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Original Article

Immunogenic multistage recombinant protein vaccine confers partial protection against experimental toxoplasmosis mimicking natural infection in murine model

Yaprak Gedik [a,b,c], Sultan Gülçe İz [a], Hüseyin Can [d], Aysu Değirmenci Döşkaya [b], S. İsmet Deliloğlu Gürhan [a], Yüksel Gürüz [b], Mert Döşkaya [b,*]

[a] Ege University, Faculty of Engineering, Department of Bioengineering, Bornova, İzmir 35100, Turkey
[b] Ege University, Faculty of Medicine, Department of Parasitology, Vaccine Research and Development Laboratory, Bornova, İzmir 35100, Turkey
[c] AtaFen Inc., Veterinary Vaccine Production Plant, Kemalpaşa, İzmir, Turkey
[d] Department of Molecular Biology, Ege University Faculty of Sciences, Bornova, İzmir 35100, Turkey

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ABSTRACT

Toxoplasma gondii is a protozoan parasite that can infect warm-blooded animals including humans. Vaccination studies mostly use tachyzoite specific proteins however in natural route of infection, toxoplasmosis initiates with tissue cysts (bradyzoites) or oocysts (sporozoites) and thereafter stage conversion takes place where the tachyzoites take action and cause acute infection continues with tachyzoites. Despite this knowledge, challenging models used in the vaccination studies prefer administration of tissue cyst forming strains intraperitoneally or subcutaneously instead of oral administration which is the natural route of infection. In the present study, a multivalent adjuvanted recombinant protein vaccine that contains bradyzoite specific BAG1 and tachyzoite specific GRA1 protein and controls were administered to female *Swiss Webster outbred* mice. Humoral and cellular immune responses were analyzed by Rec-ELISA, Western blot, and flow cytometry. Mice were infected orally with *T. gondii* PRU strain tissue cysts using feeding needle to mimic the natural route of infection. 40 days after challenging microscopy and Real Time PCR were performed to determine the protection level.

Analysis of sera obtained from vaccinated mice showed strong *anti*-BAG1 and *anti*-GRA1 IgG responses. The IgG2a response was significantly higher ($P < 0.0001$) and the ratio of CD8 + T lymphocytes secreting IFN-γ almost doubled compared to PBS control which are indicative of protection against toxoplasmosis. The amount of tissue cysts in vaccinated group was reduced 10.5% compared to control group.

To generate a protective vaccine against toxoplasmosis, multistage vaccines and usage of challenging models mimicking natural route of infection are critical cornerstones. In this study, we generated a BAG1 and GRA1 multistage vaccine that induced strong immune response in which the protection was not at anticipated level. In addition, the murine model was orally challenged with tissue cysts to mimic natural route of infection.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a broad range hosts including nucleated cell types from all warm-blooded vertebrate hosts [1,2]. *T. gondii* infects human and causes serious problems including congenital toxoplasmosis in newborns which displays as malformations in unborn children, toxoplasmic retinochoroiditis which leads to blindness, and reactivation of the parasite in immunologically impaired individuals leading to fatal toxoplasmic encephalitis. Currently, toxoplasmosis is linked to schizophrenia and other mental disorders [1,2]. *T. gondii* is acquired via eating tissue cyst contaminated raw meat and water or food contaminated with oocysts released from the infected cats. On the other hand, the veterinary importance of toxoplasmosis is very high, since abortion and neonatal loss in sheep are important problems resulting with economic loss. Toxoplasmosis is an important disease for not only sheep but also pigs. Transmission from pork meat is one of the most important transmission

* Corresponding author at: Ege University, Faculty of Medicine, Department of Parasitology, Vaccine Research and Development Laboratory, Bornova, Izmir 35100, Turkey.
E-mail address: mert.doskaya@ege.edu.tr (M. Döşkaya).

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routes [3,4]. Therefore, development of an effective vaccine against toxoplasmosis that can be used in animals or humans is valuable.

Natural infection with *T. gondii* initiates with sporozoites (in oocysts) or bradyzoites (in tissue cysts). In a couple of days, with stage conversion, tachyzoites formed from either bradyzoites or sporozoites expand the acute illness [5]. After the acute illness, tachyzoites change into bradyzoites and ultimately become tissue cysts to be dormant in tissues especially central nervous system or retina. Although there are three stages of the parasite taking action during the acute illness, almost all of the vaccine studies aim to develop protection against tachyzoite form of the parasite which invade the host cell shortly and embed themselves in a protective parasitophorous vacuole in the host cell straightaway whereby avoid from the host immune response. Thus, a vaccine against toxoplasmosis, caused by tissue cysts is required to protect the initially from bradyzoite and subsequently tachyzoite invasion and must contain specific antigenic proteins from both stages of the parasite.

