

***Toxoplasma gondii* Antigenuria in Patients with Acquired Immune Deficiency Syndrome**

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A longitudinal study was performed with sera and urine of patients with acquired immune deficiency syndrome (AIDS), taken before, during and after clinically Toxoplasma infection. The tested patients were followed for an average of two years. The titres of the specific IgG and IgM antibodies were measured by an indirect fluorescent antibody test (IFAT), and the appearance of circulating antigens of T. gondii was determined in 36 urine samples of 13 patients with neurotoxoplasmosis by means of the coagglutination test.

The presence of T. gondii antigens in the urine of AIDS patients by this test was correlated with the immunoblot technique, with clinical symptoms and also with pathological findings.

Our results indicate that the detection of T. gondii antigens in the urine of AIDS patients can be regarded as a rapid and efficient method for the diagnosis of acute toxoplasmosis.

Key words: *Toxoplasma gondii* antigens - coagglutination test - AIDS patients - neurotoxoplasmosis

Toxoplasma gondii is recognized as an important pathogen during pregnancy and the perinatal period (Remington & Desmont 1983). It has recently gained additional interest as a cause of life threatening opportunistic diseases in immunocompromised individuals, including patients with acquired immune deficiency syndrome (AIDS) and transplant recipients (Postman et al. 1988). Toxoplasmosis is a major opportunistic parasitic disease in AIDS patients. Toxoplasmic encephalitis (TE) can only be diagnosed very late since the classical serological tests based on the detection of anti-*T. gondii* IgG and IgM are inefficient in these patients (Luft & Remington 1988). More sensitive diagnostic tests for the early diagnosis of TE are needed.

The detection of antigens or parasites of *T. gondii* in different biological fluids is an accurate and rapid diagnosis. Recently, some researchers have described the use of *T. gondii* DNA sequence for the diagnosis of toxoplasmosis using the polymerase chain reaction (PCR) (Dupcuy-Camet et al. 1993). We have reported the use of coagglutination assay to detect *T. gondii* antigens in the urine of infected mice (Fachado et al. 1990) and also in the urine of AIDS patients (Fachado et al. 1992).

We have developed the coagglutination technique using urine of AIDS patients with acute cerebral toxoplasmosis. In order to confirm the toxoplasmic origin of the antigens detected by CoA-Toxo, the immunoblot was employed. The results also were correlated with clinical symptoms of toxoplasmosis, response to specific chemotherapy and anatomopathologic exam if the patient died.

MATERIALS AND METHODS

Production of the soluble antigenic extract - A soluble antigenic extract was prepared from the RH strain of *T. gondii*. The trophozoites were taken from peritoneal exudates of mice, three days after infection. The exudate was centrifuged at 650xg for 10 min in 2 ml of 0.1M phosphate buffer saline (PBS), pH 7.2-7.4 to eliminate host cell residues. The supernatant was discarded and the pellet resuspended with 2ml PBS and repeatedly passed through a 26 gauge needle in order to break the macrophages. It was again centrifuged at 160xg during 10 min at 4°C and the supernatant centrifuged at 650xg during 10 min at 4°C. The pellet was washed three times in 50 ml PBS. The suspension was then sonicated at 4°C to obtain the parasites antigens, and centrifuged at 650xg during 2 hr at 4°C. The protein concentration of the *T. gondii* antigens suspension was determined by the method of Lowry et al. (1951).

Production of antiserum - The anti-*Toxoplasma* antiserum was prepared by dilution of 0.5 ml of the *T. gondii* antigens (1.3 mg/ml of protein) in the same volume of PBS. The diluted antigenic solu-

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tion was mixed in an equivalent volume of Freund's complete adjuvant for subcutaneous inoculation into rabbits (approximately 3 kg body weight). Four inoculations at weekly intervals were performed. Seven days after the f

inal inoculation, the animals were bled by cardiac puncture and the sera stored at -20°C. The anti-*Toxoplasma* antibody titre in the hyperimmune sera was determined by counterimmunoelectrophoresis (1/64).

