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Full Length Research Paper

The role of various proteins of *Toxoplasma gondii* in provoking immune response at different stages of infection

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***Toxoplasma gondii* is the causative protozoan of toxoplasmosis which has a worldwide distribution among humans and warm-blooded animals. The diagnosis generally depends on serologic tests but the persistently high Immunoglobulin M (IgM) or low IgG avidity complicate diagnosis. It is essential to identify acute-stage-specific antigen to use in a single test for the definitive diagnosis of the acute disease, especially in pregnant women and immunocompromised individuals. In this study, we investigated the recognition of proteins of lysate antigen (LA) and excretory-secretory antigen (ESA) fractions of the virulent RH strain of *T. gondii* by the sera of patients who had recent or past infections and the sera of experimentally infected mice. As a result, no specific bands for discrimination of recent infection from the past one could be detected in our study but the results confirmed that proteins of approximately 20, 30 and 40 kDa were among the major targets for IgG responses during acute and chronic infection with *T. gondii* in humans.**

Key words: *Toxoplasma gondii*, acute, antigen, immunoblotting, human, mice.

INTRODUCTION

Toxoplasma gondii is an obligatory intracellular protozoan which has a worldwide distribution among humans and warm-blooded animals (Ferguson, 2009). The protozoan can infect the host by its bradizoite (tissue cyst), oocyst and tachyzoite forms. The main routes of transmission are eating raw or undercooked meat of the infected animals and consuming fruits or vegetables contaminated with the oocysts in the cat stool. Toxoplasmosis can have serious outcomes in humans, especially in immuno-compromised patients and in the fetus of the pregnant women who are primarily infected during their pregnancies (Montaya and Liesenfeld, 2004). The disease also has detrimental effects on economy by aborts and stillbirths among livestock.

The direct diagnosis of this parasite is quite difficult as isolation is expensive and time consuming. That is why, diagnosis generally depends on serologic tests. However, as Immunoglobulin M (IgM) positivity and low IgG avidity index can persist more than a year, it is recommended to use more than one serologic test to confirm acute toxoplasmosis which is not only costly but also labor-intensive (Bertozzi et al., 1999). Therefore, it is essential to identify specific antigens to use in serologic tests for the rapid and definitive diagnosis of acute toxoplasmosis especially in pregnant women and immunocompromised patients.

Among *T. gondii* strains, one of the most studied is RH strain (1941) and it is classified to be a type-1 virulent

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Abbreviations: ESA, Excretory-secretory antigen; **LA,** lysate antigen.

strain. The antigen structures of *T. gondii* are described as surface, cytoplasmic and excretory-secretory antigens (ESA). The degree of immune response against different antigenic structures of the parasite gains importance in the improvement of both diagnostic tests and vaccine development. Especially, the problems existing in the diagnosis of acute infection in pregnant women and the reactivation in immunocompromised patients can be solved by highly immunogenic and early stage-specific antigens. (Lekutis et al., 2001; Costa-Silva et al., 2008). Several antigens that are targeted by IgM and IgG antibodies in acute and chronically infected patient sera have been described in various studies (Huskinson et al., 1989; Suzuki et al., 1990; Delibas et al., 2006).

In this study, in order to characterize the antigens that are immunogenic for different stages of infection, the onset period and type of the antibody response against lysate antigen (LA) and ESA fractions of the virulent RH strain of *T. gondii* were investigated by probing with the sera of both experimentally infected mice and naturally infected humans.

MATERIALS AND METHODS

Human sera

Serum samples of patients who had recent and latent infections were used in order to investigate the role of different proteins from LA and ESA antigenic preparations in antibody production. Human sera belonging to 11 patients who had a serologic profile of recent infection (Group I: IgM and IgG positive), 6 patients who had past infection (Group II: IgG positive) and 5 control patients who were seronegative for toxoplasmosis were enrolled in this study. All of the human sera were previously screened for IgG and IgM at the diagnostic laboratory by using anti-toxoplasma IgG Enzyme-linked immunosorbent assay (ELISA) and anti-toxoplasma IgM ELISA (immunocapture) tests, respectively. All serum samples were also tested for specific antibodies by using Sabin Feldman test.

