Detection of Antibodies to the 97 kDa Component of *Toxoplasma gondii* in Samples of Human Serum

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This study was carried out to investigate the immune response against 97 kDa (p97) molecular marker of Toxoplasma gondii *that has been characterized as a cytosolic protein and a component of the excreted-secreted antigens from this parasite. A total of 60 serum samples from patients were analyzed by enzime-linked immunosorbent assay and Western blot for toxoplasmosis. These samples were organized in three groups, based on clinical symptoms and results of serological tests. Group I: 20 samples reactive to IgG and IgM (acute phase); group II: 20 non-reactive samples (control group); and group III: 20 samples reactive only to IgG (chronic phase). Western blot was performed with total antigenic extracts or with excreted and secreted antigen from* T. gondii *to identify the fraction correspondent to p97. It was observed that this cytosolic component from* T. gondii *stimulates the immunologic system to produce both IgM and IgG antibodies in the beginning of the acute infection and IgG throughout the chronic stage of the asymptomatic toxoplasmosis.*

Key words: *Toxoplasma* - enzime-linked immunosorbent assay - Western blot - p97

Toxoplasma gondii is an obligate intracellular parasite, which belongs to the phylum Apicomplexa, to the class Sporozoea and to the genus *Toxoplasma*. In the present classifications it is considered part of the Sarcocystidae family. In 1908, the parasite was discovered by Splendore in Brazil in a rabbit, and also by Nicole and Manceaux in North Africa in a Northern African rodent called gondi (Rey 2001).

Toxoplasmosis is a zoonosis from a variety of vertebrates including man. In humans, due to the action of the immune system, chronic asymptomatic infection frequently occurs. Two groups of risk, however, do exist, newborns of mothers who had toxoplasmosis during pregnancy and immunocompromised individuals. The individuals of both these groups frequently develop fatal toxoplasmic encephalitis (Chaves-Borges et al. 1999).

The multiplication of tachyzoites is normally inhibited by immunologic mechanisms that favor the formation of cysts containing bradyzoites (Soete et al.1993). Nevertheless when individuals are immunocompromised, *T. gondii* produces a severe disease due to infection reactivity. In these cases, the cysts rupture and tachyzoites multiply rapidly (Gazzinelli et al. 1992).

Several proteins of *T. gondii* have been studied regarding their biological functions such as their role in inducing immune response by the host organism and their roles as diagnostic reagents. It is interesting to determine which antigens are useful for diagnosis, as well as which ones are responsible for the development of protective immunologic mechanisms of toxoplasmosis (McLeod et al. 1991). Through molecular weight, various proteins such as p30, p22, p23, p60, p76, p97 and others that may be or not anchored to the membrane surface of the parasite via glycosilphosphatidilinositol (McLeod et al. 1991) have been identified. Many studies look for molecular markers associated with their biological role, as well as trying to relate them to the acute or chronic phases of the disease (Makioka et al. 1991, Soete et al. 1993).

One of the recently studied components of the parasite (p97) has been reported to be very important for *T. gondii* replication in the cell of the host. This antigen is not found on the surface of this protozoan, but in immersed vesicles in the cytosol. Blocking a determined epitope of this component through monoclonal antibody 1B8 inhibits *T. gondii* replication in vitro (Mineo et al. 1994).

Matsuura and Kasper (1997) reported the isolation of the sequence of cDNA encoding this 97-kDa protein and characterized the biochemical nature of this molecule. The analysis of five clones reactive to mAb 1B8, against p97, suggested that the second ATG matched closely as a putative translation initiation site. The coding sequence encodes a peptide with 892 amino acid residues. The peptide sequence of this molecule is composed of 15 repeating units. They had demonstrated a homologous gene sequence in two closely related Apicomplexa, *Neospora caninum* and *Besnoitia jellisoni*, suggesting this protein is conserved among some species of the Sarcocystidae.

A study conducted in France highlighted the importance of the immune response against excreted and secreted antigens (ESA) by tachyzoites, suggesting that the use of these antigens for future diagnosis of toxoplasmosis. Serum analysis of the acute phase of the disease demonstrated that the sera recognizes 69 and 97 kDa bands of ESA. The 97 kDA antigen was recognized precociously by the IgM antibody (Decoster et al. 1988).

