

Construction, Expression and Preliminary Immunological Evaluation of a DNA Plasmid Encoding the GRA2 Protein of *Toxoplasma gondii*

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ABSTRACT

Toxoplasmosis is a worldwide infection which is commonly asymptomatic but can cause serious medical problems in immunocompromised individuals and fetus. The infection also causes considerable economic loss because of abortion in livestock, mostly in sheep and goats. DNA vaccination may be a powerful approach against intracellular parasites such as *Toxoplasma gondii*. The goal of this study was to construct and evaluate the functionality of an eukaryotic expression plasmid pRC/CMV-GRA2, harboring dense granule antigen-2 (GRA2) gene of *T. gondii* and to perform preliminary studies on its immunogenicity in a mouse model. The GRA2 complete cDNA was inserted in PCR2.1 plasmid, sequenced, then cut and inserted in pRC/CMV plasmid, to produce the recombinant plasmid pRC/CMV-GRA2 (pGRA2). To verify that the plasmid construct pGRA2 was capable of expressing GRA2 in mammalian cells, it was transfected into 293-T cells, an embryonic kidney cell line. Western-blot analysis of the transfected cells using a monoclonal antibody specific for GRA2 indicated specific expression of GRA2 protein. CBA/J mice were subcutaneously immunized three times with 100 µg of pGRA2 plasmid. The obtained sera recognized GRA2 that is shown by Western-blotting. These findings indicate that pGRA2 plasmid directs high-level expression of antigenic GRA2 protein in mammalian cells and is immunogenic in CBA/J mice. *Iran. Biomed. J.* 9 (1): 1-8, 2005

Keywords: *Toxoplasma gondii*, Dense granule antigen-2 (GRA2), DNA vaccine, Transfection, Immunization

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects human and other warm-blooded animals. Although usually asymptomatic in immunocompetent individuals, toxoplasmosis may cause severe disorders in immunocompromised patients (e.g., AIDS patients and organ transplant recipients) and in pregnant women because of the high risk of transplacental transmission and the occurrence of multiple congenital lesions in the fetus [1]. Toxoplasmosis can be effectively treated with a combination of pyrimethamine plus sulfadiazine. However, a significant occurrence of adverse reactions to this therapy, particularly in AIDS patients often results in discontinuation of therapy

and relapse of the disease. In pregnant women, serologic screening is necessary to identify newly acquired infection, because primary infection is largely asymptomatic and therapy with pyrimethamine must be avoided during the first 16 weeks because of potential teratogenicity [2]. These considerations are compelling arguments for the development of a vaccine against toxoplasmosis.

So far, the only developed vaccine is the live, attenuated tachyzoite S48 [3]. However, this vaccine is not widely accepted because of its adverse effects, short shelf-life, and high cost. Live vaccines also carry a risk of accidental infection of humans and unexpected harmful reverse mutations. To overcome these problems, current research is investigating subunit, recombinant and DNA vaccines, but they do not provide complete

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protection against *T. gondii* infection. It has been hypothesized that “naked” DNA vaccines in combination with a potent adjuvant and targeted administration, via appropriate delivery system, may result in a significant level of protective immune response against pathogenic effects of *T. gondii* [reviewed in 4 and 5]. Such vaccines are likely to be useful for preventing the spread of toxoplasmosis worldwide since an available panel of field isolates from Asia, Europe and American continent exhibited the prevalence of only one immunotype of Toxoplasma [6, 7].

We have focused on the development of a DNA-based vaccine because such vaccines have been shown to elicit a potent, long-lasting humoral and cell-mediated immunity, as well as providing protection against viral, bacterial, and parasitic infections. Several trials of DNA-based vaccines against toxoplasmosis have been conducted, mainly in mice and using various *T. gondii* antigens, such as membrane-associated surface antigen SAG1 [2, 8], excreted-secreted dense-granule proteins GRA1 [9, 10], GRA4 [1] and GRA7 [10], and rhoptry proteins ROP1 and ROP2 [10-12]. These trials have been encouraging, in that they have demonstrated the development of different levels of protection in mice.

