Protective Role of Antigens from Peritoneal Exudates of Infected Mice against Toxoplasmosis

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ABSTRACT

Background: Toxoplasma gondii is an obligate intracellular parasite that infects all mammalian cells. Several antigens such as excreted/secreted antigens have been identified as a potential vaccine candidate. **Objective:** To determine how excreted/secreted antigens from peritoneal exudates of infected mice (mESA) stimulate cell-mediated immune responses and induce protective immunity against toxoplasmosis in the murine model. **Methods:** The supernatants produced from the peritoneal fluids, were fractionated by precipitation in ammonium sulphate solution (30-80% saturated). For induction of cell-mediated immune responses, delayed type hypersensitivity was measured, in injected footpad. Response to purified antigen was measured by lymphocyte proliferation assay. Nitric oxide was measured by Griess method. For immunization, Balb/c mice were immunized 2 times with mESA, mESA-40% and Toxoplasma Lysate Antigen (TLA). The virulent RH strain of Toxoplasma gondii was used for challenging. **Results:** The pattern of lymphocyte responsiveness was dependent on the antigen employed. In sensitized mice, those received mESA-40% displayed higher lymphocyte response than mice stimulated by mESA ($p<0.05$). The highest amounts of nitric oxide were observed in macrophages, which received mESA-40% and mESA ($p<0.05$). Mice immunized with mESA-40% survived longer than those immunized with mESA and other antigens ($p<0.05$). **Conclusion:** As fraction 40% (mESA-40%) showed a good result in induction of cell-mediated responses in the murine model, the purification and isolation of the mESA 40% is highly recommended for future study.

Keywords: Toxoplasma gondii, Immune Responses, Excreted/secreted Antigens, Mice Peritoneal Exudates

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects all mammalian cells (1). Human infection is generally asymptomatic and self-limiting in immunocompetent hosts. These individuals remain chronically infected, the parasites persist encysted in brain and muscles, and develop life-long protective immunity against reinfection (1,2). Toxoplasmosis may cause abortion or neonatal malformations if contracted during pregnancy. Furthermore, this disease is often lethal for immunocompromised patients such as those with AIDS, neoplastic diseases, and bone marrow or heart transplant recipients (3). The role of cell mediated immunity against acute and chronic Toxoplasma infection is well characterized (4-6). Vaccines based on killed organisms have been unsuccessful in producing effective immunity even against a challenge with an avirulent strain of the parasite (7).

Several T. gondii antigens, such as the major immunodominant surface antigen SAG-1 (8-10) and excreted/secreted antigens (ESA) have been identified as potential vaccine candidates. They are also thought to play an important role in the pathogenesis and immune escape of the parasite (11).

The ESA confers a significant protection against a lethal challenge with the 76 K strain cysts in mice by direct immunization and in nude rats by passive transfer of immune sera or T cells (12,13).

The ESA is able to stimulate a better cell mediated immune response compared with soluble or cyst antigens. Therefore, this antigen is a good candidate for immunizing against T. gondii infection (14). These studies have been performed on total–ESA of T. gondii.

Supernatants of cell cultures infected with T. gondii have been the main source of ESA for investigations. The main goal of the present study was to determine how excreted/secreted antigens from peritoneal exudates of infected mice (mESA) stimulate cell-mediated immune response and induce protective immunity against toxoplasmosis in the murine model.

MATERIALS AND METHODS

Parasite. Tachyzoites of the highly virulent RH strain of T. gondii were maintained in our laboratory by intraperitoneal passage in albino mice. Parasites were passed 10 times through a 27-gauge needle to release the intracellular tachyzoites, harvested in RPMI-1640 medium, filtered on 3 µm polycarbonate membranes (Nucleopore, Pleasanton, Ca, USA), and washed twice in the same medium containing 100 IU/ml penicillin and 100µg/ml streptomycin. The concentration of tachyzoites was determined after adequate dilution in RPMI-1640 medium by enumeration in a Neubauer counting chamber at 400× magnification (15).

Mice. Balb/c female mice, 8-10-week old were obtained from Razi institute of Iran, and 8-10-week-old albino female mice were obtained from animal house of Tarbiat Modarres University (Tehran, Iran). The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the human use of laboratory animals.

Toxoplasma Lyzate Antigen (TLA). 2×10^9 parasites of RH strain harvested in PBS were filtered and centrifuged at 750×g, 3 times for 15 min. The pellet was solubilized

by adding distilled water, and then the solution was supplemented with protease inhibitor, 5mM phenylmethylsulphonyl fluoride (PMSF). The suspension was freeze-thawed five times. The protein content of TLA was determined using Bradford method then stored at-20^{o}C (15,16).

