Antigenaemia and antibody response to *Toxoplasma gondii* in human immunodeficiency virus-infected patients

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ABSTRACT

Toxoplasma encephalitis in immunocompromised patients results from reactivation of previously acquired (latent) infection. The aim of the study is to assess the antigenaemia and antibody response to *Toxoplasma gondii* in human immunodeficiency virus (HIV)-infected patients to determine the best marker for early diagnosis of toxoplasmosis in such patients. Indirect enzyme-linked immunosorbent assay (ELISA) for detection of IgG, IgM and IgA anti-toxoplasma antibodies and double-sandwich ELISA for toxoplasma antigen is carried out in serum samples collected from 100 HIV seropositive patients and 75 controls. Toxoplasma-specific IgG, IgM and IgA antibody response and antigenaemia were detected in 12%, 6%, 7% and 14% of HIV-infected patients, respectively. On retrospective analysis of 14 patients with antigenaemia only one had central nervous system (CNS) symptoms attributable to toxoplasma infection. In this patient, the $CD4^+$ cell count was below 50/µL and none of the specific immunoglobulin isotype responses could be detected. The patient showed clinical improvement following specific chemotherapy for toxoplasmosis. In 25 HIV-negative and anti-toxoplasma IgG antibody-positive controls, IgM was detected in two (8%), IgA in five (20%) and antigenaemia in 10 (40%), while 50 HIV seronegative healthy controls were negative for both antigen and antibody responses. The study indicates that detection of toxoplasma antigen in addition to IgG antibody response may prove to be a useful indicator in the early diagnosis of reactivated toxoplasmosis in HIV/AIDS patients.

KEY WORDS: Antibodies. Antigens. HIV. Toxoplasma gondii.

Introduction

In the majority of normal, healthy (immunocompetent) subjects, infection with *Toxoplasma gondii* is asymptomatic and frequently results in the chronic persistence of cysts within host tissues. The cysts normally lie dormant, probably for life, but in immunocompromised states such as in human immunodeficiency virus (HIV) infection/acquired immune deficiency syndrome (AIDS) this latent infection is reactivated and can cause severe disease. Without prophylaxis, a third of HIV-infected persons with *T. gondii* infection develop clinical toxoplasmosis, and the risk of developing toxoplasma encephalitis is highest when $CD4^+$ cell counts drop below 100 cells/µL.

Toxoplasmosis is the most common cause of secondary central nervous system (CNS) infection in AIDS, accounting for 38% of all infections,[1] and the most commonly recognised cause of CNS mass lesions in patients with AIDS.[2,3] It is the initial AIDS-defining condition in 2% of the AIDS patients in the US. Overall, toxoplasmosis is present in about 15% of patients with AIDS and is most commonly reported in patients from the Caribbean and France.[1]

Diagnosis of toxoplasmosis is based mainly on clinical suspicion aided by radiological and immunological findings. Computed tomography (CT) scanning and magnetic resonance imaging (MRI) are the key methods for identifying lesions suggestive of CNS toxoplasmosis. However, scanning results may be inconclusive and the non-availability of CT and MRI facilities necessitate diagnosis by immunological methods.

The prevalence of toxoplasma antibodies varies considerably among different populations and ranges from 10–40% in the US up to 70–80% in Central America, France, Turkey and Brazil.[4] In HIV-infected individuals, raised antibody titre can be asymptomatic or precede the development of clinical manifestations. However, in such patients, the antibody response (first IgM, then IgG) is generally normal and therefore serological detection of antibodies cannot be relied upon to rule out toxoplasmosis. IgM antibodies are usually undetectable, while IgG antibodies are present at low titres (<1:100),[5] and does not necessarily indicate an acute infection. Detection of toxoplasma antigen shows greater sensitivity over antibody detection in such patients.[2]

As HIV-positive cases continue to increase in India, it is imperative to be cautious about opportunistic parasitic infections in such patients. Reports indicate that cryptosporidiosis (27.48%) is the most common parasitic infection in AIDS cases in India, followed by toxoplasmosis

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Table 1. Antigenaemia and anti-toxoplasma IgG, IgM, IgA antibody response in HIV/AIDS patients and controls.

