kit (Qiagen). The pCISP41 construct was digested with endonucleases and DNA sequenced to confirm the presence and the orientation of the SP41 gene. For large-scale plasmid DNA isolation, a colony of *E. coli* TOP10 containing pCISP41 was cultured in LB containing 100 µg/ml ampicillin and DNA isolation was performed using an EndoFree Plasmid Giga kit (Qiagen) according to the manufacturer's directions. The DNA was finally resuspended in PBS at a concentration of 1 mg/ml. The DNA concentration and purity were determined by the OD, and the A260/A280 ratio was typically >1.8.

Antigen expression in Vero cell line

Monkey kidney Vero cells were grown at 37°C in 5% CO2 in six-well plates (TTP, Trasadingen, Swaziland) containing Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Essen, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and gentamicin (50 µg/ml), and subconfluence monolayers were washed once with serum-free DMEM. Afterwards, 500 µl of DMEM (supplemented as above, but without FBS) was added. Then 100 µl of transfection mixture (100 µl of serum-free DMEM containing 6 µg of Fugene6 Boehringer, Ingelheim, Germany) and 1 µg of plasmid DNA were kept at room temperature (RT) for 5 minutes and added to the cells, which were then incubated at 37°C in 5% CO_2 overnight [17]. Expression of SP41 protein was detected by immunoblotting.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed using standard procedures for Western blot analysis, and proteins were transferred from the gel (12% wt/vol polyacrylamide) to 0.2 µm-pore-size nitrocellulose membranes (Bio-Rad, Hercules, USA).The assay was achieved using the rabbit anti-SP41 polyclonal antibody and the purified recombinant SP41 protein (rSP41) as a control. Membranes were probed with an anti-SP41 and then with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Bethyl Laboratories, Inc., Montgomery, USA). Detection of SP41 antigen was achieved upon development with

the TMB membrane substrate (3,3,5,5-tetramethyl benzidine) (Amresco, Solon, OH, USA) in citrate-phosphate buffer (0.05 M Na2HPO4, 0.025 M citric acid, pH 5.0) and 2 mM H_2O_2 were added to monitor the peroxidase activity.

Mice DNA vaccination and challenge

Mice randomly allocated in three groups of 20 mice each received intramuscular (i.m.) injections in the tibialis anterior muscles as follows: one group received 100 µg of pCISP41, one group received pCI as a negative control in 50 µl sterile saline (PBS), and one group was treated with 50 µl of saline by using a 1-ml insulin syringe with a 28-gauge needle. Three vaccinations at three-week intervals were performed. The immune response (five mice per group) was analyzed four and eight weeks after the last DNA vaccination. Positive control mice (n = 10) received an intraperitoneal (i.p.) injection with 1 x 105 CFU of Rev.1 in 100 µl of sterile PBS four weeks before the challenge. Thirty days after the last DNA injection, 10 mice per group were challenged i.p. with approximately 3 x 10^5 CFU of *B. melitensis* 16M in 100 µl of sterile PBS, and ten mice per group were sacrificed to conduct an analysis of immune responses, including IgG, proliferation cells, and cytokine production.

Quantification of bacteria in the spleen

Protection experiments were performed as previously described [32]. Briefly, at four and eight weeks post-challenge, five mice were killed by cervical dislocation, and their spleens were removed, homogenized, and dilutions were plated to determine the number of *Brucella* spp CFU per spleen. Three repeats were performed for each mouse, and then the mean of these repeats was calculated and considered as the average CFU number of each mouse. Log10 units of protection were obtained by subtracting the mean $\log_{10}$ CFU for the experimental group from the mean $\log_{10}$ CFU of the corresponding control group.

ELISA

The presence of serum IgG specific to rSP41 was determined by indirect ELISA on the 30th day after the final immunization. Purified rSP41 was diluted to 0.01 mg/ml in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene plate (100 µl/well; (MaxiSorp surface; Nunc, Waltham, USA) or with *B. melitensis* 16M bacterial lysate at a concentration of 3 µg/ml. After overnight incubation at 4°C, the plates were washed, blocked, and incubated with serially diluted sera for three hours at RT. Following another washing, IgG goat anti-mouse horseradish peroxidase conjugates were added (100 µl/well) at the appropriate dilutions. After one hour of incubation at RT, the plates were washed, and TMB in citrate-phosphate buffer (pH 5.0) and 2 mM H_2O_2 were added to each well to monitor peroxidase activity. The enzymatic reaction was allowed to proceed for 20 minutes at room temperature; then the reaction was stopped with the addition of 50 µl of 1M sulfuric acid per well. The absorbance of the developed color was measured at 450 nm in a Multiskan, Thermo-lab Systems Reader, Vantaa, Finland. The titer of each serum was calculated as the log10 of the reciprocal of the highest serum dilution yielding a specific OD higher than the cutoff value. All assays were performed in triplicate for each mouse, and their mean was considered as the average of each mouse.