The GRA proteins were first described as components of the excretory secretory antigens (ESA) released by the parasites when incubated with serum [13]. These proteins may be important protective antigens since they are secreted in abundance and are major components of both the vacuole surrounding tachyzoite and the cyst wall surrounding the more slowly growing bradyzoite [14]. GRA2 is expressed by both tachyzoite and bradyzoite stages, and immunization with purified GRA2 has been shown to induce both a vigorous antibody and T-cell response and partially protects against acute infection. [15-18]. To date, there is no data regarding immunogenicity and vaccine potential of naked recombinant GRA2 gene.

In this article, the expression and immunogenicity of the recombinant GRA2 in the form of antigen encoding plasmid DNA were evaluated as a novel basis toward developing a DNA-based vaccine for prevention and/or treatment of toxoplasmosis. Hence, a construct harboring the full-length GRA2 cDNA (pGRA2) was cloned and tested for expression in mammalian cell culture and immunogenicity in susceptible mice injected subcutaneously with the naked plasmid DNA.

MATERIALS AND METHODS

Parasite. *T. gondii* tachyzoite of the RH wild-type strain was propagated in human foreskin fibroblast (HFF) using DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamine and 10% FCS (all from Gibco, USA). Parasites were harvested after complete lysis of the monolayer, purified through 3.0 µm filters, and washed in phosphate-buffered saline (Gibco, USA).

Plasmid construction and purification. The full-length GRA2 cDNA was amplified using a pBluescript-GRA2 plasmid [19] (kindly provided by Marie-France Cesbron-Delow). To introduce the Kozak translational consensus sequence [20] in amplified product, the following primers were designed:

G2F: 5' GCC ACC ATG TTC GCC GTA AAA CAT TG 3'

G2R: 5' TCA TGT CAA TAA TTC GTC TGC C 3'

Cycling conditions for amplification was 95°C for 4 min, followed by 25 cycles at 94°C for 1 min, 65°C for 30 s, and 72°C for 1 min and the final primer extension at 72°C for 20 min. PCR products were electrophoresed on 0.8% agarose gel, the corresponding band was cut and the PCR product was purified from agarose gel (Kilobase DNA Marker from Amersham Pharmacia Biotech, USA). Restriction analysis was performed on the purified PCR product using Taq I and *Rsa* I enzymes. Fresh purified PCR products were ligated into vector PCR2.1 (Invitrogen, USA) provided in a T/A cloning kit and then transformed into competent DH5α bacterial cells. Recombinant clones were confirmed by restriction analysis; one positive clone was sequenced and subcloned into pRC/CMV eukaryotic expression plasmid (Invitrogen, USA) at *Hind* III and *Bst* XI restriction sites to generate the final construct pGRA2.

The plasmids were purified from transformed *Escherichia coli* DH5α by anion exchange chromatography (Endofree plasmid Mega kit; Qiagen, Germany) as specified by the manufacturer. The purified plasmids were dissolved in sterile endotoxin free PBS, pH 7.2 (Sigma, Germany) and stored at -20°C. The integrity of the DNA plasmids was checked by agarose gel electrophoresis after digestion with appropriate restriction enzymes. The DNA concentration was determined by measuring the optical density at A₂₆₀ nm wavelength. The

A260/280 ratio for the purified plasmids was 1.8 to 1.95 indicating that preparation was free of any major protein contamination.

Expression of pGRA2 in vitro. The 293-T cells (NCBI C-498, National Cell Bank of Iran, Tehran, Iran) were transfected with either pGRA2, or a control plasmid, pRC/CMV, using Polyfect, a polycationic liposome reagent (Qiagen, Germany) according to the company instructions: (i) The day before transfection, approximately 3.5×10^5 cells were seeded in 35 mm wells containing 2 ml of DMEM medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamine and 10% FCS (all from Gibco, USA) and incubated at 37°C in a humidified 5% CO₂ atmosphere. (ii) The cells were incubated until they were about 50 to 70% confluent. (iii) On the day of transfection, the culture medium was replaced shortly before adding polyfect/DNA mixture with 1.5 ml of complete fresh medium. (iv) The mixture of polyfect/ DNA was prepared as follows: 4 µg DNA was diluted with the culture medium without serum and antibiotics to a final concentration of ≈ 50 ng/µl and was mixed with 10 µl of Polyfect reagent. (v) The mixture was then incubated at room temperature for 15 min to let the Polyfect/DNA complex to form. (vi) The Polyfect/DNA complex was diluted with about 400 µl of complete DMEM medium and was added quickly and dropwise to the culture. (vii) The cells were incubated for 16 to 24 h before replacing the transfection medium with 2 ml of fresh complete growth medium. After 3 days, cell monolayers were washed 3 times with 5 ml of PBS and scraped into 1 ml of PBS. The cells were then pelleted by centrifugation at 13,000 g for 15 min and cell pellets were stored at -20°C until further analysis.

