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

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Abstract: *Toxoplasma gondii* is an obligate intracellular parasite that infects all mammalian cells. Several antigens such as excreted/secreted antigens have been identified as potential vaccine candidates. 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 responses and protective immunity against toxoplasmosis in the murine model. The supernatants produced from the peritoneal fluids were fractionated by precipitation with ammonium sulphate solution (30-80% saturated). For induction of cell-mediated immune responses, Delayed type hypersensitivity was measured in injected footpad measured in injected footpad. By adding [^{3}H] thymidine to cell cultures, their radioactivities were measured. 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. The pattern of lymphocyte responsiveness was dependent on the kind of antigen employed for induction of the blastogenesis. In sensitized mice, those received mESA-40% displayed higher counts per minute than mice that 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 ($p<0.05$). As fraction 40% (mESA-40%) showed a good result in induction of cell-mediated responses in the murine model, for getting better response, this antigen have to be purified further.

Key words: *Toxoplasma gondii*, immune responses, excreted / secreted antigens, mice peritoneal exudate

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 persisting 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 disease, bone marrow or heart transplant recipients[3]. The central role of cell mediated immunity in host defense against the acute infection as well as in the control of the chronic state is well recognized[4-6]. Vaccines based on killed organisms have been unsuccessful in producing effective immunity even against a challenge with an avirulent strain of 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 confer a significant protection against a lethal challenge with the 76 K strain cysts in both mice, by direct immunization and nude rats, by the passive transfer of immune sera or T cells[12,13].

The ESA is able to stimulate a better cell mediated immune response as compared to soluble or cysts antigen. Therefore, this antigen is a good candidate for research into immunizing agents against *T. gondii* infection[14]. These studies have performed on Total-ESA of *T. gondii*.

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Supernatants of cell cultures infected with *T. gondii* have been the main source of ESA for scientific investigation. There appear to be a little information on the comparison of ESA from peritoneal exudates of infected mice. 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 responses and protective immunity against toxoplasmosis in the murine model.

MATERIALS AND METHODS

Tachyzoites of the highly virulent RH strain of *T. gondii* were maintained in our laboratory by intraperitoneal passages in albino mice. Parasites were passed 10 times through a 27-gauge needle to release the intracellular tachyzoites and were harvested in RPMI-1640 medium, filtered on 3 μm polycarbonate membranes (Nucleopore, Pleasanton, Ca, USA) and washed twice in the same medium containing 100 Iμ mL^{-1} penicillin and 100 μg mL^{-1} 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].

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

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 the distilled water. 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°C[15,16].

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

The supernatants produced from the peritoneal fluids were fractionated by precipitation with ammonium sulfate 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°C to remove the ammonium sulphate residue.

Five groups (n = 5) of female Balb/c mice (8-10-week–old) were sensitized subcutaneously (SC) with 100 μL of TLA containing 30 μg 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 subcutaneously 30 μg of TLA, total–ESA, mESA and mESA–40% in the left footpad area. About 100μL of PBS was also injected to the right footpad of the same mice (negative control). One group received only 100 μL of PBS. The injections site was examined for erythema and induration after 6, 24, 48 and 72 hrs. Results were reported as footpad assay: the difference in thickness (in millimeters) between the footpad injected with antigen and those injected with PBS[19].

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^{-1}), TLA, total–ESA, mESA or ESA–40% (5, 10 and 20μg/well). Experiments were performed in triplicate wells in a final volume of 200 μl/well. After 72 hrs of incubation at 37°C under 5% CO_2, cultures were pulsed for 18 hrs with 1μCi of [^{3}H] thymidine (1μCi/well). Then the cells were harvested onto glass fiber filter strips using a cell harvester. Incorporation of [^{3}H] TdR was determined with a scintillation counter. Results of triplicate cultures were expressed as Counts Per Minute (CPM).

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°C in a humidified atmosphere containing 5% CO_2. After 2 hrs, 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); rIFN-ã (20ng mL^{-1}) and lypopolysaccharid (10 μg mL^{-1}) were added to related wells. Experiments were performed in duplicate wells in a final volume of 250 μL per well. After 24 hrs of incubation at 37°C under 5%CO_2, supernatants were collected. Nitrite concentration in cell culture supernatants was measured by the Griess

Table 1: Mean of the results of DTH reaction in different groups of Balb/c mice examined by footpad[1]

	Kind of antigen or material	Mean percent of swelling response in:		
Mice group	Injected to footpad of mice	24 hrs after injection	48 hrs after injection	72 hrs after injection
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: Each data indicates the difference in thickness (in millimeter) between the footpad injected with antigen and the footpad injected with PBS 2. TLA.*Toxoplasma* Lysate Antigen (as positive control group) 3. PBS.Phosphate Buffer Saline (as negative control group)

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

	Kind of	Counts per minute in different groups of:	
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	PHA[4]	1328±87	630±65

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

assay[20,21]. Briefly; 100 µL of the sample was added to 96-well plates; 100 µL of a 1:1 mixture of 1% sulfanilamide dihydrochloride in 5% H_3PO_4 and 0.1% naphthylethylenediamide dihydrochloride in 5% H_3PO_4 wMarch 13, 2006 as 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.

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 50 µg 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 subcutaneosly with 2×10^3 RH parasites.

