African Journal of Biotechnology Vol. 11(47), pp. 10632-10639, 12 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB12.350
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Partial immunity in murine by immunization with a toxoplasmic DNA vaccine

Z. Eslamirad[1]*, A. Dalimi[2], F. Ghaffarifar[2], Z. Sharifi[3] and S. Ghasemi Nikoo[2]

[1]Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran.
[2]Department of Parasitology, Faculty of Medical sciences, University of Tarbiat Modarres, Tehran, Iran.
[3]Research Center of Iranian Blood Transfusion Organization, Tehran, Iran.

Accepted 8 May, 2012

***Toxoplasma gondii* is an obligate intracellular protozoan that is causative agent of atoxoplasmosis, a disease which may result in a spectrum of consequences. Previous studies have reported that DNA vaccine can be effective in partial protection against this parasite. In this study, we constructed a single DNA vaccine containing rhoptry protein 1 (ROP1) and evaluated its immune response in Balb/c mice. We used alum as an adjuvant to enhance the immune response. After intramuscular injection, we evaluated the immune response using cytokine and antibody assay and mortality rate. The results show that mice immunized by pcROP1 with or without alum produced high Th1 immune response compared with the control groups. This kind of DNA vaccine prolonged survival time. The current study showed that ROP1 DNA vaccine could induce partial protective response against toxoplasmosis.**

Key words: *Toxoplasma gondii*, DNA, vaccine, Rhoptry protein 1 (ROP1).

INTRODUCTION

Toxoplasma gondii is an obligating intracellular protozoan belonging to *Apicomplexa* that is causative agent of toxoplasmosis, a disease which may result in a spectrum of consequences including severe congenital defects, blindness or death. This parasite could infect a wide variety of host species and cell types (Ossorio et al., 1992; Dubey, 2007). Host cell penetration is an active process that requires parasite motility, orientation and energy expenditure. For invasion, the apical end of the parasite must contact with host cell. It seems the specialize organelles at the apical end, including the conoid, rhoptries, micronemes and dens granules, to play a role in the invasion process (Dubey, 2007; Werk, 1985). Like other unicellular organisms *Toxoplasma gondii* is composed of various antigens. Moreover, various DNA vaccines against *Toxoplasma* composed of single or cocktail antigens have been investigated (Hafid et al., 2005; Bhopale, 2003).

Nowadays interest to the somatic antigen has been reduced and the studies focused on the antigens known as excreted/secreted antigens or exoantigens. *T. gondii* secretory proteins are effective antigens that can activate strong immune responses. Several parasite products are secreted during and after invasion (Dubey, 2007; Lerche and Dubremetz, 1991). Rhoptry proteins are a variety of secretory antigens that secreted from rhoptry organelle. So far eleven rhoptry proteins have been identified (Lerche and Dubremetz, 1991). The time of the release of these molecules, as well as targeting to the host cell surface or parasitophorous vacuole, suggests their role for invasion of parasite. Rhoptry proteins may facilitate formation of the vacuole and mediate its clustering with host cell organelles. Rhoptry protein 1 (ROP1) has been associated with a molecular activity that can enhances the invasion *in vitro*. This property makes ROP1 a vaccine candidate (Reichman et al., 2002; Bradley et al., 2002). ROP1 is a single copy gene encoding a 2.1 kb transcript (Ossorio et al., 1992).

The mature protein has a mass of -33.6 kDa but migrates as if -60 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bradley et al., 2002) probably because of the octa peptide repeats which are rich in proline-glutamic acid residues

*Corresponding author. E-mail: z-eslami64@arakmu.ac.ir. Tel: +98 861 4173503 7. Fax: +98 861 4173521.

(Soldati et al., 1998). This protein is expressed in tachyzoite, bradyzoite and sporozoite (Garcia et al., 2004). The molecular weight of rhoptry protein1 from full length gene is - 46 kDa, but because of amino acids composition, it has strong charge asymmetry (Bradley et al., 2002) and migrates unusually slowly on SDS-PAGE (Garcia et al., 2004). The potential capacities of some excreted-secreted proteins to elicit protective immune responses against toxoplasmosis were investigated. We have attempted to evaluate the ability of ROP1 to elicit a protective immune response by cloning of ROP1 into the plasmid expression vector pcDNA3. The protein synthesized from this recombinant plasmid could be producing a mature ROP1 antigen. Intramuscular injection was used to activate a Th1-type response (Montgomery et al., 1997). We evaluate the protective effect of this plasmid by peritoneal challenge of mice with lethal dose of RH strain of *Toxoplasma* and also analyzed antibody response, cytokine patterns of *in vitro* restimulated splenocytes in mice.

