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Enhancement of Antibody Immune Response to a *Toxoplasma gondii* SAG1-Encoded DNA Vaccine by Formulation with Aluminum Phosphate

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In the present study, we evaluated aluminum phosphate as an adjuvant of SAG1-encoded DNA vaccine against *T. gondii* in BALB/c mice. Anti-*T. gondii* IgG values increased markedly in the pcSAG1 and pcSAG1+$AlPO_4$ groups, which were significantly higher than those of control groups ($p<0.05$). Although pcSAG1+$AlPO_4$ elicited IgG antibody values were greater than that of the sera of mice immunized with pcSAG1 alone, there was no statistically significant difference between these two groups ($p>0.05$). In spite of remaining alive of one mouse more than 45 days in pcSAG1+$AlPO_4$ group after challenge with *T. gondii* tachyzoites, all of the experimental mice died and the survival time of the mice immunized with pcSAG1+$AlPO_4$ was markedly longer than those of control and pcSAG1 groups ($p<0.05$). All mice in the control groups died within 7-9 days after challenge, whereas the average survival time of pcSAG1 and pcSAG1+$AlPO_4$ groups were up to 14 and 21 days after infection, respectively. Indeed its low cost, ease of use and safety record, these findings indicate that the $AlPO_4$ adjuvant can be used to combine DNA-based vaccination to prime an enhanced and balanced specific immunity.

Key words: DNA vaccine, *Toxoplasma gondii*, SAG1, $AlPO_4$

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INTRODUCTION

Toxoplasmosis, caused by an intracellular protozoan parasite, *Toxoplasma gondii*, is widespread throughout the world (Bhopale, 2003). The disease is of major medical and veterinary importance, being a cause of congenital disease and abortion in humans and in domestic animals (Bhopale, 2003). Toxoplasmosis is life-threatening in immunocompromised states, such as AIDS, organ transplantations and the fetus during pregnancy, while *T. gondii* infection is asymptomatic in grown hosts with intact immunity (Saito *et al*., 2001). Treatment of this disease is difficult due to toxic effects of available drugs and reinfection occurs rapidly. Under the present scenario, development of either new antitoxoplasma drugs or a vaccine is an attractive alternative (Bhopale, 2003). In recent years, significant progress has been made in the identification of vaccine candidates which can induce a protective response. Most of the works has focused on surface antigens of tachyzoites (Bhopale, 2003). Among the vaccine candidates, SAG1 (30 kDa) is one of the predominant vaccine candidates (Bhopale, 2003). SAG1 or P30 protein is 30 kDa (Kasper *et al*., 1985) and the main surface antigen of *T. gondii* tachyzoites (Letscher-Bru *et al*., 2003) and is highly conserved in *T. gondii* strains (Letscher-Bru *et al*., 2003; Burg *et al*., 1988) and constitutes 3-5% of the total tachyzoite protein (Burg *et al*., 1988; Zinecker *et al*., 2001). This antigen has two glycoforms (Zinecker *et al*., 2001) and is a highly conformational antigens (Chen *et al*., 2001). The gene encoding SAG1 occurs as a single copy, without introns (Kimbita *et al*., 2001; Biemans *et al*., 1998). SAG1 is the immunodominant antigen of *T. gondii* tachyzoites (Zinecker *et al*., 2001) and for this reason, basis of several widely used diagnostic assays (Kimbita *et al*., 2001; Biemans *et al*., 1998; Nigro *et al*., 2003; Meek *et al*., 2003) as well as being considered the most promising molecule for a native (Velge-Roussel *et al*., 2000; Debard *et al*., 1996; Bonenfant *et al*., 2001; Kasper *et al*., 1985; Bulow *et al*., 1991; Letscher-Bru *et al*., 1998) or recombinant vaccine (Letscher-Bru *et al*., 2003; Peterson *et al*., 1998) and such as DNA vaccine (Saito *et al*., 2001; Nielsen *et al*., 1999; Mohamed *et al*., 2003; Couper *et al*., 2003; Haifeng *et al*., 2003; Fachado *et al*., 2003; Gunjin *et al*., 2002) against toxoplasmosis. Purified P30 has been successfully tested as a single antigen for the serodiagnosis of acute and chronic toxoplasmosis (Burg *et al*., 1988; Nigro *et al*., 2003) and SAG1 is one of the first antigens recognized by IgM during acute infection (Rojas *et al*., 2000).