Western-blot analysis. Western-blot analysis of transfected 293-T cells was done on cell pellets of single 35-mm wells. The pellet was suspended in 0.5 ml of SDS-PAGE sample buffer, sonicated, boiled for 5 min, and 20 µL was loaded onto a 13% polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane via electrophoresis, which was carried at 90 V for 1 h, using a transfer system (Bio-Rad, Hercules, CA) according to the conditions suggested by the supplier. The membranes were saturated for 1 h with 5% fat-free dried milk in PBS and probed with the monoclonal antibody Tg17-179 [21] diluted 1:15,000 against GRA2 in 5% fat-free dried milk. Bound antibodies were detected using peroxidase-conjugated goat anti-mouse secondary antibody (Jackson immuno-

research laboratories, West Grove, PA) diluted 1:20,000 in fat-free dried milk, and signals were detected using super signal ECL (Enhanced Chemiluminescence) system (Pierce chemical, Rockford, IL).

For Western-blot analysis of RH tachyzoites, $\approx 5 \times 10^6$ tachyzoites were loaded onto each lane. Electrophoresis and transfer were as described above, and the membrane was probed either with monoclonal antibody Tg17-179 (1:15,000 dilution) or with 1:50 dilutions of sera from immunized mice taken 2 weeks after the third immunization.

DNA immunization. Female CBA/J mice (H-2^k), 6 to 8 weeks old, were obtained from IFFA Credo (L'Arbresle, France) and maintained under conventional conditions in our animal house. The mice (5 per group) were injected subcutaneously (s.c.) in their hind footpad three times, three weeks apart using syringes with 301/2-gauge needle (Microlance; Becton Dickinson), with 100 µg of pGRA2 or pRC/CMV empty plasmid in 50 µl of sterile endotoxin-free PBS, pH 7.2 (Gibco, USA). A group of mice remained untreated as a negative control group. Mice were bled two weeks after the third immunization and sera were stored at -20°C until Western-blotting.

RESULTS

Cloning of GRA2 gene. GRA2 gene was amplified by PCR using a pBlue script plasmid [19] containing GRA2 cDNA as template and a pair of specific primers. The sequence preceding the initiation codon was modified from the original sequence of GRA2 gene to introduce the Kozak consensus sequence for optimal protein expression in mammalian cells.

Specific PCR product of 1,000 bp was obtained and viewed on 0.8% agarose gel (Fig. 1A). The PCR product was confirmed using Taq I and *Rsa* I restriction enzymes. Restriction analysis exhibited the presence of Taq I at 120 and 360 sites and of *Rsa* I at 120 site, respectively, (Fig. 1B and C) which are in agreement with GRA2 gene sequence. The amplified GRA2 cDNA was cloned in T/A cloning site of PCR2.1 plasmid and the recombinant PCR2.1-GRA2 was confirmed by both restriction analysis and sequencing.

The final candidate vaccine DNA clone, i.e., pGRA2, was obtained after subcloning GRA2 gene in the *Hind* III and *Bst*X I restriction sites of the eukaryotic shuttle expression vector, the pRC/CMV plasmid (Fig. 2).