MATERIALS AND METHODS

Parasite

The RH strain of Toxoplasma gondii was kindly provided by the Department of Parasitology in Health Faculty of Tehran University of Medical Science (Tehran, Iran). The parasite maintained by serial intraperitoneal passage in mice and the tachyzoites were harvested from peritoneal fluids, washed and suspended in sterile phosphate buffered salin (PBS).

Bacterial strain

Escherichia coli TOP10 strain was used as host cell for all plasmid manipulations. The bacteria were propagated in Luria Bertani broth or on Luria Bertani agar supplemented with ampicillin (100 mg/ml).

Plasmid constructions

The gene expression plasmid pcDNA3-ROP1, encoding the ROP1 (760 bp) antigen, was constructed by the following methods. We used standard method for DNA extraction (Sambrook et al., 1989; Hosseinian Khosroshahi et al., 2008). We designed a pair of primers according to the DNA sequence of the ROP1 gene which was obtained from the GenBank database (Accession number M71274). Forward primer introduced EcoR1 recognition site, underlined: 5′- CA GAA TTC ATG GAC TTC GCC TCC GAC GAC - 3′ and reverse primer introduced Xho1 recognition site, underlined: 5′- CG CTCGAG TTA CAG ACT GGC ACC ACT TGT - 3′. The purified polymerase chain reaction (PCR) products were ligated into pTZ57R/T plasmid vector (InsT/A clone ^{TM} PCR product cloning Kit, Fermentas) according to the manufacturing protocol. The recombinant plasmid (pT-ROP1) was used to transform competent *Escherichia coli* TOP10 strain. The cloned ROP1 was confirmed by PCR, restriction enzymes (EcoRI and Xho1) digestion and nucleotide sequencing. The coding region of ROP1 was subcloned from pT-ROP1 into the EcoRI and Xho sites of the pcDNA3 to produce recombinant eukaryotic expression vector pcROP1 (Sambrook et al., 1989). The plasmid which used for immunization was extracted by Endofree plasmid Giga kit Qiagen and was stored at -20°C.

Expression of ROP1 *in vitro*

The recombinant eukaryotic expression vector pc-ROP1 was used to transfect CHO cell. CHO cells were transfected with pcROP1 and control plasmid pcDNA3, using FuGENE6 Transfection Reagent (Roche) according to the instructions of the manufacturer. Six-well tissue culture plates were seeded with CHO cells in 2 ml Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/µl streptomycin, and the cells were grown until they were about 50 to 80%. Transfection was performed according to the manufacturing protocol. After 48 and 72 h, transfected cells were removed separately. The samples (transfected and non-transfected control cells) were concentrated by centrifugation. SDS-PAGE was performed as described by Laemmeli (Laemmeli 1970), using 10% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membrane for immunoblotting analysis. Membrane was blocked with 2% skim milk in PBST20 (PH 7.4) and probed with primary antibody (polyclonal antibodies) and peroxidase-conjugated secondary antiserum diluted in blocker respectively (1/10 and 1/3000). Specific bands were revealed with 3,3-Diaminobenzidine (DAB,Sigma).

Immunization

All the studied were done on 6 to 8 weeks old female Balb/c mice (Institute Razi, Iran). Mice were separated in 6 groups of 10 to 14 animals for injected PBS, Alum (Adjuvant), pcDNA3, pcDNA3 + Alum, pcROP1, pcROP1 + Alum. Intramuscular injection was done in the quadriceps muscles as each muscle was injected with 50 µg plasmid in 50 µl volume (each mouse 100 µg plasmid). The mice were boosted in the same way on 21 and 42 days.

Antibody assay

Blood were collected from the retro-orbital plexus of mice on day 21st, 42nd and 63rd after the first immunization. To measure total antigen-specific antibodies, 96-well costar plates were coated overnight at 4°C with 20 µg/ml solution of total *T. gondii* antigens in 0.05 M potassium phosphate buffer at pH 8 (50 µl per well). Blocking was carried out with 3% skim milk in PBST20 for 2 h at 37°C. After washing with PBST20, sera were diluted 1/10 in blocker added to wells (100 µl per well) and incubated 2 h at 37°C. After washing, peroxidase-labeled anti-mouse immunoglobulin (Sigma) was diluted 1/3000 in blocker added to wells (100 µl per well) and incubated for 2 h at 37°C. One hundred microliter (100) µl TMB was added per well (10 mg/ml) and after 20 min the reaction was stopped with 100 µl per well 2 M H_2SO_4 and OD was read at 450 nm in an enzyme linked immunosorbent assay (ELISA) micro-plate reader (Bio-Rad, USA). All samples were run in triplicates.

