

Use of the serum reactivity against *Toxoplasma gondii* excreted–secreted antigens in cerebral toxoplasmosis diagnosis in human immunodeficiency virus-infected patients

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Despite the development of serological and molecular methods in recent years, the diagnosis of cerebral toxoplasmosis in human immunodeficiency virus (HIV)-infected patients still presents difficulties. In the present study, we investigated whether cerebral toxoplasmosis induced changes in the reactivity of serum toward *Toxoplasma gondii* excreted–secreted antigens (ESA) in order to develop an assay for evaluating HIV-infected patients with cerebral toxoplasmosis. The antigen selection was based on those produced by tachyzoites, since it is the form of the organism responsible for disseminating the infection, as well as stimulation of the humoral and cellular immune responses. By using an ELISA containing pooled ESA recovered from infected culture supernatants with tachyzoites-RH strain (ESA-ELISA), we found that ESA had a high specificity for sera from patients with cerebral toxoplasmosis. The reactions were compared with an ELISA using crude tachyzoites antigen, widely used in traditional serology. The assays were performed on 293 serum samples separated as follows: 100 sera from patients with cerebral toxoplasmosis and AIDS (symptomatic), 99 sera from individuals with chronic toxoplasmosis (asymptomatic) and 94 sera from healthy individuals without toxoplasmosis (control). The crude tachyzoite antigen in ELISA was able to distinguish both groups of sera with toxoplasmosis, as similar reactivity were observed in sera from patients with cerebral toxoplasmosis and those from chronic individuals. In contrast, ESA-ELISA distinguished sera from symptomatic and asymptomatic individuals (three times more reactive in the former group, 12.6 versus 4.2). The assays were reproducible based on immunoblotting and statistical analysis. These data suggest the utility of ESA-ELISA in the diagnosis of cerebral toxoplasmosis in HIV-infected patients, since it provided clear evidence that anti-ESA antibodies are present principally in patients with active infection. The absence of a significant amount of antibodies distinguished the patients without clinical symptoms of infection.

Received 11 October 2007

Accepted 7 March 2008

INTRODUCTION

Toxoplasma gondii is an intracellular parasite that is able to infect all mammalian cells. During the chronic phase, the parasites persist encysted in brain and muscle, and the host develops life-long protective immunity against reinfection (Dubey, 1996, 1998; Hill *et al.*, 2005). The active secretion of antigens by *T. gondii* may be an essential part of the low-grade stimulation or boosting of the immune system, as these antigens have been shown to stimulate an antibody as well as a T-cell response (Duquesne *et al.*, 1990; Carruthers, 2002). A group of the most important of these antigens has

been documented as parasite excreted–secreted antigens (ESA), which represent the majority of the circulating antigens in sera from hosts with acute toxoplasmosis (Cesbron-Delauw & Capron, 1993; Tilley *et al.*, 1997). When released by tachyzoites they are highly immunogenic (Prigione *et al.*, 2000; Carruthers, 2002) and induce protective immunity, either antibody dependent or cell mediated (Zenner *et al.*, 1999). CD4⁺ T cells specific for ESA may be involved in the maintenance of long-term immunity in healthy chronically infected individuals. However, when an immunodeficiency appears, such as AIDS, the low levels of CD4⁺ T cells cause parasite proliferation and a symptomatic disease such as cerebral toxoplasmosis develops; this being the most frequent

Abbreviations: ESA, excreted–secreted antigens; HIV, human immunodeficiency virus.

reactivation of the latent infection (Montoya & Liesenfeld, 2004; Hoffmann, 2005).

Cerebral toxoplasmosis remains a prevalent disorder of the central nervous system, particularly among severely immunosuppressed human immunodeficiency virus (HIV)-infected patients (Antinori *et al.*, 2004). This situation is critical in resource-limited settings, where the highly active antiretroviral therapy (HAART) is not available. In Brazil, despite a HAART programme being used, cerebral toxoplasmosis still accounts for high mortality and morbidity (Vidal *et al.*, 2005).