Another study characterized antigenic markers recognized by human serum samples from patients with acute

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Received 21 December 2001
Accepted 29 July 2002

and chronic toxoplasmosis, by IgG avidity assays. An avidity immunoblotting assay described by Marcolino et al. (2000), recognized that bands p16, p32, p38, p40, p43, p54, p60, p66 and p97 were more frequently recognized by low-avidity IgG in recent infection and by high-avidity IgG in chronic toxoplasmosis. From these antigenic bands, p38 can be characterized as an optimal antigenic marker of low avidity for the recent form of toxoplasmosis.

The present study aimed to detect the presence of antibodies against 97 kDa proteins of *T. gondii* in serum samples of patients from the Hospital de Clínicas, Universidade Federal de Uberlândia, by using immunoblotting assay with different antigenic fractions: total antigen and ESA of *T. gondii.*

MATERIALS AND METHODS

Patients - The patients attended at the Hospital de Clínicas, Universidade Federal de Uberlândia (HC-UFU) made up the group study. Serum samples were supplied by the Clinical Analysis Laboratory of HC-UFU and previously screened by enzyme-linked immunosorbent assay (ELISA) for *T. gondii*. Sixty samples were selected and divided into three groups of 20 samples, according to the clinical symptoms and the results of serological tests that were also carried out at the Laboratory of Immunology, UFU.

The groups of serum samples were defined as follows: group I: 20 human serum samples from patients with typical clinical symptoms of infectious mononucleosis-like syndrome, with fever, fatigue, and enlargement of the cervical lymph nodes. The sera from these patients exhibited specific IgM (titer ≥ 64) and IgG (titer ≥ 256) antibodies (acute phase); group II: 20 human serum samples from individuals serologically negative and asymptomatic healthy controls (negative controls); group III: 20 human serum samples from asymptomatic but serologically positive individuals for IgG antibodies only (chronic phase).

Total antigen of T. gondii - Tachyzoites of *T. gondii* (RH strain) were grown intraperitoneally in Swiss mice for 48 to 72 h. The peritoneal exudates were obtained from infected mice and then washed in sterile PBS. The concentration of the parasite was adjusted for 10^7 a 10^8 parasites/ml and these samples were solubilized in a SDS/PBS buffer (1:1). The suspension was heated to 100°C for 3 min and aliquoted.

ESA of T. gondii - RH strain tachyzoites were harvested from mice peritoneal exsudates two days after the inoculation of parasites. The suspension was selected when parasite content ranged from 10^7 to 10^8 parasites/ml, followed by centrifugation at 1,000 g for 15 min. The sediment was resuspended in 10 ml of sterile PBS at 4°C and centrifuged again as described above. The sediment was resuspended in 0.5 ml of sterile PBS, incubated at 37°C for 45 min under eventual stirrings and centrifuged at 4,000 g for 15 min. The supernatant was filtered in 0.2 µm membrane (Sterile Acrodisc 13, Gelman Sciences, USA) and was stored at -20°C.

ELISA for T. gondii - Polyvinylchloride (PVC) plates were sensibilized overnight at 4°C with *T. gondii* suspensions (10^5 tachyzoites/well) and dried at 37°C for the IgG test and with soluble parasite antigens obtained by cryolysis for the IgM test (5 µg of protein/well). The plates were blocked with PBS-Tween plus 5% nonfat dry milk for 30 min and then washed with PBS-Tween 1% of block solution. Serum samples were incubated at 37°C for 45 min and the plates washed again (3 times for 5 min each) and incubated at 37°C with an immunoenzymatic conjugate (human anti-IgG or human anti-IgM labeled with peroxidase). Afterwards, the plates were washed and an immunoenzymatic substrate (OPD solution and 30% hydrogen peroxide) was added for revelation. Tests were read by a photometer at 492 nm (Titertek Multiskan Plus, Flow Laboratories, Geneva, Switzerland).

SDS-PAGE of T. gondii proteins - Electrophoresis of *T. gondii* extracts were conducted using 12% separating gel and 5% stacking gel at a 20 mA constant current for approximately 2 h as described by Laemmli (1970). Samples of total antigen extract and ESA extract were obtained according to the protocol described previously. Standard markers of molecular weight were used in all experiments (LMV and HMW, Pharmacia, Uppsala, Sweden).