Preparation of the coagglutination reagent - Cowan I strain *Staphylococcus aureus*, rich in protein A, was sensitized with anti-*Toxoplasma* rabbit antiserum (positive reagent) as previously described (Arvidson et al. 1971). Briefly, a stock solution of *S. aureus* was prepared by suspending lyophilized cell powder (Pasteur Institute) with phosphate buffered saline (PBS) at pH 7.2-7.4, 1 ml of a 10% (v/v) *S. aureus* suspension in PBS was mixed with 0.5 ml of the anti *T. gondii* rabbit hyperimmune serum and was then incubated for 3 hr at 37°C. Afterwards, the mixture was centrifuged at 700xg for 15 min and the supernatant was removed. A volume of 4 ml of PBS containing 0.4% methylene blue and 0.1% sodium azide was added to the packed cells, and the cells were re-suspended by mixing them gently with a Pasteur pipette. This antibody coated staphylococci solution, now in a 5% suspension was stored at 4°C. Before use, the positive reagent may be diluted to about 1%. The optimal dilution depended upon the degree of agglutination required to better distinguish positive from negative controls. Staphylococci coated with the rabbit pre-immune serum were used as a negative reagent.

The coagglutination test - For the coagglutination test, two separated drops of an antigenic solution (sample control) and two drops of urine sample were placed on a white cardboard. A drop of a suspension of staphylococci coated with hyperimmune serum was added to one of the drops of antigenic solution and one drop of the negative reagent to the other sample control. This same operation was performed with the two drops of a urine sample, in which was added the above reagent.

Preparation of the immunoblot - In order to determine the *T. gondii* antigens in urine, the immunoblot technique was performed. The urine samples and soluble antigenic extract controls were solubilized in buffer sample and subjected to electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) on 10% polyacrylamide gel. Gels were run at 35mA at room temperature and were stained with 0.05% coomassie brilliant blue R. Bovine serum albumin (BSA 66kD),

ovalbumin (45kD), pepsin (34,7kD), trypsinogen (24kD), β -lactoglobulin (18,4kD) and lysozyme (14.3kD), Sigma Chemical Co., were used as molecular weight marker. The proteins separated by SDS-PAGE were then transferred to nitrocellulose paper (Schleider & Schull) 0.45 μ m porosite according to the protocol of Towbin et al. (1979). Briefly, the nitrocellulose sheets were treated with 4% skim milk in 0.05% tween 20-PBS to saturate protein binding sites and then cut into strips. The strips were incubated overnight in *T. gondii* specific rabbit polyclonal serum diluted 1:100 with 4% skim milk in 0.05% tween 20-PBS and then incubated during 1 hr with horseradish peroxidase-labelled goat anti-rabbit immunoglobulins G, Sigma Chemical Co., diluted 1:1000. A control strip of conjugate was included. The blotted strips were washed several times and stained with diaminobenzidine (0.004g) in 10 ml PBS with 0.1% hydrogen peroxide.

Patients - From 1989 to 1991, amongst the HIV seropositive at Sanatorio Santiago de Las Vegas (AIDS clinic), 65 patients were classified as stage IV of HIV infection, according to CDC-system (1989). All of them were included in this study. Their written consent was obtained.

At the beginning of the study some of the patients were suspected of having toxoplasmic encephalitis because they presented neurologic manifestations as well as suggestive lesions of cerebral abscess by computerized axial tomography. The detection of *T. gondii* antibodies or antigens was performed in all patients independently of the presence of clinical manifestations suggesting neurotoxoplasmosis.

Sera for toxoplasma serology was obtained at monthly intervals during two years. *Toxoplasma* specific IgG antibodies were measured by IFAT as previously described by Machín et al. (1986). The presence of IgM anti-*T. gondii* antibodies was tested by using a commercial kit (Behring).

Urine was collected monthly in sterile flasks. The detection of *T. gondii* antigen was done by CoA-Toxo. Patients that presented toxoplasmic clinical suspicion were treated until the neurological symptoms have disappeared. Chemotherapy with pyrimethamine-sulphadiazine combination was employed. This combination therapy produced prompt improvement with objective clinical occurring within two to four weeks. The attack effective dose of sulphadiazine was 6 to 8 g/day together with pyrimethamine 75 mg initially followed by 25 to 50 mg daily. The prophylactic regimen of the recurrence with sulphadiazine 1 to 2 g/day plus pyrimethamine 25 mg was employed lifelong.

If the patient died, during the autopsy the confirmation of *T. gondii* in cerebral hemispheres or

other organs was tried to do.

RESULTS

Sixty-five AIDS patients studied had low titres of IgG (<1/128) by IFAT. None of the patients had anti-*Toxoplasma* IgM antibodies.

Fifty-two from 65 AIDS patients were negative by CoA-toxo and immunoblot. Neither at the clinical examination nor the autopsy demonstrated the presence of *T. gondii*.