Using recombinant proteins as vaccine antigens against toxoplasmosis has become popular after 1990s. Initially SAG1 which is SRS (SAG1 related sequences) family protein presented on the surface of the parasite and dense granule protein GRA1 were evaluated [6,7]. After year 2000, GRA7 and rhoptry protein ROP2 [8], other SRS family members SAG2, SAG3, SRS1 [9], some heat shock proteins HSP70, HSP30 (BAG1) [10], microneme proteins MIC1, MIC2, MIC3, MIC4, MIC6 [11–14] were added to the list. Some more dense granule proteins (GRA2, GRA4, GRA5, GRA6) [15–17], AMA1 (Apical membrane antigen-1) [13], MAG1 (Cyst matrix protein) [18], serine protease inhibitor-1 [19], and finally rhoptry protein ROP4 [20] were also used as vaccine antigens. After 2010, the variety of proteins increased rapidly by addition of more microneme proteins (MIC8, MIC11, and MIC13) [21–23], rhoptry proteins (ROP1, ROP5, ROP8, ROP9, ROP 13, ROP16, ROP17, ROP18, and ROP38) [24–32], RON2, RON4 [33,34], SAG2CDX, SAG5D, SRS4, and SRS9 from SRS family [35–38] and some others such as PLP1 (perforin-like protein 1) [39], IMP1 (immune mapped protein-1) [40], 14-3-3 protein [41], ROM1 (rhomboid protein 1) [42], CDPK3 (calcium-dependent protein kinase 3) [43], eIF4A (Eukaryotic translation initiation factor) [44], eIF2α (eukaryotic initiation factor-2α) [45], CyP (cyclophilin), [46] cathepsin B and L like proteases [47], aspartic protease 1 [48], cyclophilin [49], PDI (protein disulfide isomerase) [50], ACT (Actin) [51], CDPK5 (calcium-dependent protein kinase 5) [52], DPA (Deoxyribose Phosphate Aldolase) [53], RACK-1 (receptor for activated C kinase 1) [54], and GST (Glutathione-S Transferase) [55]. Most of these vaccine antigens were chosen randomly without taking into account of *T. gondii*'s multistage property and consequently did not confer the desired immune responses.

Overall, development of a multivalent vaccine against different life cycle stages of *T. gondii* could be more efficient. *T. gondii* BAG1 and GRA1 proteins have important roles in parasite's life cycle. It is well known that BAG1 is expressed by the bradyzoite form of the parasite which promotes the differentiation of tachyzoites to bradyzoites and takes role at inducing dendritic cells and activating cellular immune response [13,56,57]. GRA1 protein is expressed by tachyzoite, bradyzoite and sporozoite forms which is excreted to parasitophorous vacuole during the invasion of host cell for stability of the parasite through parasitophorous network [58]. Previously, GRA1 and BAG1 genes were used individually as vaccine candidate antigens in DNA vaccination or recombinant protein vaccination studies and induced protective immune responses [7,8,10,13,18,36,59–62].

The other cornerstone to generate a protective vaccine is the selection of appropriate challenging model. The main route of challenging differs in each study. However, naturally acquired infection occurs through ingestion of tissue cysts and oocyst in animals and humans [63,64]. Thus, when tissue cyst forming strains of *T. gondii* are used, oral infection through curved feeding needle can be preferred which is demonstrated in this article.

In the present study, due to their active role during the invasion of host cell as well as their immunogenic and protective efficacy in vaccination studies, GRA1 and BAG1 proteins were selected as vaccine candidate antigens to generate a multivalent recombinant protein vaccine to sequentially block the bradyzoite and tachyzoite stages of the parasite. In addition, vaccinated and control group mice were challenged orally with tissue cyst forming *T. gondii* PRU strain to mimic natural route of infection.

2. Materials and methods

2.1. Ethic statement and animal studies

All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms. For vaccination studies, 6–8 weeks old female *Swiss Webster outbred* mice were obtained from the Bornova Veterinary Control Institute Animal Production Facility and housed under standard and suitable conditions during the experiments.

2.2. Plasmids

pET28a/BAG1 and pET28a/GRA1 plasmids (Novagen, USA) used to express recombinant BAG1 protein (rBAG1) and GRA1 protein (rGRA1) were generated as described [65]. pET28a/BAG1 and pET28a/GRA1 plasmids are transformed into *E. coli* BL21 Star (DE3) pLysS chemically competent cells (Invitrogen, USA) as described [59]. Plasmids from overnight culture were isolated using purification kit according to the manufacturer's protocol (Qiagen, USA), visualized by agarose gel electrophoresis.

2.3. Protein expression and purification

E. coli BL21 Star (DE3) pLysS cells containing pET28a/BAG1 and pET28a/GRA1 were inoculated into 500 ml LB medium supplemented with antibiotics (50 μg/ml kanamycin and 50 μg/ml chloramphenicol) and incubated overnight at 37 °C with vigorous shaking (220 rpm). Next day, overnight culture was inoculated to bioreactor (Bioflo 110, New Brunswick, USA) with enrichment medium. The dissolved oxygen and pH levels were maintained at 40–60 and 7.0 ± 0.4 with vigorous mixing (400 rpm) at 37 °C until OD_{600} reached 0.4. Then, the cells were induced with isopropyl-D-thiogalactopyranoside (IPTG) to the final concentration of 0.5 mM overnight at 18 °C.

The cells were harvested by centrifugation at 5000×*g* after 16 h and the resuspended pellet with 50 ml pre-chilled loading buffer (50 mM Tris–Cl pH: 7.5 and 0.3 M NaCl) was homogenized using a blender (Waring, USA). The homogenized cells were disrupted twice with an M-110L microfluidizer processor (Microfluidics, USA) at a low temperature under internal pressure of 18,000 psi and centrifuged at 30,000×*g* for ½ h at 4 °C. The clarified supernatant was filtered through 0.45 μm filter (Corning, USA). Purification of the protein was performed by AKTA FPLC, a liquid chromatography system, which is controlled by UNICORN™ software (GE Health, USA). Clarified supernatant was applied to the HiTrap column (GE Health, USA) with loading buffer. Then, the column was washed with 125 mM imidazole containing 50 mM Tris–Cl, pH 7.5, and 0.3 M NaCl. The recombinant proteins were eluted by raising the imidazole concentration to 375 mM. The protein fractions were detected by UV280, confirmed by 12% SDS–PAGE, pooled, and concentrated with Vivaspin 20 (Sartorius, Germany)