A number of strategies to potentiate DNA vaccines have been investigated in recent years, ranging from eletroporation, antigen-targeting, cytokines, viral vectors, liposomes or microparticles, among others. While several of these strategies have been able to significantly increase the immune response induced by DNA vaccines, their general use as vaccine adjuvant present some limitations. For example, electroporation is highly effective but may not be easily applicable to humans and cytokines and viral vectors may induce side effects and face some safety issues. On the other hand, aluminum salts have been extensively used as an adjuvant for commercial human and veterinary vaccines and are still the most commonly used adjuvants. The adsorption of protein antigens to aluminum salts potentiates the induction of strong Th2 type immune responses in immunized hosts. However, recent studies suggest that aluminum salts used with DNA vaccines could allow to reduce the plasmid dose required to induce an immune response and favor or at least maintain the Th1 bias of the immune response. However, this latter point remains unclear as some authors only focused on the enhancement of the humoral response (Rosado-Vallado *et al*., 2005). In the present study we evaluated the usefulness of aluminum phosphate as an adjuvant with *Toxoplasma gondii* major surface antigen (SAG1)-encoded DNA vaccine in BALB/c mice in an effort to optimize the efficacy of this vaccine against toxoplasmosis.

MATERIALS AND METHODS

Parasites: The tachyzoites of the highly virulent RH strain of *T. gondii* used for genomic DNA extraction and challenge of immunized mice. The parasites were maintained and propagated in BALB/c mice by serial intraperitoneal passages. The tachyzoites from peritoneal aspiration were washed and resuspended in phosphate-buffered saline (PBS), followed by freezing and thawing (10 cycles). The extract was centrifuged (10,000 x g), the supernatant filtrated in a 0.2 μm pore filter (Nalge Company, USA) and protein concentration determined by Lowry's method (Lowry *et al*., 1951) and the extract was used for ELISA.

Genomic DNA extraction: About 5×10^7 *T. gondii* tachyzoites were concentrated by centrifugation, washed with phosphate buffer saline (PBS), then lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% Sodium dodecyl sulphate (SDS), 0.1 M NaCl and 10 mM EDTA and then treated with proteinase K (100 μg mL^{-1}) at 55°C for 2 h (Kimbita *et al*., 2001). The genomic DNA was extracted by phenol/chloroform method followed by ethanol precipitation. After centrifugation the pellet was dissolved in sterile distilled water and stored at -20°C until use (Sambrook *et al*., 1989).

PCR amplification: Genomic DNA isolated from tachyzoites was used as a template to amplify the SAG1 gene by PCR performed in 25 μL of solution containing 3 μL of template DNA, 0.5 μL dNTP (Fermentas), 0.5 μL Taq DNA polymerase (Fermentas), 2.5 μL 10X PCR buffer (Fermentas), 0.75 μL $MgCl_2$(Fermentas), 15.75 μL distilled water and 1 μL each of primers [Forward, 27nt: introduced HindIII recognition site, underlined: 5′-ATT AAG CTT ATG TTT CCG AAG GCA GTG-3′ (1-18nt); Reverse, 26nt; introduced EcoRI recognition site, underlined: 5′-ATT GAA TTC TCA CGC GAC ACA AGC TG-3′(960-943nt)] under the following conditions: After an initial 5 min denaturation at 94°C, each cycle consisted of 60 sec at 94°C, 30 sec at 54°C and 45 sec at 72°C at the end of the 30 cycles of amplification, a final extension was continued for 5 min at 72°C. The DNA sequence of gene encoding the surface antigen P30 (SAG1) of *T. gondii* was obtained from the Gene Bank database (http//www.ncbi.com) with accession No. AY217784.1 and 960 base pairs. The forward and reverse primers were designed according to the nucleotide sequence in Gene Bank database and GenRuner software.