	Antigenaemia	Anti-toxoplasma antibodies		
		IgG	IgM	IgA
Group I HIV seropositive population (n=100)	14 (14%)*	12 (12%)	6 (6%)	7 (7%)
Group II Toxoplasma IgG seropositive patients, HIV seronegative (n=25)	10 (40%)*	25 (100%)	2 (8%)	5 (20%)
Group III Other parasitic infections (n=25)	0	0	0	0
Group IV HIV seronegative healthy controls (n=25)	0	0	0	0

* P <0.02

Table 2. Anti-toxoplasma IgG, IgM, IgA antibody response and toxoplasma antigen in HIV/AIDS patients (n=100).

Number of patients	Antigenaemia	Antibody response IgG	IgM	IgA
3	+	+	+	+
2	+	+	–	–
0	+	–	+	+
5	–	+	–	–
9	+	–	–	–
1	–	–	–	+
0	–	+	+	–
80	–	–	–	–

(4.95%).[6] Toxoplasma infection has been reported from almost all areas of India, with increasing seropositivity.[7–11] The overall seroprevalence of toxoplasmosis in AIDS patients was found to be 67.4% in Bombay, and it is suggested that the seroprevalence may vary according to geographical locations.[12]

Wadia *et al*.[13] suggest that in absence of biopsy confirmation, empirical anti-toxoplasma treatment should be initiated for all HIV patients with mass lesions. Those HIV-infected persons at risk of toxoplasmosis should receive prophylaxis as a high priority. Indications for prophylaxis include a positive IgG antibody response and $CD4^+$ cell count less than 100 cells/μL.[14] However, early diagnosis followed by prompt treatment is important in such patients.

The present study aims to assess the antigenaemia and IgG, IgM and IgA antibody response to *T. gondii* in HIV infected/AIDS patients. Furthermore, retrospective analysis of antigen and antibody responses with $CD4^+$ cell counts is performed in HIV and toxoplasma seropositive and/or antigen-positive patients in order to define the best marker for early diagnosis of toxoplasmosis in such patients.

Materials and methods

Subjects and samples

One hundred HIV seropositive individuals with or without AIDS, as defined by the CDC classification,[15] were included in the study. Seventy-five other patients/subjects were included as controls. All patients/subjects gave informed consent and were grouped as follows: 100 HIV seropositive individuals (group I); 25 apparently normal healthy individuals who were positive for *T. gondii*-specific IgG antibody on routine testing and who were HIV seronegative (group II); 25 patients comprising five each suffering from malaria, hydatidosis, cysticercosis, amoebiasis and leishmaniasis (group III); 25 normal healthy individuals, seronegative for HIV-I by enzyme- linked immunosorbent assay (ELISA) (group IV). Blood samples were drawn from all the patients and controls and separated serum was stored at –20°C.

Parasite strain and antigen preparation

Antigen used for ELISA was prepared from tachyzoites of an RH strain of *T. gondii* maintained in Swiss albino mice. The crude soluble toxoplasma antigen was prepared as described previously.[11] Briefly, the harvested organisms (tachyzoites) were suspended in a small volume of phosphate-buffered saline (PBS). After three cycles of freezing and thawing, the parasite suspension was sonicated in an ultrasonic disintegrator (MSE Soniprep) for 20 seconds. The material was centrifuged at 12,000 rpm for 30 min to sediment the debris. The supernatant was used as the stock antigen. Protein concentration was estimated[16] and the antigen stored at –70°C. This antigen was used for raising hyperimmune serum in rabbits and also for coating the wells of ELISA plates to detect toxoplasma antibodies.

Antibody detection

Antibody response (IgG and IgM) to *T. gondii* was detected by an in-house standardised ELISA technique. IgA response was detected by a commercially available kit.

IgG and IgM: Indirect ELISA was carried out according to slightly modified standard technique.[11,17] Optimum dilutions of the antigen, test sera and conjugate were determined by checkerboard titration with known positive and negative sera. Each microtitre plate well was coated by overnight incubation at 4°C with 100 μL antigen (1 μg/well) and washed (x3) with PBS-Tween (PBS-T). The wells were blocked with 2% bovine serum albumin (BSA), incubated at 37°C for 1 h, and then washed (x3). Each serum sample was prepared as a 1 in 800 dilution (predetermined) and

Table 3. Antibody response, antigenaemia and clinical profile in relation to CD_4^+ cell counts in HIV/AIDS patients (n = 9).