In spite of the fact that a definitive diagnosis of cerebral toxoplasmosis requires demonstration of tachyzoites in brain biopsy or necropsy, in clinical practice treatment is usually initiated upon a presumptive diagnosis (based on clinical and radiological features). Recently, the inclusion of PCR in cerebral toxoplasmosis diagnosis has been shown to be an important tool (Cohen, 1999; Bastien, 2002; Bretagne, 2003; Vidal *et al.*, 2004; Colombo *et al.*, 2005), but it is still unavailable in most of the developing countries. As a significant portion of the world population is infected with *T. gondii*, the serological diagnosis is not so relevant in defining a cerebral toxoplasmosis diagnosis, but some studies suggested that high titres might be indicative of the active disease or a higher risk of developing it (Derouin *et al.*, 1996; Hellerbrand *et al.*, 1996; Colombo *et al.*, 2005).

Currently, there is no information about the utility of ESA in distinguishing the cerebral toxoplasmosis in HIV-infected patients. Thus, we evaluated whether sera from patients with cerebral toxoplasmosis specifically recognize ESA and whether this recognition decreases in asymptomatic individuals. The assays were carried out by ELISA using tissue culture cell supernatants infected with tachyzoites RH strain.

METHODS

Patients and samples. The capability of ESA proteins in distinguishing symptomatic toxoplasmosis was determined by assaying 293 serum samples separated in three groups. Group I, called 'symptomatic sera', was composed of sera from 100 patients with cerebral toxoplasmosis and AIDS. Group II, 'asymptomatic sera', was composed of sera from 99 individuals with asymptomatic toxoplasmosis. Group III, 'control sera', was composed of sera from 94 healthy individuals with negative serology for toxoplasmosis. All HIV-infected patients were admitted and treated at Emilio Ribas Institute of Infectious Diseases, a tertiary teaching hospital in Sao Paulo, Brazil. The sera were previously analyzed by indirect immunofluorescence test and ELISA in the Laboratory of Toxoplasmosis, Instituto Adolfo Lutz. Each serum sample was dissolved in 90% (v/v) glycerol buffered with 0.5 M carbonate/bicarbonate, pH 9.5, as additive, and stored at -20°C until use. ELISA and immunoblotting standardizations were determined using a panel of 30 well-known sera (from 10 healthy individuals, 10 sera-positive individuals and 10 cerebral toxoplasmosis patients). The diagnosis of cerebral toxoplasmosis in HIV-infected patients was based on: (1) progressive neurological deficits, (2) contrast-enhancing mass lesion(s) on computed tomography scans, and (3) successful response within 2 weeks to specific treatment (Portegies *et al.*, 2004). In addition, all patients had at least one

positive PCR of blood and/or cerebrospinal fluid for *T. gondii*, as previously described (Vidal *et al.*, 2004; Colombo *et al.*, 2005). Blood samples for serological diagnosis and PCR were collected from AIDS patients before or until the third day of the specific therapy for toxoplasmosis. The institutional review boards of the ethics committees of the Instituto de Infectologia Emilio Ribas and Instituto Adolfo Lutz ethically approved this study.

***T. gondii* and antigens.** *T. gondii* RH tachyzoites were grown and maintained in Swiss mice ascites by intraperitoneal inoculation. Every 3 or 4 days after infection, the peritoneal fluids from infected mice were collected in PBS, pooled and centrifuged at 1000 g for 10 min. The parasite pellets were washed twice, counted, and suspended in PBS pH 7.2, at different concentrations for infecting tissue cultures and preparing antigens for conventional ELISA. Alternatively, tachyzoites were maintained in Vero cells (ATCC CCL - 81), at 37°C , 5% CO_2 , in Eagle's medium (Gibco) containing 292 mg L-glutamine l^{-1} , 110 mg sodium pyruvate l^{-1} , 1 g glucose l^{-1} , 2.2 g sodium bicarbonate l^{-1} , 100 000 U penicillin l^{-1} , 133 mg streptomycin l^{-1} and 10% fetal bovine serum. The tissue culture parasites were used for immunoblotting and the culture infected cell supernatants were used in ESA recovery.