Plasmid constructions: The SAG1 coding sequence was amplified by PCR from the genomic DNA of *Toxoplasma gondii* RH strain and cloned into HindIII/EcoRI sites in the polylinker of plasmid pTZ57R/T (Fermentas) by InsT/Aclone™ PCR product cloning kit (Fermentas), according to the manufacturer's protocol to produce cloning vector pT-SAG1. Competent *E. coli* (TG1 strain) cells were transformed with the ligation mixture by calcium chloride (Sambrook *et al*., 1989). *E. coli* (TG1 strain) was provided by the Iranian Institute Pasteur, Biotechnology Department. The recombinant plasmid DNA was purified from transformed *E. coli* by plasmid extraction kit (Bioneer, Germany), dissolved in sterile deionized distilled water and stored at -20°C until use. The recombinant plasmid pT-SAG1 was detected by restriction analysis with EcoRI and HindIII and PCR amplification of SAG1 (pT-SAG1 as a template). The coding region for the *T. gondii* SAG1 was subcloned from the plasmid cloning vector pT-SAG1, in which the SAG1 gene has been cloned, with linkers to join to the HindIII and EcoRI sites of the pcDNA3 (Invitrogen, USA) to produce recombinant eukaryotic expression vector pcSAG1. The upstream primer for the major SAG1 gene contains a HindIII site and the ATG start codon. The downstream primer contains an EcoRI site and the stop codon. Competent *E. coli* cells were transformed with the ligation mixture by calcium chloride (Sambrook *et al*., 1989). The plasmid with the correct insert orientation was detected by restriction analysis with EcoRI and HindIII. The plasmids were purified by plasmid extraction kit (Bioneer, Germany) according to instructions of the manufacturer and sequenced. The nucleotide sequencing showed 100% similarity with the sequences reported at Gene Bank for SAG1/P30 (AY217784.1). DNA concentrations were measured by absorbance at 260 nm. The $OD_{260/280}$ ratios for purified DNA were 1.80-1.95, indicating preparations were free from protein contamination.

***In vitro* transient transfection of CHO cells:** Plasmid expression was analyzed in transfected CHO cells. CHO cells were grown to 60-70% confluence at 37°C and 5% CO_2 in 35 mm wells in Dulbecco's modified Eagle's medium (DMEM, Gibco) each containing 100 U mL^{-1} penicillin and streptomycin and 10% fetal calf serum (FCS). Cells were washed in a serum-free medium and the transfection was performed with a transfection kit (Genejuice Transfection Kit, Novagene, USA) according to instructions of the manufacturer.

SDS-PAGE and Western blot analysis: The cells (transfected and non-transfected control cells) were harvested for 48 h following the transfection and lysed in sample buffer. After sonication, the cells were concentrated by centrifugation and their protein profile was resolved in 12.5% reducing SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose membrane. Membrane strips were blocked with 1% BSA-PBST20 overnigth and sequentially probed with toxoplasma antibody-positive human sera (high titers, IgM4+, were found by IgM-ELISA) (Martin *et al*., 1998) and a peroxidase-conjugated anti-human IgM (DAKO, Denmark) diluted in 1% BSA–PBST20 (1/200 and 1/2000, respectively), specific binding was revealed with diamino benzidine (DAB) (DAKO, Denmark). The Western-blotting analysis showed that the mature proteins produced *in vitro* in CHO cells upon transfection with pcSAG1/P30 plasmid is of the expected molecular mass and recognized by specific polyclonal antibodies, whereas no *T. gondii* proteins were detected in non-transfected control cells.

Immunization Preparation of pcSAG1 plasmid vaccine formulated with aluminum phosphate: Aluminum phosphate was purchased from Iran's Razi Serum and Vaccine Production Research Institute. Preparation of pcSAG1 plasmid vaccine formulated with aluminum phosphate was carried out according to the method of Sasaki (2003) in that the kinetic of adsorption was determined after mixing 100 μg of pcSAG1 plasmid with 45 μg of aluminum phosphate in 100 μL of PBS solution for each injection.