Splenocyte culture and lymphocyte proliferation

Four to eight weeks after the last immunization, mice were sacrificed, and their spleens removed under aseptic conditions. Single-cell suspensions were prepared from the spleens, and red blood cells were lysed with ACK (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na2EDTA, pH 7.3). Splenocytes were cultured at 37°C in 5% CO_2 in a 96-well flat-bottom plate at a concentration of 2 x 105 cells per well in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated FBS (Eurobio, Courtaboeuf, France), in the presence of concanavalin A (ConA; 3 µg/ml), rSP41 (1 µg/ml), bacteria lysate (4 µg/ml), or no additive in culture medium for a total volume of 0.1 ml per well. Cell proliferation was determined in triplicate using a ALEXIS Biochemicals Cell Counting Kit-F (Lausen, Switzerland). After incubation for 72 hours at 37°C in 5% CO_2, the plates were washed three times with D-PBS(-) to remove esterase and phenol red, then 100 µl of D-PBS(-) was left in each well. Next 10 µl of CCK-F working solution was added to each well and plates were incubated at 37°C for 30 minutes [32]. Measurement of the fluorescence intensity of each well was performed at 535 nm (excitation at 485 nm) using a fluorescence plate reader (FluoroSkan Ascent FL, Thermo-Electron Corporation, Helsinki, Finland). The mean number of cells count and the standard error of the mean for each mouse group were also determined.

In vitro *assay for cytokine production by spleen cells*

Levels of IFN-γ and interleukin-5 (IL-5) in murine splenocyte culture supernatants were measured after

Table. Protection of BALB/c mice against challenge with *B. melitensis* 16M after immunization with pCISP41 or attenuated *B. melitensis* strain Rev. 1[a]

mice group (n=5)	Vaccine	$\log_{10}$ CFU of *B. melitensis* 16M in spleen (mean + SD)		$\log_{10}$ units ofprotection	
		4w	8w	4w	8w
1	PBS	5.67±0.42	5.26±0.13	-	-
2	pCI	5.71±0.29	5.36±0.11	-	-
3	pCIsp41	4.42±0.35	4.12±0.17	1.25[b]	1.14[c]
4	Rev.1	3.88±0.31	2.09±0.18	1.79[b]	3.17[c]

[a] Mice were challenged intraperitoneally with 10^5 CFU of strain *B. melitensis*16M after 4 weeks of the last immunization
[b]$p < 0.05$, [c]$p < 0.005$, (significant) compared to the control group

96 hours of incubation with antigen or mitogen as described for the lymphocyte proliferation assay. IFN-γ and IL-5 were assayed by specific ELISA kits (Euro-Clone, CITY, Italy), and samples were tested in duplicate. The concentration of IFN-γ or IL-5 in the culture supernatants was calculated via a linear-regression equation obtained from the absorbance values of the standards as indicated by the manufacturer. Values of less than 40 and 10 pg/ml were considered negative for IFN-γ and IL-5, respectively.

Statistical methods

Statistical analyses were performed with student's t-test. Log units of protection were obtained by subtracting the mean counts of the vaccinated group from the mean of the corresponding control group. A mean value for each spleen count was obtained by averaging the triplicate values after log conversion.