In 36 urine samples from 13 of all the patients included in this study were detected *T. gondii* anti-

gens by CoA-Toxo test. These results were confirmed by the immunoblot technique.

Focal abnormalities including hemiparesis, cerebella tremor, hyporeflexie, visual disorders, hemiplegia, severe headache as well as adenopathy, vomits, confusion, disorientation were the most frequently presented in this patients' group. In all cases when therapy was given, not only symptomatology, but antigens in urine as well, disappeared at different time intervals.

Toxoplasmic encephalitis often occurred with other diverse disease processes like cryptococcosis, mycobacteriosis, candidiasis, *Pneumocystis carinii*

TABLE
Summary results of the detection *Toxoplasma gondii* antigens in urine of 13 AIDS patients

Patient No.	Sample date	Urinary Test Coa-toxo	Neurological symptoms	Specific therapy	Associatted pathologies	Authopsy presence of <i>T. gondii</i>
1	08/08/91	Pos	Yes	Yes	-	-
	11/08/91	Neg	Yes	Yes	-	-
2	22/06/89	Pos	Yes	-	-	-
	09/04/91	Neg	No	No	-	-
	06/05/91	Pos	No	No	-	-
	10/05/91	Pos	No	No	-	-
	18/05/91	Pos	Yes	Yes	HSV, Cerebral tremus no Hodgkin	Brain
4	11/10/91	Neg	No	No	-	-
	20/10/91	Pos	No	No	-	-
	23/10/91	Pos	Yes	Yes	PCP	-
	26/10/91	Pos	Yes	Yes	Cryptococcosis	-
	15/01/92	Pos	Yes	Yes	-	-
5	20/06/91	Neg	No	No	Cryptosporidiasis	-
	21/09/91	Pos	Yes	Yes	Histoplasmosis ganglionar	-
	14/11/91	Neg	No	Yes	-	Brain calcification
6	01/03/90	Pos	Yes	Yes	-	-
	15/03/90	Pos	Yes	Yes	-	Brain
7	11/03/90	Pos	Yes	Yes	Cryptococcosis cerebral	-
	21/10/91	Neg	No	Yes	-	-
	11/11/91	Pos	Yes	Yes	-	-
	04/12/91	Neg	No	Yes	-	Brain
8	09/01/91	Neg	No	No	-	-
	03/04/91	Pos	No	No	HSV, Candidiasis oral	-
	09/04/91	Pos	Yes	Yes	-	Brain
	22/08/89	Pos	Yes	Yes	Cryptosporidiasis	-
9	09/11/89	Pos	Yes	Yes	PCP, Candidiasis oral	-
	08/03/90	Neg	No	Yes	-	-
	21/01/91	Neg	No	Yes	-	Dissemination in blood
	22/06/89	Pos	Yes	Yes	Cryptosporidiasis, HSV	Brain and heart
11	05/12/91	Pos	No	No	-	-
	09/12/91	Pos	Yes	Yes	-	-
	13/01/92	Pos	Yes	Yes	-	-
12	12/02/91	Pos	Yes	Yes	-	-
	05/03/91	Pos	Yes	Yes	-	Meningoencephalitis by <i>Mycobacterium</i>
13	14/04/90	Pos	No	No	-	-
	08/05/90	Pos	Yes	Yes	PCP	Subacute encephalitis

HSV: Herpes simplex Virus; PCP: *Pneumocystis carinii pneumonia*; Pos: positive; Neg: negative

pneumonia. The clinical data are summarized in Table.

Eleven patients died during the acute illness. The autopsy confirmed the presence of *T. gondii* in the cerebral hemispheres of seven patients. In one, neurologic lesions cured with calcification were found and in another case the parasites on Wright-Giemsa blood stained slides were observed.

In the patients 12 and 13 had agreement between the CoA-Toxo, immunoblot test and clinical symptoms of neurotoxoplasmosis, but parasites were not found in the brain at autopsy. Using a specific serum, in techniques described by detection *T. gondii* antigen in urine, was found as positive the patients who died from *Mycobacterium* and subacute encephalitis, respectively.

Twenty-one urine samples presented a band at 66 kD and 19 had it at 61 kD by immunochemical analyses, using specific serum anti-*T. gondii*. Some minor bands between 58 kD and 18 kD were detected in two urine samples. Background caused by the use of conjugate was eliminated (results not shown).