Cytokine assay

Mice were killed and the spleens were isolated and cut into small pieces, rinsed twice with phosphate buffered saline (PBS) and minced by a forceps and scalpel. The suspension was passed through a 100-l m stainless steel mesh to obtain a single cell suspension. Erythrocytes were lysed at 4°C using lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.001 mM Na2-EDTA). The cells were washed and resuspended in RPMI (Gibco) supplemented with 10% FCS and then were plated into 24 microplates at 1.5 × 10^6 /500 µl/well. The cells were stimulated by 50 µg/ml of *T.gondii*

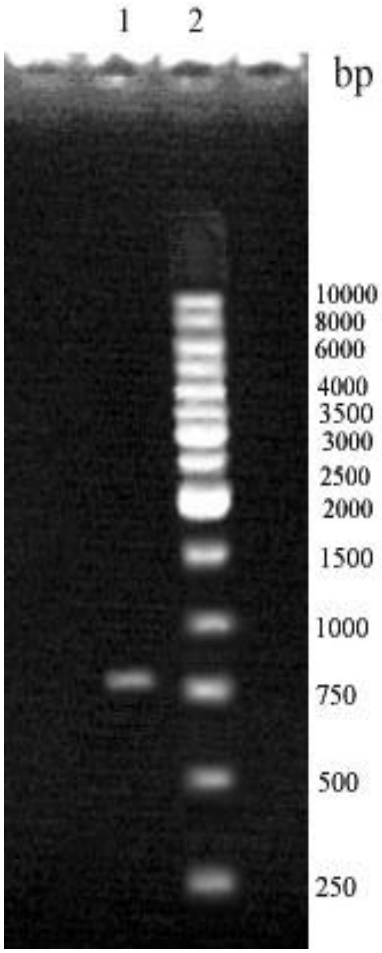

Figure 1a. Identification of the ROP1 fragment of Toxoplasma gondii amplification. Lane1: ROP1 fragment (760 bp), lane 2: 1 kb ladder.

Antigen. After 72 h, culture supernatants were harvested to test the presence of interferon IFN-γ and interleukin IL-4 (Hosseinian Khosroshahi et al., 2008). Assays for IFN-γ and IL-4 were performed using ELISA procedures according to the manufactures instruction ((R&D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm and results were expressed as pg/ml in the samples on the basis of the standard curve.

Challenge

Three weeks after last immunization, in each group half of the mice were infected intraperitoneal (i.p) with 5×10^5 tachyzoite of *T.gondii*. Mortality was recorded after the challenge.

Statistical analysis

The level of significance for the differences between groups of mice was determined by the Mann-Whitney test. All statistical analyses were performed using SPSS 16.0 for windows (statistical package for the social sciences, SPSS Inc.,IL, USA). Differences were considered significant when $P < 0.05$.

RESULTS

The ROP1 gene was amplified by PCR and using genomic DNA from the RH strain of *T.gondii* as template. The PCR product was ligated successfully into PTZ57R/T and pcDNA3, respectively. After digestion of this recombinant plasmid with restriction enzyme, the electrophoresis was performed and shown that the size of digested fragment was the same as that of the PCR product (Figure 1a and Figure 1b). The results were as expected. The synthesis of ROP1 protein in a eukaryotic system was investigated. The CHO cells were transfected with pcROP1 or pcDNA3 for 72 h. Identification of this protein was performed by SDS-PAGE and western blotting. It was detected that a protein about 26 kDa (as expected) when using polyclonal antibodies of mice that were immunized with plasmid encoding ROP1 *T. gondii* (Figure 2).

To compare the survival rates and induce protection

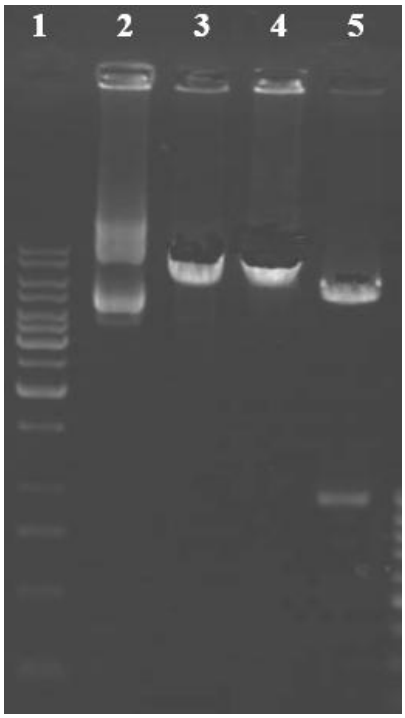

Figure 1b. Identification of the recombinant expression plasmid pc-ROP1 with restriction enzyme digestion. Lane 1: Ladder 1 kb, lane 2: pcROP1, lanes 3 - 4: pc-ROP1 after digestion with 1 enzyme, Lane 5: pcROP1 after digestion with 2 enzyme (ROP1 760 bp).