Results

Construction and testing of the plasmid construct pCISP41

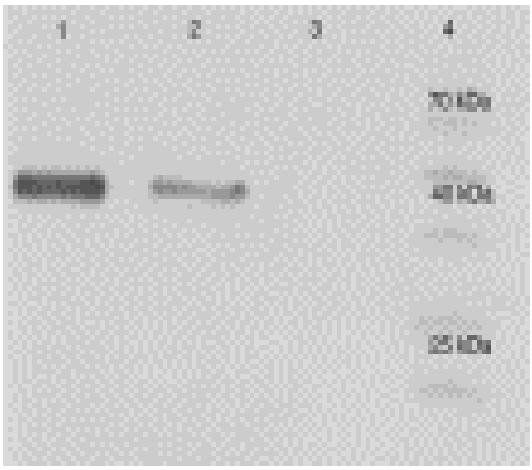
To study the roles of SP41 as a potential DNA vaccine in immune response and protective immunity induced against brucellosis, we prepared a plasmid construct able to express SP41 in mammalian cells, pCISP41. The recombinant plasmid was verified by restriction digestions and sequencing. To verify the ability of the constructed DNA vaccine to be correctly expressed in mammalian cells, we transformed Vero cells with pCISP41 and the expression of exogenous SP41 was shown by Western blot assay using a homemade SP41-specific polyclonal antibody (Figure 1). As expected, a 41-kDa protein, similar to the detected rSP41, was found in the lysate of pCI-SP41 transformed Vero cells and not in the control cells transformed with empty plasmid pCI (Figure 1).

Immune response of BALB/c mice vaccinated with pCISP41

To evaluate the induction of humoral immunity using pCISP41 as DNA vaccination in mice, the titers of anti-SP41 antibodies in mouse sera were measured by ELISA, using total *B. melitensis* lysate or rSP41 as immobilized antigens. As expected, IgGresponse in mice immunized with pCISP41 against both types of antigens were seen four weeks after vaccination (reciprocal dilutions of sera were 1500 and 2000, respectively). On the contrary, specific anti-SP41 antibodies were not detected in sera of mice inoculated with pCI or PBS (Figure 2). Significant differences were observed in the amount of IgG produced by DNA vaccine and by the pCI vector control ($P < 0.01$).

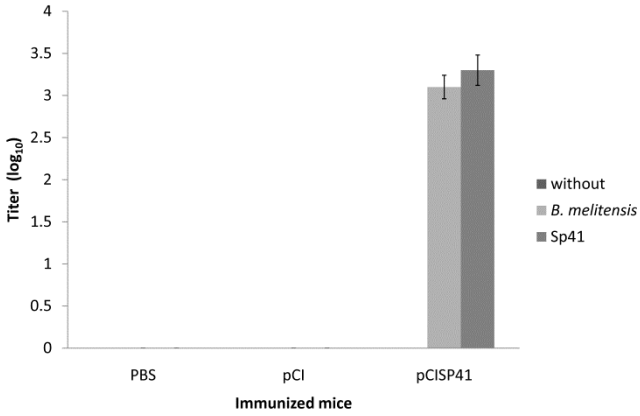
To examine the CMI response to rSP41 protein and crude *B. melitensis* proteins, the proliferation response and cytokine profile of spleen cells from mice immunized with pCISP41, pCI, and PBS were determined. As shown in Figure 3A, significant T-cell proliferation response to rSP41 protein and lysed bacteria was seen four weeks after immunization ($P < 0.05$) compared with the PBS group in the lymphocytes of mice immunized with pCISP41, whereas, Figure 3B shows a significant splenocytes proliferation response to specific antigens eight weeks after mice immunization with pCISP41 *in vitro*. With respect to cytokine profile, supernatants of spleen cell cultures from pCISP41-immunized animals contained high levels of IFN-γ ($P < 0.05$) compared to the negative control groups; however, IL-5 has not been produced in all culture supernatants of splenocytes stimulated with specific antigens (data not shown). Splenocytes stimulated with concanavalin A have produced the same high levels of IFN-γ in all mice groups, including the control group (Figure 4).

Figure 1. Expression of recombinant plasmid pCISP41 in Vero cells



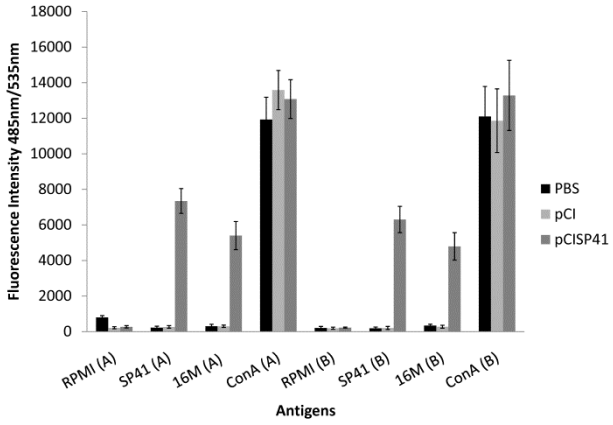
The lysates of Vero cells transformed with the recombinant plasmids were analyzed for the respective target protein expression by Western blot assay. Vero cells were transformed with pCISP41 (lane 2) or pCI (lane 3) as negative control. Loaded sample containing rSP41 (0.5 µg) was used as positive control (lane 1). Lane (4), molecular size protein markers. A molecular mass of the target protein is indicated beside the figure.

