African Journal of Biotechnology Vol. 11(35), pp. 8735-8741, 1 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1860
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Induction of protective immunity against toxoplasmosis in mice by immunization with a plasmid encoding *Toxoplama gondii* ROP1 gene

Z. Eslamirad[2], A. Dalimi[1]*, F. Ghaffarifar[1], Z. Sharifi[3] and A. Zavaran Hosseini[4]

[1]Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
[2]Department of Parasitology, Faculty of Medical Sciences, University of Tarbiat Modares, Tehran, Iran.
[3]Research Center of Virology, Iranian Blood Transfusion Organization, Tehran, Iran.
[4]Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Accepted 18 February, 2011

Toxoplasma gondii **is an obligate intracellular protozoan that is a causative agent of toxoplasmosis, a disease which may result in a spectrum of consequences. It has been shown that DNA vaccine can be effective in partial protection against this parasite. In the present work, a single DNA vaccine containing ROP1 was evaluated against *T. gondii* infection in Balb/c mice. To enhance the immune response, alum was used as an adjuvant. After intramuscular immunization, cytokine and antibody assays and mortality rate were evaluated. The results showed that mice immunized by pcROP1 with or without alum produced high Th1 immune response compared with control groups. This type of DNA vaccine prolonged slightly the survival time. The current study showed that ROP1 DNA vaccine can induced partial protective response against toxoplasmosis.**

Key words: *Toxoplasma gondii,* DNA vaccine, ROP1gene, immunity, mice.

INTRODUCTION

Toxoplasma gondii is an obligate 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 can infect a wide variety of host species and cell types (Ossorio et al., 1992; Dubey, 2007). Host cell penetration is an active process requiring parasite motility, orientation and energy expenditure. For invasion, the apical end of the parasite must contact with host cell. Specialized organelles at the apical end, including the conoid, rhoptries, micronemes and dens granules, are believed to play a role in the invasion process (Dubey, 2007; Werk, 1985).

Like other unicellular organisms *T. gondii* is composed of various antigens. The most important *Toxoplasma* antigens are somatic and excreted/secreted antigens. Some surface antigens like SAG1 is proposed as candidate for vaccine production against toxoplasmosis (Bhopale, 2003). Numerous studies have been performed on the structure, function and immunity of SAG1.

Moreover, various DNA vaccines against *Toxoplasma* compose of single or cocktail antigens have been investigated (Hafid et al., 2005). Nowadays, interest on 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 kinds of secretory antigens that are 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 their targeting to the host cell

*Corresponding author. E-mail: dalimi_a@modares.ac.ir.

Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **FCS,** fetal calf serum; **DMEM,** Dulbecco's modified Eagles medium; **DAB,** 3,3-diaminobenzidine; **RPMI,** Roswell Park Memorial Institute; **FBS,** fetal bovine serum; **IFN-γ,** gamma interferon; **IL,** interleukin

surface or parasitophorous vacuole, suggests their role in invasion of parasite. Rhoptry proteins may facilitate formation of the vacuole and mediate its clustering with host cell organelles. ROP1 has been associated with a molecular activity that can enhance invasion *in vitro*. This property makes ROP1 as 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 mass ~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 (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 have been investigated.

In the present study, we have attempted to improve the ability of ROP1 to elicit a protective immune response by cloning ROP1 into the plasmid expression vector pcDNA3. The antibody response, the lymphoproliferative response to ROP1 antigen and the cytokine patterns of *in vitro* re-stimulated splenocytes were analyzed. Lastly, the effects of injecting adjuvant with pcROP1 on the immune responses were evaluated in mice.

MATERIALS AND METHODS

Parasite

The *RH* strain of *T. gondii* was provided by Department of Parasitology, Faculty of Health, Tehran Medical Sciences University. Tachyzoites of parasite were harvested from peritoneal fluid of mice which 3 to 4 days earlier had been experimentally infected.

Bacterial strain

Escherichia coli strain TOP10 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) where appropriate.

Plasmid constructions

Standard methods were used for DNA extraction (Sambrook et al., 2001; Eslamirad et al., 2008). The *ROP1* gene of *Toxoplasma* was amplified by polymerase chain reaction (PCR). According to the DNA sequence of the ROP1 gene (GenBank database, accession number M71274) a pair of primers were designed. Forward: 5´- CA GAA TTC ATG GAC TTC GCC TCC GAC GAC - 3´; Reverse: 5´- CG CTCGAG TTA CAG ACT GGC ACC ACT TGT - 3´ The purified PCR products were ligated into pTZ57R/T plasmid vector (InsT/A cloneTM PCR product cloning kit, Fermentas ®) according to the manufacturing protocol and then, transformed and screened according to the protocol (Sambrook et al., 2001; Eslamirad et al., 2008). The plasmid was extracted by Accuprep plasmid extraction kit (Roche®) according to the protocol.