Western blotting - The Western blot technique described by Towbin et al. (1979) was applied to evaluate the *T. gondii* surface antigens recognized by polyclonal and monoclonal antibodies and by the serum samples of the patients. Antigenic fractions on polycrylamide gel (SDS-PAGE) were transferred to a nitrocellulose membrane (Sigma-Aldrich Co, St. Louis, MO, USA). After transferring the fractions to nitrocellulose, the reactions were stopped with a protein solution (0.05% PBS - Tween + 5% nonfat dry milk) and incubated with diluted 1:15 monoclonal antibody 1B8 (anti-p97) (Mineo 1994) or with diluted 1:50 blood samples overnight at 4°C under slow horizontal agitation. Binding of antibodies was verified using immunoenzymatic conjugate (rabbit IgG anti mouse gamma globulin, human anti-IgG or anti-IgM bound to peroxidase-Sigma) and revealed with a diaminobenzidine (DAB) solution containing 5 µl of 30% hydrogen peroxide per ml of PBS.

Statistical analysis - The Chi-square test (Siegel 1975) was applied to verify if there were any significant differences between the frequencies of the presence or the absence of antibodies against p97 in blood samples of patients from group I and group III. Data were compared for Western blot results to total *T. gondii* antigen results as well as for Western blot results to ESA results. Differences were considered significant when P was < 0.05.

RESULTS

ELISA - The ELISA analysis standardized at the Laboratory of Immunology confirmed the existence of a very close relation to those results obtained at the HC-UFU. In group I, IgM and IgG antibodies were positive with dilutions from 1:16 to 1:4096 and from 1:64 to 1:8192, respectively. For group III, IgG antibody was positive with dilutions from 1:256 to 1:4096.

Western blot - Representative data from Western blot with total antigen and ESA of *T. gondii* are demonstrated in Figs 1 and 2. In these assays, proteins from *T. gondii* recognized by human sera were revelated with immunoenzymatic conjugate anti-human IgM and anti-human IgG-peroxidase.

