Bivalent DNA vaccination with genes encoding *Leishmania major* cysteine proteinases type I and II protects mice against infectious challenge

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Abstract
Cysteine proteinases (CPs) of Leishmania are considered to be attractive vaccine candidate in which their immunogenicity and immuno-modulatory effects have been confirmed. We have previously reported that a cocktail of two DNA plasmids encoding Leishmania major cysteine proteinases type I (CPB) and type II (CPA) induces a partial protective response in murine model of cutaneous leishmaniasis. The results also showed that the induced protective response was better than the responses given by each one the plasmids alone. However, in view of the capability of DNA plasmid for encoding several antigens, we investigated the possibility of using a single bivalent DNA vaccine, based on CP genes as an alternative mean of inducing protective immunity. Here we present evidence favoring that CPA and CPB delivered in the same plasmid DNA backbone either in separate locus or as a tandem fused gene induce partial protection against Leishmania major infection in susceptible BALB/c mice. Immunization of mice with these constructs promoted specific T-cell response of Th1 phenotype that is characterized by an increase in production of IFN-γ. Our results confirm the previous observation about the possibility of DNA immunization against leishmaniasis using CP genes and lend support to the idea of using a single polyvalent plasmid DNA construct to elicit immune responses to several distinct antigens.
Keywords*: Bivalent DNA Vaccination, Cysteine Proteinases, Type I and II, Leishmania major.*

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INTRODUCTION

Leishmaniasis is an endemic disease in more than 80 countries which represents a significant worldwide health problem. Approximately, 350 million people are at risk of acquiring the various forms of the disease, and 2 million new cases each year are believed to be due to the lack of both an efficacious vaccine and safe methods to control the vectors that transmit the disease (http://www.who.org).

Leishmania major, one of the causative agents of cutaneous leishmaniasis in humans, has been extensively studied in mouse models to understand the requirements for an effective vaccine against healing and non-healing forms of leishmaniasis. It has been widely accepted that the development of protective immunity to *Leishmania* is dependent on the capacity to mount an IL-12-driven Th1 response, while the non-healing phenotype is associated with the expansion of the Th2 subset of lymphocytes (Sacks *et al*., 2002).

During the last decade, DNA vaccines have been developed against several viral, bacterial, and parasitic infections (Ulmer *et al*., 1993; Lowrie *et al*., 1994; Vercommen *et al*., 2000; Davis *et al*., 1994; Mor *et al*., 1995). It is believed that this form of antigen delivery can induce both CD_4^+ and CD_8^+ T-cell responses to the immunizing antigen. The CpG motifs present in plasmid DNA are immunomodulators and like an adjuvant switches on a Th1 response (Ertl *et al*., 2003). These characteristics suggest that DNA vaccinations would be particularly suitable for use in development of a vaccine against diseases that require cell-mediated

immunity, such as leishmaniasis.

Vaccination with DNA encoding *Leishmania* antigens has been examined in murine model, such as LACK (Gurunathan *et al*., 1997), gp63 (Xu *et al*., 1995), and PSA2 (Sjolander *et al*., 1998). DNA vaccines were also shown to induce partial protection against *L. major* infection (Mendez *et al*., 2001*;* Gurunathan *et al*., 2000).

An advantage for the use of DNA in immunizations is that a multivalent vaccine could be developed where a single plasmid would carry more than one gene encoding protective proteins. For example, Compose-Neto *et al*. (2003) showed that immunizations of mice with a plasmid DNA encoding TSA and LmSTI1 in tandem induced a solid protection in BALB/c mice challenged with *L. major.* In addition, a single multivalent DNA vaccine would be more cost-effective and easier to produce.

We have previously cloned and characterized two cysteine proteinases from *L. major* (CPA and CPB) and examined their potential for eliciting a protective response in the form of recombinant proteins and in a DNA vaccine in the mouse model (Rafati *et al*., 2001a; Rafati *et al*., 2002). We have reported that a cocktail vaccine containing CPA and CPB encoding plasmids induces long lasting protection against the *L. major* infection (Rafati *et al*., 2001a). These data also showed that a bacterially expressed recombinant CPA/B hybrid protein administered with poloxamer 407 as adjuvant, elicited a partial protective response against infection with *Leishmania* in BALB/c mice that was better than the response given by recombinant versions of CPA and CPB expressed as individual proteins (Zadeh-Vakili *et al*., 2004). To test the privilege of DNA vaccination and maintenance the conjunction of CPA and CPB without requiring the production of two distinct DNA constructs or the difficult process of protein purification and adjuvant administration, we designed CPs containing bivalent DNA vaccines. This investigation report on the efficacy of co-delivery of CPA and CPB genes in a single construct at eliciting a protective immune response in BALB/c mice. These data showed that immunization of mice with either co-expressing CPA and CPB DNA plasmid or the fusion construct encoding CPA/B hybrid protein, induced partial protection against *L. major* infection.