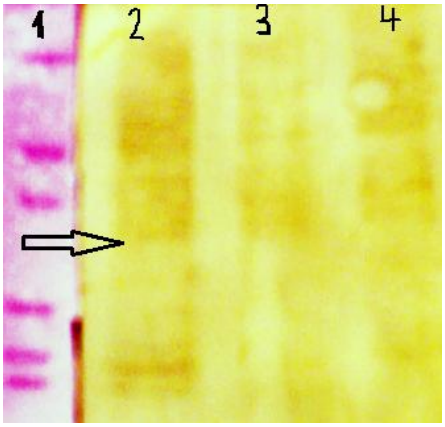

Figure 2. Western blot analysis of expressed gene product. Western blotting showed mice sera that were immunized with plasmid encoding ROP1 T. gondii recognizing ROP1 protein from transfected CHO cells. It was not detected in non-transfected control cells. Lane 1, Protein molecular weight marker (top to down 66.2, 45, 35, 25, 18.4, 14.4 kDa). Lanes 2 and 3, transfected cells containing Pc-ROP1 plasmid. Lane 4, transfected cell containing Pc-DNA 3 (negative control).

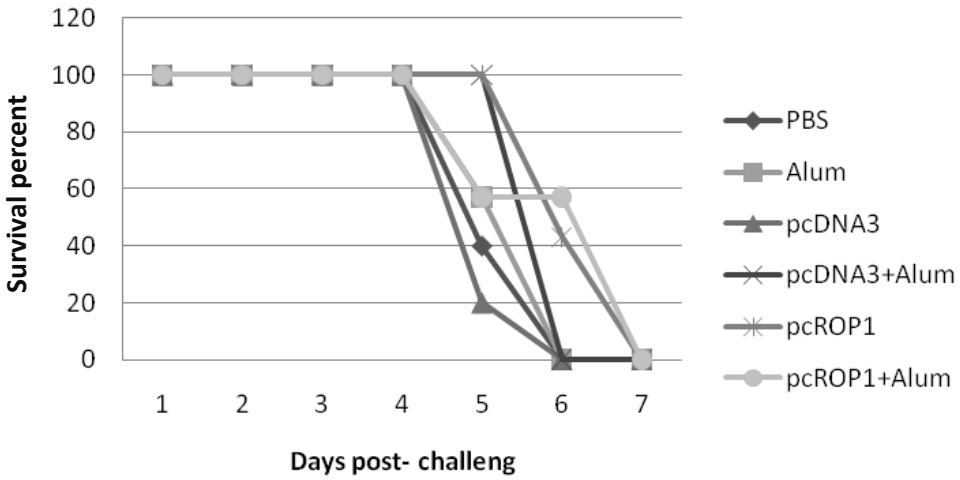

Figure 3. Survival curves of immunized BALB/c mice after challenge with 5 × 105 tachyzoite form of RH strain of T. gondii, 3 weeks after the last immunization. There were 5 to 7 mice in each group.

against mortality, 3 weeks after the last immunization 5 to 7 mice in each group (half of mice in each group) were challenged with 5×10^5 tachyzoites of virulent RH strain. The mortality was recorded daily. In pcROP1 and pcROP1 + Alum groups had high survival rate against control groups but it was not significant (Figure 3). To determine the level of anti-*T.gondii* antibodies, all sera were tested by using ELISA (Figure 4). The level of IgG antibody in the sera of mice immunized with pcROP1 and pcROP1+Alum were significantly higher than the control groups ($P < 0.05$). Higher antibody response was observed when animals were immunized with pcROP1 alone (Figure 4a) ($P < 0.05$).The level of IgG2a antibody in sera of mice immunized with pcROP1 and pcROP1+ Alum were significantly higher than the control groups (Figure 4b) ($P < 0.05$). Five weeks after the final immunization, splenocytes of five mice were cultured and cytokine products were studied *in vitro* (Table 1). A large amount of IFN-γ was detected in the splenocyte cultures of pcROP -immunized mice and significant difference was observed between amount of IFN-γ in pcROP1-immunized mice and pcROP1 + Alum-immunized mice with the control groups ($P < 0.05$). The amount of IL4 was detected in the splenocyte cultures significantly lower than IFN-γ.

DISCUSSION

Parasites are complex and immunologic responses change during the course of infection and stage specific antigens too. A DNA vaccine presents researchers to design with the tools effective vaccines with specific purposes (Ivory and Chadee, 2004). It is approved that DNA vaccine is a promising approach to protect animals and humans against pathogenic microorganism in particular intracellular parasites (Fachado et al., 2003). In this respect, progress has been made to develop vaccines against some protozoa, for example, malaria, leishmania and Toxoplasma (Kofta and Wedrychowicz, 2001; Crampton and Vanniasinkam, 2007; Schaap et al., 2007). In the present study, we targeted ROP1 protein of *T.gondii* as a vaccine candidate, because it is a penetration enhancing factor that is expressed by tachyzoites, bradyzoites and sporozoites (Smith, 1995). This antigen is secreted by apical organelles called rhoptries and secretion of these organelles is part of parasite invasion (Striepen et al., 2001).