For conventional ELISA, the crude tachyzoite antigen was obtained by sonication of tachyzoites (10 cycles of 1.0 A min^{-1} , for 5 min, with 2 min intervals). The crude antigen was dissolved in 0.3 M NaCl and the protein concentration was determined. For immunoblotting, the crude tachyzoite antigen was prepared from 1×10^7 tissue culture tachyzoites solubilized in 50 μl SDS-PAGE sample buffer, containing 2% SDS, 50 mM Tris/HCl, 5% 2-mercaptoethanol, 10% (v/v) glycerol, and 0.01% bromophenol blue, boiled and run in 10% polyacrylamide-SDS gels. ESA were used in immunoblotting and ELISA (ESA-ELISA). The proteins were recovered from tachyzoite-infected culture supernatant. For infection with 1×10^7 tachyzoites ml^{-1} , Vero cells were previously washed three times and the medium was replaced by another aliquot of medium without fetal bovine serum. After 48 h post-infection, culture supernatants were harvested, filtered through a 0.22 μm -pore-size filter and 10 μg protease-inhibitor cocktail ml^{-1} added, containing (per ml): 20 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μM EDTA, 1.3 μM bestatin, 0.14 μM E-64, 10 nm leupeptin, 3 nm aprotinin (Sigma). The supernatants, referred to as ESA were concentrated in a Speed Vac (RC 10.09; Jouan) for 4 h and dialysed against PBS overnight at 4°C . Traces of serum were further observed in ESA preparations, by SDS 10% PAGE, despite washing the Vero cells before *T. gondii* infection. Thus, ESA concentrations were determined by dilution for use in ESA-ELISA and immunoblotting.

ELISA and immunoblotting. The sera were tested by ELISA using two antigens. ESA was used at dilution 1:5–1:40 (for each batch of ESA prepared) (ESA-ELISA). The conventional ELISA was carried out using the crude tachyzoite antigen at a concentration of $1\ \mu\text{g ml}^{-1}$ (this antigen is frequently used in conventional diagnosis). The reactions were performed with microtitre polystyrene plates (flat bottom, low binding; Corning). Each plate was incubated overnight at 4°C with the wells containing the antigen dissolved in 0.1 ml 0.1 M NaHCO_3 , pH 8.5. Unbound antigen was removed by washing the plates with PBS pH 7.2 containing 0.05% Tween 20 (PBST). The free binding sites were blocked by treating the wells with 5% skimmed milk/PBS. After 30 min, 50 μl each serum sample diluted 1:200 in 5% skimmed milk/PBS was added to the wells and incubated for 60 min at 37°C . After five washes with PBST, the plates were incubated for a further 60 min at 37°C with horseradish peroxidase-conjugated goat anti-human IgG (Sigma) diluted 1:10 000 in 5% skimmed milk/PBS. After a new wash cycle with PBST, substrate solution (0.1 M citric acid, 0.2 M Na_2HPO_4 , 0.05% *o*-phenylenediamine, 0.1% H_2O_2) was added to each well, and the plates were left to stand at room temperature in the dark for

30 min. Colour development was stopped by adding 50 μ l 2 M H_2SO_4 , and the absorbance was measured with an ELISA reader (Multiscan; Labsystems) with a 492 nm filter. The reactions were previously standardized with 30 sera described above. Different dilutions of ESA (1:10 to 1:80) on the microtitre plates, different conjugate dilutions (1:2500 to 1:20 000) and different serum dilutions (1:50 to 1:400) were tested (data not shown). Each serum sample was assayed in duplicate. The absorbance values were subtracted from the background, and the arithmetic mean was calculated. The cut-off was calculated in each reaction using a sera panel from 20 healthy individuals.

