Antigenaemia and antibody response to *Leishmania donovani* stage-specific antigens and rk39 antigen in human immunodeficiency virus-infected patients

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Accepted: 26 August 2003

ABSTRACT

In order to define the possible markers for the early diagnosis of asymptomatic visceral leishmaniasis in human immunodeficiency virus (HIV)-infected individuals, the antigenaemia and antibody response to stage-specific *Leishmania donovani* and rk39 antigens is assessed by enzyme-linked immunosorbent assay (ELISA) and immunoreactivity to stage-specific antigens analysed by Western blot. Serum samples from two out of 100 HIV-infected individuals were found positive for antigenaemia, antibody response to stage-specific *L. donovani* antigens and rk39 antigen, and one sample was also positive for antigenaemia and antibody response to *L. donovani* antigens, while antibody detection to rk39 antigen was not carried on this sample. Additionally, one sample was found positive for amastigote antigenaemia and antibody response to amastigote antigen, while in this patient promastigote antigenaemia and antibody response to promastigote *L. donovani* and rk39 antigen could not be detected. One sample was found positive for antigenaemia, antibody response to amastigote antigen and negative for antibody response to promastigote antigen, while in this patient response to rk39 antigen was borderline. Although antibody response to rk39 antigen could be detected in 9/88 (10%) HIV-infected individuals, in six of these nine patients neither antigenaemia nor antibody response to stage-specific *L. donovani* antigens could be detected. All 10 confirmed visceral leishmaniasis and HIV-negative control patients had positive antigenaemia and antibody response to *L. donovani* amastigote and promastigote antigens, while all the normal healthy individuals were negative. The study indicated that detection of antibody response to rk39 antigen, amastigote antigenaemia and antibody response to amastigote antigen may prove to be better markers than detection of promastigote antigenaemia, antibody response to promastigote antigen and immunoblot reactivity.

KEY WORDS: Human immunodeficiency virus. Leishmania donovani. Visceral leishmaniasis. Western blot.

Introduction

Visceral leishmaniasis (VL) has become an important opportunistic infection among individuals infected with human immunodeficiency virus type I (HIV-I), particularly in those geographical areas where both infections are endemic.[1,2] To date, most co-infections have been reported from southern Europe, with a clear predominance in Spain, where *Leishmania infantum* is endemic.[3] India falls into the rapidly progressing zone for HIV-I positive individuals; therefore it is imperative to be cautious about the opportunistic infections in such individuals. The gold standard clinical criteria for the early diagnosis of HIV-VL co-infection is not well defined and may vary in different geographical locations.

Foolproof laboratory diagnosis of VL is the demonstration of parasites in tissue samples and/or its isolation by culture techniques. However, these methods have limitations, being invasive, time consuming and less sensitive, and can be complicated by the general deterioration of these patients in advanced stages.

Serology techniques are simple, non-invasive approaches and useful in the diagnosis of VL in immunocompetent individuals. However, reports on the serological response to *L. donovani* in HIV-I infected individuals are scarce and often incomplete and discrepant. Although the conventional serodiagnostic techniques for antibody detection (using rk39 protein) have shown high sensitivity for the diagnosis of VL,[4,5] there are conflicting reports in leishmaniasis and HIV co-infected patients, and immunoblotting has proved a useful tool for studying the natural course of leishmaniasis in HIV-I-infected patients.[6]

It has been suggested that antigen detection is a better option than antibody detection in such patients with a suppressed immune response.[7] Moreover, an earlier study reported the use of promastigote antigen, and it is felt that the use of an amastigote antigen may yield better results as the amastigote stage leads to active infection and the development of an immune response.[5]

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Although, the amplification of specific sequences of leishmania DNA by a polymerase chain reaction (PCR) technique may prove useful in the diagnosis of HIV-VL co-infected patients but problems of standardisation[8,9] and non-availability at all the centres has hampered its use for routine diagnosis.

This study aims to detect stage-specific *L. donovani* antigens, antibody response using stage-specific and rk39 antigens, and to identify the immunoreactive amastigote and promastigote antigenic components by Western blot in HIV-I-infected individuals in northern India, in order to define the best possible marker for the early diagnosis of HIV-VL co-infection.

Materials and methods

Subjects and samples

Blood samples were collected from the following groups of patients and controls and serum stored at –20°C until used.