MATERIALS AND METHODS

Mice and parasites

Female BALB/c mice (6-8 weeks old) obtained from the breeding stock maintained at the Pasture Institute of Iran. The *L. major* strain, MRHO/IR/75/ER parasites were kept in a virulent state by continuous passage in BALB/c mice. Two month after infection, the spleen or lymph node was isolated, homogenized and cultured in NNN media in the presence of 100 μg/ml gentamycin. Promastigotes were collected by centrifugation (270 ×g, 10 min, 4°C), washed three times in PBS (8 mM Na_2HPO_4, 1.75 mM K_2HPO_4 , 0.25 mM KCl, 137 mM NaCl) and resuspended at a concentration of 2-4 × 10^7 cells/ml. Frozen/thawed (F/T) promastigote antigen was prepared by 10 cycles of alternative freeze/thawing of the parasites.

Plasmid construction and purification

The full-length coding sequences of CPA and CPB were PCR amplified (694 and 950 bp respectively) from pGEM-*cpa* and pGEM-*cpb* (Rafati *et al*., 2001a) using specific primer pairs. Primers were designed on the basis of published data (Rafati *et al*., 2001a; Sakanari *et al*., 1997) and contained Kozak sequence and restriction endonuclease sites used in the subsequent cloning into the dicistronic expression vector pTCAE (*cpa* primers: upstream; 5´GAC GGATCCACCATGCCCAGTGGTGTGATGTCG3´, downstream; 5´GG GGAATTCCTAGGCCGTTGTCGTCGGCAC3´, *cpb* primers: upstream; 5´CACCATGGATGCGGTGGACTGGCG CGAGAA3´, downstream; 5´GCGCGGATCCCTACACGTACTGGCAAATG3´). Dicistronic pTCAE vectors allow the co-expression of two distinct genes from two transcripts. They contain two cytomegalovirus (CMV) promoters followed by restriction cloning sites. The amplified CPA open reading frame was inserted into the *Bgl*II/*Eco*RI sites of the pTCAE vector (pTCAE-*cpa*). The CPB open reading frame was amplified with *pwo* polymerase to produce a blunt ended fragment. This fragment was digested with *Bam*HI to create a *cpb* insert, then cloned into the pTCAE-*cpa* that was cut with *Sal*I, treated with klenow, to produce blunt ends, and subsequently digested with *Bam*HI, to generate pTCAE-*cpa/b*. The fused gene containing *cpa* and *cpb* in tandem has been previously prepared and subcloned into the pUC18 (Zadeh-Vakili *et al*., 2004). This fragment was amplified by PCR (1644 bp) using primer pairs containing the Kozak sequence and restriction endonuclease sites for cloning in eukaryotic expression vector pCB6 (upstream primer: 5´GACGGATCCACCATGCCCCCAGTGGTGTGATGTCG3´, downstream primer: 5´GCGCTCTAGACTGCAGCTACACGTACTGGCAAATG3´). The amplified fragment was subcloned into the pCB6 vector (*Bgl*II/*Xba*I sites) downstream to a CMV promoter (pCB6-*cpa/b*). The integrity of cloning was determined by restriction analysis and one clone for each

gene was selected for large-scale DNA preparation. All plasmids were propagated in *Escherichia coli* DH5α. Plasmid DNAs were purified by using EndoFree Plasmid Mega kits according to the instruction given by the manufacturer (QIAGEN, Hilden, Germany). In the case of pCB6-*cpa/b* purification, since the quality of purified pCB6-*cpa/b* was not as expected, the host strain was changed to XL1-Blue as suggested by the manufactarer.