Then, this fragment was subcloned into expression plasmid pcDNA3.The pcDNA3 and the pT-ROP1 was digested by EcoR1 and Xho1. Then, digested plasmids were isolated and purified from agarose gel using DNA extraction kit (Roche®). The digested pT-ROP1 ligated, into the digested pcDNA3 by T4 DNA ligase and then, transformed and screened according to the protocol (Sambrook et al., 2001).

The plasmid used for immunization was extracted by Endofree plasmid giga kit Qiagen GmBH and stored at -20°C. The integrity of the DNA plasmids was checked by agarose gel electrophoresis, PCR amplification and restriction digestion. DNA concentration was determined by UV absorbance at 260 nm.

Expression of ROP1 *in vitro*

CHO cells were transfected with pcROP1 and control plasmid pcDNA3, using FuGENE® 6 transfection reagent (Roche®). Six-well tissue culture plates were seeded with CHO cells in 2 ml Dulbecco's modified Eagles medium (DMEM) (ready to use); 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 (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 phosphate buffered saline Tween 20 (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, respectively). Specific binds was revealed with 3,3-diaminobenzidine (DAB) (SIGMA).

Immunization

All the studied were done on 6 to 8 week old female Balb/c mice (Razi Institute, Iran). The mice were divided into 5 groups of 10 to 14 animals receiving PBS, pcDNA3, pcDNA3+alum, pcROP1, pcROP1+ alum. Intramuscularly injection was done in the quadriceps muscles. Each mouse received 100 µg plasmid. The mice were boosted in the same way on days 21 and 42.

Antibody assay

Blood were collected from the retro-orbital plexus on day 21, 42 and 63 after the first immunization. To measure total antigen-specific antibodies, plates (Nunc-Immuno plate, Maxisorp) were coated with 20 µg/ml solution of total *T. gondii* antigens (ST-Ag) in carbonate-bicarbonate buffer pH 9.6 (100 µl per well) overnight at 4°C. Antibody assay was carried out using ELISA technique and OD was read at 450 nm in ELISA micro plate reader.

Cytokine assay

Mice were dislocated and their spleens were isolated, cut into small pieces, rinsed twice with PBS and minced with a forceps and scalpel. The suspensions were passed through a 100 lm stainless

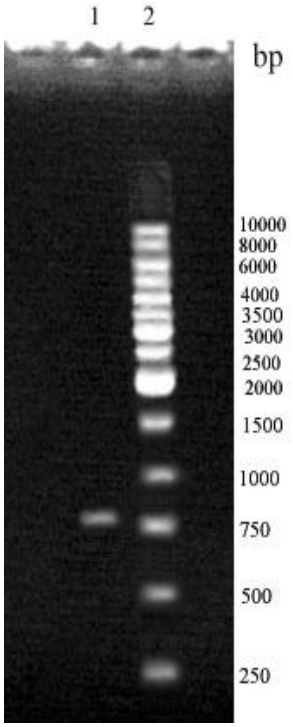

Figure 1. ROP1 fragment of *T. gondii* PCR amplification and gel electrophoresis. Lanes 1, PCR product (approximately 760 bp); Lane 2, 1 Kbp DNA ladder