Group I: 100 hundred HIV seropositive individuals (71 males, 29 females; age range 15–55 years [mean 33.3 years]), with or without acquired immune deficiency syndrome (AIDS) as defined by the CDC classification,[10] either attending the out-patients department or admitted to the Department of Internal Medicine and allied specialties at Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. All gave informed consent.

Group II: 10 confirmed VL patients from endemic areas.[11]

Group III: 10 patients, comprising two each suffering from amoebiasis, toxoplasmosis, malaria, hydatidosis and cysticercosis.

Group IV: 25 normal healthy individuals, seronegative for HIV-I by enzyme-linked immunosorbent assay (ELISA).

Parasite strain and antigen preparation

The Indian strain of *L. donovani* (MHOM/IN/80/DD8) maintained in both promastigote and amastigote stages by serial subculturing in NNN[12] and RPMI-1640[13] media, respectively, in the Department of Parasitology was used for the preparation of promastigote and amastigote antigens.[11] Protein concentrations were estimated[14] and the antigens stored at –20°C until used.

Antibody detection

Antibody responses to *L. donovani* amastigote and promastigote antigens were detected by an in-house ELISA technique, and to the promastigote antigen also by a commercially available kit.

ELISA technique: This was carried out according to a standard method,[15] with slight modification.[16] Briefly, optimum promastigotes, amastigote antigen and serum dilutions were determined by a checkerboard titration method with five positive and five negative sera. Each well of a microtitre plate was coated by overnight incubation at 4°C with 100 µL antigen (1 µg/well) and washed (x3) with phosphate-buffered saline-Tween (PBS-T). The wells were blocked with 2% bovine serum albumin and plates were incubated at 37°C for 1 h, and then washed (x3). Each serum sample was prepared as a 1 in 400 dilution (predetermined) and 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 (Dakopatts, Denmark) diluted 1 in 4500 (predetermined). The plate was incubated at 37°C for 1 h, washed and then 100 µL substrate solution consisting of orthophenylene diamine (OPD) and hydrogen peroxide 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 (A_4 Eurogenetics, Tessenderle, Belgium) at 492 nm. Each sample was assayed in duplicate and positive and negative serum samples were included in each plate, together with substrate and buffer blanks. The samples were also analysed for antibody response to promastigote antigen by a commercially available kit (R-Biopharm, Darmstadt, Germany), following the manufacturer's instructions.

Antibody response to rk39 antigen: Samples collected from 88 HIV-I-seropositive patients (Group I) were transported to the Molecular Biology Laboratory, Institute of Pathology (ICMR) Safdarjung Hospital, New Delhi, under refrigerated conditions for detection of the antibody response to recombinant K39 antigen. Recombinant K39 antigen was a gift from Steve Reed (Corixa, Seattle). Polystyrene microtitre plates (Corning NY) were coated with 10 ng rk39 protein per well and ELISA was carried out as described previously.[5] Each sample was assayed in triplicate, along with appropriate positive and negative controls.

Antigen detection

Hyperimmune sera: This was raised in adult male white New Zealand rabbits, 6-8 months old by immunising two rabbits with 1 mg promastigote and two rabbits with 1.2 mg/mL amastigote *L. donovani* antigen (protein concentration 1.2 mg/mL), with an equal amount of Freund's complete adjuvant, subcutaneously at multiple sites once a week for four weeks, followed by two boosters. The rabbits were bled first from an ear vein and sera were checked for anti-leishmania antibodies by a gel diffusion test. This was followed by collection of blood from heart without sacrificing the animal.[17] Sera were separated and stored at –70°C. IgM was removed by the addition of 1% 2-ME and IgG was purified by ammonium sulphate precipitation and subsequent dialysis.[17] Pure IgG was then used to coat the microtitre plates.

Double sandwich ELISA: Double antibody sandwich ELISA was carried out according to a standard technique,[15] with slight modification. Optimum dilutions of hyperimmune sera, test samples and conjugates were determined by checkerboard titration techniques. Each well of a microtitre plate was coated by overnight incubation with 100 µL purified antibody against promastigote and amastigote antigen separately (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) followed by washing (x3). Then, 100 µL secondary purified

Table 1. Stage-specific antigenaemia and antibody responses to stage-specific antigens and rk39 antigen (number positive/number tested).

