Evaluation of the Protective Effect of Deoxyribonucleic Acid Vaccines Encoding Granule Antigen 2 and 5 against Acute Toxoplasmosis in BALB/c Mice

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Abstract. Toxoplasma gondii infects a broad range of warm-blooded hosts, including humans. Important clinical manifestations include encephalitis in immunocompromised patients as well as miscarriage and fetal damage during early pregnancy. Toxoplasma gondii dense granule antigen 2 and 5 (GRA2 and GRA5) are essential for parasitophorous vacuole development of the parasite. To evaluate the potential of GRA2 and GRA5 as recombinant DNA vaccine candidates, these antigens were cloned into eukaryotic expression vector (pcDNA 3.1C) and evaluated in vaccination experiments. Recombinant DNA vaccines constructed with genes encoding GRAs were validated in Chinese hamster ovary cells before evaluation using lethal challenge of the virulent T. gondii RH strain in BALB/c mice. The DNA vaccines of pcGRA2 and pcGRA5 elicited cellular-mediated immune response with significantly higher levels of interferon-gamma, interleukin-2 (IL-2), IL-4, and IL-10 (P < 0.05) compared with controls. A mixed T-helper cell 1 (Th1)/Th2 response was associated with slightly prolonged survival. These findings provide evidence that DNA vaccination with GRA2 and GRA5 is associated with Th1-like cell-mediated immune responses. It will be worthwhile to construct recombinant multitaintgen combining full-length GRA2 or/and GRA5 with various antigenic proteins such as the surface antigens and rhoptry antigens to improve vaccination efficacy.

INTRODUCTION

Toxoplasma gondii is a ubiquitous and obligate intracellular protozoan parasite which infects a broad range of warm-blooded hosts,† causing a disease known as toxoplasmosis. Toxoplasmosis is globally distributed and affects up to one-third of the world’s human population.‡ Acute toxoplasmosis is correlated with intracellular growth of the rapidly replicating tachyzoites, causing the death of infected host cell by rupturing to liberate more tachyzoites to continue invading neighboring cells.§ Chronic toxoplasmosis is related to the formation of tissue cysts containing bradyzoites as a result of the parasite’s response to the host immune mechanism.¶ Tissue cysts are found predominantly in the brain and skeletal muscle of the host. The cysts do not trigger any inflammation and remain dormant throughout the entire life of the host.‖ Encystation of the bradyzoites protects them from being detected by the host’s immune system.

Interconversion between tachyzoites and bradyzoites is a reversible process. Suppressed level of nitric oxide, T lymphocytes, interferon-gamma (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α), especially in immunocompromised patients, can cause reactivation of T. gondii infection through the rupture of tissue cysts. This releases bradyzoites that will convert into active tachyzoites.¶ Disease reactivation, toxic effects, and possibility of drug resistance in the parasites makes drug treatment unreliable for long term.¶ As a result, there is a need to develop vaccines that confer lifelong protection against T. gondii primary infection (during pregnancy), reactivation (immunocompromised patients), and reinfection.‖

One of the approaches to produce safe vaccines is through DNA vaccine technology. In this approach, a DNA plasmid encoding the protein of interest is taken up by muscle cells of the host. Protein expression is then driven by the host’s cellular machinery. The expressed protein may then be degraded by host proteases into smaller peptides before being transported into the endoplasmic reticulum and binds to major histocompatibility complex (MHC) class I molecules. The peptides are then presented on the cell surface for recognition by CD8+ cytotoxic T cells, thereby inducing cellular-mediated immunity. The expressed proteins may also be directly delivered out from the muscle cell via exocytosis and taken up by antigen presenting cells (APCs) such as macrophage. The protein will then be processed into peptide-MHC class II complex within the APC before being presented on the cell surface to CD4+ helper T cells for stimulation of humoral-mediated immunity.¶ DNA plasmid vaccine has been observed to elicit mechanism of immune responses similar to that triggered in natural T. gondii infection.