The result of the present study shows that animals that were immunized using the pcROP1 *T.gondii* generated a humoral immune response (total IgG). We also observed animals were immunized by pcROP1 alone compared to other groups that were immunized by pcROP1 plus Alum produced higher titer of IgG. These findings are different to previous studies on similar gene that indicated mice immunized by ROP1 alone or plus genetic adjuvant (IFN-γ) until 90^{th} days after immunization, no significant increased was observed in level of IgG and after this time no significant difference was observed between case and control groups (Chen et al., 2001; Guo et al., 2001). Also in the case of other exoantigens that is, ROP2, GRA1 and GRA7 no significant difference was observed between case and control groups (Chen et al., 2003; Vercammen et al., 2000).

Furthermore, we were interested that the animals immunized by pcROP1, induce the types of immune responses Th1 or Th2. In natural infection of toxoplasmosis, it has been demonstrated that a Th1 response is responsible for effective protection and seems that a good immunization protocol should be able

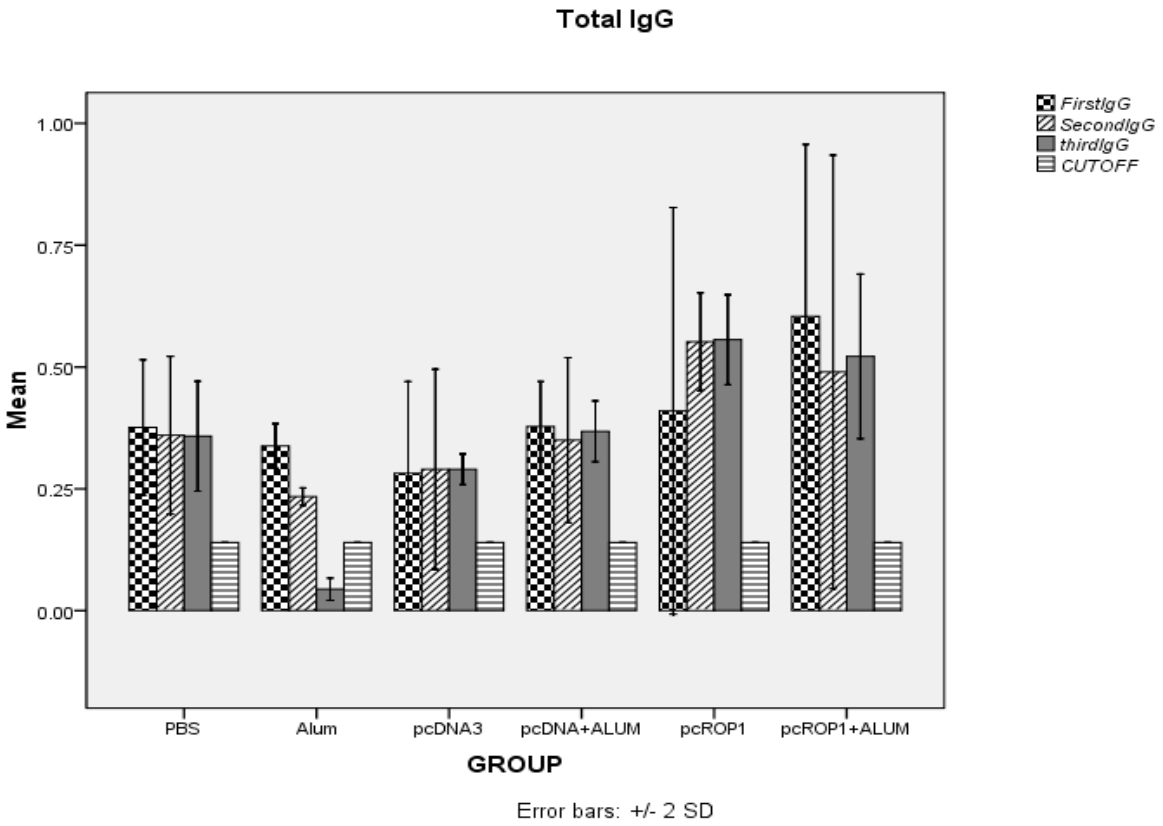

Figure 4a. ROP1-specific IgG in the sera of immunized mice. Sera were collected at 21, 42 and 63 days after first injection.

to switch responses toward Th1 type (Miller et al., 2009; Denkers and Gazzinelli, 1998). In this respect, we evaluated IgG2a in animal sera. The results determined that IgG2a level of pcROP1 immunized mice were significant higher than control mice. These findings are similar to studies with single or multiantigen DNA vaccine including other exoantigens that is, ROP2, GRA2, GRA4, GRA6 and GRA7(Fachado et al., 2003; Vercammen et al., 2000; Martin et al., 2004; Leyva et al., 2001; Golkar et al., 2007; Xue et al., 2008a).