Immunoblot analysis
Expression of CPs from monocistronic and bicistronic vectors was tested by transiently transfection of Cos-7 cells. Cos-7 cells (NCBI-C143) were kindly provided by Dr. Shokrgozar (The National Cell Bank of Iran, Pasteur institute of Iran) and transfected using the transfecting reagent Dotap (Roche) according to the manufacturer's instruction. Briefly, the cells were seeded at 8×10^5 in 60 mm culture plates grown overnight until they reached 60-70% confluence, then transfected with 5 µg of either pTCAE-*cpa/b*, pCB6-*cpa/b* or vector alone (pCB6 or pTCAE) as control. After 48 h, cells were harvested, washed three times with PBS, and immediately lysed by addition of sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Lysates derived from equivalent number of cells were resolved by SDS-PAGE and transferred to nitrocellulose, and the blots were probed with rabbit antiserum against either hybrid protein (CPA/B) (Zadeh-Vakili *et al.*, 2004) or CPA or CPB (Rafati *et al.*, 2001b).

Immunization and challenge
Five groups of mice (n=10) were immunized intramuscularly (i.m.) twice, 3 weeks apart, with 100 µg of each plasmid DNA containing the gene of interest or with vector alone (without the antigen encoding insert), all were prepared in PBS. Group I was injected with pCB6-*cpa/b*, group II with pTCAE-*cpa/b,* group III with pCB6, group IV with pTCAE and group V with PBS. Mice were infected 3 weeks after completion of the immunization protocol. They were then challenged with 2×10^5 *L. major* MRHO/IR/75/ER metacyclic promastigotes suspended in 50 µl PBS and injected subcutaneously into the left footpad. The progress of infection was followed by measuring footpad size with a metric caliper. Footpad size was defined as the mean of thickness and width of footpad in mm. Animals were sacrificed when the lesions became necrotic.

Cytokine assay
Seven weeks after infection, 2 mice from each group were sacrificed and the spleens removed. Single cell suspensions of splenocytes were obtained by gentle squeezing of whole spleens in erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM EDTA pH 7.2). Residual debris was removed and the recovered cell suspension was washed twice in RPMI 1640. Then cells were resuspended and plated in RPMI-1640 medium supplemented with 5% FCS (GIBCO), 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES and 40 µg/ml gentamycin. Cells were incubated in 96-well plates at 2×10^5 cells per well. Cells were stimulated *in vitro* at 37ºC in 5% CO_2 with rCPA/B at 10 µg/ml, F/T antigen (10 µg/ml) or medium alone. T-cell mitogen Concanavalin A (Con A), at a concentration of 2 µg/ml, was used in all experiments as a positive control. A dose- response curve was carried out using up to 20 µg/ml rCPA/B; the optimum response was obtained with 10 µg/ml. After 120 h, supernatants were removed and tested for the IFN-γ and IL-5 elicitation by commercially available sandwich based ELISA kits (R&D, Minneapolis, MN USA) as described in the kit manual. The lower detection limits of IFN-γ and IL-5 were 2 and 7 pg/ml respectively. All tests were performed in triplicate and the median was recorded for each set of sample.

Measurement of CP-specific antibody responses
Mice were bled before immunization and also immediately before and 7 weeks after infection by orbital plexus puncture and the pooled sera from each group were stored at -20ºC until use. The antigen-specific enzyme-linked immunosorbent assays (ELISA) were performed for the identification of specific anti-rCPA/B, anti-CPA and anti-CPB total IgG and its subclasses (IgG1 and IgG2a). Briefly, 96 well microtiter plates (Nunc) were coated (100 µl/well) with each recombinant antigen at 10 µg/ml in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were washed and blocked with 100 µl of 1% BSA in PBS-Tween per well for 2 h at 37°C. Serum samples were diluted to 1:50 with PBS-Tween-1% BSA. Plates were incubated at 37°C for 2 h and washed. Biotinylated rabbit anti-mouse IgG1 or IgG2a (Pharmingen, San Diego, CA) was added at a 1:200 dilution, and then incubated for 1 h at 37°C. After washing, streptavidine-horseradish peroxidase (Gibco BRL, Gaithersburg, MD) was added at 1:1000 dilution and incubated at 37°C for 1 h.

After washing, detection was done with the substrate O-phenylenediamine (OPD). Optical density at 492 nm was determined on a titertek multiscan plate reader.