Toxoplasma gondii

T. gondii propagation and the experimental infections were carried out by using Swiss Albino mice. For maintenance of the protozoan, mice were infected intraperitoneally by *T. gondii* RH strain (ATCC, 50174) and tachyzoites were collected by peritoneal washing with 3 ml phosphate-buffered saline (PBS), pH 7.2 at day 3 post-inoculation. The exudate including tachyzoites was centrifuged at 50 x*g* for 5 min to remove host cells. The parasites in the supernatant were washed twice at 1000 x*g* for 10 min. The pellet was suspended in PBS and used immediately for experimental infection of mice or to inoculate vero cell cultures.

Experimental infection of mice

Mice enrolled in the experiments were 8 to 10 weeks old female Swiss Albino mice. Each of 50 mice were infected intraperitoneally with 500 µl RH strain suspension at a concentration of 5×10^2 tachyzoites/ml. In order to observe the antibody response of the experimentally infected mice, they were bled twice daily for a period of 5 days. Blood of the mice from each group was pooled and their sera were stored at -20°C until used. They were all tested with ESA and LA. The mice experiments were undertaken according to the approved procedures by the local ethical committee.

Cell culture

T. gondii tachyzoites were obtained in large quantities by growing them in vero cell culture. A high glucose Hepes modification of Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich D1152) supplemented with 3.7 g/l Na_2CO_3, 10% inactivated fetal calf serum (FCS), penicilline (100 U/ml) and streptomycin (100 µg/ml) was used (Lei et al., 2005). When vero cells were 80% confluent at 37°C and 5% CO_2, *T. gondii* tachyzoites obtained from peritoneal fluid of mice were inoculated in cell culture with a maintenance medium containing 2% FCS. After all the cells were lysed by tachyzoites, the grown tachyzoites were centrifuged at 50 x*g* for 5 min and 1000 x*g* for 10 min, respectively. The pellet was resuspended in PBS and used to prepare LA fraction.

Antigenic preparations

LA and ESA were used in our study because they contain all the major antigens that could have diagnostic importance. For obtaining LA, after 4 cyles of freezing and thawing, the suspension was sonicated five times at 60 Hz for 15 s by using ultrasonicator (Branson). Cell lysate was also prepared following the same procedure to be used as control in immunoblotting. Lysed tachyzoite and cell samples were aliquoted and stored at -20°C. ESA fraction was prepared as follows: tachyzoites were suspended in a cell-free DMEM at a concentration of 1×10^8 tachyzoites/ml and incubated with mild agitation at 37°C for 3 h. Following centrifugation at 1000 x*g* for 10 min, the supernatant was filtered through 0.2 µm pore-sized filter and stored at -20°C until used (Son and Nam, 2001; Daryani et al., 2003). Protein concentration of the antigenic preparations was determined following the Bradford method using bovine serum albumin (BSA) as a standard.

Immunoblotting

LA and ESA fractions of *T. gondii* were analyzed for their ability to be recognized by the sera of experimentally infected mice and naturally infected humans. As LA fraction was obtained from tachyzoites grown in cell culture, vero cell lysate was used as control to evaluate antigenic contamination. For immunoblotting, initially, LA and ESA proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Antigens were run in 4 to 12% polyacrylamide SDS gels (Invitrogen) according to standart procedures (Laemmli, 1970). Prestained molecular weight marker (Invitrogen) and the antigen preparations (13 µl per lane) were mixed with 5 µl sample buffer (Invitrogen) and 2 µl reducing agent (Invitrogen) in a total volume of 20 µl. After denaturating the samples at 70°C for 10 min, they were run in a X-cell SureLock (Invitrogen) electrophoresis tank with the running buffer (NuPage MES 20X-Invitrogen) by applying a constant current of 20 mA.

The gels were then transferred to nitrocellulose membranes by using I-blot western (Invitrogen). The membranes were cut into strips containing all antigens (LA, ESA and cell lysate). Unbound sites on strips were bloked with 1% BSA in Tris-buffered saline with Tween (TBS-T) 0.05% buffer for 1 h at room temperature. The strips were subsequently washed 3 times at 15, 5 and 5 min interval in washing buffer (TBS-T). Each of the strips was incubated overnight with a different serum sample (1:200) belonging either to mouse or human (Towbin et al., 1979). After another washing step, diluted secondary conjugates-goat HRP-conjugated anti-mouse IgG (1:2000), anti-human IgG (1:2000), anti- human IgM (1:1000) and