Figure 2. Antibody profile of mice immunized with various DNA vaccines



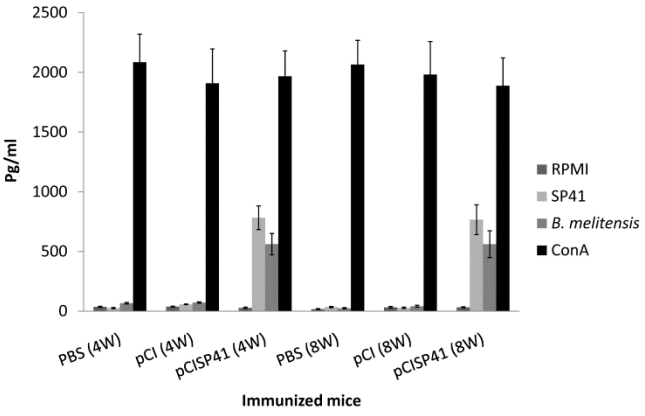
Mice (five per group) were injected either by pCISP41 (as a vaccine), or by pCI or PBS as negative controls. Four weeks after the last immunization, sera were collected from the experimental mice, and antibody titers were evaluated by ELISA in the absence (without) or the presence of immobilized rSP41 or *Brucella* lysate as antigens. The IgG amount was higher in pCISP41 group compared with pCI-immunized group, $P < 0.01$. Data represent the mean ± standard deviation (error bars) from the five mice.

Figure 3. Lymphocytes proliferation assay



BALB/c mice were immunized with PBS, parental plasmid pCI or pCISP41. At week 4 (to the left - A) or 8 (to the right - B) after immunization, splenocytes at 2 x 10^5 per well from each mouse were prepared and stimulated *in vitro* with rSP41 (1 µg/ml), bacterial lysate of *B. melitensis* 16M (4 µg/ml), ConA (3 µg/ml) as positive control or without antigen as negative control (RPMI). Cell proliferation was determined using Cell Counting kit-F. Data represent the mean ± standard deviation (error bars) from the five mice.

Figure 4. *In vitro* splenocytes secretion of IFN-γ upon stimulation with different antigens



BALB/c mice were inoculated i.m. with pCISP41, pCI or with PBS and killed 4 weeks after the last injection. 2 x 10^5 CFU of splenocytes were isolated and cultured in 96-well plates in triplicate without antigen (RPMI) as negative control, or with stimulation with rSP41 (1 µg/ml), or bacterial lysate of *B. melitensis* 16M (4 µg/ml), or ConA (3 µg/ml) as positive control of the assay. After 96 h, supernatants were collected and tested for IFN-γ production by sandwich ELISA. *Statistically significant differences compared to RPMI1640 ($P < 0.05$). Data represent the mean ± standard deviation (error bars) from the five mice.

Protective efficacy

Immunization with pCISP41 resulted in a significantly higher degree of protective efficacy, compared with that done by pCI or PBS, four and eight weeks post-injection ($P < 0.05$ and $P < 0.005$, respectively). However, vaccination with Rev.1 induced a higher level of protection, four and eight weeks post-infection, compared to mice injected with pCISP41 [1.79 and 3.17 log, respectively ($P < 0.05$)]. No significant differences in CFU counts were seen among mice injected with pCI or PBS (Table).