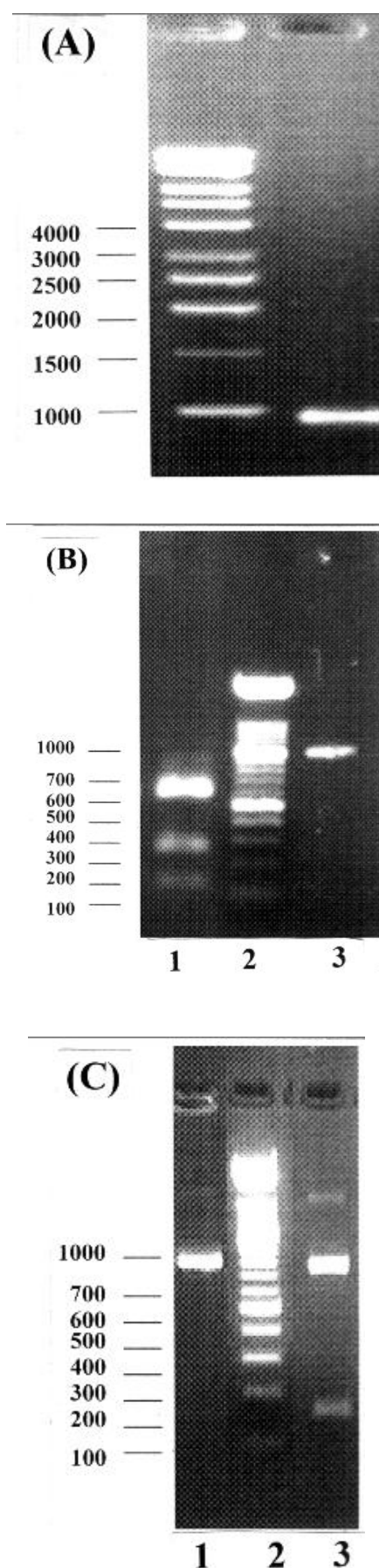


Fig. 1. (A), PCR amplification of GRA2 full length cDNA; (B), Restriction digestion of amplified segment by Taq I enzyme. Lane 1, digestion of PCR product by Taq I enzyme; lane 2, DNA molecular weight marker XIV (Roche molecular biochemical); lane 3, PCR product. (C), Restriction digestion of amplified segment by *Rsa I* enzyme. Lane 1, PCR product; lane 2, DNA molecular weight marker XIV (Roche Molecular Biochemicals); lane 3, digestion of PCR product by *Rsa I* enzyme.

Expression of GRA2 in mammalian cells. To confirm that the plasmid construct pGRA2 is functional and can direct expression of GRA2 in mammalian cells, it was transfected into 293-T epithelial cells, an embryonic kidney cell line. Empty plasmid pRC/CMV was transfected into 293-T cells as a negative control. The transfected cells were cultured for three days and washed; then the expression of GRA2 was assessed by SDS-PAGE and Western-blot analyses.

Western-blot analysis of the lysate from pGRA2-transfected 293-T cells showed two bands of ≈ 28 kDa (which is the same size as the native glycosylated GRA2) and ≈ 24 kDa (Fig. 3). The ≈ 24 kDa band probably corresponded to mature GRA2 protein without its putative signal peptide which has not been glycosylated. In contrast, no band was found in lysate of 293-T cells transfected with empty plasmid pRC/CMV (Fig. 3). Hence, pGRA2 appeared to direct the synthesis of antigenic GRA2 protein by mammalian cells *in vitro*.

Immunogenicity of pGRA2 in CBA/J mice. To examine immunogenicity of GRA2 gene, CBA/J mice were immunized with pGRA2 plasmid construct or the empty plasmid pRC/CMV and another group of mice remained untreated. Two weeks after the last immunization, 5 mice from each group were anaesthetized and sera were obtained from their retro-orbital sinus. SDS-PAGE and Western-blot analyses were performed to assess the presence of anti-GRA2 antibody. In the immunoblot analysis, a pool of sera from 5 mice immunized with pcGRA2 reacted strongly with a protein band at the expected molecular mass of GRA2 (28 kDa) (Fig. 4). In contrast, antibodies recognizing the 28-kDa antigen were absent in the sera of mice injected with control plasmid pRC/CMV or in non-immunized mice.