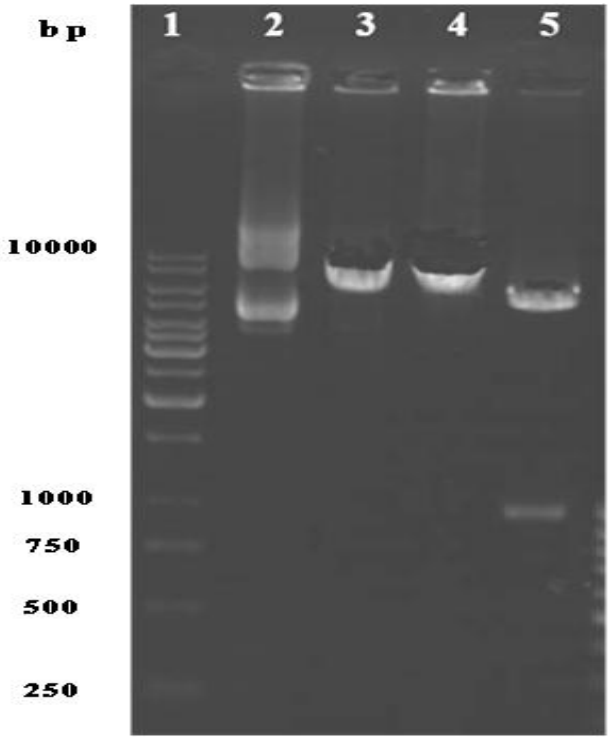

Figure 2. Digestion of extracted pc-ROP1 after transformation: Lane 1: Ladder 1 kb, Lane 2: pcROP1, Lane 3 to 4: pc-ROP1 after digestion with 1 enzyme, Lane 5: pcROP1 after digestion with, enzyme (760 bp).

steel mesh to obtain a single cell suspension. Erythrocytes were lysed at 4°C using ACK lysis buffer (150 mM NH4Cl, 1 mM KHCO3, and 0.001 mM Na2-EDTA). The cells were washed and resuspended in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and then, plated into 24 microplates at 1.5×10^6 /500 µl/well. The cells were stimulated by 50 µg /ml of *T. gondii* antigen. After 72 h, culture supernatants were harvested to test the presence of) and IL-4 (Farnandez-Botran et al., 2001). Assays for gamma interferon (IFN-γ) and interleukin (IL)-4 were performed using ELISA procedures according to manufacture's instruction (R&D). Absorbance was measured at 450 nm and results expressed as pg/ml IFN-γ or IL-4 in the samples, as determined from the standard curve.

Challenge

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

Statistical analysis

Levels of significancy for the differences between groups of mice were determined by the Mann-Whitney test. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 15 software.

RESULTS AND DISCUSSION

The ROP1 gene was amplified by PCR using genomic DNA from the RH strain of *T. gondii* as template (Figure 1). The PCR product was ligated successfully into PTZ57R/T and pcDNA3, respectively. After digestion of this recombinant plasmid with restriction enzyme, the size of digested fragment was the same as that of the PCR product (760 bp) (Figure 2).

The synthesis of ROP1 protein in a eukaryotic system shows the CHO cells are transfected with pcROP1 or pcDNA3 within 72 h. Identification of this protein were performed by SDS-PAGE and western blotting. A protein about 26 kDa (as expected) was detected when polyclonal antibodies of mice that were immunized with plasmid encoding ROP1 *T. gondii* were used, whereas, the control group do not show any band upon incubation with the same antibodies. In Figure 3, western blotting shows that, mice *T. gondii* positive sera recognizing ROP1 protein from transfected CHO cells whereas, it was not detected in non-transfected control cells.

To compare the survival rates and induce protection 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. Mortality was recorded daily. High survival rate were

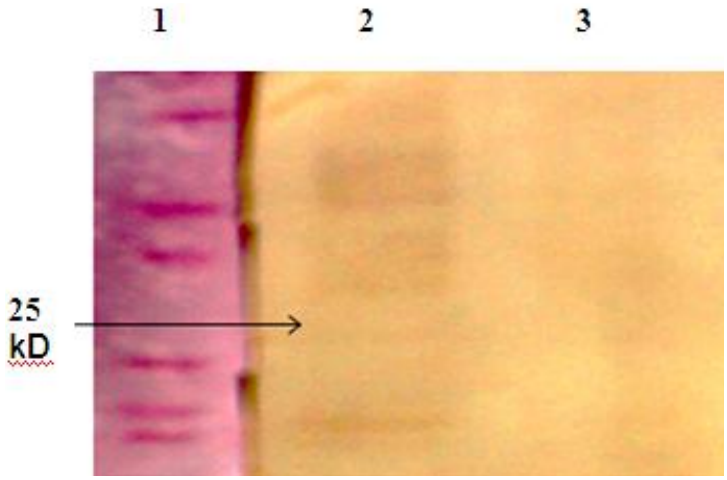

Figure 3. Western blot analysis of expressed gene product. Lane 1, Protein molecular weight marker (top to down 66.2, 45, 35, 25, 18.4, 14.4 kDa); Lanes 2, transfected cells containing Pc-ROP1 plasmid (25 to 35 KDa); Lane 3, CHO cells (negative control).