Humoral and cellular immune responses accompanied by significant increase in the survival rates, commonly were observed when mice vaccinated with DNA vaccine cocktail and results of our study was different. Three weeks after the last immunization with 3 doses of 100 µg of single pcROP1 plasmid we challenged immunized mice with virulent strain of *T. gondii* and observed low partial protection. All mice in control groups died after six days, but mice in the case groups were alive until seven days, therefore we observed very low increase in survival rate of the case groups that are similar to the results obtained in some studies that single recombinant plasmid were used for immunization. In previous studies challenged with avirulent *T. gondii* strains produced restrict partial protection in mice while challenged with virulent *T. gondii* strains (RH) was produced, no protection in mice (Fachado et al., 2003; Vercammen et al., 2000; Martin et al.,2 004; Leyva et al., 2001). In the other studies that multiantigene or cocktail vaccines were used, significant increase in survival rate were observed (Fachado et al., 2003; Vercammen et al., 2000; Martin et al., 2004; Xue et al., 2008a, b).

DNA-based immunization strategies have been used, particularly useful in inducing cytotoxic T-cells likely via IFN-γ dependent mechanisms (Miller et al., 2009; Denkers and Gazzinelli, 1998). Several studies on cytokine profile of mice that were immunized by multiantigene or cocktail vaccines were revealed that this kind of immunization generated antigen-specific IFN-γ – producing cells that it is possible at least part of detected IFN-γ results of CD8 + CTL activation that further experiments are required to elucidate this question (Miller et al., 2009; Denkers and Gazzinelli, 1998). In our study, single DNA vaccine used for immunization, in the case groups of the level of IFN-γ were notably raised as compared to the control groups and in the all groups of mice, the level of IL4 were clearly low. The ability of splenocytes of pcROP1 immunized mice to release large amount of IFN-γ and low amount of IL4 indicate that response to switch toward Th1 type (Desolme et al., 2000).

It has been reported that in mice doses as low as 1 µg,

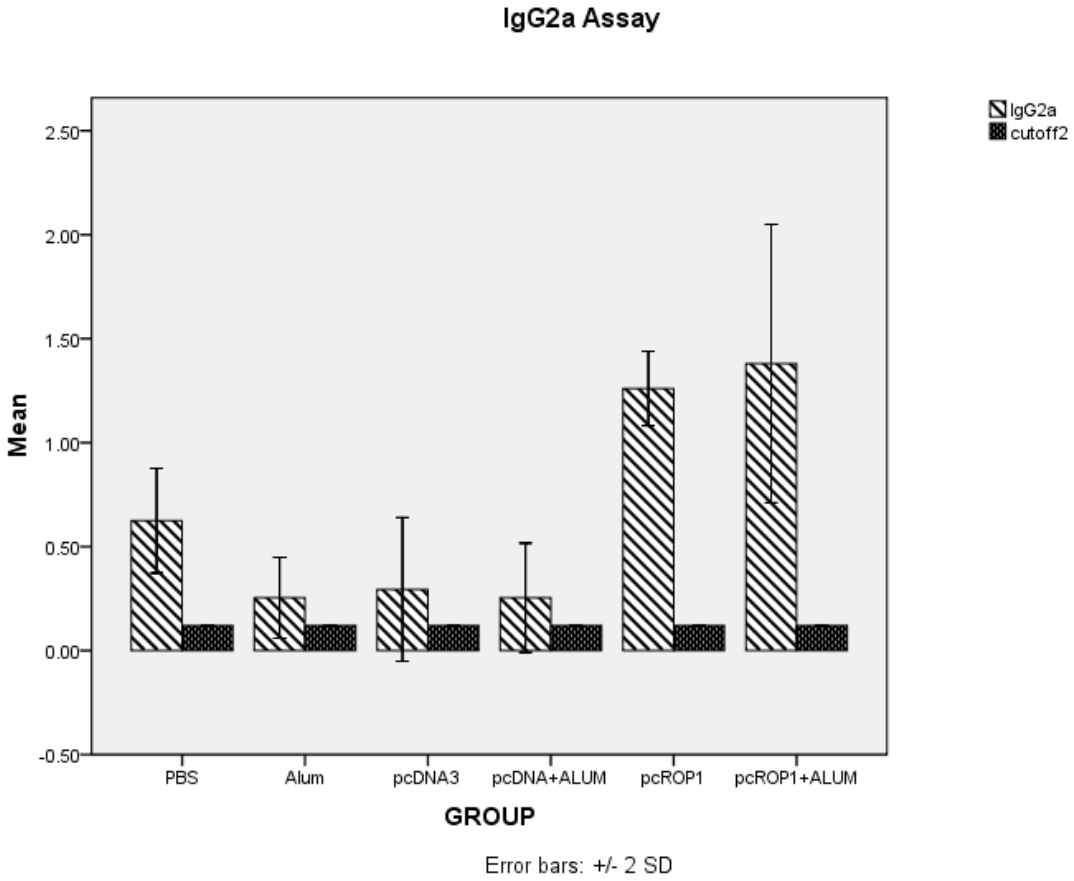

Figure 4b. Determination of IgG subclass in the sera of immunized mice. Sera were collected at 21, 42 and 63 days after first injection.