Statistical analysis

The differences in the level of protection, cytokine and antibody production were determined by one-way ANOVA and student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression of CPA and CPB in mammalian cells

In order to determine if co-delivery of *cpa* and *cpb* coding sequence on the same plasmid backbone enhance the protective immune response, two DNA vaccines were constructed. Including *cpa* and *cpb* genes driven by separate CMV promoters (pTCAE-*cpa/b*) and the other included *cpa/b* fused gene driven by a single CMV promoter (pCB6-*cpa/b*) as shown in figure 1. Cos-7 cells were used in order to confirm the correct expression of the recombinant CPA/B, CPA and CPB proteins by transfection with either pTCAE-*cpa/b* or pCB6-*cpa/b.* Cell extracts were analyzed in western blot technique using specific antibodies. Transfected cells produced high levels of recombinant proteins as shown in figure 2. The pCB6-*cpa/b* produced 60 kDa hybrid protein. The lysate prepared from transfected Cos-7 cell with empty vector and bacterially expressed recombinant CPA/B were used as negative and positive controls respectively. In the case of transfected Cos-7 cells with pTCAE-*cpa/b*, a 27 and 40 kDa proteins which correspond to CPA and CPB respectively were expressed. Similarly, the transfected Cos-7 cells with empty pTCAE vector and rCPA and rCPB were used as negative and positive controls. Additional bands presented in the bacterially expressed rCPB are brobably the breakdown product of rCPB.

Immunization of BALB/c mice with CPA and CPB co-expressing plasmids

Vaccinated animals were challenged 3 weeks after the last boost with $2x10^5$ promastigote forms of *L. major.* The course of infection was followed for 9 weeks and the results are shown in figure 3. Significant delays in lesion development ($P<0.05$), in comparison to control groups, indicating that mice immunized with the plasmid DNAs containing the CPs genes were partially protected against infection with *L. major* (Fig. 3a). Average lesion size (expressed as the increase in infected footpad size versus uninfected one), was reduced in immunized groups with CPs expressing vectors. The reduction at 7 weeks post-infection was almost 71% for group I (pCB6-*cpa/b*), and 81% for group II (pTCAE-*cpa/b*) in comparison to the unimmunized PBS control group (Fig. 3b). No protective effect was seen when mice were immunized with the control vector pCB6. Although the development of lesions in pCB6 and pTCAE recipients was not significantly different, but for pTCAE vector recipients

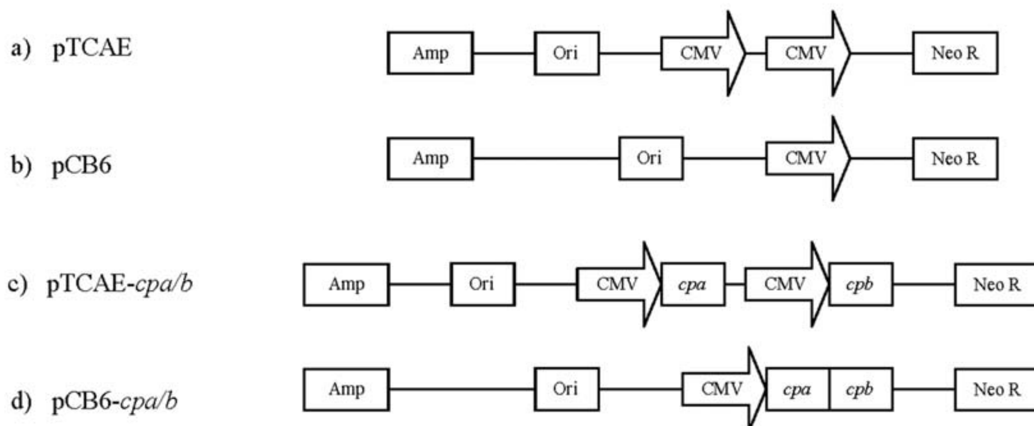


Figure 1: Schematic representation of the plasmids used in the generation of a DNA vaccine. The vector backbones (a and b) used in the construction of the DNA vaccines (c and d) are shown. (c) pTCAE-*cpa/b* plasmid includes the *cpa* and *cpb* genes each inserted downstream of a CMV promoter. (d) pCB6-*cpa/b* plasmid includes the *cpa/b* gene fused, downstream of the CMV promoter. These schematic representation plasmids do not reflect the relative size of each of the plasmids.