For immunoblotting, ESA and lysed tachyzoites (20 μ l per strip) were boiled and fractionated by run 10% SDS-PAGE (Laemmli, 1970). The proteins were transferred to nitrocellulose membranes (Towbin *et al.*, 1979), cut into 3–4 mm wide strips, blocked for 1 h with 5% skimmed milk PBS and incubated with serum diluted 1:50 at room temperature. After 1 h, the strips were washed with PBS and incubated for 1 h at room temperature with goat horseradish peroxidase-conjugated anti-human IgG diluted (1:500) in 5% skimmed milk/PBS. Bound antibodies were visualized after incubation with 0.03% 4-chloro-1-naphthol (Sigma) in methanol, 50 mM Tris/HCl pH 7.6, 0.1% H_2O_2 .

Data analysis. The cut-off of each reaction corresponded to the mean plus two times the SD of the A_{492} . Next, the absorbance results were transformed into ELISA relative values as described previously (Pereira-Chioccola *et al.*, 2003) and shown in Figs 1 and 2. The ELISA relative values represent the ratio of the absorbance of each serum sample at 492 nm (A_{492}) to the cut-off value (serum absorbance/cut-off absorbance). Values greater than 1.0 were considered reactive.

The results were statistically evaluated by the computer program Sigma Plot 9.0. Differences between the means of the ELISA relative values in symptomatic and asymptomatic groups for ESA and crude tachyzoite antigen were tested for significance by using variance analyses, calculated by *F* test and single factor ANOVA. The coefficient of similarity between the sera groups was calculated by Student's *t*-test (one-tailed and variances). Differences or similarities were considered statistically significant when the *P* value was <0.05.

RESULTS

The first step was to set up the conditions for ELISA and immunoblotting. The checkerboard assay performed with both antigens in an ELISA determined the optimal working dilutions of the crude tachyzoite antigen (1 μ g ml^{-1}) and ESA (dilution 1:5–1:40 depending on the batch) in alkaline buffer (0.1 M $NaHCO_3$, pH 8.5), using the microtitre polystyrene plates type (low binding). Next, the optimal working dilutions were determined for serum samples (1:200) and conjugate (1:10 000), and the intra- and inter-assay reproducibility was determined. These experimental conditions yielded the greatest difference in absorbance between positive and negative samples. For immunoblotting, the standardization assays were performed with ESA. The optimal working dilution for solubilization (20–30 μ l) was 4:1 in SDS-PAGE sample buffer. The optimal working dilution was also determined for serum samples (1:50) and conjugate (1:500).

Next, we analysed the reactivity of the sera for both antigens. The same batch of sera was assayed in parallel for both antigens in order to maintain the same laboratory conditions. The crude tachyzoite antigen is largely used for

'in house' conventional ELISA and commercial kits. We assayed the serum samples from all groups and the results, shown in Fig. 1(a), were expressed in ELISA relative values as described in Methods. The mean ELISA relative value for sera from the control group was 0.5 ± 0.02 (mean \pm SE). In contrast, the values for serum samples from patients of groups I and II were 8.7 ± 0.29 and 8.2 ± 0.26 , respectively. Student's *t*-test revealed that both groups are statistically similar ($P > 0.05$). As expected, serum samples collected from groups I or II patients were reactive with crude tachyzoite antigen (Fig. 1b, strip 1) in immunoblotting, giving similar intensities (Fig. 1b, strips I and II).

At the same time, we tested the same sera in ESA-ELISA. The ELISA relative values are shown in Fig. 1(c). The mean value for sera from control group were 0.5 ± 0.02 . Group I (symptomatic) had high values (12.6 ± 0.74). In contrast, group II (asymptomatic) showed a mean value of 4.2 ± 0.21 . Student's *t*-test showed that both groups are statistically different ($P < 0.05$). These results were con-