The key element in protection against T. gondii in the infected host is the triggering of T-helper cell 1 (Th1) cellular-mediated immune response via the production of proinflammatory cytokines such as IL-12, TNF-α, and IFN-γ. However, an overwhelming production of these cytokines may lead to severe inflammation at the infected sites causing severe tissue damages. Therefore, anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta have to be secreted at the same time to ensure equilibrium.¶,§

When the T. gondii tachyzoite invades a cell, a parasitophorous vacuole (PV) will be formed to enclose and protect the parasite within the infected cell. The T. gondii dense granules (GRAs) are specialized secretory organelles involved in PV development. The GRA-related proteins help in the maturation and modification of the PV and its membrane.¶ These proteins are found in the vacuole which surrounds the tachyzoite and encysted bradyzoites.‖,¶ Some of these proteins have been identified as potential vaccines.¶,§ Two of the potential vaccines are GRA2 and GRA5. GRA2 is involved in the formation of intravacuolar network in PV, whereas GRA5 helps to inhibit apoptosis of the infected cells, thereby protecting the parasite during cell invasion.¶,§ Both GRA2 and GRA5 are expressed throughout the whole intermediate host life cycle of T. gondii, thus preventing stage-limited protection against toxoplasmosis.‖,¶

Several studies have been conducted to evaluate multi-component vaccines, which incorporate GRA2 or GRA5 with
other proteins. However, only limited number of studies have been performed using GRA2 or GRA5 as single antigen vaccine. The objective of this study was to evaluate the protective effect of DNA vaccines encoding GRA2 and GRA5 against acute toxoplasmosis in a mouse model.

MATERIALS AND METHODS

Mice and ethics statement. Six- to eight-week old female BALB/c mice were purchased from Monash University Sunway Campus. The mice were maintained in a pathogen-free environment and were fed ad lib with commercial food pellets and water. Experiments were carried out in compliance with the animal ethics approved by Institutional Animal Care and Use Committee of the University of Malaya, Faculty of Medicine (2014-06-03/ PARA/R/CXT).

Parasites propagation and harvest. Toxoplasma gondii tachyzoites of the virulent wild-type RH strain were provided by the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia. They were maintained according to the procedures described in our previous study.

DNA plasmid transfection. Six-well flat-bottom microplate was seeded with 0.8–2.4 × 10⁵ Chinese hamster ovary (CHO) cells in 4 mL of DMEM (Dulbecco’s modified Eagle’s medium) complete medium 24 hours before transfection. When the cells reached 70–90% confluence, 4 μg of the isolated endotoxin-free DNA plasmid was diluted in 400 μL of serum-free DMEM. Diluted DNA plasmid was then mixed immediately with 6 μL Turbofect™ (Thermo Scientific, Waltham, MA) Protein Transfection reagent through vortexing. The Turbofect/DNA mixture was incubated for 20 minutes at room temperature before adding 400 μL of the mixture into each well containing CHO cells. The cells were then incubated in carbon dioxide (CO₂) incubator at 37°C for 24–48 hours before harvested for the analysis of recombinant protein expressions by western blot assay.

Immunization regimen. Six- to eight-week old female inbred BALB/c mice were divided into four immunization groups with 13 mice in each group. Four different groups of BALB/c mice were given intramuscular injection at tibialis anterior muscle of both leg with 100 μL (50 μL in each leg) of phosphate-buffered saline (PBS) (negative control), empty vector (negative control), 100 μg of pcGRA2, and 100 μg of pcGRA5. A total of three injections were carried out at 3 weeks interval. Blood samples (50–100 μL) were collected from the injected mice through tail-bleeding on day 0, 21, 42, and 63.

Evaluation of humoral response. Mice serum samples harvested were analyzed by western blot assay and in-house enzyme-linked immunosorbent assay (ELISA) against Toxoplasma total lysate antigen (TLA) to detect the presence of antigen-specific immunoglobulin G (IgG) antibodies. IgG antibody titers and subclass determination were carried out as described previously with TLA as the coating protein.