Animals and immunization: Female 6-8-week-old BALB/c (susceptible strain) mice were purchased from Iran's Razi Serum and Vaccine Production Research Institute and maintained under standard conventional conditions. The mice were grouped to be based on administration content as follows: 1-PBS, 2-$AlPO_4$, 3-pcDNA3, 4-pcDAN3+$AlPO_4$, 5-pcSAG1, 6-pcSAG1+$AlPO_4$. Five mice were used for each experimental group. Mice in each group were anesthetized with 25 μL g^{-1} of mixture of ketamin 10% and xylazin 2% via intraperitoneal (i.p.) and immunized via intramuscular (i.m.) into both quadricepses with 100 μL of administration content according to their grouping. It was not injected more than 50 μL a muscle (Sasaki *et al*., 2003). Two inoculations were employed with the same DNA and $AlPO_4$ doses and the same immunization schedule was applied at three weeks intervals.

Measurement of humoral antibody response: Blood samples were collected by retro-orbital puncture from immunized mice two weeks after the final booster injection and sera were tested for the presence of total anti-*T. gondii* IgG antibodies by ELISA (Crowther, 1995), using soluble *T. gondii* antigen (10 μg mL^{-1}) and 1/100 and 1/200 serum dilutions. Briefly, microtitre plates were coated overnight at 4°C with solution of soluble *T. gondii* antigens in 1 M carbonate-bicarbonate buffer pH 9.6 (100 μL per well). The plates were then washed three times in PBS (pH 7.4), 0.05% Tween 20 (PBST20). Blocking was carried out with 1% dried skimmed milk in PBS (pH 7.2) for 1 h at 37°C. After washing with PBS containing 0.05% Tween 20 (PBST20), sera were diluted 1/100 and 1/200 in 1% dried skimmed milk-PBST20 (100 μL per well) and incubated for 1 h at 37°C. After washing, bound antibodies were detected by incubation at 37°C for 1 h with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (DAKO, Denmark) at 1/2000 dilution in 1% dried skimmed milk-PBST20 (100 μL per well). Peroxidase activity was revealed by adding 50 μL per well of a solution containing 12.5% H_2O_2, 0.1 M citrate-phosphate pH 4 and 10 mg mL^{-1} of ortho-phenylen diamine (OPD). The reaction was stopped adding 50 μL of 2 M H_2SO_4 and the optical density (OD) was read at 492 nm in an ELISA micro plate reader (Bio-Rad, USA).

Challenge infection: Immunized and control mice were infected by the intraperitoeal (i.p.) route with 10^5 tachyzoites forms of *T. gondii* RH strain, 2 weeks after the last immunization.

Statistical analysis: For statistical comparison between groups, the Multiple Comparisons; LSD was used and $p<0.05$ was considered statistically significant.

RESULTS

Construction of eukaryotic expression vector: The SAG1 gene was cloned into pcDNA3 vector by using the restriction enzymes HindIII and EcoRI. The resulting vector consisted of a cytomegalovirus immediate-early promoter that can drive the transcription. The sequence analysis proved that no PCR-induced mutation occurred and the resulting vector consisted of 960 base pairs (positions1 to 960 according to Gene bank, AY217784.1) from SAG1 gene.

SAG1 expressed *in vitro* by transfected CHO cells: SAG1 was synthesized in a eukaryotic system. The CHO cells (transfected and non-transfected control cells) were harvested for 48 h following the transfection and lysed in sample buffer. The protein extracts were then analyzed by SDS-PAGE and western blotting. A band at about 30 kDa was recognized by toxoplasma antibody-positive human sera in protein extracts of cells transfected with pcSAG1. SAG1 protein was not detected in non-transfected control cells. Thus the functionality of the vector, in terms of in vitro production of the SAG1 protein, was confirmed (Fig. 1).