Patient No.	CD4+ cell counts (/μL)	Antigenaemia	Antibody response			Symptoms attributable to toxoplasma infection	
			IgG	IgM	IgA	Headache, fever	Altered sensorium
1	<50	+	–	–	–	+	+
2	100–200	+	–	–	–	+	–
3	100–200	+	+	–	–	+	–
4	100–200	+	–	–	–	+	–
5	200–400	–	+	–	–	+	–
6	200–400	+	+	–	–	+	–
7	200–400	+	–	–	–	+	–
8	200–400	–	–	–	+	+	–
9	>400	+	+	+	+	+	–

100 μL was added to each well. Plates were incubated at 37°C for 1 h, followed by a wash and the addition of 100 μL horseradish peroxidase (HRP)-labelled antihuman IgG/IgM (Dakopatts, Denmark) diluted 1 in 4500 (predetermined). The plate was incubated at 37°C for 1 h, washed and then 100 μL substrate solution (orthophenylene diamine [OPD] and hydrogen peroxidase) was added to each well. The plates were kept at room temperature for 15 min and the enzyme-substrate reaction was stopped with 1 mol/L H_2SO_4. Absorbance (*A*) was read in an automated ELISA reader (Eurogenetics, Tessenderle, Belgium) at 492 nm. Each sample was assayed in duplicate and positive and negative samples were included in each plate, together with substrate and buffer blanks. The cut-off A value for a positive reaction was calculated as the mean + 2 standard deviations (+2SD) of the *A* values of five negative controls.

IgA: ELISA was performed with a commercially available kit (Enzywell Toxoplasma IgA ELISA kit, Diagnostica Sense, Italy) as per the manufacturer's instructions.

Antigen detection

Hyperimmune serum: Two adult male New Zealand rabbits (6–8 months old) were immunised (1 mg toxoplasma antigen mixed with an equal amount of Freund's complete adjuvant) subcutaneously at multiple sites, once a week for 4 weeks, followed by two boosters. Blood was obtained from an ear vein two weeks after the last injection and checked for anti-toxoplasma antibodies by a gel diffusion test. Subsequently, the rabbits were bled intracardially to collect as much blood as possible without sacrificing the animal. The sera was separated and stored at –70°C. IgM was removed by the addition of 1% 2-mercaptoethanol. IgG was purified by ammonium sulphate precipitation and subsequent dialysis.[18] Purified IgG was used to coat the microtitre plates (1 μg/well) for the double- sandwich ELISA method

Double-sandwich ELISA: The technique was performed by a slightly modified standard technique.[11,17] Optimum dilutions of hyperimmune serum, test samples and conjugates were determined by checkerboard titration. Each microtitre plate well was coated by overnight incubation with 100 μL purified *T. gondii* antibody (1 μg/well), followed by washing (x3) with washing buffer (PBS-T). The wells were blocked with 2% BSA for 1 h at 37°C, followed by washing (x3). The plates were incubated with 100 μl serum per well, diluted 1 in 200 (predetermined), and then washed (x3). Then, 100 μL secondary purified human anti-toxoplasma IgG antibody (serum pooled from five toxoplasma seropositive subjects with high IgG titres) was added to each well (0.1 μg/well), incubated at 37°C for 1 h and then washed (x3). HRP-conjugated antihuman IgG/IgM (100 μL; diluted 1 in 4500 [predetermined]; Dakopatts) was added to each well and the plate was incubated at 37°C for 1 h, washed (x3) and substrate was added. Plates were kept at room temperature for 15 min and reaction was stopped with 1 mol/L H_2SO_4. Absorbance values were read in an ELISA reader at 492nm. Each sample was assayed in duplicate and positive and negative serum samples were included in each plate, together with substrate and buffer blanks.

CD4+ cell count

Absolute $CD4^+$ T lymphocytes were measured by flow cytometry (FACS COUNT; Becton Dickinson, USA) and commercially available monoclonal antibody (Becton Dickinson) in HIV seropositive patients (group I) wherever possible. Briefly, 100 μL EDTA whole blood was incubated with anti-CD4 monoclonal antibody (antihuman, FITC-labelled) for 30 min in the dark at room temperature. After washing and lysis steps, the cells were fixed in 0.5% paraformaldehyde. A total of 10,000 cells were counted and the percentage $CD4^+$ cell count was obtained.