Evaluation of cellular response. In vitro splenocyte proliferation assay and cytokines (IFN-γ, IL-2, IL-4, and IL-10) assay were performed according to the protocols described previously. Cultured mice splenocytes were induced with culture medium alone (negative control), 10 μg/mL TLA, or 5 μg/mL con A (positive control) before incubated at 37°C in 5% CO₂ incubator for 24, 72, and 96 hours.

Mice challenge. The remaining vaccinated and control mice were injected intraperitoneally with 1,000 live tachyzoites. Mortality rate of the mice was monitored and recorded twice daily whereby the infected mice were observed for end-point criteria; heavily infected with symptoms of sluggish movement, hunched back posture, ruffled, and thinning hair coat as well as obvious reduced food and water consumption. The heavily infected mice that reached end-point criteria were humanely killed by exposure to a gradually increasing concentration of CO₂ inside a closed chamber.

Statistical analysis. Significance levels of the differences between groups of mice were analyzed through Student’s t test or analysis of variance. P < 0.05 indicates statistical significance. The survival rate was calculated based on χ² test, whereas the survival graph was drawn based on Kaplan–Meier method.

RESULTS

Mammalian cell expression of pcDNA 3.1C constructs. Transfection study involved two negative controls: pcDNA 3.1C empty vector and nontransfected cells, one positive control: pcDNA 3.1/His/lacZ, and two target genes: pcGRA2 and pcGRA5. Protein expressions of the constructs were analyzed through western blot assay using Xpress mouse monoclonal antibody as illustrated in Figure 1. Results obtained showed that pcGRA2 and pcGRA5-transfected CHO cells produced antigenic proteins with their respective expected protein sizes of 30 and 20 kDa. Positive control–transfected CHO cells produced β-galactosidase protein with approximate protein size of 120 kDa. Meanwhile, no

![Figure 1](image-url)
protein bands were detected in both the negative control-transfected and nontransfected CHO cells.

**IgG antibody detection.** Specific anti-TLA IgG antibody was at an undetectable level in the sera collected from all the injected mice at 0, 3, 6, and 9 weeks after first injection (data not shown). The cutoff (mean ± 2 standard deviation) optical density (OD) value of the IgG level in the sera of pcGRA2- and pcGRA5-immunized mice groups was approximately the same as that of both PBS- and pcDNA 3.1C-injected mice groups (data not shown). On the other hand, antibody titers and IgG isotypes were unable to determine as well.

**In vitro splenocytes proliferation assay.** Significantly higher SI value was observed in the recombinant DNA plasmid-vaccinated groups compared with the control groups (P < 0.05) (Figure 2 and Table 1). There was no statistical difference between two vaccinated groups (P > 0.05) and also between two control groups (P > 0.05). These results indicated that T lymphocytes of the vaccinated mice were successfully stimulated.

**Cytokine production assay.** Results showed that the vaccinated mice produced significantly higher level of IFN-γ and IL-2 compared with the control groups (P < 0.05) (Figure 3 and Table 1). No statistical difference was observed between two vaccinated groups (P > 0.05) and between two control groups (P > 0.05). Relatively low levels of IL-4 and IL-10 were secreted by the stimulated splenocytes of the mice immunized with pcGRA2 and pcGRA5 (Figure 4 and Table 1). In contrast, these two cytokines levels were undetectable in the control groups. These results showed that predominantly Th1 immune response was favored in the vaccinated mice.

**Protective efficacy of DNA plasmid vaccination in BALB/c mice.** Results (Figure 5) indicated that two vaccinated mice groups were only managed to prolong the survival days up to 2–3 days as compared with the two control mice groups (PBS and pcDNA 3.1C) (P < 0.05). All PBS-injected mice died on day 6 (median survival of 6 days), whereas pcDNA 3.1C-injected mice died within 6–7 days (median survival of 7 days). On the other hand, pcGRA2- and pcGRA5-immunized mice succumbed to the parasite infection on 6–9 days postinfection with the median survival of 8 days.