Excreted/Secreted Antigens (ESA). For preparing ESA in cell–free incubation media, each 1.5×10^{8} filtered RH strain tachyzoites per milliliter was aliquated into ten tubes and incubated at 37^{o}C for 3 hours under mild agitation. Tubes were centrifuged at 1000×g for 10 min, their supernatants were filtered by passing through 0.22 µm millipore membrane filter (Millipore Corp., Bedford, MA, USA), and stored at –20^{o}C until used (12,14,17,18).

Mice Excreted/Secreted Antigens (mESA). The supernatants produced from the peritoneal fluids were fractionated by precipitation with ammonium sulphate solution (30-80%). After precipitation, each fraction was washed twice with the corresponding precipitant solution, dissolved in phosphate buffered saline (PBS), and dialysed against PBS for 2 days at 4^{o}C to remove the ammonium sulphate residue.

Delayed–type Hypersensitivity Response (DTH). Five groups (n=5) of female Balb/c mice (8-10-week–old) were sensitized subcutaneously (SC) with 100µl of TLA containing 30µg of protein mixed (1:1) with adjuvant. Each mouse received 3 immunizations at 10-day intervals. The first time with Freund's complete adjuvant (FCA) and the second and third time with Freund's incomplete adjuvant (FIA). One week after the last injection, each mouse received 30µg of TLA, total–ESA, mESA, and mESA–40% subcutaneously in the left footpad area. 100µl of PBS was also injected to the right footpad of the same mice (negative control). One group received only 100µl of PBS. The injections site was examined for erythema and induration after 6, 24, 48, and 72 h. Results were reported as footpad assay: the difference in thickness (in millimeters) between the footpad injected with antigen and those injected with PBS (19).

Lymphocyte Transformation Test (LTT). Assays were performed using techniques described in principle elsewhere (18). Balb/c mice were sensitized with TLA. After 1 week lymph nodes from mice were aseptically removed and lymphocytes were washed twice in RPMI–1640 containing heat–inactivated fetal calf serum (FCS), counted, and added at a cell density of 5×10^{5} cells per well of 96–well flat–bottom tissue culture plates. Cells were stimulated by phytohemaglutinine A (PHA) (10µg/ml), TLA, total–ESA, mESA, or ESA–40% (5, 10 and 20µg/well). Experiments were performed in triplicate wells in a final volume of 200µl/well. After 72 h of incubation at 37^{o}C under 5% CO_2, cultures were pulsed for 18 h with 1µCi of [^{3}H] thymidine (1µCi/well). Then cells were harvested onto glass fiber filter strips using a cell harvester. Incorporation of [^{3}H] TdR was determined by a scintillation counter. Results of triplicate cultures were expressed as counts per minute (CPM).

Nitric Oxide Assay (NO). Resident peritoneal macrophages of sensitized mice were aseptically removed. Macrophages were centrifuged at 250×g twice for 10 min in RPMI–FCS, counted and added at a cell density of 2×10^{5} cells per well of 96–well flat–bottom tissue culture plates, and were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. After 2 hours, supernatants containing the nonadherent cell were collected and replaced with RPMI containing FCS 20%. Five micrograms of TLA and 10 µg of Total-ESA, mESA and mESA–40%, Aminoguanidine (1mM), r-IFN-γ (20ng/ml), and lipopolysaccharide (10µg/ml) were added to related wells. Experiments were performed in duplicate wells in a final volume of 250µl per well.

After 24 h of incubation at 37°C under 5%CO_2, supernatants were collected. Nitrite concentration in cell culture supernatants was measured by the Griess assay (20,21). Briefly, 100µl of the sample was added to 96-well plates, 100µl of a 1:1 mixture of 1% sulphanilamide dihydrochloride in 5%H_3PO_4, and 0.1% naphthylethylenediamide dihydrochloride in 5% H_3PO_4 were then added to the samples. After standing at room temperature for 10 min, the A 540 was determined with a microplate reader with reference to a standard curve for concentrations of sodium nitrite from 100 to 1000 nmol.

Immunization Procedure and Challenge of Immunized Mice. Five groups (n=10) of female 8-10–week–old Balb/c mice were immunized subcutaneously with 100µl of TLA, total–ESA, mESA or mESA–40% containing 50µg of protein mixed (1:1) with adjuvant. One group was also injected with adjuvant (Negative control). Each mouse received two immunizations administered at 2-week intervals (The first time with FCA and the second time with FIA). One week after the second immunization, the mice were challenged subcutaneously with 2×10^3 RH parasites.

Statistics. Statistical analyses were performed by parametric (ANOVA and LSD) and nonparametric tests (Wilcoxon, Mann–whitney and Kruskal–Wallis). The Wilcoxon test was utilized for testing the difference between survival curves.