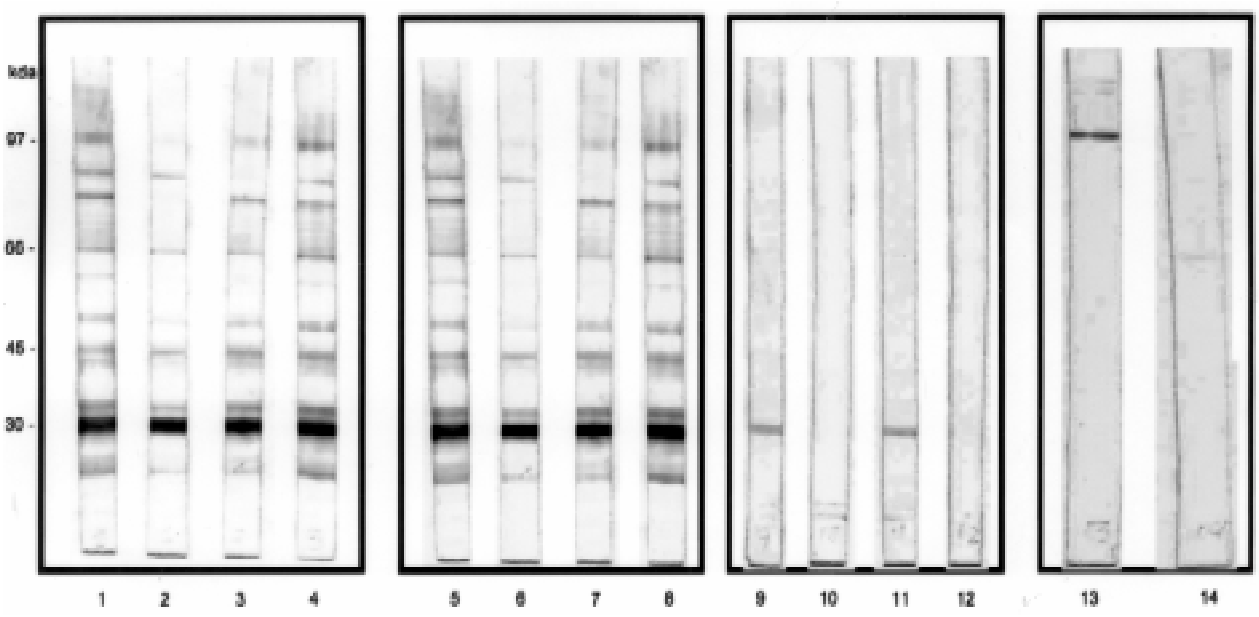

Fig 1: detection of p97 antibodies by Western blot with total antigen of *Toxoplasma gondii* in serum samples of patients, with immunoenzymatic conjugate anti-human IgG. Lanes - 1 to 4: samples of group III (IgG+IgM-); 5 to 8: samples of group I (IgG+IgM+); 9 to 12: samples of group II (IgG-IgM-); 13: mAb 1B8 (monoclonal antibody anti-p97), with immunoenzymatic conjugate anti-mouse IgG; 14: immunoenzymatic conjugate control (without serum).

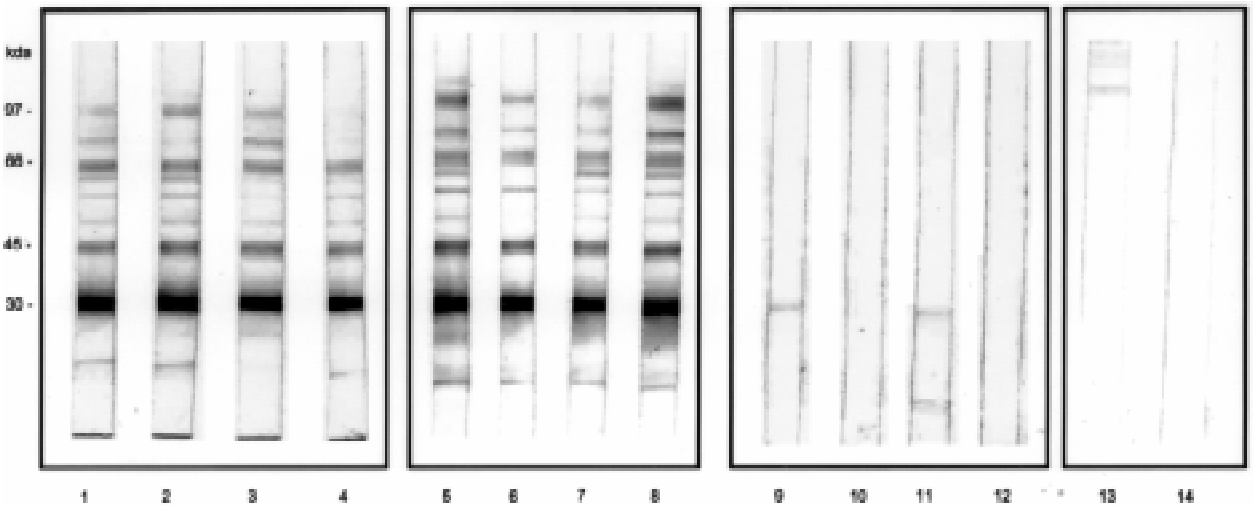

Fig 2: detection of p97 antibodies by Western blot with excreted and secreted antigens (ESA) of *Toxoplasma gondii* in serum samples of patients, with immunoenzymatic conjugate anti-human IgG. Lanes - 1 to 4: samples of group III (IgG+IgM-); 5 to 8: samples of group I (IgG+IgM+); 9 to 12: samples of group II (IgG-IgM-); 13: mAb 1B8 (monoclonal antibody anti-p97), with immunoenzymatic conjugate anti-mouse IgG; 14: immunoenzymatic conjugate control (without serum).

When the total antigen was used, p97 was detected in only two samples from group I (recent phase) and eight samples from group III (chronic phase) (Table). However, it was recognized in these groups only by IgG antibodies. Results obtained with Western blot and total antigens of *T. gondii* indicated that there were significant differences between the frequencies relative to the presence and absence of the band. The frequencies relative to the absence were higher than the frequencies relative to the presence ($P < 0.05$).

With the use of ESA, p97 was detected in 15 serum samples from group I and 18 serum samples from group III (Table). Only one sample of group I identified the p97 through IgM antibodies. The other samples identified this band only by IgG antibodies. Results obtained with Western blot and ESA indicated that frequencies relative to the presence of antibodies were higher than the frequencies relative to the absence of antibodies ($P < 0.05$).

In group II (control group), p97 was not revealed with any antigenic extract.