**DISCUSSION**

Mammalian cells such as CHO cells have been widely used for expression of *Toxoplasma* recombinant protein,\(^{26}\) functional validation of *Toxoplasma* recombinant DNA plasmid\(^{27–29}\) as well as growth investigation of the parasite.\(^{30}\) In this study, CHO cell was used to validate the expression of GRA2 and GRA5 by the recombinant DNA construct prior to DNA vaccination study. Heterologous expression of recombinant protein in mammalian cell involves gene transfection into the host cell, delivery into host cell nucleus and subsequently protein translation in the cytoplasm.\(^{31}\) The Transfection Reagent used in this study is a water-based cationic polymer, which forms a positively charged complex with the recombinant DNA plasmid. This stable complex is essential for the uptake by cell through endocytosis against the negatively charged cell membrane. Apart from providing the positive charge, the transfection reagent also helps to protect the DNA from degradation.\(^{31}\)

The successful transfection and expression of pcGRA2 and pcGRA5 in CHO cells were confirmed in the western blot analysis. However, protein expression levels were too low for detection in Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The transcription was transient as the plasmid DNA was not integrated into the CHO cell genome. The GRA genes would not be propagated but occurred in the host cell for only a few days. The genes would eventually be diluted on host cell divisions and degradation by host nucleases.\(^{32}\)

Transient transfection usually serves as a preliminary experiment before the actual DNA vaccination in live animal. A previous study reported pcGRA2- and pGR5-transfected

<table>
<thead>
<tr>
<th>Group (N = 3)</th>
<th>Proliferation (SI)&gt;</th>
<th>IFN-γ (pg/mL)</th>
<th>IL-2 (pg/mL)</th>
<th>IL-4 (pg/mL)</th>
<th>IL-10 (pg/mL)</th>
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<tr>
<td>pcGRA2</td>
<td>1.679 ± 0.0828*</td>
<td>5341 ± 79.7*</td>
<td>359.1 ± 74.5*</td>
<td>21.51 ± 11.2*</td>
<td>57.27 ± 25.29*</td>
</tr>
<tr>
<td>pcGRA5</td>
<td>1.672 ± 0.1136*</td>
<td>4669 ± 453.2*</td>
<td>360 ± 63.48*</td>
<td>15.12 ± 7.38*</td>
<td>51.47 ± 20.06*</td>
</tr>
<tr>
<td>pcDNA3.1C</td>
<td>1.289 ± 0.0812</td>
<td>757.5 ± 0.005</td>
<td>17.99 ± 12.4*</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>PBS</td>
<td>1.222 ± 0.0019</td>
<td>625.1 ± 367.8</td>
<td>143.1 ± 34.9*</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
</tbody>
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\(^{*}\)P < 0.05 in comparison with the control groups (PBS or pcDNA 3.1C).
HEK 293-T cells produced 28 kDa GRA2 and 18 kDa GRA5,33,34 which were almost similar to that obtained in this study. Another study reported the in vitro expression of pVAX1-GRA2 in HFF mammalian cells, producing an antigenic protein at 20 kDa.35 Expression of several other *T. gondii* genes in mammalian cells has also been evaluated.13,22,35–43 In this study, DNA vaccination of BALB/c mice with pcGRA2 and pcGRA5 triggered mixed Th1/Th2-like cellular immune responses, predominantly of Th1. Humoral immunity was not successfully elicited as anti-TLA IgG was not detected either in western blot and ELISA analyses. Furthermore, the vaccinated mice were not protected against lethal challenge of *T. gondii* infection.

These results demonstrated stimulation and proliferation of T lymphocytes in the vaccinated mice. High level of the pro-inflammatory cytokines IFN-γ and IL-2 was achieved. However, relatively low levels of anti-inflammatory cytokines IL-4 and IL-10 were obtained. Collectively, it suggested that a predominantly Th1 immune response was elicited, which prolonged median survival days of the injected mice from 1 to 2 days.