DISCUSSION

Toxoplasmosis is one of the several opportunistic infectious diseases that affect patients with AIDS. Acute toxoplasmosis, specially with lesions in the central nervous system, has emerged as a major cause of death in AIDS patients suffering from infections due to opportunistic agents. Antibody titration is not the usual diagnostic procedure for the detection of *Toxoplasma* infection in AIDS patients. However, an alternative serological procedure, the detection of circulating antigens, is considered to be useful for serodiagnosis (Hassl et al. 1988).

Huskinson et al. (1989) reported the detection of *T. gondii* antigens in urine samples of AIDS patients by ELISA. Nevertheless, the clinical significance of *T. gondii* antigenuria remains to be studied.

According to our results, serological data is not a good marker of acute phase as shown by the immunofluorescence test. This is probably associated with the altered capacity of these patients to develop a normal antibody response not even including the possibility of detecting the local intrathecal production of antibodies (Luft & Remington 1988).

The experiments reported here confirmed that *T. gondii* antigens can be detected in the urine of AIDS patients, group IV, had been developed symptomatology suggestive of *T. gondii* infection. Their characteristics in relation to the infection were different. Some ones had evident neurologic manifestations of toxoplasmosis from the begin-

ning of the study. Nevertheless, five patients without specific symptomatology of toxoplasmosis, but included in this study because they were classified as stage IV, developed later clinical manifestations of neurotoxoplasmosis and in their urine could be detected antigens by CoA-Toxo and immunoblot before clinical symptoms.

When therapy was administrated, specific antigens disappeared at different intervals. This is possible because of the characteristic of the patients in relation to the degree of damage of the immune system and the opportunistic diseases that they were facing. In addition, the parasite could be controlled by drugs and the secretion-excretion antigens were low, so the sensibility of the technique could not detect this concentration, how was observed in the patient 1, who presented toxoplasmosis alone as an opportunistic infection. This patient left the clinic and was impossible to follow him.

Of the 13 AIDS patients screened which presented clinical symptoms of neurotoxoplasmosis at different stages of the study, 11 died. All of them were positive for *T. gondii* antigens in urine. Trophozoites in the brain of five patients were observed using histologic examination. The presence of calcifications in the brain have been associated with neurotoxoplasmosis. One of the patients in which were not found trophozoites in the brain, calcified lesions were observed.

Consent was not obtained in order to perform the autopsy on patient 9. The parasites had been observed on Wright-Giemsa stained slides of the blood and there is the hypothesis that this patient presented a disseminated infection.

Generalized abnormalities including confusion and disorientation, typical of *Mycobacterium* infection, were presented in patient 12. The possibility of *T. gondii* infection is not excluded because of the presence of persistent adenopathy. In addition there has been an increasing number of reports in which toxoplasmic encephalitis occurred concurrently with other pathogens including *Cryptococcus* neoformans, *Aspergillus* spp. and *M. tuberculosis* (Luft & Remington 1988, Bell et al. 1992).

The diagnosis of *T. gondii* could not be confirmed or discarded because the impossibility of using the immunohistochemical assay. For this reason, the patients 12 and 13 could not be discarded as a positive false. Nevertheless, the subacute encephalitis can be caused by *T. gondii* parasite.

Many studies have been done to characterize excreted-secreted antigens that range from 69 kD to 20 kD of molecular weight (Decoster et al. 1988, Darcy et al. 1988, 1992, Charif et al. 1990, Cabazone et al. 1994). Electrophoretic bands in this

same range have been detected in serum of AIDS patients (Hassl et al. 1988).

With an antiserum against a soluble antigen extract of *T. gondii*, was detected a 66 kD band in the urine samples of all patients with neurotoxoplasmosis. A band of 61 kD was found in 11 of 13 patients. Using an antiserum against the same antigens, the presence of *T. gondii* antigens were demonstrated in all the urine samples by the coagglutination test. Deeper studies are necessary to characterize these molecules. It is important to note that the detection of these antigens were associated with an active *T. gondii* infection as shown by clinical information and anatomopathological examination. It will be of particular importance the positive results to *T. gondii* antigens obtained in patients without symptomatology suggestive of it, moreover when autopsy confirmed the toxoplasmic origin of the antigens.

Since the number of AIDS patients with neurotoxoplasmosis is not high our results should be regarded as preliminary. Nevertheless, because the diagnosis of toxoplasmic encephalitis requires a high index of suspicion the detection of antigens in urine may be a useful tool in the detection of acute human infection in this patients' group.

Further studies are need to determine whether methods for antigens detection will be useful for predicting the prognosis of the disease and response to therapy against *T. gondii* infection.

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