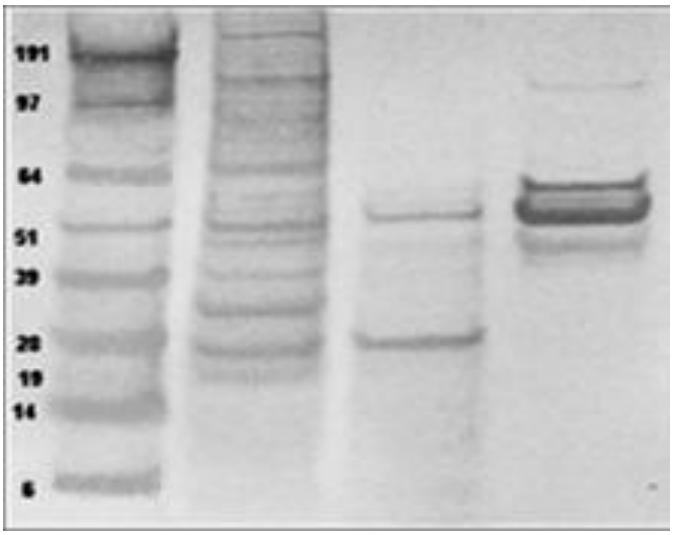

Figure 1. IgG immunoblot of IgG positive and IgM negative human serum with LA, vero cell lysate and ESA fractions (Lane 1, mw standarts; Lane 2, LA; Lane 3, cell lysate; Lane 4, ESA).

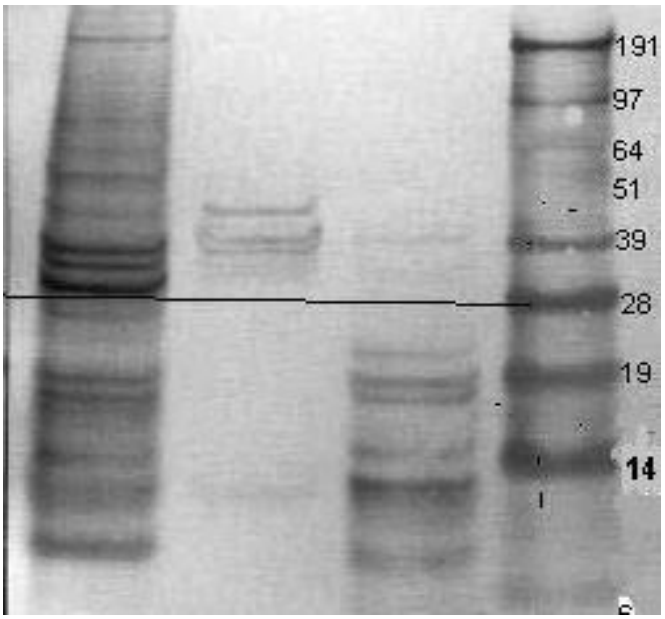

Figure 2. IgG immunoblot of IgG and IgM positive patient sera (Lane 1, LA; Lane 2, cell lysate; Lane 3, ESA; Lane 4, mw standarts).

donkey HRP-conjugated anti-mouse IgM (1:1000) were added. After 1 h, strips were washed again and developed using the substrate 3,3′ diaminobenzidine tetrahydrochloride.

RESULTS

By using IgG immunoblotting with LA, 100, 75, 70, 64, 57, 49, 46, 40, 37, 30, 26 and 18 kDa bands were observed by more than 40% frequency in Group I. Predominant reactive proteins in Group II were 85, 75, 40, 37, 30 and 20 kDa. With the same method, 57 kDa protein were seen in most of the Group I patients reacting with ESA, while 85 and 64 kDa bands were observed in Group II. The common bands obtained both with the cell lysate and either one of the antigenic fractions (LA or ESA) were ignored as these bands were thought to be non-specific.

IgM immunoblotting revealed reactive bands of 130, 85, 65, 55 and 37 kDa with LA. IgM blotting with ESA revealed reaction against 85 and 75 kDa proteins in Group I. When analyzing the IgM reactivity of Group I, common bands with Group II were not taken into consideration.

The results of the experimentally infected mice sera showed that, reactive bands were first observed on day 3 post-infection. By using IgM immunoblotting against LA, 100, 85 and 75 kDa proteins were recognized. The bands detected in IgG blotting with LA appeared on day 5 post-infection and they were 100, 85, 55 and 28 kDa. The IgM immunoblotting of mice sera with ESA revealed a 55 kDa protein. No band was observed as a result of IgG immunoblotting of mice sera with ESA. Some samples of the immunoblots are shown in Figures 1, 2, 3 and 4.