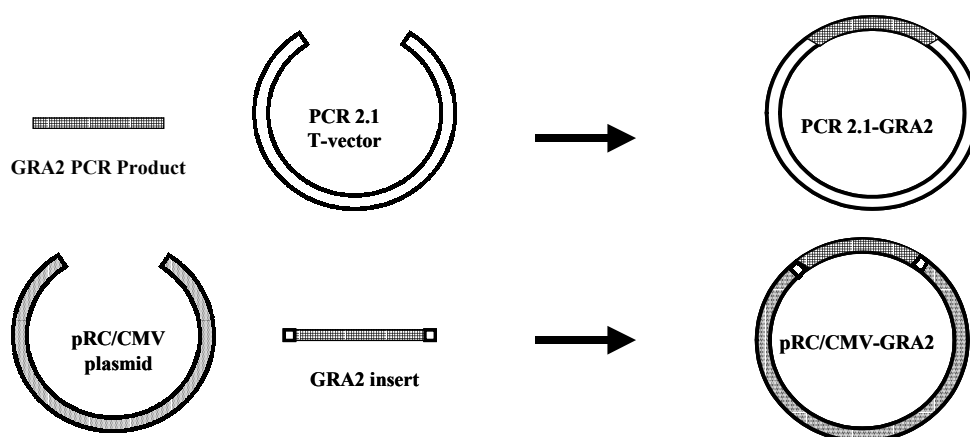


Fig. 2. Cloning steps of GRA2 gene. Schematic presentation of cloning steps of GRA2 PCR product in PCR2.1 plasmid and subcloning in pRC/CMV plasmid. For detail explanation see the materials and methods.

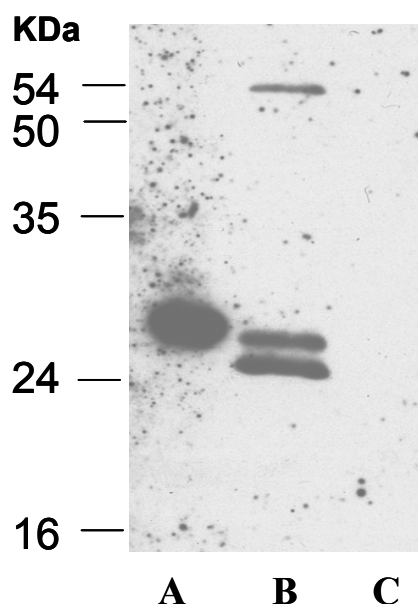


Fig. 3. Western-blot analysis of recombinant GRA2 protein of *Toxoplasma gondii* expressed in 293-T cells. Membrane was probed with GRA2-specific monoclonal antibody Tg 17-179 and developed with horseradish peroxidase-conjugated goat anti-mouse antibody and signals were detected using super signal ECL (Enhanced Chemiluminescence) system. Lane A, native RH strain SAG1; lane B, Lysate of 293-T cells transfected with pGRA2; lane C, lysate of 293-T cells transfected with pRC/CMV (negative control).

DISCUSSION

A GRA2-based DNA vaccine against toxoplasmosis was made since the previous works showed immunogenicity and vaccine potential of native GRA2 protein [15-18]. It has been shown

that immunization of mice with native GRA2 protein combined with Freund's complete adjuvant induced long term and almost complete protection against a lethal infection with tachyzoite of the moderately virulent C56 strain of *T. gondii* [15, 18].

The first steps in developing a DNA vaccine is making an antigen-encoding plasmid DNA followed by confirming expression of the corresponding antigen *in vitro* and *in vivo*. To permit optimal expression in mammalian cells, Kozak consensus sequence was introduced before GRA2 initiation codon. GRA2 complete cDNA was cloned in pRC/CMV plasmid under transcriptional control of the cytomegalovirus early promoter.

Transfection of human embryonic kidney cells, 293-T cells, with pGRA2 plasmid resulted in production of two protein bands of ≈ 28 and ≈ 24 kDa that were shown to be immunoreactive on Western-blot analysis with GRA2-specific monoclonal antibody Tg 17-179 (Fig. 3). We hypothesized that 293-T cell cellular machinery can recognize and cut GRA2 signal peptide and also glycosylates part of the mature GRA2. The considerable difference between sizes of the two bands probably refers to o-glycosylation of GRA2 which has been shown in other studies [22, 23]. To test the above explanation, we cloned GRA2 cDNA without its putative signal sequence in pRC/CMV plasmid and transfected the resulting plasmid construct pWSGRA2 in 293-T cells. Western-blot analysis of the transfected cells showed only one band at ≈ 24 kDa (data not shown) that is the same size of the lower band in the blot of pGRA2-transfected cells and represent mature GRA2 protein without glycosylation.