Statistical analyses were performed by parametric (ANOVA and LSD) and nonparametric tests (Wilcoxon, Mann-whitney and kruskal-wallis). The wilcoxon test was the utilized for testing the difference between survival curves. In this test the null hypothesis is that there is no real difference between survival curves for the five compared groups.

RESULTS

After sensitization of mice with TLA and injection of antigens to different groups of mice, footpad assay reported at 6, 24, 48 and 72 hrs was shown in Table 1. After 24, 48 and 72 hrs, swelling response in the first group that received TLA, was higher than negative control group ($p<0.05$). After 24 hrs, this response in the second group that stimulated by mESA-40% compared with negative control group showed significant difference ($p<0.05$).

The lymphocyte transformation test was performed in non-immunized mice and in mice immunized with different antigens as TLA, Total-ESA, mESA and mESA-40%. The results of this test are presented in Table 2. All immunized groups following in vitro stimulation in comparison with control group displayed significant proliferative responses ($p<0.05$). 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 that 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.

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

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

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

Groups	1	2	3	4	5	6	7	8	9	10	11
Added materials to cells mice group	LPS[1]	TLA	TLA +AG	Total -ESA	Total-ESA+AG	mESA	mESA +AG	mESA -40%	mES -40%+AG	AG[2]	BSA[3]
Sensitized mice	226.50 ±47.53	189.89 ±66.71	89.93 ±15.17	200.89 ±50.58	98.48 ±84.44	191.19 ±34.17	68.42 ±17.28	212.25 ±30.14	85.29 ±13.49	49.26 ±12.03	5.8 ±0.8
Non sensitized mice	179.77 ±60.26	177.53 ±53.88	81.17 ±28.86	180.45 ±52.53	86.55 ±37.86	174.32 ±22.19	59.86 ±10.21	188.63 ±27.16	76.27 ±14.20	45.0 ±27.61	4.8 ±0.83

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

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

Days after infection	Cumulative mortality frequency percent in mice groups injected with					Percent survival[1] in mice groups injected with				
	TLA	mESA	mESA-40%	Total-ESA	Adj°	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%. °Adj. Adjuvant

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 of mice immunized with total– ESA, mESA–40% and mESA in association with adjuvant ($p<0.05$) in contrast to a lack of protection observed with mice immunized with adjuvant only. Finally, no significant protection was observed with the groups of mice immunized with TLA in association with adjuvant.

DISCUSION

Among the *T. gondii* antigens characterized thus far, ESA are peculiar since, at least for most of them, expression is maintained duringboth the acute and the chronic stages of the parasite[22,23]. Thus, in principle, ESA could play a role in the persistent stimulation of cell-mediated immunity in chronically infected healthy subjects[23].

Although excretory/secretory antigens may be the best form of antigen for stimulation of the cell-mediated immune response and hence it appears to be a good candidate for vaccine in toxoplasmosis[14], only a few studies have been focused on its fractions[24,25].

In a study, 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 in induction of 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 the studies of others[12]. As mice are very sensitive to *T. gondii* infection, there are problems in challenge experiments and either low virulent strain or high virulent strain of *T. gondii* with low dose must be used. In this study such as Yap, Kersten and Ferguson 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 potentialities in inducing cellular immunity system. After sensitization of mice by TLA, their lymphocytes were contacted with Total-ESA, mESA and mESA-40%. The highest proliferative responses were shown in lymphocytes induced with ESA-40%. Probably, this fraction has antigens that only induce lymphocytes but TLA, Total-ESA and mESA-40% that inhibit lymphocytes.

In nitric oxide assay, macrophages induced with purified fraction, mESA-40%, produced higher amounts of nitric oxide than those induced with total-ESA and TLA. Probably, these fractions have antigens that only induce macrophages but mESA and TLA are total antigens contain some antigens that inhabit macrophages. Therefore production of nitric oxide by these antigens is lower than mESA-40%.

Some workers have reported the involvement of macrophages in the immunosuppression observed during toxoplasmosis[29,30].

As nitric oxide has previously been shown to play an important role in inhibition of intracellular proliferation of tachyzoites in macrophages activated by IFN-ã plus TNF-á *In vitro*[31,32] and in conferring resistance against development of toxoplasmic encephalitis during the chronic stage of the infection[33,34], mESA and mESA-40% by macrophages induction and nitric oxide production can play an important role in protection of host against toxoplasmosis.

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 the immunization of mice with various Toxoplasma crude antigens[19,35,36], may confer resistance against acute *T. gondii* infection. Among *T. gondii* antigens, the most studied 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 potentialities of ESA would suggest an alternative approach for vaccine development. As *Toxoplasma* 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 immune system.

The ESA have been shown to be protective in the models of acute infection of mice by direct immunization[10]. Their protective role has also been shown in nude rats passively transferred with serum or T cells from infected or ESA-immunized euthimic rats[12,13,37]. Therefore, immunization with ESA provides a high level of protection. Cazabonne, Bessieres, Pipy and Seguela evaluated the effect of ESA fractions on macrophages infected by *T. gondii*. The different antigens used did not change the rate of penetration and proliferation of the parasites. Therefore, the secreted products, which are capable of provoking an immune response, could not directly activate the macrophages[24]. Some workers have reported that experiments carried out with two purified ESA, namely GRA2 and GRA5, had a significant degree of protection[18].

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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