Discussion

Conventional attenuated or inactivated vaccines have the disadvantage that they can produce adverse side-effects. The improvement to the methods of cloning and purifying proteins has led to the use of purified recombinant proteins as cellular vaccines in experimental trials. These preparations as well as the synthetic peptides are more convenient to use than attenuated vaccines but are not able to confer a high degree of protection or induce a strong CMI response [23]. In view of this situation, recent vaccine research has concentrated on the development of alternative vaccines, such as DNA vaccines. These involve the inoculation with an expression vector that encodes an antigenic protein; the encoded antigen is then produced *in situ* and elicits an immune response [33]. Vaccination with plasmid DNA has several advantages compared to the traditional formulations that are applicable to the development of vaccines for biodefense [34]. DNA vaccines, also known as dgeneticT, dnucleicacidT or dpolynucleotideT vaccines, deliver genes encoding protein antigens into host cells, enabling antigen production to occur *in vivo* [35]. Consequently, both strong cellular and humoral immune responses may be induced. In addition, the ability to genetically manipulate DNA offers the advantage of vaccines designed to produce co-stimulatory molecules, or the ability to target protein production to specific cell compartments to modulate the specificity of the immune response [35]. However, the most serious disadvantages of DNA vaccination are the possibility to induce either: (i) the transformation due to integration of DNA into the host cell genome, or (ii) the development of autoimmunity due to induction of T cells to cryptic epitopes [33].

The i.m. injection is the most common route of DNA administration. It seems that dendritic cells, professional antigen-presenting cells, play a major role in the initiation of an immune response in DNA vaccination by efficiently priming T cells with exogenous or endogenous antigens [14].

In DNA vaccination against brucellosis, injection of vector encoding either P39 [17] or SOD [36] by this route elicits protection against pathogen infection. In this study, we showed that i.m. injection of a DNA vector containing the DNA insert of *B. melitensis* SP41 was able to generate a moderate protective immune response. It is well documented that CMI plays a major role in the establishment of a protective response against *Brucella* spp [3], and for that reason the design of a preventive vaccine against brucellosis must be based on its capacity to generate a strong Th1-type immune response, with high levels of IFN-γ and T-cytotoxic activity involved in the immune process [37]. Our results show that the mice immunized with the DNA-SP41 vaccine exhibited remarkable titers of total IgG; and the mean antibody endpoint titers against rSP41 and lysed *B. melitensis* were respectively 9- and 7- folds higher than those induced by the pCI vaccine (Figure 2). These titers were similar to those previously found in several described *Brucella*spp DNA vaccines [17,29]. T-cell immune response induction after DNA immunization was evaluated by measuring lymphocyte proliferation and cytokine production after *in vitro* stimulation of splenic cells with purified rSP41 or crude *B. melitensis*extract. Both conditions induced a higher T-cell level against *B. melitensis* proliferative response (Figure 3) and high levels of secreted IFN-γ (Figure 4). By contrast, no detectable levels of IL-5 were present in the supernatants (data not shown). Our present results suggest that immunization with a SP41-based DNA vaccine was able to induce a strong Th1-type response that could be detected in T-cell cultures from vaccinated BALB/c mice. However, theoretically, we suppose that our vaccine was not able to elicit a strong CMI response, which is necessary to provide a sufficient protection against *B. melitensis*16M infection, compared to a live *Brucella* vaccine (Rev.1). This finding concurs with those of other studies, which reported that a DNA vaccine encoding L7-L12 [38], SOD [36] or P39 [17] induced an appropriate immune response, and conferred a moderate protection in BALB/c mice challenged with *Brucella* spp compared to that observed in positive control mice vaccinated with live vaccine S19. In contrast, Cassataro *et al*. found that the vaccination of BALB/c mice with DNA vaccine coding for the chimera BLSOmp31 (pCIBLSOmp31) provided similar protection as Rev.1 against *B. melitensis* [28].

In spite of the relative failure of the pCISP41 as a competitor vaccine, we remain confident of the potentials of SP41, because of its important role in *Brucella*spp adhesion and invasion.

Castañeda-Roldán*et al*. suggested that SP41 protein is produced *in vivo* and that it elicits an antibody immune response [30]. The antibodies directed against the SP41 inhibited bacterial adherence and invasion of HeLa or epithelial cells [30,31]. Luo *et al*. found that divalent DNA vaccine encoding both the L7/L12 and Omp16 genes elicit protective immunity against *B. abortus* in BALB/c mice [39]. Moreover, Yu *et al*. showed that combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *B. abortus* 2308 [40]. Such interesting propositions are compelling to test our vaccine candidate SP41 in different vaccine formulations (*e.g.*, adding CpG ODN as adjuvant or using a live delivery vector) or in combination with several already-used DNA-encoded vaccines (*e.g.*, P39, L7/L12 or/and SOD). These studies are currently under investigation in our laboratory.