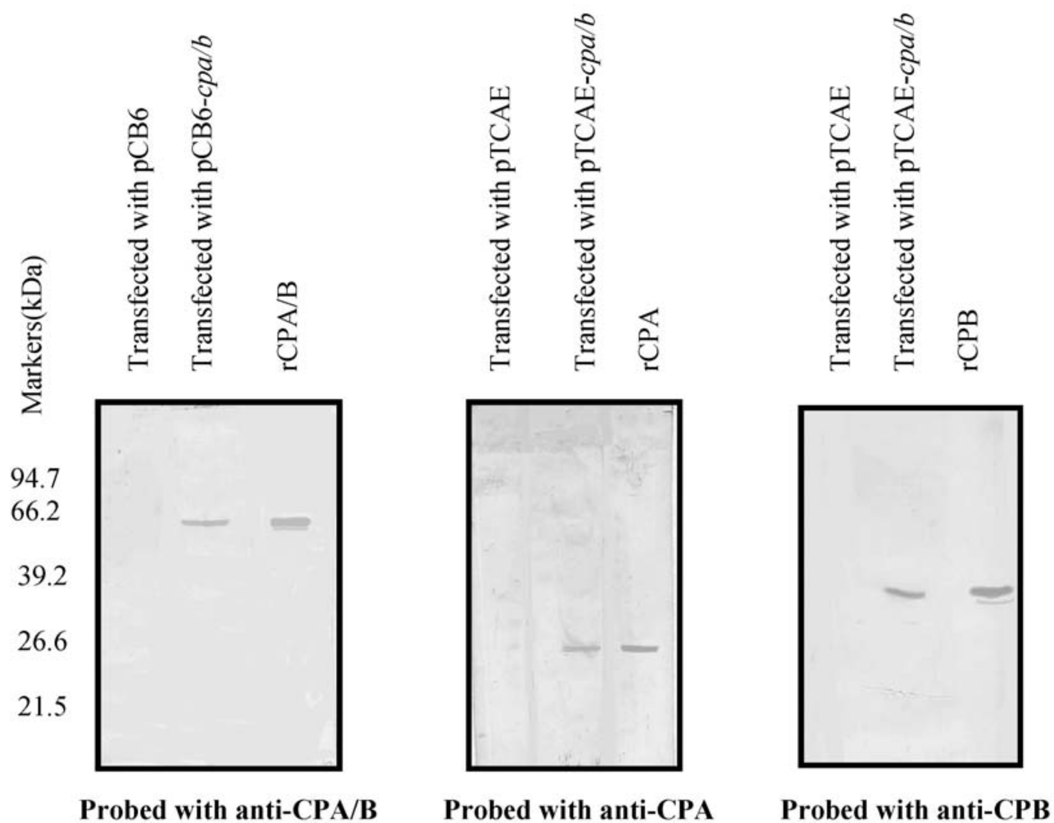
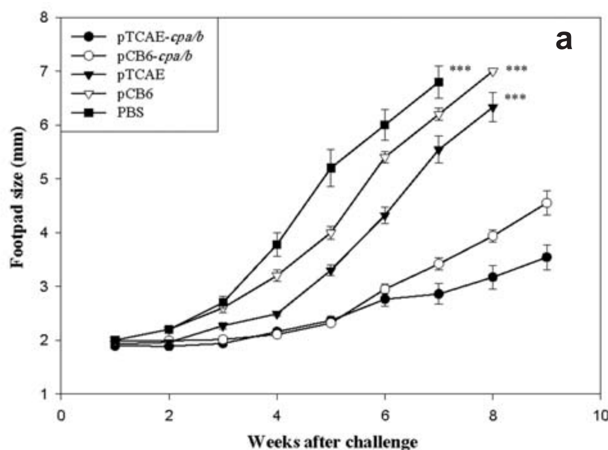


Figure 2: Expression of cysteine proteinases in Cos-7 cells. Cos-7 cells growing in 60 mm dishes were transfected with DNA constructs (5 µg/dish) encoding the *L. major* antigen CPA/B, CPA or CPB under the control of a constitutive CMV promoter. Forty-eight hours post-transfection, the cells were harvested, lysed, and analyzed by immunoblotting using anti-CPA/B, anti-CPA or anti-CPB polyclonal antiserum to determine the protein expression. Lysates prepared from transfected Cos-7 cells with empty vector and bacterially expressed recombinant proteins were used as negative and positive controls.