The predominant Th1-like response in the vaccinated mice was most likely attributed to the presence of unmethylated CpG motifs in the vaccine vector pcDNA 3.1C.15,44 The immunostimulatory CpG motifs possess Th1-directing adjuvant activity and are associated with IFN-γ secretion as well as enhancing the immunogenicity of DNA vaccines.45–48

The DNA vaccines in this study were constructed without signal peptide at the N-terminal to ensure the translated proteins remained in the cytosol for antigen processing and presentation through the MHC class I complex to trigger cellular-mediated immune response.49 The exclusion of signal peptide from these two DNA vaccine constructs might be the reason contributing to the failure to develop antigen-specific antibody response (humoral immune response) as the translated proteins were not signaled out from the cells for the formation of peptide-MHC class II complex.7 A similar phenomenon has been observed in previous studies in which DNA vaccine candidates encoding *T. gondii* MIC2-MIC3-SAG1 and GRA3-GRA7-M2AP without the signal peptides stimulated only weak antibody production.44 No antibody production was elicited by DNA vaccines expressing MIC2 and MIC3 antigens.50 In contrast, full-length recombinant DNA vaccine constructs have been shown to produce strong antigen-specific antibody response.51

Several earlier investigations reported GRA2 DNA vaccine success against acute *T. gondii* infection. Multi- or single antigen elicited IgG antibody production with high

![Figure 3](image-url) **FIGURE 3.** Interferon-gamma (IFN-γ) and interleukin-2 (IL-2) production by the stimulated splenocytes of the immunized mice. Culture supernatants from the total lysate antigen–stimulated immunized mice splenocytes were collected at 96 and 24 hours postincubation for the evaluation of (A) IFN-γ and (B) IL-2 production, respectively, via enzyme-linked immunosorbent assay. Data are expressed as mean ± standard deviation (N = 3). Statistical difference is represented by * (P < 0.05) in comparison with the control groups (phosphate-buffered saline [PBS] or pcDNA 3.1C).

![Figure 4](image-url) **FIGURE 4.** Interleukin-4 (IL-4) and IL-10 production by the stimulated splenocytes of the immunized mice. Culture supernatants from the total lysate antigen–stimulated immunized mice splenocytes were collected at 24 and 72 hours postincubation for the evaluation of (A) IL-4 and (B) IL-10 production, respectively, via enzyme-linked immunosorbent assay. Data are expressed as mean ± standard deviation (N = 3). Statistical difference is represented by * (P < 0.05) in comparison with the control groups (phosphate-buffered saline [PBS] or pcDNA 3.1C).
Our previous studies using subunit vaccines GRA2 and GRA5 showed partial protection in infected mice.23 In these instances, both humoral and cellular immunity were successfully mounted with relatively lower IFN-γ and higher IL-10 levels in comparison with DNA vaccination. This observation is supported further by the various investigations that observed increased mortality and susceptibility rates toward *T. gondii* infection in mice lacking anti-inflammatory cytokines (IL-4 and IL-10) but high level of pro-inflammatory cytokine (IFN-γ).57–59

**CONCLUSION**

Intramuscular vaccination of mice with DNA vaccine triggered Th1/Th2 response with predominant Th1-directed response associated with significant elevation of IFN-γ and IL-2 levels, but relatively low levels of IL-4 and IL-10. Humoral immunity was not elicited in the vaccinated mice, which subsequently succumbed against *Toxoplasma* challenge. Despite the successful induction of cell-mediated immunity, failure to trigger humoral immunity has become one of the limitations in this study. Therefore, in the future studies, it will be worthwhile to evaluate the efficacy of recombinant multi-antigen incorporating full-length GRA2 or/and GRA5 with several other antigenic proteins such as the surface and rhoptry antigens.