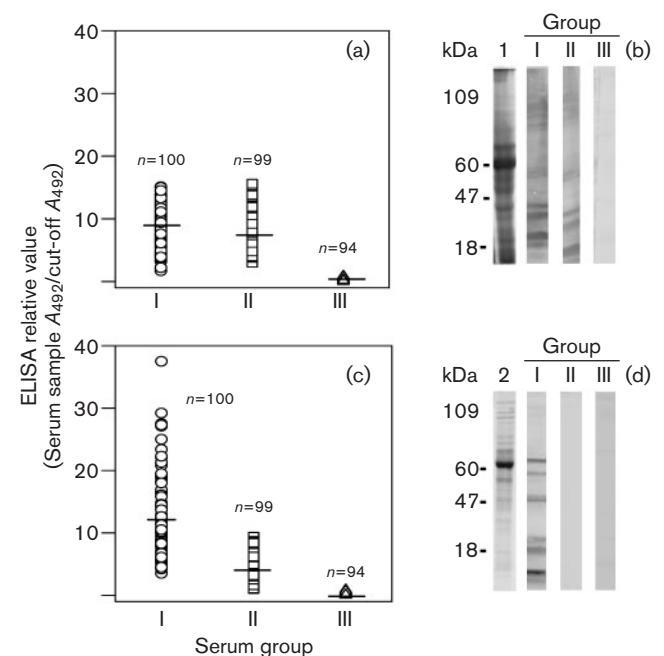


Fig. 1. Serological reactivity of crude tachyzoite lysate (a) and *T. gondii* ESA (c) against sera from patients with cerebral toxoplasmosis and AIDS (○), individuals with chronic toxoplasmosis (□) and healthy individuals (△) by ELISA. The results were calculated as the ratio of the absorbance of each serum sample at 492 nm to the cut-off absorbance value. Values greater than 1.0 were considered reactive. The horizontal lines represent the arithmetic means: 8.7 and 12.7 in group I, 8.2 and 4.3 in group II, 0.5 and 0.4 in group III. Student's *t*-test revealed that groups I and II are statistically similar in (a) and different in (c) at $P < 0.05$. (b, d) Immunoblotting analysis of a lysate- 1×10^7 tachyzoites (b, strip 1) and ESA (d, strip 2), separated by 10% SDS-PAGE and transferred to nitrocellulose. Serum from each group (I, II and III) was incubated with the nitrocellulose strips.

firmed by immunoblotting, as shown in Fig. 1(d). Sera from group I reacted well with ESA (Fig. 1d, strip I), while sera collected from group II failed to do so.

The data presented in Fig. 2 show the distribution of ELISA relative values for sera obtained after testing the reactivity using the crude tachyzoite lysate and ESA in ELISA. The linear regression clearly indicates that the sera from chronic individuals were more reactive against the crude tachyzoite lysate than ESA. The correlation index was 0.48 (Fig. 2a). In contrast, the linear regression shown in Fig. 2(b) indicates that sera from patients with cerebral toxoplasmosis had high reactivity with ESA compared with those with crude tachyzoite lysate. The correlation index was 1.4.

DISCUSSION

Despite development of serological and molecular methods in recent years, the diagnosis of cerebral toxoplasmosis in

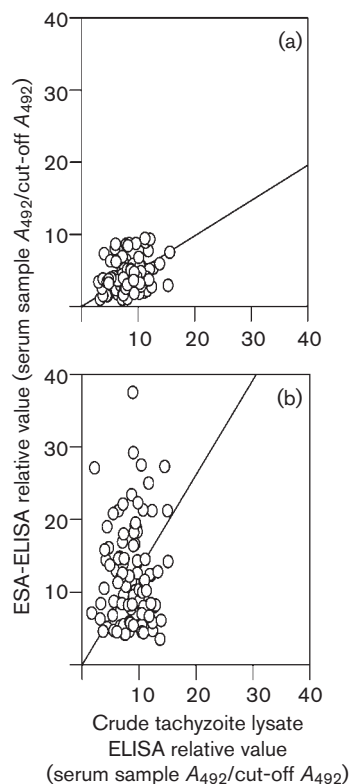


Fig. 2. Correlation between the results of ELISA employing crude tachyzoite lysate and ESA-ELISA. Each point represents the reactivity of each serum sample of chronic individuals (a) and cerebral toxoplasmosis patients (b) using ESA (y-axis) and the crude tachyzoite lysate antigen (x-axis). Each serum result was calculated as the ratio of the absorbance of each serum sample at 492 nm (A_{492}) to the cut-off value. Values greater than 1.0 were considered reactive. r (the correlation index for each test) was 0.48 and 1.4 for (a) and (b), respectively.

