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Enhancing immune responses to a DNA vaccine encoding *Toxoplasma gondii* GRA14 by calcium phosphate nanoparticles as an adjuvant

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ABSTRACT

Several approaches have been used to improve the immunogenicity of DNA vaccines. In the current study, we constructed the plasmid encoding *T. gondii* dense granule 14 (GRA14) and investigated the immunological properties of calcium phosphate nanoparticles (CaPNs) as nano-adjuvant to enhance the protective efficacy of pcGRA14. BALB/c mice intramuscularly injected three times at two-week intervals and the immune responses were evaluated using lymphocyte proliferation assay, cytokine and antibody measurements, survival times, and parasite load of mice challenged with the virulent *T. gondii* RH strain. The results showed that the immune responses were induced in mice receiving pcGRA14 DNA vaccine. Interestingly, pcGRA14 coated with nanoparticles led to statistically significant enhancements of cellular and humoral immune responses against *Toxoplasma* infection ($P < 0.05$). After challenge with RH strain of *T. gondii,* immunized mice with pcGRA14 showed prolong survival time compared to control groups ($P < 0.05$). In addition, pcGRA14 coated with nano-adjuvant exhibited the lowest parasitic load in the infected mice tissues. For the first time, our data indicate that the pcGRA14 coated with CaPN was more effective for stimulation of immune responses and should be considered as an adjuvant in the design of vaccines against toxoplasmosis.

1. Introduction

Toxoplasma infection as a public health problem imposes a major burden on human and animal health [1–3]. Moreover, *T. gondii* infection during pregnancy, particularly in the first trimester, can lead to either death or serious pathological complications in fetuses such as nervous system involvement, mental retardation, neonatal growth retardation, ocular defects, and blindness in later life [3,4]. The progression of infection and parasite pathogenesis may be related to hosts genetics and age, intensity and frequency of infection, transmission manner, and the parasite strain [3–5].

DNA vaccines are of great interest in immunization against *T. gondii*. In recent years, studies showed that among the potential vaccine antigens, dense granules such as GRA1, GRA4 and GRA7, provided significant stimulation of the immune system and protection against *T. gondii* infection in mice [6–8].

Although the DNA vaccine can induce the suitable level of immune responses, the immunogenicity of most DNA vaccines was not satisfactory, and the protection from immunization was not complete. Mainly, due to the difficulties associated with the poor DNA stability and delivery, as well as insufficient antigen presentation and immune activation, most DNA vaccines exhibit low immunogenicity [9–11]. Although adjuvants improve immune responses using stabilization of antigen, delivery of antigen, increase of innate immunity activation, but they are not immunogenic by themselves. The list of FDA (Food and Drug Administration) approved that adjuvants are very limited and alum (aluminum hydroxide) is the most widely used vaccine adju-

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vant, remained as the only approved adjuvant for several decades. Although alum's adjuvant induces strong humoral responses, it does not improve the induction of Th1 antibody isotypes (cellular immune responses) [10,12]. Therefore, alum component has not been effective in preventing infection due to the presence of intracellular pathogens such as *Plasmodium*, *Leishmania* and *Toxoplasma.* Hence, efforts have been done to develop alternative adjuvants [12]. Nanoparticles as the adjuvants have been shown to modulate humoral and cellular immune responses [13,14]. Recently, nano-adjuvants have received special attention, commonly used in vaccination against infectious diseases [15–17]. Among different type of nanoparticles, calcium phosphate nanoparticles (CaPN) has been used for many years as a DNA delivery system and approved to utilize in the vaccine as the adjuvant. CaPN has also been found to be safe and non-causing site-specific reactions [18,19].

In our previous study (Ahmadpour et al., unpublished data), a DNA vaccine encoding *T. gondii* GRA14 was cloned in pcDNA3 and expressed in CHO cells. The BALB/c mice were immunized with pcGRA14, which induced both strong cell mediated and humoral immune responses. To investigate further immunogenicity of *T. gondii* GRA14 antigen, we constructed the recombinant DNA vaccine encoding the GRA14 antigen of *T. gondii* and coated with CaPN, as delivery systems (nano-adjuvant), to evaluate its efficacy to improve immune response and protective effect in BALB/c mice against acute toxoplasmosis.

2. Materials and methods

2.1. Animals

The inbred male BALB/c mice (8–10 weeks old) were used in this study. All mice were maintained under standard conventional conditions. The experiments were performed according to institutional animal ethics guidelines. The animal protocols were approved by Animal Research Center, Mazandaran University of Medical Sciences (ARCMUMS). Ethical approval was obtained from the Mazandaran University of Medical Sciences Ethics Committee (MUMSEC) (No. 91.327).

