

Levels of Transforming Growth Factor-Beta After Immunization of Mice With *in vivo* prepared *Toxoplasma gondii* Excretory/Secretory Proteins

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Background: Zoonotic parasite *Toxoplasma gondii* has a high prevalence in human populations. A suitable vaccine for animals can stop the transmission of infection between animal and human.

Objectives: The aim of this study was to evaluate *in vivo* prepared excretory/secretory antigens (E/SA) as a potential candidate for immunization against the parasite and its effect on the production of transforming growth factor-beta (TGF- β).

Materials and Methods: *Toxoplasma gondii* tachyzoites were inoculated in the peritoneal cavity of mice and E/SA was harvested and used in animal immunization with and without adjuvant. Serum levels of anti-E/SA antibodies and TGF- β were measured in days 0, 3, 7, 14, 28 and 56 after immunization using ELISA technique. The measurements were statistically analyzed.

Results: Our results showed that the serum levels of anti-E/SA immunoglobulins significantly increased in all of the immunized groups. The differences of the serum levels of TGF- β between the groups were statistically significant at days 28 and 56 after immunization with E/SA.

Conclusions: Based on our study, *in vivo* prepared E/SA may be considered as a good candidate for animal immunization.

Keywords: *Toxoplasma*; Immunization; Transforming Growth Factor-beta; Excretory-Secretory Antigen

1. Background

Toxoplasmosis is a common worldwide parasitic infection caused by *Toxoplasma gondii* (1, 2). While toxoplasmosis has mild clinical presentations in immuno-competent individuals, in immuno-suppressed adults or congenitally-infected infants the disease is associated with severe pathological complications and even death (3). *Toxoplasma gondii* can be mainly transmitted to humans through ingesting undercooked warm-blooded livestock meats, containing tissue cysts, as well as foods or water contaminated with cat feces harboring oocysts (4). The transmission can also occur via contaminated milk (5). Therefore, immunization of animals against *T. gondii* may decline the risk of human infections. Previous studies have used whole inactive tachyzoites (6), purified and recombinant *T. gondii* proteins, and DNA Molecules (7-9) to make an appropriate immunization against *T. gondii* in animals. None of the abovementioned vaccines could lead to a successful immunization against the parasite.

Toxoplasma gondii excretory-secretory antigens (E/SA), comprising more than 90% of the total parasite circulating antigens, are the primary targets for the host im-

mune system (2, 10). E/SAs are the most important toxoplasma antigens which form the majority of circulating antigens in the sera from hosts with acute toxoplasmosis. It seems that E/SA plays an important role in inducing appropriate humoral and cellular immune responses against *T. gondii*. Although E/SAs are proteins identified in tachyzoites, sporozoites and encysted bradyzoite stages, there is evidence that ESAs released by tachyzoites are highly immunogenic compounds which induce both antibody-dependent and cell-mediated protective immunity, whereas E/SA secreted by bradyzoite causes long-lasting immunity to the parasite (10-13). Jongert et al., Hafid et al. and Darcy et al. have evaluated an E/SA derived vaccine candidate from an *in vitro* cell culture of *T. gondii* parasites (6, 13, 14). They have shown that E/SA can be considered as a good candidate for inducing immunization against *T. gondii*; however, not yielding 100% protection (6, 13, 14). Interestingly, Costa-Silva et al. demonstrated that E/SA derived from *in vitro* cell culture of *T. gondii* led to high IgG1, IFN- γ and TNF- α production (12). E/SA also induces apoptosis in T-regulatory lymphocytes which is

an important mechanism to elicit a potential immunization against infectious agents (15).

It seems that *in vitro* production of antigens cannot lead to appropriate glycosylation (16), and hence, the humoral immunity is unable to recognize native E/SA recovered from *in vitro* cultures. We previously reported that anti-*T. gondii* IgG antibody can recognize *in vivo* prepared E/SA (ivE/SA) (2). Therefore, using E/SA prepared from *in vivo* culture systems may be considered as a useful vaccine candidate for researchers in this field. Additionally, since cytokines are crucial factors that mediate many immune responses (17, 18), an appropriate immunization has to induce suitable cytokine levels. Transforming growth factor-beta (TGF- β) is a cytokines which plays key roles in the regulation of immune cell functions (19). This cytokine leads to inhibition of B and T lymphocytes proliferation and induces homeostasis (19). TGF- β also contributes to tissue remodeling which happens after infections and injuries (20). This cytokine also contributes to development of Th17 and T-regulatory lymphocytes, which play significant roles in activation and suppression of immune responses, respectively, against parasite infections (20). Evaluation of TGF- β could be useful to follow up the status of immune responses in experimental animal models and a good immunization should decrease TGF- β production at the first days after immunization.

