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

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ABSTRACT

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

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

INTRODUCTION

T*oxoplasma 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 transplacental transmission and tl of multiple congenital lesions in Toxoplasmosis can be effectively t combination of pyrimethamine plu However, a significant occurrenc reactions to this therapy, particul patients often results in disco therapy and relapse of the disease women, serologic screening is

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identify newly acquired infection, because primary infection is largely asymptomatic and therapy with pyri-methamine 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 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.

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The GRA proteins were first described as components of the excretory secretary 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 pu
has been shown to induce both
antibody and T-cell response a
protects against acute infection. [15
there is no data regarding immun
vaccine potential of naked recom
gene.

In this article, the expr
immunogenicity of the recombinant
form of antigen encoding plasmi
evaluated as a novel basis toward
DNA-based vaccine for preven
treatment of toxoplasmosis. Henc
harboring the full-length GRA2 cD
was cloned and tested for e
mammalian cell culture and immu
susceptible mice injected subcuta
the naked plasmid DNA.

MATERIALS AND METH

Parasite. *T. gondii* tachyzoite of
type strain was propagated in hu
fibroblast (HFF) using DMEM
with 100 U/ml penicillin,
streptomycin, 2 mM Glutamine a
(all from Gibco, USA). Parasites w
after complete lysis of the monola
through 3.0 µm filters, and washed
buffered saline (Gibco, USA).

Plasmid construction and purifica
length GRA2 cDNA was ampli
pBluescript-GRA2 plasmid [19] (ki
by Marie-France Cesbron-Delow).
the Kozak translational consensus
in amplified product, the following
designed:

G2F: 5′ GCC[m1] ACC ATG TT
AAA CAT TG 3′

G2R: 5′ TCA TGT CAA TAA TT
C 3′

Cycling conditions for amp
95°C for 4 min, followed by 25 cycl
1 min, 65°C for 30 s, and 72°C for
final primer extension at 72°C for
products were electrophoresed on
gel, the corresponding band was
PCR product was purified from
(Kilobase DNA Marker from
Pharmacia Biotech, USA). Restri

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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 A_{260} 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% CO_2 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 th
The cells were incubated for 16 t
replacing the transfection medium
fresh complete growth medium. Aft
monolayers were washed 3 times
PBS and scraped into 1 ml of P
were then pelleted by centrifugati
for 15 min and cell pellets were st
until further analysis.

Western-blot analysis. Western-b
transfected 293-T cells was done on
single 35-mm wells. The pellet was
0.5 ml of SDS-PAGE sample buff
boiled for 5 min, and 20 µL was
13% polyacrylamide gel. P
transferred onto nitrocellulose m
electrophoresis, which was carried
h, using a transfer system (Bio-R
CA) according to the conditions su
supplier. The membranes were sat
with 5% fat-free dried milk in PB
with the monoclonal antibody T
diluted 1:15,000 against GRA2 in
dried milk. Bound antibodies v
using peroxidase-conjugated goa
secondary antibody (Jackson imm
laboratories, West Grove, PA) dil
in fat-free dried milk, and signals
using super signal ECL
Chemiluminescence) system (Pie
Rockford, IL).

For Western-blot analysis of Rl
≈5 × 10^6 tachyzoites were loaded o
Electrophoresis and transfer were
above, and the membrane was
with monoclonal antibody Tg17-
dilution) or with 1:50 dilutions
immunized mice taken 2 weeks a
immunization.

DNA immunization. Female CB
2^k), 6 to 8 weeks old, were obtaine
Credo (L'Arbresle, France) an
under conventional conditions in
house. The mice (5 per group)
subcutaneously (s.c.) in their hind
times, three weeks apart using
301/2-gauge needle (Microlar
Dickinson), with 100 µg of pGRA2
empty plasmid in 50 µl of sterile e
PBS, pH 7.2 (Gibco, USA). A g
remained untreated as a negative c
Mice were bled two weeks aft

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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 *BstX 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 biochemicals); 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 kid Empty plasmid pRC/CMV was tr 293-T cells as a negative co transfected cells were cultured fo and washed; then the expression assessed by SDS-PAGE and analyses.

Western-blot analysis of the pGRA2-transfected 293-T cells bands of ≈ 28 kDa (which is the sa native glycosylated GRA2) and ≈ 24 The ≈ 24 kDa band probably correspon GRA2 protein without its putative which has not been glycosylated. no band was found in lysate o transfected with empty plasmid pR 3). Hence, pGRA2 appeared t synthesis of antigenic GRA2 mammalian *in vitro*.

Immunogenicity of pGRA2 in CB examine immunogenicity of GRA2 mice were immunized with pG construct or the empty plasmid pl another group of mice remained ur weeks after the last immunization each group were anaesthetized a obtained from their retro-orbital PAGE and Western-blot an performed to assess the presence antibody. In the immunoblot analy sera from 5 mice immunized w reacted strongly with a protein expected molecular mass of GRA2 4). In contrast, antibodies recogn kDa antigen were absent in the injected with control plasmid pR non-immunized mice.

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Fig. 2. Cloning steps of GRA2 gene. Schematic presentation of cloning steps of GRA2 PCR product in PCR2.1 plasmid 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

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A GRA2-based DNA vaccine against to[m2]xoplasmosis 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 plasmi production of two protein bands of kDa that were shown to be immu Western-blot analysis with GRA2-s clonal antibody Tg 17-179 (Fig. thesized that 293-T cell cellular m recognize and cut GRA2 signal pe glycosylates part of the mature considerable difference between si bands probably refers to o-gly GRA2 which has been shown in [22, 23]. To test the above explanati GRA2 cDNA without its putative si in pRC/CMV plasmid and tra resulting plasmid construct pWSG cells. Western-blot analysis of th cells showed only one band at ≈ 24 shown) that is the same size of the the blot of pGRA2-transfecte represent mature GRA2 pro glycosylation.

Fig. 4. Detection of specific anti-GRA2 I sera of CBA/J mice immunized with pGR (5×10^6) were loaded on each lane. La probed with GRA2-specific monoclonal 179; lane B, membrane probed with ser mouse; lane C, membrane probed with injected with control plasmid pRC/ membrane probed with sera from mi recombinant plasmid pGRA2. Membranes with horseradish peroxidase-conjugated antibody and signals were detected using s (Enhanced Chemiluminescence) system. M markers are shown at left. CMV, cytomega

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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 in were specific for SAG1 and GRA2 emphasized the ability of GRA2 specific cellular immune response T-cell production. Furthermor probably an important virulen Mercier *et al.* [32] showed targeted the GRA2 locus in *T. gondii* de virulence in mice.

In conclusion, this study shows time that making a plasmid DNA GRA2 protein of *T. gondii* is a rea that pGRA2 plasmid is able to dire antigenic GRA2 protein in man Plasmid pGRA2 is also capable specific humeral response in immu mice. The next step would be e pGRA2 plasmid as a potential DN protect against Toxoplasmosis infec

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