Table 1. Cytokines detected by ELISA in splenocyte cultures from immunized mice after stimulation with ST-Ag.

Immunization regimen	IFN-γ (pg/ml)	IL4 (pg/ml)
PBS	46.61 ± 1.79	13.73 ± 3.61
Alum	43.21± 3.56	12/11± 1.76
pcDNA3	133 ± 36.6	14.96 ± 1.38
pcDNA3+Alum	36.35 ± 14.4	34.81 ± 12.7
pcROP1	1161 ± 76.1	19.446 ± 13.1
pcROP1+Alum	433 ± 51	12.1 ± 2.36

Values are expressed as mean ± SD Values for IFN-γ and IL4 are for 72 h. A splenocyte from mice were harvested 5 weeks after the last immunization. $P < 0.05$.

DNA plasmid is sufficient to induce immune responses but in most animals, we needed >50 µg DNA plasmid to induce immune responses. Probably, requirement to high dose is related to the low expression level of protein, because it was detected that the low expression of ROP1 in CHO eukaryotic cell lines transfected *in vitro* with the pcROP1 plasmid and unable to detect this protein in other eukaryotic cell lines(COS7 and Hek 293) (Leyva et al., 2001). Manipulation of a gene and cloning in a plasmid that leads to the secretion of the protein can modify the type and strength of the immune responses (Leyva et al., 2001). The initial goal of this study was to evaluate the ability of ROP1 to induce protective immune responses by DNA vaccination. Our results indicate that the protective immune responses induced in Balb/C mice by injecting a plasmid containing ROP1 DNA is

modulated by Th1-type response elements. The inoculations of pcROP1 which enhance Th1 response decrease the survival rate after lethal challenge confirmed the modulation of Th1 response.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Seyedtabaee, Dr. Sadraie and Dr. Shojaee for their technical assistance to us.

REFERENCES

Bhopale GM (2003). Development of a vaccine for toxoplasmosis: current status. Microbes Infect., 5: 457-462.

Bradley PJ, Hsieh CL, Boothroyd JC (2002). Unprocessed *Toxoplasma* ROP1 is effectively targeted and secreted into the nascent parasitophorous vacuole. Mol. Biochem. Parasitol., 125: 189-193.

Chen G, Guo H, Lu F, Zheng H (2001). Construction of a recombinant plasmid harbouring the rhoptry protein 1 gene of *Toxoplasma gondii* and preliminary observations on DNA immunity. Chin. Med. J., 114(8): 837-840.

Chen H, Chen G, Zheng H, Guo H (2003). Induction of immune responses in mice by vaccination with liposome- entrapped DNA complexes encoding *Toxoplasma gondii* SAG1 and ROP1 genes. Chin. Med. J. (Eng), 116(10):1561-1566.

Crampton A, Vanniasinkam T (2007). Parasite vaccines: The new generation. Infec. Genet. Evol., 7: 664-673.

Denkers EY, Gazzinelli RT (1998). Regulation and function of T-cell-mediated Immunity during *Toxoplasma gondii* infection. Clin. Microbiol. Rev., 11(4): 569-588.

Desolme B, Mevelec MN, Buzoni – Getel D, Bout D (2000). Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding *Toxoplasma gondii* GRA4 gene. Vaccine, 18: 2512-2521.

Dubey JP (2007).The History and Life Cycle of *Toxoplasma gondii*. In: Weiss LM, Kim K. *Toxoplasma gondii*: The Model Apicomplexan Perspectives and Methods.Chapter 1. Elsevier, pp. 1-12.

Fachado A, Rodriguez A, Angel SO, Pinto DC, Vila I (2003).Protective effect of a naked DNA vaccine cocktail against lethal toxoplasmosis in mice. Vaccine, 21: 1327-1335.

Garcia JL, Gennari SM, Navarro IT, Machado RZ, Sinhorini IL (2004).*Toxoplasma gondii*: isolation of tachyzoites rhoptries and incorporation into ISCOM. Exp. Parasitol., 108:40-46.

Golkar M, Shokrgozar MA, Rafati S, Musset K, Assmar M (2007). Evaluation of protective effect of recombinant dense granule antigens GRA2 and GRA6 formulated in monophosphoryl lipid A (MPL) adjuvant against *Toxoplasma* chronic infection in mice. Vaccine, 25: 4301-4311.