2. Objectives

The aim of this study was to evaluate *in vivo* prepared E/SA as an immunization candidate and its effects on the production of TGF- β as an anti-inflammatory cytokine in animal models.

3. Materials and Methods

3.1. Production of *in vivo* Prepared Excretory/Secretory Antigens

Five mice were infected with tachyzoites of *T. gondii* (RH strain) by peritoneal cavity inoculation. In day three after the inoculation, peritoneal fluids were taken and centrifuged (1000 \times g for 15 minutes). Afterwards, the supernatants were filtered and stored at -20°C as ivE/SAs. The ivE/SA was measured using the Bradford method.

3.2. Animal Models

The study was carried out on 8-10-week-old Balb/C female inbred mice, obtained from the Rafsanjan University of Medical Sciences animal house and kept under standard conditions. The mice were randomly divided into eight groups (with 8-10 mice in each group) as follows; group A was designated as control with no treatment; group B received 10 μ g of ivE/SA; group C received a solution of 10 μ g ivE/SA and 100 μ L complete Freund's adjuvant (CFA); group D received 100 μ L CFA; group E included mice infected with *T. gondii* which received 10

μ g ivE/SA; group F included mice infected with *T. gondii* which received a solution of 10 μ g ivE/SA and 100 μ L CFA; group G were infected with *T. gondii* and received 100 μ L CFA; and finally, group H received 100 mL normal saline solution. The mice were immunized with ivE/SA in days 0, 3, 7, 14, 28 and 56 and blood samples were obtained three days after the immunization from the tail veins of seven mice per each group and their sera were stored at -20°C until used for the specific antibodies and TGF- β analysis. Serum samples taken before the immunization at day 0 were utilized as negative controls.

3.3. Antibody Assay

Antibody against E/SA was measured using ELISA technique. ivE/SA was used for capturing specific antibodies against *T. gondii* in ELISA plates. The following protocol was used for antibody detection: after 16 hours incubation with ivE/SA at 4°C, the plates were washed five times and incubated for two hours with blocking buffer (including BSA) at room temperature, then washed five times. Serum samples including negative and positive controls were added to the plates after two hours of incubation at room temperature and the plates washed five times. Thereafter, horseradish peroxidase (HRP)-conjugated antimouse antibody was added to the plate wells and incubated for two hours at room temperature and washed five times. Finally, the substrates (Tetramethylbenzidine (TMB) and H₂O₂) were added for color development for 15 minutes and the reaction was stopped with addition of H₂SO₄ (2N). The cutoff value was calculated using optical density (OD) values of positive and negative controls. A serial dilution for the positive control was also used for efficacy calculation.

3.4. Cytokine Assay

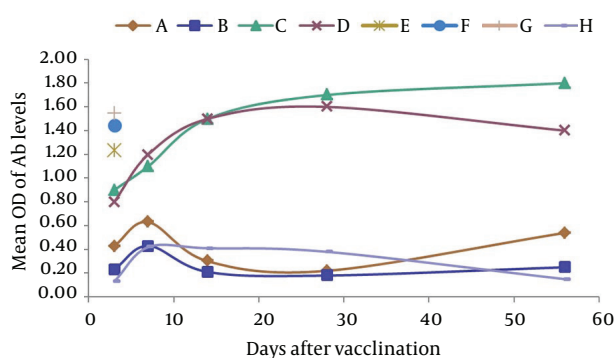
TGF- β serum levels of the mice were detected using a commercial ELISA kit (eBioscience, Spain) immediately after the blood collection, according to the manufacturer's guidelines. The sensitivity of the kit was 2 pg/mL and inter- and intra-assay assessments of reliability of the kit were conducted.

3.5. Statistical Analysis

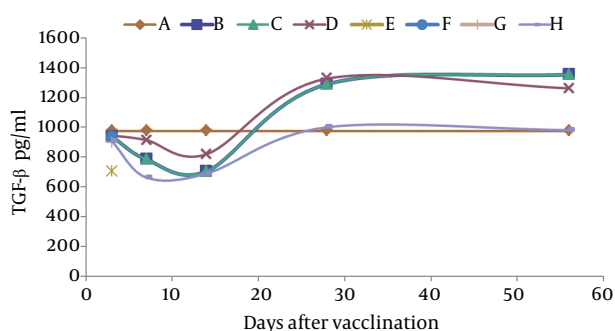
The mean differences of the serum levels of TGF- β and specific antibodies against *T. gondii* between the groups in a given time after immunization and in a group in different times were respectively analyzed by analysis of variance (ANOVA) and repeated ANOVA tests, using SPSS 20 software. When P value was less than 0.05, the difference was considered statistically significant.

4. Results

Results of the study showed that the serum levels of anti-E/SA immunoglobulins increased in all the groups, except for group H, compared to the control group A (Figure 1).