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References

1. Meng XJ, Lindsay DS, Sriranganathan N (2009) Wild boars as sources for infectious diseases in livestock and humans. Philos Trans R Soc Lond B Biol Sci 27: 2697-2707.
2. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, Schwarzenberger P, Shellito JE, Kolls JK (2001) Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. Am J Respir Cell Mol Biol 25: 335-340.
3. Glynn MK and Lynn TV (2008) Brucellosis. J Am Vet Med Assoc 15: 900-908.
4. Blasco JM (2010) Control and eradication strategies for *Brucella melitensis* infection in sheep and goats. Prilozi 31: 145-165.
5. Blasco JM (1997) A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. Prev Vet Med 31: 275-83.
6. Neubauer H (2010) Brucellosis: new demands in a changing world. Prilozi 31: 209-217.
7. Baldwin CL, Goenka R (2006) Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection? Crit Rev Immunol 26: 407-442.
8. Pasquevich KA, Ibañez AE, Coria LM, García Samartino C, Estein SM,Zwerdling A, Barrionuevo P, Oliveira FS, Seither C, Warzecha H, Oliveira SC, Giambartolomei GH, Cassataro J. (2011) An oral vaccine based on U-Omp19 induces protection against *B. abortus* mucosal challenge by inducing an adaptive IL-17 immune response in mice. PLoS One 146: e16203.
9. de Bagüés Jiménez MP, Ouahrani-Bettache S, Quintana JF, Mitjana O, Hanna N, Bessoles S, Sanchez F, Scholz HC, Lafont V, Köhler S, Occhialini A (2010) The new species *Brucella*microti replicates in macrophages and causes death in murine models of infection. J Infect Dis 202: 3-10.
10. Zhan Y and Cheers C (1993) Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. Infect Immun 61: 4899-4901.
11. Stevenson FK (2004) DNA vaccines and adjuvants. Immunological Reviews 199: 5-8.
12. Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE (2006) Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 24: 345-54.
13. Liu F, Shollenberger LM, Conwell CC, Yuan X, Huang L (2007) Mechanism of naked DNA clearance after intravenous injection. J Gene Med 9: 613-619.
14. Ingolotti M, Kawalekar O, Shedlock DJ, Muthumani K, Weiner DB (2010) DNA vaccines for targeting bacterial infections. Expert Rev Vaccines 9: 747-763.
15. Peng S, Monie A, Kang TH, Hung CF, Roden R, Wu TC (2010) Efficient delivery of DNA vaccines using human papillomavirus pseudovirions. Gene Ther 17: 1453-1464.
16. Commander NJ, Brewer JM, Wren BW, Spencer SA, Macmillan AP, Stack JA (2010) Liposomal delivery of p-ialB and p-omp25 DNA vaccines improves immunogenicity but fails to provide full protection against *B. melitensis* challenge. Genet Vaccines Ther 16: 5.
17. Al-Mariri A, Tibor A, Mertens P, DeBolle X, Michel P, Godfroid J,Walravens K, Letesson JJ (2001) Induction of immune response in BALB/c mice with a DNA vaccine encoding bacterioferritin or P39 of *Brucella* spp. Infect Immun 69: 6264-6270.
18. Roop RM, Fletcher TW, Sriranganathan NM, Boyle SM, Schurig GG (1994) Identification of an immunoreactive *Brucella abortus* HtrA stress response protein homolog. Infect Immun 62: 1000-1007.
19. Baloglu S, Toth TE, Schurig GG, Sriranganathan N, Boyle SM (2000) Humoral immune response of BALB/c mice to a vaccinia virus recombinant expressing *Brucella abortus* GroEL does not correlate with protection against a *B. abortus* challenge. Vet Microbiol 76: 193-199.
20. Lin J, Adams LG, Ficht TA (1996) Immunological response to the *Brucella abortus* GroEL homolog. Infect Immun 64: 4396-4400.
21. Leclerq S, Harms JS, Rosinha GM, Azevedo V, Oliveira SC (2002) Induction of a Th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus* GroEL heat-shock gene. J Med Microbiol 51: 20-26.
22. Singha H, Mallick AI, Jana C, Isore DP, Goswami TK, Srivastava SK, Azevedo VA, Chaudhuri P, Owais M (2008) Escheriosomes entrapped DNA vaccine co-expressing Cu-Zn superoxide dismutase and IL-18 confers protection against *Brucella abortus*. Microbes Infect 10: 1089-1096.
23. Tabatabai LB and Pugh GW (1994) Modulation of immune responses in BALB/c mice vaccinated with *Brucella abortus* Cu-Zn superoxide dismutase synthetic peptide vaccine. Vaccine 12: 919-924.