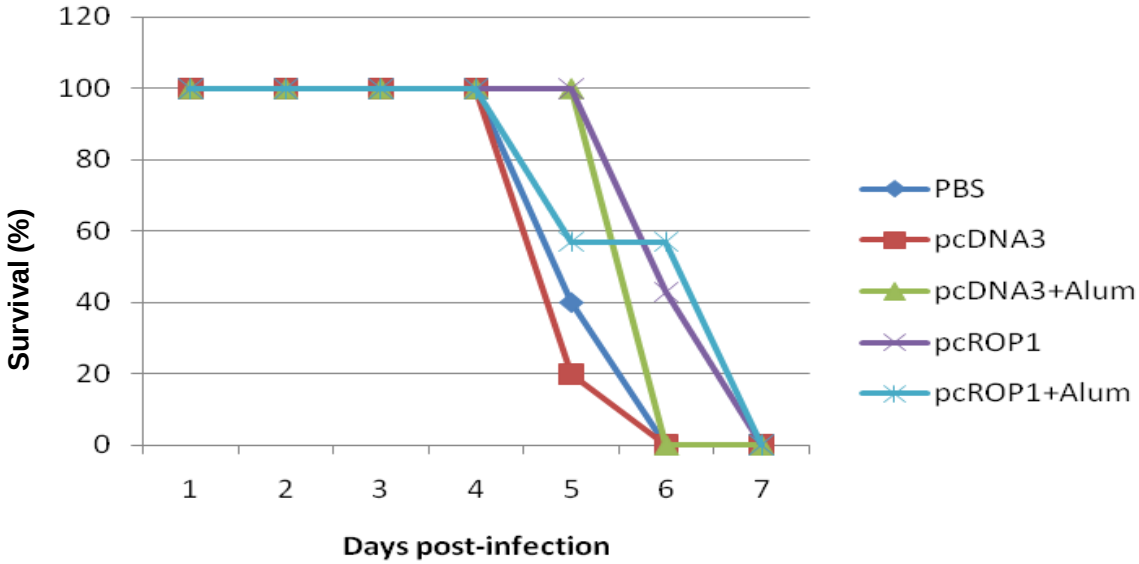

Figure 4. Survival curves of immunized BALB/c mice after being challenge with 5×10^5 tachyzoites with RH strain of *T. gondii*, 3 weeks after the last immunization. There were 5 to 7 mice in each group ($P > 0.05$ in Mann-Whitney test .

Seen in pcROP1 and pcROP1+alum groups in comparison with control groups but the difference was not significant (Figure 4; $P > 0.05$).

The levels of IgG antibody in the sera of mice immunized with pcROP1 and pcROP1+alum were significantly higher than control groups. Higher antibody response was observed when animals were immunized with pcROP1 alone (Figure 5; $P < 0.05$). The levels of IgG2a antibody in sera of mice immunized with pcROP1 and pcROP1+ alum were significantly higher than control groups (Figure 6; $P < 0.05$).

Five weeks after the final immunization, splenocytes of five mice were cultured and cytokine productions were studied *in vitro* (Table 1). Large amount of IFN-γ were detected in the splenocyte cultures of pcROP1-immunized mice and significantly different observed between amount of IFN-γ in pcROP1-immunized mice and pcROP1+alum-immunized mice with control groups ($P < 0.05$. The amount of IL4 detected in the splenocyte cultures was significantly lower than IFN-γ ($P < 0.05$.

DNA vaccines present researchers with the tools to design effective vaccines with specific purposes (Ivory