RESULTS

Delayed–type Hypersensitivity (DTH) Test. After sensitization of mice with TLA and injection of antigens to different groups of mice, footpad assays were reported at 6, 24, 48, and 72 h. Table 1. After 24, 48, and 72 h, swelling response in the first group that received TLA, was higher than negative control group ($P<0.05$). After 24 h this response in the second group that was stimulated by mESA–40% compared with negative control group showed significant difference ($P<0.05$).

Table 1. Results of DTH reaction in different groups of Balb/c mice examined by footpad assay[1]

Antigen or material injected		Mean percent of swelling response		
Mice group		after 24 h	after 48 h	after 72 h
1	TLA[2]	29.88±3.64	11.95±2.57	7.17±0.94
2	mESA	14.24±2.30	10.13±1.65	4.95±1.23
3	mESA-40%	17.12±3.29	12.631±3.7	3.64±0.85
4	Total – ESA	19.38±2.73	11.04±1.96	2.72±0.91
5	PBS[3]	3.9±0.44	2.88±0.6	0.79±0.47

1. Footpad assay: Data indicate the difference in thickness (in millimeter) between footpad injected with antigen and footpad injected with PBS.
2. TLA.*Toxoplasma* Lysate Antigen (positive control group).
3. PBS.Phosphate Buffer Saline (negative control group).

Lymphocyte Transforming Test (LTT). The lymphocyte transformation test was performed in non–immunized and immunized mice. The results are presented in Table 2. All immunized groups, following in vitro stimulation, displayed significant proliferative responses ($P<0.05$) in comparison with control group. In each group sensitized mice showed higher proliferative response than non– sensitized mice ($P<0.05$). In sensitized mice those received mESA–40%, displayed higher counts per minute than mice stimulated by mESA and TLA. Significant difference was observed

between two groups (P<0.05). The mice that obtained Total–ESA showed higher counts per minute than those received TLA.

Table 2. In vitro proliferation of mice lymph node cells stimulated by TLA, mESA, mESA-40% and Total-ESA

Mice group	Stimulator	Sensitized mice[1]	Non sensitized mice
1	TLA	1448±44[2]	655±19
2	MESA	1625±39	915±23
3	mESA-40%	4497±26	1021±47
4	Total – ESA	4683±34	1180±74
5	RPMI[3]	646±29	812±86
6	PHA4	1328±87	630±65

1. Sensitized mice already have sensitized by TLA
2. Mean counts per minute and SD of triplicate wells
3. RPMI. (negative control)
4. PHA.Phytohemaglutinin A (positive control)

Nitric Oxide Assay. Results of nitric oxide assay were shown in Table 3. All test groups, in comparison with negative control group (49.26nM) displayed significant responses following in vitro stimulation (P<0.05). The highest amounts of nitric oxide were observed in macrophages, which received mESA-40% (212.25nM). In sensitized mice macrophages induced with mESA-40%, total-ESA, and mESA produced the highest amounts of nitric oxide.

Table 3. Mean of nitric oxide concentration produced (nM) in test and control groups 24 hours after cell culture*

groups	1	2	3	4	5	6	7	8	9	10	11
	LPS[1]	TLA	TLA+ AG	Total-ESA	Total-ESA +AG	mESA	mESA +AG	mESA-40%	mESA-40%+ AG	AG[2]	BSA[3]
Sensitized mice	226.50 ±47.53	189.8± 66.71	89.93± 15.17	200.8± 50.58	98.48± 84.44	191.1± 34.17	68.42± 17.28	212.2± 30.14	85.29± 13.49	49.2± 12.03	5.8± 0.8
Non sensitized mice	179.77 ±60.26	177.5± 53.88	81.17± 28.86	180.4± 52.53	86.55± 37.86	174.3± 22.19	59.86± 10.21	188.6± 27.16	76.27± 14.20	45.0± 27.61	4.8± 0.83

*rIFN-γ was added to all groups.
1. LPS. Lipopolysaccharide (positive control)
2. AG.Aminoguanidine (an inhibitor as negative control)
3. BSA. Bovin Serum Albumin

Immunization of Mice and Challenge Experiments. The results of challenge experiments in mice immunized with total–ESA, mESA, mESA-40%, and TLA are presented in Table 4. Non-vaccinated animals or control mice immunized with adjuvant alone died within 10 days when challenged subcutaneously with 2000 tachyzoites of RH strain. The percentage of survival in the other groups on day 10 was as follows: 70% in mice immunized with TLA and 90% in mice immunized with total-ESA. These results indicate a significant protection in mice immunized with total–ESA, mESA–40% and mESA in association with adjuvant (P<0.05), compared with those immunized with adjuvant alone. Finally, no significant protection was observed in the group of mice immunized with TLA in association with adjuvant.