Immunization Group	Lesion size 7 w.p.i.	% of reduction
1. pCB6-*cpa/b*	1.44± 0.1	71
2. pTCAE-*cpa/b*	0.94± 0.15	81
3. pCB6	4.28 ± 0.14	12
4. pTCAE	3.64 ± 0.2	25
5. PBS	4.89 ± 0.25	0

b

Figure 3: The DNA vaccines confer partial protection to mice upon challenge with *L. major* promastigotes. Mice (10 per group) were immunized two times i.m. (three week intervals) with 100 µg of pCB6-*cpa/b*, pTCAE-*cpa/b*, control DNAs (pCB6 or pTCAE) or PBS. Three weeks after the last immunization, the animals were infected in the left hind footpad with 2x10^5 *L. major* promastigotes and footpad size was measured weekly thereafter. (a) Increase in footpad size at weekly intervals of each immunized group infected with *L. major* is presented. (b) Lesion size for each immunization group with S.E. at week 7 post-infection. Lesion size was expressed as the increase in footpad size (in mm) of infected versus uninfected hind foot. The percent reduction in lesion size is compared to the unimmunized group. ***Destruction of footpad and mice were sacrificed; S.E. Standard Error; 7 w.p.i. seven weeks post infection.

there was a slight but significant difference compared to PBS group. Reduction of lesion size for this group (pTCAE group) at 7 weeks after challenge was 25%. This observation may be attributed to the differences in the bacterial backbone of these two plasmids.

Effect of multivalent DNA vaccine on IFN-γ production

In order to characterize the cellular immune response throughout the infection, splenic mononuclear cells obtained 7 weeks after challenge were stimulated *in vitro* with the recombinant CPA/B and F/T antigen. After 5 days incubation, supernatants were harvested and assayed for both IFN-γ and IL-5. The results are shown in figure 4 and indicate that DNA vaccination with CPs containing constructs stimulated the production of considerable quantities of IFN-γ by mononuclear spleen cells. High levels of cytokines in response to mitogen ConA (>600 pg/ml for IFN-γ and >80 pg/ml for IL-5 in all groups), confirmed the accuracy of the tests. In response to stimulation with CPA/B, splenocytes from mice that received CP encoding plasmids produced IFN-γ six to ten times more than IL-5 (Fig. 4a). The ratio of IFN-γ /IL–5 is approximately 1 for mice that were injected with pCB6 or PBS. In control mice that were injected with pTCAE, this ratio was about 2.5, which may responsible for delayed footpad swelling observed in this group as it is shown in figure 3a. Similar ratios were also observed in response to F/T antigen (Fig. 4b). The obtained results are in corroborated with the induced protection in immunized mice and suggest that the T-cell response elicited by the DNA immunization with these two genes is preferentially of the Th1 type.

Effect of DNA vaccination on humoral immune response

To monitor the antigen-specific humoral response primed by immunization with CPs containing plasmids, specific anti-CPA/B IgG was measured in the sera of un-immunized (before immunization, BI) and immunized animals (three weeks after the last immunization, AI). As it is shown in figure 5a, there is no significant differences in the level of total IgG and its subclasses in all groups before immunization (BI). However, three weeks after immunization (AI), the mice immunized with either pCB6-*cpa/b* or pTCAE-*cpa/b* developed significant level of total IgG and IgG2a against rCPA/B in compare to control groups (p<0.05). IgG1 specific antibodies were similar in all vaccinated and control groups. No anti CPA/B antibody response was detected in the sera of mice immunized with empty vectors and/or PBS groups. The level of total IgG was also measured 7 weeks after challenge and showed five-fold increase compared to before challenge in CPs recipient groups (Fig. 5b). However, the overall levels of IgG2a and IgG1 in pCB6-*cpa/b* and pTCAE-cpa/b were not high, but the ratios (IgG2a/IgG1) indicated the excess of IgG2a. These ratios for pCB6-*cpa/b* and pTCAE-*cpa/b* recipient groups are 2.2 and 2.3, respectively whereas for control groups is ≤0.5.