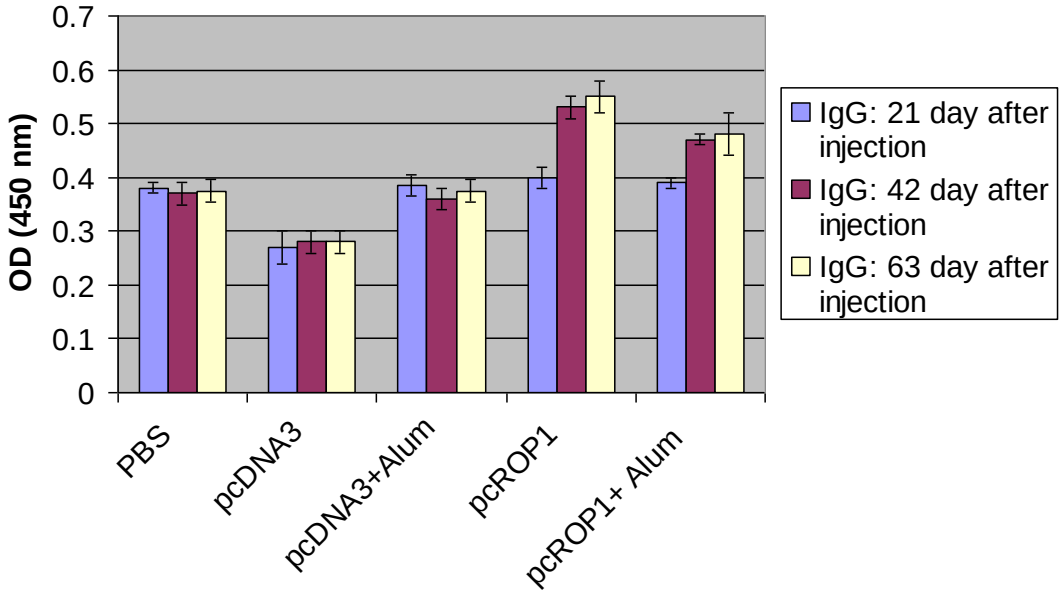

Figure 5. ROP1-specific IgG production in the serum of the different mice of each group. Sera were collected at 21, 42 and 63 days after first injection ($P < 0.05$ in Mann-Whitney test .

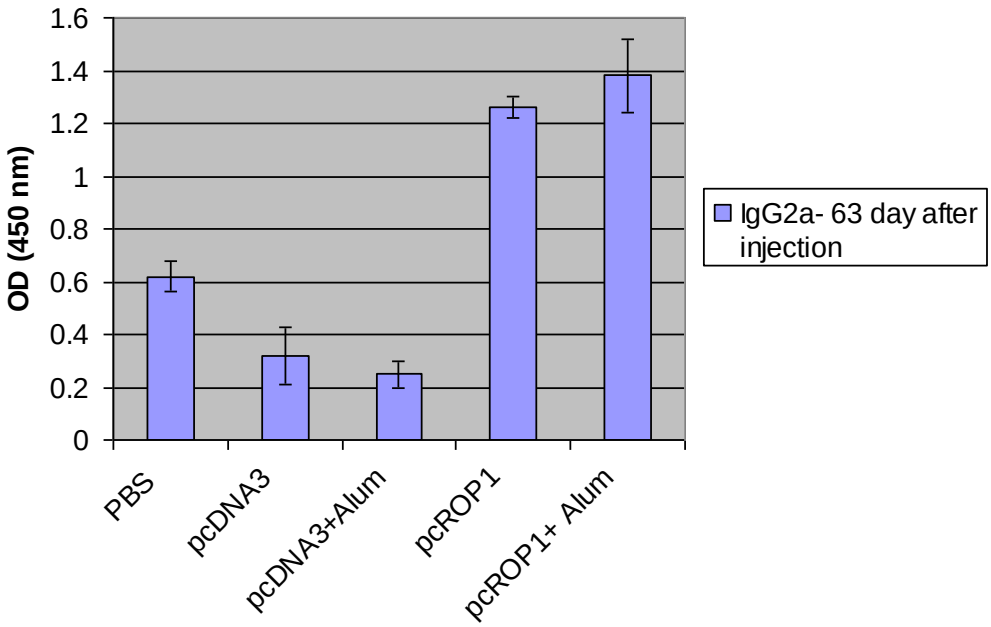

Figure 6. ROP1-specific IgG2a production in the serum of the different mice of each group. Sera were collected at 63 days after first injection ($P < 0.05$ in Mann-Whitney test .

and Chadee, 2004). It is approved that DNA vaccine is a promising approach to protect animals and humans against pathogenic microorganisms' particularly intra-cellular 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 specific antigen for 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

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

Immunization regimen	IFN-γ (pg/ml) (Mean ± SE)	IL4 (pg/ml) (Mean ± SE)
PBS	46.61 ± 1.79	13.73 ± 3.61
pcDNA3	133.00 ± 36.60	14.96 ± 1.38
pcDNA3+alum	36.35 ± 14.40	34.81 ± 12.70
pcROP1	1161.00 ± 76.10	19.44 ± 13.10
pcROP1+alum	433.00 ± 51	12.10 ± 2.36

Values for IFN-γ and IL4 are for 72 h; splenocyte from mice were harvested 5 weeks after the last immunization.

parasite invasion (Striepen et al., 2001). Our result indicated that, animals that were immunized with the pcROP1 generated a humoral immune response (total IgG) and when compare with other groups that were immunized by pcROP1 plus alum produced higher titer of IgG. These findings are different from previous studies on similar gene that mice have been immunized by ROP1 alone or plus genetic adjuvant (IFN-γ), whereas, no significant increased in levels of IgG was observed and 90 days after immunization no significant differences between case and control groups were observed (Chen et al., 2001; Gue et al., 2001). Similarly, 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). The reasons for these differences may be due to different adjuvant used and protocol of immunization.