		Antigenaemia		Antibody responses			
Group				Amastigote	Promastigote	Kit	rk39
		Amastigote	Promastigote	in-house ELISA			
I	HIV +ve	5/100	4/100	4/100	3/100	3/100	9/88
II	HIV–ve, VL confirmed	10/10	10/10	10/10	10/10	10/10	ND
III	Other parasitic diseases	0/10	0/10	0/10	0/10	0/10	ND
IV	**Healthy subjects**	**0/25**	**0/25**	**0/25**	**0/25**	**Nil**	**ND**
ND:	Not done						

human anti-leishmania IgG antibody (serum pooled from five confirmed kala-azar patients) was added to each well (0.1 µg/well), which was incubated at 37°C for 1 h and then washed (x3). Horseradish peroxidase (HRP)-conjugated antihuman IgG (100 µL; diluted 1 in 4500, predetermined; Dakopatts, Denmark) was added to each well and the plate was incubated at 37°C for 1 h, followed by washing (x3) and the addition of substrate. Plates were kept at room temperature for 15 min and reaction was stopped with 1 mol/L H2SO4. Absorbance (*A*) values were read in an ELISA reader at 492 nm. Each samples was assayed in duplicate and positive and negative serum samples were included in each plate, together with substrate and buffer blanks.

SDS–PAGE and immunoblotting: Soluble amastigote and promastigote *L. donovani* antigens were separated electrophoretically by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli,[18] and separated proteins were subjected to immunoblotting as detailed previously[19] using 50 serum samples from HIV and VL-seropositive patients (n=5), HIV-negative and confirmed VL patients (n=10), HIV-seropositive and VL-seronegative patients (n=20), patients with other parasitic infections (n=10) and healthy normal individuals (n=5). The predetermined optimum serum dilution used was 1 in 400, as assessed by testing doubling dilutions of sera (from 1 in 100).

Results

Enzyme-linked immunosorbent assay
Antibody response to amastigote antigen: The threshold *A* value calculated as the mean +2SD of the five negative controls was 0.333. Samples equal to and above this value were regarded as positive. Antibody response was positive in five (5%) and 10 (100%) samples in group I and II patients, respectively, while none of the samples in groups III and IV showed a positive response (Table 1).

Antibody response to promastigotes antigen – in-house ELISA: Threshold *A* value was found to be 0.288 and samples equal to or above this value were considered positive. The antibody response was positive in three (3%) and 10 (100%) samples in group I and II patients, respectively, while none of the samples in groups III and IV showed a positive response (Table 1).

Antibody response to promastigote antigen – kit method: Antibody response to promastigote antigen was positive in three (30%) samples in group I and 10 (100%) samples in group II patients. None of the samples in groups III and IV showed a positive response (Table 1).

Antibody response to rk39 antigen: Out of 88 samples collected from HIV-I infected patients (group I), nine (10%) were positive, one of which showed a borderline *A* value. Samples from groups II, III and IV were not tested for anti rk39 antibody response (Table 1).

Amastigote antigenaemia: Threshold *A* value calculated as mean +2SD of the negative controls was 0.219. Amastigote antigenaemia was detected in five (5%) and 10 (100%) samples from group I and II patients, respectively, while none of the samples in groups III and IV showed a positive response (Table 1).

Promastigote antigenaemia: Threshold *A* value calculated as mean +2SD was 0.168. Promastigote antigenaemia was detected in four (4%) and 10 (100%) samples from group I and group II patients, respectively. None of the controls (groups III and IV) showed antigen positivity (Table 1).

Immunoblotting: Western blot analysis showed multiple immunoreactive antigenic fractions, ranging in molecular mass from 9–205 kDa for the amastigote antigen and 18–180 kDa for the promastigote antigen. Reactivity was similar in samples from five HIV-I-infected patients who were VL seropositive (group I) and 10 VL-confirmed patients who were HIV-negative (group II). The majority of the samples in groups I and II recognised 9, 31, 62, 66, 116, 138, 200–205 and 62, 84, 138, 200–205 kDa amastigote antigenic fractions, respectively, while samples in both groups reacted with lower molecular mass promastigote antigenic fractions (45, 55–60 and 66–70 kDa, respectively).