Antibody response: Sera were collected from immunized and control mice two weeks after the final booster injection and tested for the presence of total IgG antibodies by ELISA. Figure 2 shows that the humoral response was elicited by the immunization. Anti-*T. gondii*

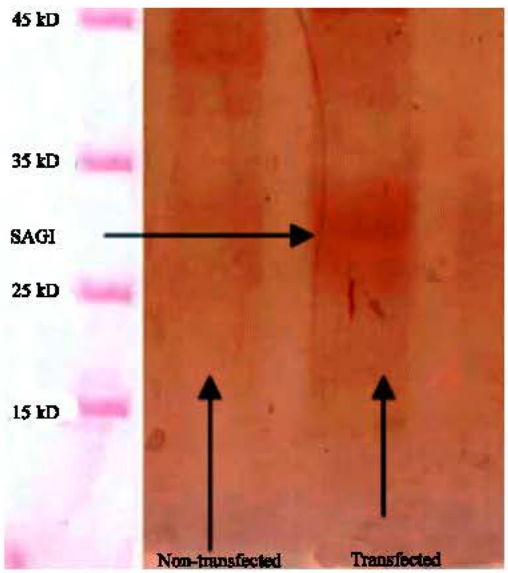

Fig. 1: Western blotting was showed human *T. gondii*-positive sera recognizing SAG1 protein from transfected CHO cells. It was not detected in non-transfected control cells

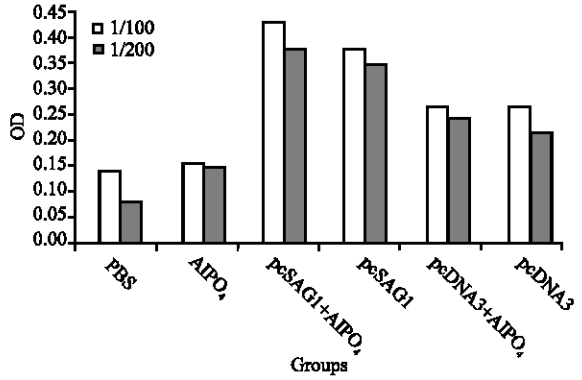

Fig. 2: Effect of aluminum phosphate on antibody response to *T. gondii* infection induced by DNA vaccination

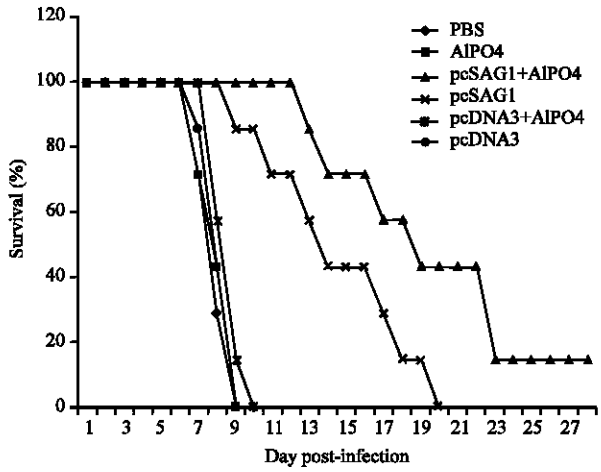

Fig. 3: Effect of aluminum phosphate on protection against *T. gondii* infection induced by DNA vaccination

IgG values increased markedly in the pcSAG1 and pcSAG1+$AlPO_4$ groups, which were significantly higher than those of PBS, $AlPO_4$, pcDNA3 and pcDNA3+$AlPO_4$ groups ($p<0.05$). Although pcSAG1+$AlPO_4$ elicited IgG antibody values were greater than that of the sera of mice immunized with pcSAG1 alone, there was no statistically significant difference between the two groups ($p>0.05$). Also results showed that pcDNA3+$AlPO_4$ elicited IgG an antibody values were greater than that of the sera of mice inoculated with pcDNA3 alone, but there was no statistically significant difference between these two groups ($p>0.05$).