HIV-infected patients still presents difficulties. In clinical practice, specific treatment is usually initiated in response to a presumptive diagnosis, which is based on clinical and radiological features (Luft & Remington, 1992). Thus, the employment of the *T. gondii* proteins of the serological diagnosis seems to be a promising tool.

It is known that *T. gondii* invasion initiates a lytic cycle leading to cell and tissue destruction that is a hallmark feature of *Toxoplasma* pathology. This process is rapid, dynamic, and relies on the secretion of numerous secretory proteins from micronemes, rhoptries and dense granules (Carruthers, 2002). Despite significant progress in studying these proteins, only a limited number of secretory proteins have been discovered (ToxoDB: the *T. gondii* genome resource, accessed in September 2007; <http://www.toxodb.org/toxo/home.jsp>) (Zhou *et al.*, 2005). In addition, as we showed recently, a great genetic variability has been observed between the *T. gondii* isolates of patients (Ferreira *et al.*, 2008). For this reason, we decided to use pooled ESA recovered from tachyzoite-infected culture supernatants as antigen because each protein could elicit the host immune system by antibody production.

The vast majority of infected immunocompetent individuals with latent infection by toxoplasmosis remain asymptomatic. Despite the presence of *T. gondii* antibodies, they have no or a low number of circulating tachyzoites. In general, these antibodies are determined in conventional serology, using total extract of tachyzoites as antigen, which consists of cytoplasmic and membrane components. When we used this antigen (crude tachyzoite antigen) in ELISA, similar reactivity was shown between sera from patients with cerebral toxoplasmosis and those from patients with latent infection (mean value of the ratio of the relative absorbance). These results suggest that this antigen, widely used in conventional diagnosis, failed to distinguish the two groups of sera.

In an earlier study we have shown that the majority of the patients with cerebral toxoplasmosis have high anti-*T. gondii* IgG titres with high avidity, suggesting that the reactivation of the latent infection observed in immunocompromised patients occurs in the secondary immune response (Colombo *et al.*, 2005). In this phase, numerous tachyzoites are released from the quiescent cysts and a considerable proportion of ESA are released, eliciting the specific immune response to these antigens. In this case, these patients present antibodies for both ESA and total crude tachyzoites antigens. ESA-ELISA distinguished sera from patients with the active disease (cerebral toxoplasmosis and positive PCR). These sera were three times more reactive than those from seropositive individuals (12.6 to 4.2). These data were reproducible based on the immunoblotting results, showing that ESA constitute an excellent serological marker for the diagnosis of cerebral toxoplasmosis in HIV-infected patients.

Even though the majority of patients with cerebral toxoplasmosis develop high titres of anti-*T. gondii*

antibodies (Derouin *et al.*, 1996; Hellerbrand *et al.*, 1996; Colombo *et al.*, 2005), some also show a humoral response deficiency and no antibodies are detected (Luft & Remington, 1992; Skiest, 2002; Vidal *et al.*, 2005). In this study, six patients with cerebral toxoplasmosis confirmed by a positive PCR of blood had a negative immunofluorescence test (for both IgG and IgM antibodies) and ELISA (both antigens) (data not shown). In this case ESA-ELISA also failed in diagnosing the active infection, confirming the caveats of serological diagnosis in a reduced subset of patients.

In summary, our results suggest that anti-ESA antibodies are present in patients with HIV-related cerebral toxoplasmosis, whereas the absence of a significant amount of antibodies distinguished the patients without clinical symptoms of infection. ESA-ELISA can be used for the diagnosis of cerebral toxoplasmosis in association with clinical and radiological information, providing a simple methodology to be used in countries with high prevalence of latent toxoplasmosis in the general population.

ACKNOWLEDGEMENTS

This study was supported by grants from the FAPESP (Fundação de Amparo à Pesquisa do Estado de Sao Paulo, Brazil), proc-05/03052-5. I. M. R. F., C. S. M. and T. A. C. S. were supported by fellowships from FAPESP: proc-06/52672-9, 06/52674-1 and 06/52673-5.

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