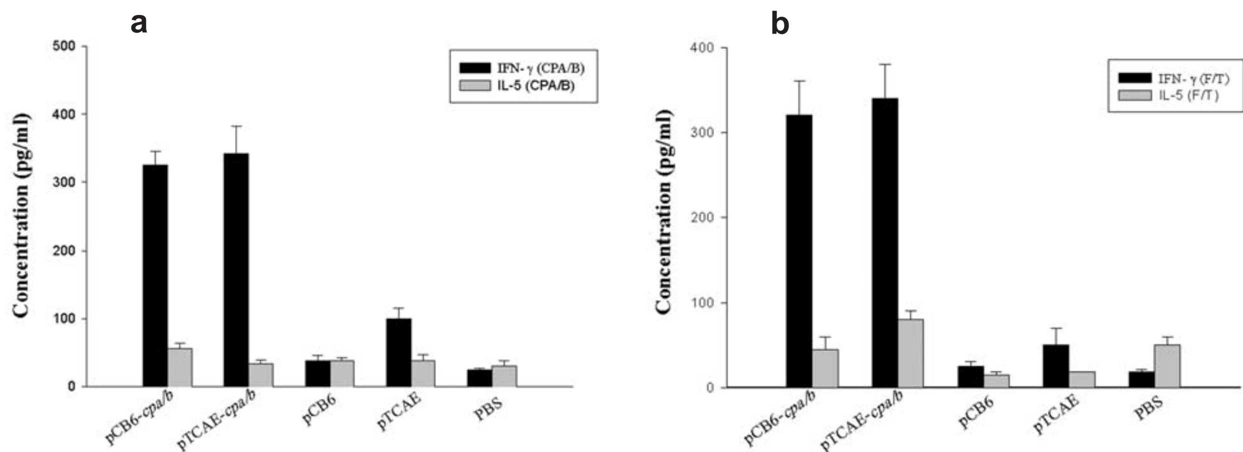


Figure 4: Cytokine production by spleen cells of BALB/c mice immunized with the cysteine proteinase DNA vaccine. Spleen cells from two mice were harvested 7 weeks after the challenge with *L. major* promastigotes and stimulated *in vitro* with (a) rCPA/B and (b) F/T antigen at 10 µg/m after 5 days, supernatants were collected and IFN-γ and IL-5 content assayed by ELISA. Each bar represents mean values and standard deviation in pg/ml for duplicates.

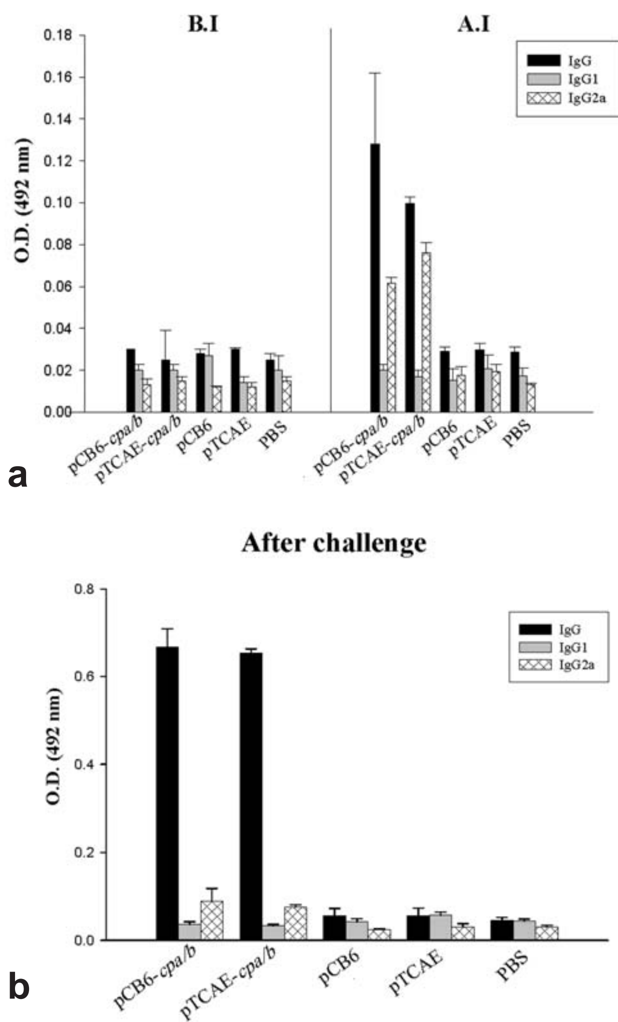


Figure 5: CP specific antibody response in DNA vaccinated groups. (a) Anti-CPA/B antibody levels were measured by ELISA and the ODs taken in the controls (Group III, VI and V) and in the CPs encoding DNA vaccinated group (Group I and II), before immunization and three weeks after the last immunization. Sera were diluted 1:50 and evaluated for the presence of total IgG, IgG1 and IgG2a. (b) Pooled sera were prepared 7 weeks after challenge with *L. major* and tested for specific anti-CPA/B antibody response of both total IgG and its subclasses (IgG1 and IgG2a).