Challenge with RH strain: For the challenge study, a highly virulent *T. gondii* RH strain was used. Mice were infected by 10^5 tachyzoites intraperitoneally 2 weeks after the last immunization. Figure 3 is a plot comparing survival of vaccinated and control mice after infection. In spite of remaining alive of one mouse more than 45 days in pcSAG1+$AlPO_4$ group, all of the experimental mice died and the survival time of the mice immunized with pcSAG1+$AlPO_4$ was markedly longer than those of PBS, $AlPO_4$, pcDNA3, pcDNA3+$AlPO_4$ and pcSAG1 groups ($p<0.05$). All mice in the PBS, $AlPO_4$, pcDNA3 and pcDNA3+$AlPO_4$ groups died within 7-9 days after challenge, whereas the average survival time of pcSAG1 and pcSAG1+$AlPO_4$ groups were up to 14 and 21 days after infection, respectively.

DISCUSSION

In attempting to develop a DNA vaccine, we have focused on the gene encoding SAG1, because previous studies for preventing toxoplasmosis have shown that immunization with SAG1 peptides, proteins or as DNA vaccine can elicit a broad range of immune responses that are capable of decreasing mortality of animals acutely infected with *T. gondii* and reducing the level of tissue cysts in the brains of infected animals, but it is still necessary to increase their efficacy.

Aluminum phosphate remains the most widely used adjuvant for human and veterinary vaccines, mostly because of their excellent track record of safety, low cost and efficacy with a large variety of protein antigens (Gupta and Rost, 2000). It is also well established that protein antigens need to be adsorbed to the adjuvant for maximum efficacy and that these formulations are very effective at increasing humoral responses but are mostly unable to increase cytotoxic T cell responses (Gupta and Rost, 2000; Brewer *et al*., 1999). The use of aluminum phosphate with DNA vaccines is much more recent and these studies suggested that it may represent an attractive alternative to increase the efficacy of this type of vaccines (Ulmer *et al*., 1999; Wang *et al*., 2000; Kwissa *et al*., 2003; Fischer *et al*., 2003; Temperton *et al*., 2003). In this study, we demonstrated that aluminum phosphate can strongly potentiate the efficacy of a DNA vaccine- encoding SAG1 against *T. gondii* infection. The results from the pcSAG1-transfected CHO cells showed that the plasmid is expressed and the recombinant SAG1 is an immunologically active form which can recognized by human antibodies from an individual with natural *T. gondii* infection. The antibody response (Fig. 2) found here after immunization with pcSAG1 and pcSAG1+$AlPO_4$ was increased, which was significantly higher than those of control groups ($p<0.05$). Although pcSAG1+$AlPO_4$ and pcDNA3+$AlPO_4$ elicited IgG antibody values were greater than those of the sera of mice immunized with pcSAG1 or pcDNA3 alone, respectively and there was no statistically significant difference between each related two groups ($p>0.05$). This indicates that pcSAG1 was able to influence the immune response toward a TH2 response by inducing an IgG response. When $AlPO_4$ is formulated with plasmid,

anti-*T. gondii* IgG values increase more than those of plasmid alone and this study suggest that $AlPO_4$ may represent an attractive alternative to increase the efficacy of this type of vaccines toward antibody production. Results from challenge with tachyzoites RH strain (Fig. 3) showed that pcSAG1+$AlPO_4$ group had a longer average survival time than control groups and pcSAG1 group and one mouse from pcSAG1+$AlPO_4$ group was alive for more 45 days. Thus $AlPO_4$ as an adjuvant was affected on efficacy of pcSAG1 DNA vaccine, because average survival time of pcSAG1 group was about 14 days after infection and all of mice in this group were dead. The mechanism of action of aluminum salts with DNA vaccines is unknown but seems to differ from those with protein vaccines. Indeed, while protein vaccines need to be adsorbed to the aluminum, previous studies suggest that DNA vaccines may need to remain mostly unadsorbed form the adjuvant. Also, it has been shown that aluminum salts do not increase neither host cells transfection nor the expression of the antigen. Alternatively, they may promote the migration of antigen-presenting cells at the site of immunization (Rosado-Vallado *et al*., 2005). Thus, aluminum salts and particularly aluminum phosphate, may be a very practical adjuvant for DNA vaccines due to its low cost, ease of use and safety record.

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