24. Vemulapalli R, Cravero S, Calvert CL, Toth TE, Sriranganathan N, Boyle SM, Rossetti OL, Schurig GG (2000) Characterization of specific immune responses of mice inoculated with recombinant vaccinia virus expressing an 18-kilodalton outer membrane protein of *Brucella abortus*. Clin Diagn Lab Immunol 7: 114-118.
25. Oliveira SC and Splitter GA (1996) Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. Vaccine 14: 959-962.
26. Cassataro J, Velikovsky CA, de la Barrera S, Estein SM, Bruno L, Bowden R, Pasquevich KA, Fossati CA, Giambartolomei GH (2005) A DNA vaccine coding for the *Brucella* outer membrane protein 31 confers protection against *B. melitensis* and *B. ovis* infection by eliciting a specific cytotoxic response. Infect Immun 73: 6537-6546.
27. Zhao Z, Li M, Luo D, Xing L, Wu S, Duan Y, Yang P, Wang X (2009) Protection of mice from *Brucella* infection by immunization with attenuated *Salmonella enterica* serovar *typhimurium* expressing A L7/L12 and BLS fusion antigen of *Brucella*. Vaccine 27: 5214-5219.
28. Cassataro J, Pasquevich KA, Estein SM, Laplagne DA, Zwerdling A, de la Barrera S, Bowden R, Fossati CA, Giambartolomei GH, Goldbaum FA (2007) A DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against *B. ovis* and a similar degree of protection against *B. melitensis* than Rev.1 vaccination. Vaccine 25: 5958-5967.
29. Velikovsky CA, Cassataro J, Giambartolomei GH, Goldbaum FA, Estein S, Bodwen RA, Bruno L, Fossati CA, Spitz M (2002) A DNA vaccine encoding lumazine synthase from *Brucella abortus* induce protective immunity in BALB/c mice. Infect Immun 70: 2507-2511.
30. Castañeda-Roldán EI, Ouahrani-Bettache S, Saldaña Z, Avelino-Flores F, Rendón MA, Dornand J, Girón JA (2006) Characterization of SP41, a surface protein of *Brucella* associated with adherence and invasion of host epithelial cells. Cell Microbio 8: 1877-1887.
31. Castañeda-Roldán EI, Avelino-Flores F, Dall'Agnol M, Freer E, Cedillo L, Dornand J, Girón JA (2004) Adherence of *Brucella* to human epithelial cells and macrophages is mediated by sialic acid residues. Cell Microbiol 6: 435-445.
32. Al-Mariri A (2010) Protection of BALB/c mice against *Brucella melitensis* 16M infection induced by vaccination with live *Escherchia coli* expression *Brucella* P39 protein. Vaccine 28: 1766-1770.
33. Gurunathan S., Klinman DM, Seder RA (2000) DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 18: 927-974.
34. Kalinna BH (1997) DNA vaccines for parasitic infections. Immunol Cell Biol 75: 370-375.
35. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines (1997) Annu Rev Immunol 15: 617-648.
36. On˜ate AA, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C,Folch H, Andrews E (2003) A DNA vaccine encoding Cu-Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun 71: 4857-4861.
37. Cutler SJ, Whatmore AM, Commander NJ (2005) Brucellosis--new aspects of an old disease. J Appl Microbiol 98: 1270-1281.
38. Kurar E and Splitter GA (1997) Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. Vaccine 15: 1851-1857.
39. Luo D, Ni B, Li P, Shi W, Zhang S, Han Y, Mao L, He Y, Wu Y, Wang X (2006) Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infect Immun 74: 2734-2741.
40. Yu DH, Hu XD, Cai H (2007) A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *Brucella abortus* 2308 by inducing specific CTL responses. DNA Cell Biol 26: 435-443.

Corresponding author

Ayman Al-Mariri
Department of Molecular Biology and Biotechnology
Atomic Energy Commission
PO Box 6091
Damascus, Syria
Telephone/Fax: 963-11-213580/963-11-6112289
Email: ascientific1@aec.org.sy

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