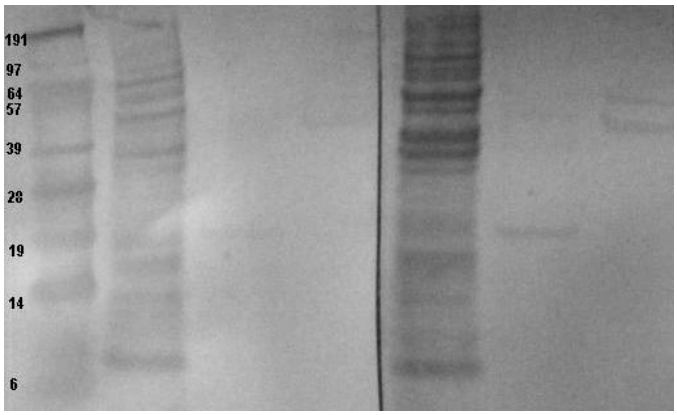

Figure 3. IgM immunoblot of two IgG and IgM positive human sera (Lane 1, mw standarts; Lane 2, LA; Lane 3, cell lysate; Lane 4, ESA).

DISCUSSION

Today, the diagnosis of acute toxoplasmosis especially in immunocompromised individuals, pregnant women, their fetuses and newborns is still a challenging issue. It mostly depends on the serologic tests undertaken by using tachyzoites or total extracts of tachyzoites. Recently, some *Toxoplasma* antigens or their recombinant forms have been used to improve serodiagnostic methods (Pfrepper et al., 2005; Araujo and Ferreira, 2008). On the other hand, the long-term

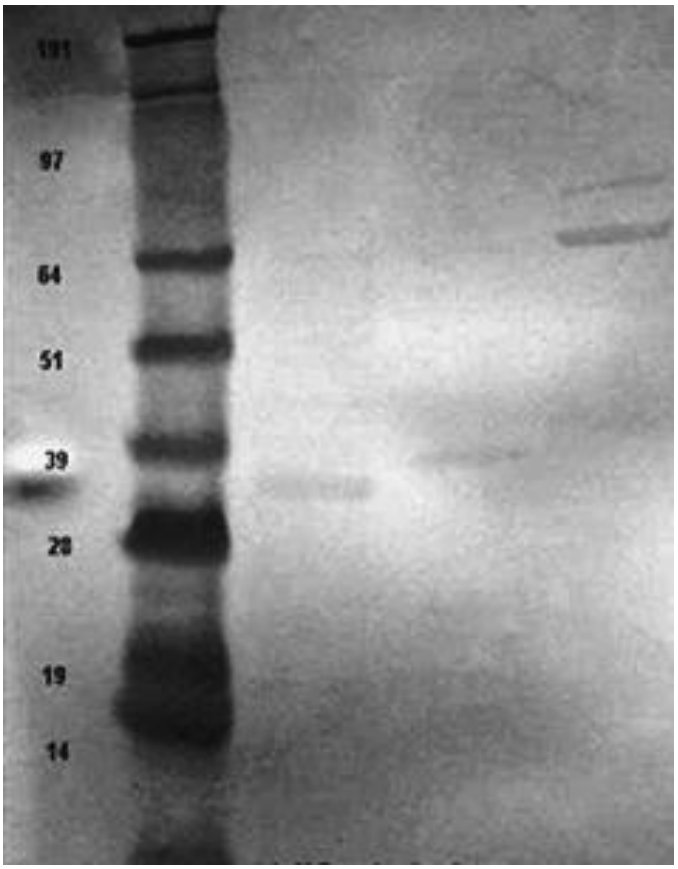

Figure 4. IgM immunoblot of mice sera on day 4 post-infection (Lane 1, mw standarts; Lane 2, LA; Lane 3, cell lysate; Lane 4, ESA).

persistence of both high levels of IgM and low levels of IgG avidity pose problems in the interpretation of the serologic results. Although IgG avidity test is a useful mean of judging past infection with a single serum sample, some studies on the kinetics of IgG avidity in pregnant women have observed high avidity in pregnant women who had been infected for 3 to 5 months and low avidity 1 year after the acute phase (Meek et al., 2003).