Statistical analysis

Results were compared between patients and control groups and evaluated by the χ^2 test.

Results

Threshold *A* value calculated as the mean (+2SD) of the five negative controls was found to be 0.323 and 0.217 for IgG and IgM, respectively. Samples showing responses equal to or above this value were considered positive. Results were interpreted as per manufacturer's instructions for IgA. Threshold *A* value calculated as mean (+2SD) of the

negative controls was 0.178 and samples equal to or above this value were considered positive (Table 1).

Correlation of antibody response and antigenaemia in HIV seropositive/AIDS patients is shown in Table 2. $CD4^+$ cell counts were available for nine patients in whom antigenaemia and/or anti-toxoplasma antibodies (IgG, IgM or IgA) were detected (Table 3).

Discussion

Almost all infection due to *T. gondii* in HIV-infected patients is due to reactivation of latent infection. The pre-existing infection is denoted by the presence of a specific IgG antibody response, while IgM and IgA usually denote active infection or reactivation in HIV-infected patients. However, due to immunosuppression, the antibody responses in HIV/AIDS patients may not be detectable, or are often low, making definition of the disease phase and therapeutic decisions difficult.

Although parasite DNA detection by the polymerase chain reaction (PCR) has been reported as a rapid and effective technique in the early diagnosis of toxoplasmosis in HIV-infected patients,[19–21] its sensitivity and specificity are variable. Moreover, lack of expertise and equipment in most diagnostic centres in developing countries poses problems in its use, and antigen detection may prove a better option in such circumstances.

Previous studies have shown that IgM anti-toxoplasma antibodies are usually undetectable in HIV-infected populations. In the USA, IgM antibody is rarely demonstrable in sera from such groups, almost all of which have IgG antibody to toxoplasma before the onset of neurological symptoms. In France, however, where toxoplasma infection is more prevalent, specific IgM antibody has been demonstrated in as many as 20% HIV/AIDS patients with acute toxoplasma encephalitis.[2]

Weiss *et al*.[22] reported that specific antibody or pattern of antibodies did not distinguish chronic from acute toxoplasmosis, with or without HIV infection, and suggested that other approaches involving antigen detection should be pursued.[22] Although IgM antibodies were detected in six (6%) patients in the present study, three of these also had detectable IgG, IgA and toxoplasma antigen. $CD4^+$ cell counts were available for only nine patients but a downward trend was observed that mirrored a decline in antibody response, particularly IgM and IgA.

Symptoms of toxoplasma encephalitis in HIV-infected patients are often vague and non-specific. Headache is present in about 50% patients, fever in 40–50% and seizures in 15–30%.[1,2] In a study conducted in Kuala Lumpur, headache was reported as the most common symptoms (67.7%).[23]

Headache and fever was present in 11 (79%) of 14 patients with detectable antigenaemia in the present study. None had a history of seizures or any focal neurological deficit. However, one patient presented with altered consciousness and toxoplasma encephalitis was suspected clinically. Magnetic resonance imaging showed multiple space-occupying lesions in the mid-brain. Antigenaemia was detected in this patient, $CD4^+$ cell count was $<50/\mu L$, but antibody response (IgG, IgM and IgA) was absent. He received high-dose sulphadiazine and trimethoprim combination therapy and showed clinical improvement after one month.

Although results from a single case are meaningless, observation suggests that toxoplasma antigenaemia could be taken as evidence of reactivation of latent toxoplasma infection in HIV-positive patients. Furthermore, those with antigenaemia may be considered for prophylactic or therapeutic administration of anti-toxoplasma drugs to limit development of toxoplasma encephalitis.

Previously, antigen and antibody responses to *Leishmania donovani* stage-specific and rk39 antigen were detected in the same 100 serum samples used in the present study, and 5% showed positive antigenaemia and/or antibody responses.[24] However, none of the HIV-infected patient was seropositive and/or antigen positive to both *L. donovani* and *T. gondii*.

In conclusion, the present study suggests that approximately 20% HIV-positive patients in north India are at risk of developing toxoplasma encephalitis, as indicated by circulating toxoplasma antigen and/or a positive specific antibody response. Toxoplasma antigen detection may be useful for the early detection of reactivation of latent toxoplasma infection and for the diagnosis of suspected toxoplasma encephalitis.

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