DISCUSSION

After the first publication about the effectiveness of DNA vaccination (Wolff *et al*., 1990), this approach for immunization has generated sustained interest because of several reasons such as its speed, simplicity, ability to elicit both humoral and cellular immune responses against native protein antigens with complex structures, without the need for complex biochemical techniques. Recently, another advantage of DNA vaccination, which is the development of a single plasmid carrying more than one gene encoding protective proteins, became attractive. These multivalent vaccines recommended because specific immunity will be promoted against increases number of epitopes. This is a desirable condition in long run since all these trials are performed to find an effective vaccine for human use; therefore a vaccine containing a broad range of different protective epitopes is more likely to cover a greater number of major histocompatibility complex phenotypes in a heterogenous population such as humans and dogs. Immunization with multivalent vaccines have been experienced using recombinant polyprotein and multivalent DNA molecules against different infectious diseases such as hepatitis B (Wild *et al*., 1998), malaria (Wunderlich *et al*., 2000) and AIDS (Hel *et al*., 2002). This approach has been also experienced against leishmaniasis (Coler *et al*., 2002; Compos-Neto *et al*., 2002).

In this study, due to our previous successful immunization using the combination of *cpa* and *cpb* genes (in different vector) against *L. major* infection in BALB/c mice (Rafati *et al*., 2001a), The efficiency of two constructs co-delivering *cpa* and *cpb* genes as DNA immunization were evaluated. pCB6-*cpa/b* that encodes CPA/B hybrid protein, and pTCAE-*cpa/b* that posses CPA and CPB under the control of two promoters and expressed them as individual proteins. The results showed that there are no significant differences between the levels of induced protection in vaccination with pTCAE-*cpa/b* bicistronic plasmid (encoding CPA and CPB separately) and pCB6-*cpa/b* monocistronic plasmid (encoding CPA/B hybrid protein). The development of lesions following the challenge with *L. major* revealed a significant delay ($p<0.05$) in CPs encoding plasmid recipient groups compared to controls. The observed reduction in lesion sizes during 7 weeks post-challenge for pCB6-*cpa/b* and pTCAE-*cpa/b* immunized groups were 71% and 81% respectively. The *in vitro* experiments indicated that induced protective response against these constructs is a typical Th1 response. This conclusion is based on the fact that spleen cells from protected mice when stimulated *in vitro* with the CP antigens, produced high levels of IFN-γ. We showed that the splenocytes from protected groups (immunized with pCB6-*cpa/b* or pTCAE-*cpa/b)* produced 6-10 times more IFN-γ than the PBS control group in response to rCPs. These bivalent constructs stimulated the production of relatively low levels of specific IgG antibody compared to previously experienced recombinant CPs (Rafati *et al*., 2002). Similar observation based on the lower antibody production in response to DNA vaccination compared to

protein vaccination has been previously reported for some other antigens (Kang *et al*., 1998). Although, the level of both produced IgG2a and IgG1 is quite low in the CP containing DNA recipient groups, but the IgG2a is two fold higher than IgG1. Interestingly, the pTCAE recipients produced slightly higher amount of IFN-γ in response to *in vitro* stimulation as compared to control group. The pTCAE immunized group also showed some degree of protection against infectious challenge with *L. major* with a reduction of 25% in lesion size at 7 weeks post-challenge, which is in accordance with slight production of IFN-γ. These may be due to the immunostimulatory sequences present in the bacterial plasmid backbone. It has been shown previously, that these specific sequence motifs elicit innate immune responses characterized by the production of IL-6, IL-12, (TNF)-α and IFN-γ which might promote resistance to leishmaniasis in a non-specific manner (Gurunathan *et al*., 2000). However, the obvious stronger promoted protective response in pTCAE-*cpa*/*b* and pCB6-*cpa/b* injected mice compared to all control groups indicates the mediation of specific immune response to CP antigens.

Although the obtained results in the present study are similar to our previous experiences with rCPA/B and DNA cocktail vaccine, we still recommend these bivalent DNA vaccines because of their advantages. The advantages of this new format of antigen delivery are; (i) as DNA vaccines, they do not need adjuvant, (ii) Production of multivalent DNA vaccines is cost-effective, and (iii) multivalent vaccines containing a broader range of protective epitopes that would cover a wide range of MHC types in a heterogeneous population.

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