2.2. Parasites and preparation of T. gondii lysate antigen (TLA)

The RH strain of *T. gondii* was provided by the Toxoplasmosis Research Center (TRC) in Mazandaran University of Medical Sciences (Sari, Iran). Tachyzoites were harvested from the peritoneal cavity of BALB/c mice 3–4 days after intraperitoneal (i.p) injection with 1×10^5 of parasite suspension in sterile Phosphate-Buffered Saline (PBS; pH = 7.4) containing 100 IU/ml penicillin and 100 mg/ml streptomycin [20]. Peritoneal cells and debris were removed by washing with PBS and centrifuging at $800 \times g$ for 10 min at 4 °C. The purified tachyzoites were used for the preparation of *Toxoplasma* lysate antigen (TLA), DNA extraction and challenge for immunized mice. For preparation of TLA, the purified tachyzoites were disrupted by freezing at −80 °C and thawing at 4 °C following sonication on ice. Finally, the lysate was centrifuged at $3000 \times g$ for 30 min at 4 °C and the supernatant containing TLA was collected. The protein concentration was determined using Bradford method. The TLA was filtered through a 0.22-μm pore sterile filter and was kept at −80 °C until use [21,22].

2.3. Plasmid construction, purification, and expression

Total genomic DNA of *T. gondii* RH strain was extracted using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea) according to manufacturer's instructions. The GRA14 gene of *Toxoplasma* was amplified by polymerase chain reaction (PCR) using the following primers: Forwards: 5′-AAGCTTATGCAGGCGATAGCG-3′; Reverse: 5′-GAATTCCTATTCGCTTGGTCTCTGGTA-3′ with the introduction of Hind III and EcoRI restriction sites (underlined). PCR products (1227 bp) were digested with Hind III and EcoRI and cloned into the cloning plasmid pTG19-T (Vivantis, Canada). Afterward, the digested fragment of GRA14 with the above corresponding restriction enzyme was purified from agarose gel and ligated into the eukaryotic expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA) vector, generating the pcGRA14 plasmid. The recombinant plasmid clones were screened by PCR and double restriction enzyme digestion and confirmed by sequencing in both directions to ensure fidelity. The plasmids (pcGRA14 and pcDNA3) were purified using EndoFree Plasmid Mega Kit (Qiagen). The extracted plasmid concentration was measured using a spectrophotometer (Biowave 11) and dissolved in sterile endotoxin-free PBS at the final concentration of 1 mg/ml. To test the in vitro expression of the recombinant plasmid, CHO (Chinese Hamster Ovary) cells were transiently transfected with pcGRA14 using TurboFect Transfection Reagent (Fermentas, Cat. No. R0531) according to the manufacturer's protocols. The empty pcDNA3 vector was used as a negative control. After 48 h, cells were lysed and proteins were collected. Finally, the expression of recombinant GRA14 proteins in a eukaryotic system was tested by RT-PCR and western blot. The polyclonal antibodies obtained from experimentally infected mice.

2.4. Synthesis of nanoparticles and CaPN-coated DNA vaccine

CaPN were synthesized according to the previously defined protocol [19]. Briefly, 12.5 mM calcium chloride, 12.5 mM dibasic sodium phosphate and 15.6 mM sodium citrate were mixed together by the slow addition and stirred for 48 h. After 30 min sonication period, particle size was analyzed by the transmission electron microscopy and zetasizer. Afterward, DNA vaccine coated with nanoparticles was prepared by vortexing the mixtures of the CaPN to pcGRA14 for 60 min (equivalent to 100 μg of pcGRA14 plus 100 μg of CaPN per dose/mice).

2.5. DNA immunization and challenge

Seven groups of BALB/c mice (14 per group) were injected intramuscularly (anterior tibial muscle). The seven groups were: PBS, empty pcDNA3 vector, pcIL-12, nano-adjuvant, pcGRA14, pcGRA14 + pcIL-12and pcGRA14 + nano-adjuvant. Plasmid DNA suspended in sterile *endo*-toxin free PBS at final concentration 100 μg/100 μl. Mice were boosted using same protocol on days 14 and 28 and tail bleeds were performed on days 0, 14, 28 and 63 to determine the presence of anti-*Toxoplasma* antibodies. Sera were stored at −20 °C until used. Five weeks after the last immunization eight BALB/c mice in all groups were intraperitoneally challenged with 1×10^5 tachyzoite of virulent *T. gondii* RH strain. The mice were monitored and deaths were recorded daily and survival time was compared to the case and control groups.

2.6. Measurement of antibody responses

The presence of specific IgG1, IgG2a and IgG2b antibodies against *T. gondii* TLA were measured by Enzyme- linked immunosorbent assay (ELISA) as previously described [23,24]. In brief, 96-well plates were coated overnight at 4 °C with 20 μg of TLA in 1 ml of PBS and then blocked with PBS containing 1% BSA for 1 h. Mice sera were diluted 1:100 in PBS prior to testing and applied to well with incubation for 2 h at 37 °C. After washing the plates three times with wash buffer (PBST), the bound antibodies were detected by horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a and IgG2b (Santa Cruz Biotechnology), diluted in 1:5000. Following incubation (2 h at 37 °C) and wash, the substrate solution was added and plates were incubated for 30 min in the dark. The reaction was