Guo H, Chen G, Lu F, Chen H, Zheng H (2001). Immunity induced by DNA vaccine of plasmid encoding the rhoptry protein 1 gene combined with the genetic adjuvant of pcIFN-γ Against *Toxoplasma gondii* in mice. Chen. Med. J. (Engl), 114 (3): 317-320.

Hafid J, Vincent N, Flori P, Bellete B, Raberin H, Manh Sung RT (2005). Production of antibodies in murine mucosal immunization with *Toxoplasma gondii* excreted/secreted antigens. Vet. Parasitol., 125: 23-28.

Hosseinian Khosroshahi K, Ghaffarifar F, Sharifi Z, Dalimi A (2008). Expression of complete rhoptry protein 2 (ROP2) gene of Toxoplasma gondii in eukaryotic cell. Afr. J. Biotechnol., 7(24): 4432-4436.

Ivory C, Chadee K (2004).DNA vaccines: designing strategies against parasitic infections.Genetic Vaccines Ther., 2: 17.

Kofta W, Wedrychowicz H (2001). c-DNA vaccination against parasitic Infection : advantages and disadvantages. Vet. Parasitol., 100: 3-12.

Lerche MA, Dubremetz JF (1991). Characterization of the protein contents of rhoptries and dense granules of *Toxoplasma gondii* tachyzoites by subcellular fractionation and monoclonal antibodies. Mol. Biochem. Parasitol., 45: 249-260.

Laemmeli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Leyva R, Herion P, Saavedra R (2001). Genetic immunization with plasmid DNA coding for the ROP2 protein of *Toxoplasma gondii*. Parasitol. Res., 87(1): 70-79.

Martin V, Supanitsky A, Echeverria PC, Litwin S, Tanis T (2004). Recombinant GRA4 or ROP2 protein combined with Alum or the gra4 gene provides partial protection in chronic murine modles of Toxoplasmosis. Clin. Diagn. Lab. Immunol., 11(4): 704-710.

Miller GM, Boulter NR, Ikin RJ, Smith NC (2009). The immunobiology of the innate response to *Toxoplasma gondii*. Int. J. Parasitol., 39(1): 23-39.

Montgomery DL, Ulmer JB, Donnelly JJ, Liu MA (1997). DNA vaccines. Pharmacol. Ther., 74: 195-207.

Ossorio PN, Schwartzman JD, Boothroyd JC (1992). A *Toxoplasma gondii* rhoptry associated with host cell penetration has unusual charge asymmetry. Mol. Biochem. Parasitol., 50: 1-16.

Reichman G, Dlugonska H, Fischer H G (2002). Characterization of TgROP9(P36), a novel rhoptry protein of *Toxoplasma gondii* tachyzoites identified by T cell clone. Mol. Biochem. Parasitol., 119: 43-54.

Sambrook J, Fritsch EF, Maniatis (1989). Molecular cloning: A laboratory manual, (2nd edn). Cold Spring Harbor Laboratory Press. Plainview, p. 25.

Schaap D, Vermeulen AN, Roberts CW, Alexander J (2007). Vaccination Against Toxoplasmosis: Current Status and Future Prospects. In: Weiss LM, Kim K. *Toxoplasma gondii* : The Model Apicomplexa Perspectives and Methods.Chapter 24. Elsevier, pp. 721-763.

Smith JE (1995).*Toxoplasma gondii* cell biology. Int. J. Parasitol. 25(11): 1301-1309.

Soldati D, Lassen A, Dubremetz JF, Boothroyd JC (1998). Processing of *Toxoplasma* ROP1 protein in nascent rhoptries. Mol. Biochem. Parasitol., 96:37-48.

Striepen B, Soldati D, Garcia N, Dubremetz JF, Roos DS (2001). Targeting of soluble proteins to the rhoptries and micronemes in *Toxoplasma gondii*. Mol. Biochem. Parasitol., 113: 45-53.

Vercammen M, Scorza T, Huygen K (2000). DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. Infect. Immun., 68(1): 38-45.

Werk R (1985). How does *Toxoplasma gondii* enter host cells? Review Infectiouse Dis., 7: 449-457.

Xue M, He S, Cui Y, Yao Y, Wang H (2008a). Evaluation of the immune response elicited by multi-antigenic DNA vaccine expressing SAG1, ROP2 and GRA2 against *Toxoplasma gondii*.. Parasitol. Int., 57: 424-429.

Xue M, He S, Zhang J, Cui Y, Yao Y (2008b). Comparison of cholera toxin A2/B and Murine interleukin-12 as adjuvants of *Toxoplasma* multi-antigenic SAG1-ROP2 DNA vaccine. Exp. Parasitol., 119: 352-357.