Figure 1. Serum Levels of Anti-E/SA Immunoglobulins in the Evaluated Groups

Y and X axis display the mean OD of the antibody levels and days after immunizations, respectively.

Figure 2. Serum Levels of TGF- β in the Groups

Y and X axis display the serum levels of TGF- β and the days after immunizations, respectively.

Additionally, all the mice in the groups E, F and G died after five days of inoculation, while the mice in other groups survived. Cytokine assays showed that the differences of the serum levels of TGF- β were not statistically significant among the groups at days 3, 7 and 14, but became significant only at days 28 and 56 after immunization with ivE/SA (Figure 2). The results also demonstrated that TGF- β serum levels significantly increased in group D only 28 and 56 days after immunization (Figure 2).

5. Discussion

Toxoplasmosis is a prevalent parasitic infections, transferred from animals to human (2). Therefore, a good vaccine for stopping the parasite life cycle in animals is essential. Our study showed that *in vivo* prepared E/SA could induce anti-E/SA antibodies in mice, and thus, it can be considered as a good vaccine candidate for animal immunization. Furthermore, we found that the serum levels of TGF- β did not changed in days 3, 7 and 14 after ivE/SA inoculation. Based on the fact that TGF- β has anti-inflammatory effects on immune cells as well as on

antibody production (19), it seems that immune cells at the early days after immunization are active and respond against E/SA.

Our data regarding anti-E/SA also confirmed this statement. After describing the elevated levels of TGF- β at days 28 and 56 after the immunization, it may be concluded that since the immune system needs to decline immune responses to E/SA and go to homeostasis, TGF- β was produced by the immune cells to achieve this goal as a normal immune system response. Previous studies showed that *T. gondii* induced TGF- β secretion by immune cells as an anti-inflammatory agent to decrease the autoimmune responses and the serum levels of the cytokine increased at early phases of infection (21, 22). Another investigation also demonstrated that *T. gondii* induced macrophages apoptosis through autocrine TGF- β signaling (23). E/SA also induced apoptosis in T regulatory lymphocytes, which are important cells to produce TGF- β (15). Therefore, it may be concluded that ivE/SA inhibited the elevation of TGF- β via induction of apoptosis in T regulatory lymphocytes.

Additionally, our results revealed that both ivE/SA and live *T. gondii* induced a good level of anti-ivE/SA production, but the *T. gondii* infected mice died after seven days. It appears that during the infection, *T. gondii* proliferates and attacks the parenchyma tissues and antibody production after *T. gondii* proliferation is unable to inhibit the disease. Therefore, *in vivo* prepared E/SA can be considered as a good candidate for animal immunization, mainly due to the good level of anti-E/SA induction as well as none-increasing TGF- β at the early phases of immunization. Other investigations have used other types of immunization including DNA vaccines (24, 25), purified protein (26), and subunit vaccines (27) against *T. gondii*. Similar to our study, antibodies against *T. gondii* increased in all the studies and cytokine assays showed increase in inflammatory and decrease in the anti-inflammatory cytokines (24-27). For instance, Costa-Silva et al. revealed that E/SA derived from *in vitro* cell culture of *T. gondii* elicited high levels of IgG1 and proinflammatory cytokines such as IFN- γ and TNF- α (12).

Costa-Silva et al. also reported that E/SA derived from *in vitro* cell culture of *T. gondii* increased the survival of infected mice (12). Several studies have also evaluated E/SA derived from cell cultures and have reported that these antigens have been useful for eliciting good levels of immune responses in immunized animals (10, 13, 28-30). The preferences of our study may include the followings: a) since E/SA was prepared from an *in vivo* approach, it may induce anti-E/SA response with higher avidity; b) the preparation of E/SA in mice peritoneal cavity is cheaper and easier than other methods. Interestingly, our previous study also showed that serum levels of IL-10, an anti-inflammatory cytokine, did not change at the early phases of immunization with ivE/SA (11).

Finally, *T. gondii* has several mechanisms to escape from the host immune responses and it is necessary to evalu-

ate more aspects of immunization such as cellular immunity and animal survival.

According to our results, iVE/SA can be considered as a good candidate for immunization against *T. gondii* and further studies on human can elucidate the immunogenicity of iVE/SA in comparison with E/SA derived from *in vitro* cell culture of *T. gondii*.

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Authors' Contributions

Seyed Hossein Abdollahi, Mohammad Kazemi Arababadi and Mohammad Zare-Bidaki: study design and supervision, literature review and manuscript writing and revision. Fateme Ayoobi, Hossein Khorramdelazad, Behzad Nasiri Ahmadabadi and Mohammadtaghi Rezayati: data collection, cytokine and immunoglobulins assays, animal practice and manuscript writing and review.

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