A serologic test with an antigen both sensitive and stage-specific for toxoplasmosis may solve this problem. In this study, we investigated the IgG and IgM antibody responses to specific proteins of LA and ESA fractions of *T. gondii* tachyzoites at different stages of infection in naturally infected humans and experimentally infected mice. We enrolled LA and ESA antigenic fractions in our experiments in order to identify antigen(s) that will be of value in discriminating toxoplasmosis phases. LA and ESA fractions of *T. gondii* play the most important roles in the virulence of the parasite and in provoking antibody response of the host. Besides major surface antigens like SAG family, there are defined major ESA proteins which are secreted by dense granules, rhoptries and micronemes of the tachyzoites (Mercier et al., 1998; Carruthers, 1999). For example, GRA2 was found to be related with the virulence of the tachyzoite while GRA7, being present in all forms of the parasite, is reported to be responsible for the long lasting antigenic stimulation of the host. Most of the targets for antibody production during the early stages of toxoplasmosis is thought to be the surface and ESA of tachyzoites (Giraldo et al., 2000). ESA of the parasite form the majority of the circulating antigens in sera from the hosts with acute toxoplasmosis (Costa-Silva et al., 2008).

We used *Toxoplasma* RH (type-1) strain in this study because it is one of the most virulent and immunogenic strains of *T. gondii* and generally is the strain of choice in the antigen studies (Araujo and Ferreira, 2010). Ware and Kasper (1986) investigated the immunoreactivity of antibodies against the strain specific antigens of *T. gondii* by using avirulent P and C strains and virulent RH strain. When rabbit antiserum and tachyzoite antigens were immunoblotted, the most prominent bands were observed against 22 and 30 kDa antigens of RH strain and had the potential to bind mostly IgG antibodies. In a study undertaken to analyse *Toxoplasma* antigens, a 60 kDa protein was reported to frequently react with IgM in acute phase of the infection (Galvan-Ramirez et al., 1998). In our study, the number of the antigens recognized by human sera IgM were lower than the ones reacted with IgG. Pattern of recognition of different fractions by IgG was more complex than the pattern generated by IgM. This may have resulted from the fact that in humans, most of the IgM responses against *T. gondii* are directed against carbohydrates, which were shown to be derived from a GIPL structure. In addition, the surface antigens have been shown to be major targets for IgG responses during chronic infection with *T. gondii* and several studies suggest a dominant response to SAG-1 (Sharma et al., 1983; Noat et al., 1983; Giraldo et al., 2000).

Human sera reacted with more LA proteins than mice sera with IgG immunoblotting in this study. This is probably because we used a virulent type-1 (RH) strain that kills mice within days. The reactive bands did not change much for the short period of infection. The antibody appearance against a number of membrane antigens is reported to be simultaneous (Handmen and Remington, 1980). If we had used a *Toxoplasma* strain like ME 49 that caused chronic toxoplasmosis in mice, we would have evaluated the antibody profile in the chronic stage of the disease. On the other hand, antibody reactivity would have been less due to low virulence.

Microneme proteins of *T. gondii* are reported to be secreted even when the parasite is not in contact with the host cell. As we used a host cell-free method to obtain ESA in our experiments, the ESA fraction bands which antibodies reacted could possibly include microneme proteins. Some of the reactive bands we observed were previously shown to elicit high antibody titer in rats and their sera transferred to other animals induced protection against RH strain (Darcy et al., 1988).

In a study undertaken to investigate avidity of IgG antibodies against ESA of *T. gondii* in order to distinguish “acute recent” from “non-acute recent” infections, it was reported that a phase-specific reactivity was observed against a 30 kDa antigen (Araujo and Ferreira, 2008). Our data does not seem to support this finding. This may have resulted from the difference between the serologic profile of the tested patients.

Some of the seronegative human sera also gave some bands in immunoblotting. The most prominent one was 85 kDa bands which is one of the bands shown to be recognized by naturally occurring human antibodies (Potasman et al., 1986). No bands were detected in control mice sera.

As a result, no specific bands unique to recent or past toxoplasmosis could be detected in our study but the results showed, in agreement with the previous studies, that protein of approximately 20, 30 and 40 kDa were among the major targets for IgG responses during acute and chronic infection with *T. gondii* in humans.

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