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 to switch response toward Th1 type (Miller et al., 2009; Denkers and Gazzinelli, 1998). In this respect, we evaluated IgG2a in animal sera. The result indicated that, IgG2a levels of pcROP1 immunized mice were higher than control mice significantly. These findings are similar to studies with single or multi-antigen DNA vaccines including other exo-antigens 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 are accompanied by significant increase in the survival rates; ordinary, were observed when mice were vaccinated with DNA vaccine cocktail but our results was different. We challenged immunized mice with virulent strain of *T. gondii*; 3 weeks after the last immunization with 3 dose of 100 µg of single pcROP1 plasmid but low partial protection were achieved. All mice in control groups died after six days but the mice in case groups remained alive until seven days. This finding is similar to the results obtained in some studies conducted using single recombinant plasmid for immunization (Fachado et al., 2003; Vercammen et al., 2000; Martin et al., 2004; Leyva et al., 2001). Contrarily, in the other studies that multi-antigens 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; Xue et al., 2008b). The reasons of these differences may be due to different adjuvant used and protocol of immunization.

DNA-based immunization strategies have been found useful particularly 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 multi-antigen or cocktail vaccines revealed that, this kind of immunization generated antigen-specific IFN-γ-producing cells that it is possible at least in part of detecting IFN-γ results of CD8+ CTL activation; further experiments are required to elucidate this question (Miller et al., 2009; Denkers and Gazzinelli, 1998). In the present study, the level of IFN-γ was notably raised in case group when compared with the control groups and in all groups of mice, the level of IL4 was clearly low. The ability of splenocytes of pcROP1 immunized mice to release large amount of IFN-γ and low amount of IL4 indicated that immune response switch toward Th1 type (Desolme et al., 2000).

Generally, our results indicated that immune responses induced in Balb/c mice by injecting a plasmid containing ROP1 DNA is modulated by Th1-type response elements. The fact is that inoculations of pcROP1 which enhance Th1 response decrease the survival rate after lethal challenge confirmed the modulation of Th1 response.

ACKNOWLEDGEMENTS

This article is part of PhD thesis supported financially by Tarbiat Modares University. The authors wish to thank research department as well as Dr. Sadraei, Head of Parasitology Department for their assistance.

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. Infect. 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 L M, Kim K. *Toxoplasma gondii*: The Model Apicomplexan Perspect. Methods Chapter 1. Elsevier, pp. 1-12.
Eslamirad Z, Dalimi A, Ghaffarifar F, Sharifi Z (2008). Cloning rhoptry protein 1(ROP1) gene of *Toxoplasma gondii* (RH) in expression vector. Arch. Razi Institute, 63(2): 11-17.
Fachado A, Rodriguez A, Angel SO, Pinto DC, Vila I, Acosta A, Amendoeira RR, Lannes-Vieira J (2003). Protective effect of a naked DNA vaccine cocktail against lethal toxoplasmosis in mice. Vaccine, 21: 1327-1335
Farnandez-Botran R, Vetyickaz V (2001). Methods in Cellular Immunology.2 edition by CRF Press LNC.
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, Sadaie R, Cesbron-Delauw MF, Mercier C (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. Chem. 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.
Ivory C, Chadee K (2004). DNA vaccines: designing strategies against parasitic infections. Genet. Vaccines Ther. 2: p. 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, Tanos T, De Roodt AR, Guarnera EA, Angel SO (2004). Recombinant GRA4 or ROP2protein combined with Alum or the gra4 gene provides partial protection in chronic murine modles of Toxoplasmosis. CVI 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.
Reichman G, Dlugonska H, Fischer HG (2002). Characterization of Tg ROP9(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 T (2001). Molecular cloning: A laboratory manual, Second Edition. Cold Spring Harbor Laboratory Press. Plainview.
Schaap D, Vermeulen AN, Roberts CW, Alexander J (2007). Vaccination Against Toxoplasmosis: Current Status and Future Prospects. In: Weiss L M, 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, De Braekeleer J, Diet R, Jacobs D, Saman E, Verschueren H (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, Wang H (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