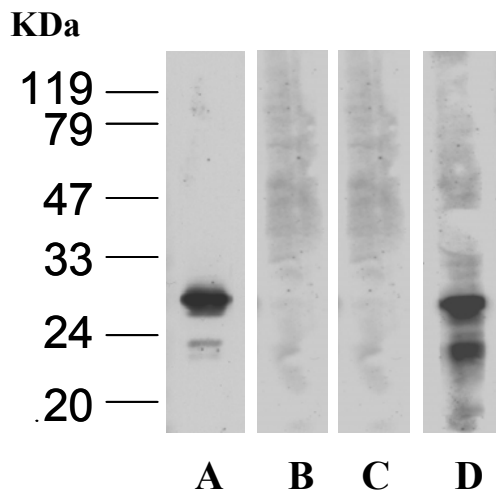


Fig. 4. Detection of specific anti-GRA2 IgG antibodies in sera of CBA/J mice immunized with pGRA2. Tachyzoites (5×10^6) were loaded on each lane. Lane A, membrane probed with GRA2-specific monoclonal antibody Tg 17-179; lane B, membrane probed with serum from a naïve mouse; lane C, membrane probed with Sera from mice injected with control plasmid pRC/CMV; lane D, membrane probed with sera from mice injected with recombinant plasmid pGRA2. Membranes were developed with horseradish peroxidase-conjugated goat anti-mouse antibody and signals were detected using super signal ECL (Enhanced Chemiluminescence) system. Molecular weight markers are shown at left. CMV, cytomegalovirus.

There is a protein band of ≈ 55 kDa in the blot of pGRA2-transfected 293-T cells that is probably related to the dimerization of GRA2 protein as it has been suggested by other researchers [19, 24]. We observed a similar protein in Western-blot analysis of *E. coli* expressed recombinant GRA2 (data not shown).

Therefore, 293-T cells are able to express antigenic GRA2 protein and recognize and cut GRA2 signal peptide. They can also glycosylate the expressed protein to rather the same extent as the native protein. Furthermore, we showed that the recombinant GRA2 protein produced *in vivo* by DNA immunization was immunogenic, because CBA/J mice immunized with pGRA2 produced anti-GRA2 antibodies that were able to recognize the native GRA2 protein found in *T. gondii*.

The observation that genetic immunization is able to elicit protective immunity [25] has fostered the development of a new generation of vaccines. DNA vaccines provide prolonged antigen expression, leading to amplification of the immune response, and appear to offer certain advantages,

such as ease of construction, low cost of mass production, high level of temperature stability, and the ability to elicit both humoral and cell-mediated immune responses [26, 27]. In addition, the endogenous expression of antigen from DNA introduced into host cells leads to peptide presentation with the major histocompatibility complex class I (MHC-I), which is ideal for induction of cytotoxic T-cell response. Therefore, DNA vaccines have been primarily considered for use against intracellular pathogens such as *T. gondii* [28].

The best candidates for vaccine antigens in protozoan parasites, such as *T. gondii*, appear to be surface and secreted antigens since these have been shown to be the major targets of the immune response in natural infections. Indeed, protective immunity has been achieved with both recombinant proteins and DNA vaccine versions of the major surface antigens SAG1 [2, 8, 29], SAG2 [30] and secreted dense granule proteins GRA1, GRA4 and GRA7 [1, 9, 10] and rhoptry proteins [10-12]. GRA2 reportedly induced strong humoral and cellular immune responses in human, as anti-GRA2 antibodies were present in about 95% of acutely infected and 80% of chronically infected people [16]. Prigione *et al.* [31] showed that most of the T-cell clones obtained from three chronically infected women were specific for SAG1 and GRA2 proteins that emphasized the ability of GRA2 in inducing specific cellular immune response and memory T-cell production. Furthermore, GRA2 is probably an important virulence factor as Mercier *et al.* [32] showed targeted disruption of the GRA2 locus in *T. gondii* decreases acute virulence in mice.

In conclusion, this study shows for the first time that making a plasmid DNA encoding the GRA2 protein of *T. gondii* is a realistic goal and that pGRA2 plasmid is able to direct synthesis of antigenic GRA2 protein in mammalian cells. Plasmid pGRA2 is also capable of inducing specific humoral response in immunized CBA/J mice. The next step would be evaluating the pGRA2 plasmid as a potential DNA vaccine to protect against Toxoplasmosis infection in mice.

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