TABLE

Western blot with total or excreted and secreted antigens of *Toxoplasma gondii* antigens of *Toxoplasma gondii*, showing the presence of p97 antibodies in serum samples collected from patients with acute toxoplasmosis (group I) and chronic toxoplasmosis (group III), from Hospital de Clínicas, Universidade Federal de Uberlândia

	Presence [a]		Absence	
Groups	Total antigen	ESA	Total antigen	ESA
I	2 (10%)	15 (75%)	18 (90%)	5 (25%)
III	8 (40%)	18 (90%)	12 (60%)	2 (10%)
Total	10 (25%)	33 (82%)	30 (75%)	18 (18%)

a: $P < 0.05$; ESA: excreted and secreted antigens from *T. gondii*

DISCUSSION

It has already been reported by several serologic studies that toxoplasmosis can be based on a classification that defines the existence of three serologic profiles in *T. gondii* infection (Camargo et al. 1978).

Profile I: the main marker is the presence of specific IgM antibodies, however a rapid ascension of IgG antibodies is observed (high titers detected by immunofluorescence and low titers by the hemagglutination test); profile II: serological phase of transition in which IgG antibodies were detected in high titers by immunofluorescence and hemagglutination tests. IgM antibodies are absent or are found in low levels during this period and tend to disappear; profile III: IgG antibodies are present in low titers detected by immunofluorescence and hemagglutination tests and anti-*Toxoplasma* IgM is absent.

In the present study, groups of serum samples were established mainly based on the clinical symptoms and the results of the ELISA assay for *T. gondii*. Group I (serum from IgG and IgM positive patients) was made up of samples that could be classified as profile I or profile II according to the titers of antibodies in each of the classes. The serum samples in group II were negative controls of the reactions, while the samples in group III corresponded to profile III infection by *T. gondii*.

The infection is generally diagnosed by demonstration of specific antibodies to *Toxoplasma* in the serum samples of infected patients. But in cases of acute toxoplasmosis, especially during pregnancy, the serologic techniques currently used have been targeted with problems, as persistent positive results for IgM antibodies, even one year more after the primary infection with *T. gondii* (Giraldo et al. 2002).

Gross et al. (2000) studied an immunoblot assay, which compares the early IgG profiles between the mother and her child directed against a total cell lysate of *T. gondii* tachyzoites. The results showed that this test is useful as an additional assay for the rapid diagnosis of congenital toxoplasmosis. The use of alternative diagnostic techniques as described in this work should be considered of great clinical relevance.

Martin et al. (1998) studied the humoral response against Rop2, based on the detection of *Toxoplasma*-specific IgG, IgM and IgA during human *T. gondii* infection. This antigen proved to be a powerful tool for development of serological diagnostic systems to diagnose either chronic or acute infections.

The IgG avidity determination is another important serological marker that can be used to distinguish between recent and chronic infections, allowing such a diagnosis of acute infection to be made from a single serum sample. High-avidity index was characteristic of the chronic phase, suggesting a progressive maturation of the affinity of *T. gondii* specific IgG antibodies after the initial antigenic challenge. Marcolino et al. (2000) observed that p97 was present among various antigenic markers, being frequently recognized by low-avidity IgG in recent infection and by high-avidity IgG in chronic toxoplasmosis.

In the present study, when we analyzed human sera from acute and chronic phases of toxoplasmosis by Western blot, the p97 antigenic fraction of *T. gondii* was detected more frequently by IgG antibodies with the total antigens of *T. gondii* and ESA. When ESA was used, the 97 kDa antigen was detected with high frequence and by IgG antibodies in almost all samples. In contrast to this, another study previously showed that the detection of IgM antibodies against an ESA 97 kDa antigen occurred very soon after infection, and suggested the use of this antigen as a good marker of early acute infection (Decoster et al. 1988). In the present study, however, p97 could not be considered a good marker to distinguish acute from chronic infections since this epitope was recognized in both phases of the infection, when using the ESA or the total antigen extracts from *T. gondii*.

Taken all together, the present results suggest that p97, an excreted and secreted component of *T. gondii*, stimulates the immune system to produce both IgM and IgG antibodies in the beginning of the infection, but with the synthesis of IgG remaining throughout the chronic phase against this antigen. Therefore, further studies are necessary to carried out an epitope mapping in the 97 kDa antigen in order to better select the components that can behave as a good marker of acute toxoplasmosis.

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