Table 4. Cumulative mortality frequency and survival percent of different groups of mice immunized by various antigens

Days after	Cumulative mortality frequency percent in mice groups injected with					Percent survival[1] in mice groups injected with				
infection	TLA	mESA	mESA-40%	Total-ESA	Adj[º]	TLA	mESA	mESA-40%	Total-ESA	Adj
8	0	0	0	0	0	100	100	100	100	100
9	10	10	0	0	20	90	90	100	100	80
10	30	10	10	10	100	70	90	90	90	0
11	50	30	20	10		50	70	80	90	
12	100	60	20	10		0	40	80	90	
13		100	20	30			30	80	70	
14			50	40			0	50	60	
15			70	100				30	0	
16			80					20		
17			100					0		
18										

Groups of 10 mice received two injects of immunogen before challenge with 2000 RH strain tachyzoites. Results plotted as number of mice surviving per day for the five groups.
1. Protection of Balb/c mice against toxoplasmosis by immunization with ESA, mESA and mESA-40%.
ºAdj. Adjuvant

DISCUSSION

Among the T. gondii antigens characterized so far, ESA is peculiar since it is expressed both during the acute and chronic phase of parasitemia (22,23). Thus, in principle, ESA could play a role in the persistent stimulation of cell-mediated immunity in chronically infected healthy subjects (23).

Excretory/secretory antigens may be the best form of antigens for stimulation of the cell–mediated immune response and a good candidate as a vaccine for toxoplasmosis prevention (14). However only few studies have been focused on the fractionated forms of ESA (24,25).

It has been shown that the fraction obtained with 40% saturation (ESA-40%) had the highest concentrations of specific proteins reacting with IgM and IgA (11). So we decided to use this antigen to induce cell-mediated immune responses against T. gondii.

Furthermore in most studies, for preparing ESA, tachyzoites have been grown in cell cultures (26,27), but in this survey, we produced them in cell-free incubation medium (RPMI–1640). Also in other surveys, RPMI–1640 was supplemented with fetal calf serum (12,14,18), but we did not add FCS to RPMI. We used 15µg of protein from 2×10^8 tachyzoites in PRMI–1640 medium and this amount was equal to other studies (12). As mice are very sensitive to T. gondii infection, there are problems in challenge experiments and either low or high virulent strain of T. gondii with low dose must be used. In this study, as in Yap et al. survey, we used 2×10^3 RH parasite (a virulent T. gondii strain) to challenge immunized mice (28). Results show that ESA, mESA and its fraction (mESA-40%) have different potentiation in inducing cellular immunity. After sensitization of mice by TLA, their lymphocytes were encountered with total–ESA, mESA and mESA–40%. The highest lymphocyte proliferative responses were shown with ESA–40%. Probably this fraction has capability to induce lymphocyte activation.

In nitric oxide assay, macrophages stimulated with purified fraction, mESA-40%, produced higher amounts of nitric oxide than those stimulated with total-ESA and TLA. Probably, these fractions contain antigenic determinants that only induce

macrophages activation, but mESA and TLA are total antigens which contain some antigens that inhabit macrophages. Therefore, production of nitric oxide by these antigens is lower than mESA–40%.

In this regard, an inhibitory function by macrophages has previously been noticed by other investigators (29,30). As it has previously shown that nitric oxide play an important role in inhibition of intracellular proliferation of tachyzoites in macrophages activated by IFN-γ plus TNF-α in vitro (31,32), and also in conferring resistance against development of toxoplasmic encephalitis during the chronic stage of the infection (33,34), therefore it is expected that mESA and mESA-40% can play an important role in protection against toxoplasmosis by macrophage activation and nitric oxide production.

Mice immunized with total ESA, mESA–40% and mESA survived longer than those immunized with TLA and adjuvant (negative control). Several reports have previously indicated that immunization of mice with various Toxoplasma crude antigens may confer resistance against acute T. gondii infection (19,35,36). Among T. gondii antigens, the most studied antigen is the major surface antigen SAG1 (P30), which confers various degrees of protection depending on the immunization protocol and the adjuvant used (8-10). The protective role of ESA would suggest an alternative approach for vaccine development. As secretion is an important event in the production of circulating antigens during the early stages of toxoplasmosis (26), ESA might be one of the first targets for the potentiation of the immune system leading hopefully to the vaccine production.

In previous study we showed that ESA-F2 protects mice against T. gondii (25) but production of ESA from cell-free culture is very difficult so in this study we used mESA from mice infected by T. gondii. Results of this study show that the mESA antigens could be used as a good candidate for the development of new immunization strategy against toxoplasmosis.

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