Discussion

Visceral leishmaniasis has re-emerged and 90% of the total cases worldwide occur in India, Nepal, Bangladesh and Sudan.[20] The disease is seen mainly in immunocompetent hosts in endemic areas but it has also been seen as a complicating infection in immunosuppressive conditions.[21-23]

As HIV-I is a frequent cause of immunosuppression, it is not surprising that an increasing number of VL cases have been described in HIV-I-infected individuals in areas where infections overlap.[24] In AIDS patients, VL is regarded as a prevalent recurrent disease with a clinical course that is

Table 2. Immunoreactivity to *Leishmania donovani* antigens by Western blotting.

Group	Promastigote antigen		Amastigote antigen	
	kDa	Samples immunoreactive	kDa	Samples immunoreactive
HIV seropositive and leishmania seropositive(ELISA) (*n*=5)	18	1	9	4
	28	1	18	1
	36	2	31	4
	45	4	62–66	5
	55–60	2	116	4
	84	2	200–205	5
	180	1		
Confirmed VL-positive and HIV negative (*n*=10)	36	6	9	5
	45	10	18	2
	55-60	6	38	3
	84	3	62	10
	94	3	66	5
	116	3	75	4
	160	3	84	10
	280	3	116	5
			138	10
			200-205	10
HIV seropositive and leishmania seronegative (*n*=20)	Nil		Nil	
Cysticercosis (*n*=2)	50–56 (faint bands) in one		Nil	
Amoebiasis (*n*=2)	sample each from			
Hydatidosis (*n*=2)	cysticercosis, toxoplasmosis			
Toxoplasmosis (*n*=2)	and malaria			
Malaria (*n*=2)				
Healthy individuals (*n*=5)	Nil			

modified by HIV.[3] Numerous cases of HIV and VL co-infection have been reported in India[25-29] and, as VL is often found in poor communities, it is regarded as imperative to have access to cheap diagnostic tests that do not require sophisticated laboratory facilities.[29]

Unfortunately, diagnosis of VL is difficult in patients with HIV-I infection,[24] and few studies have focused on the early diagnosis of asymptomatic VL in HIV-I patients, although it is estimated that between 1.5% and 9% of all patients with AIDS in southern Europe will develop VL.[30-31]

The demonstration of antibody to leishmania does not necessarily differentiate between past and active infection; thus, it is apparent that both antigen- and antibody-based tests must complement each other for a true assessment of the disease.

The present study reports the comparison of non-invasive methods (i.e., detection of stage-specific antigenaemia and antibody response to stage specific *L. donovani* antigens and rk39 antigen) for the early diagnosis of asymptomatic VL in HIV-infected patients. The data on haemoglobin level and CD4+ cell counts was available in only 43 and 23 HIV-infected patients, respectively. On retrospective analysis, mean haemoglobin was 10.77 g/dL (range: 6.4–15.3) and the mean CD4+ cell count was 183/µL (range: 10–427).

In 95 out of 100 HIV-seropositive patients, neither antigenaemia nor antibody response to stage-specific *L. donovani* antigens could be detected, while 79 out of 88 HIV-seropositive patients gave a negative response to rk39 antigen. However, five out of 100 HIV-seropositive patients were positive for antigenaemia and/or an antibody response to *L. donovani* stage-specific antigens and/or rk39 antigen.

Out of these five patients, two (3/44, 4/63) were positive for promastigote and amastigote antigenaemia and also produced an antibody response to stage-specific and rk39 antigen. Antigenaemia and antibody response to stage-specific antigens were also detected in another patient (/01) in whom anti-rk39 antibody was not carried out due to inadequate sample.

Both promastigote and amastigote antigenaemia and an antibody response to amastigote antigen were also detected in one patient (2/11) in whom antibody response to rk39 antigen was only borderline. In this patient, however, antibody response to promastigote *L. donovani* antigen was not detected by either in-house ELISA or a kit method.

The antibody response to promastigote antigen correlated well with in-house ELISA technique and commercially available kit method. On retrospective analysis, four HIV-positive, VL-seropositive patients had fever and three

Table 3. Correlation of stage-specific antigenaemia and antibody responses by ELISA in HIV-I patients ($n=5$).

Sample	CD4+(/μL)	Antigenaemia		Antibody responses			Presenting with:	
		Amastigote	Promastigote	Amastigote In-house ELISA	Promastigote Kit	rk39	Head-ache	Fever
11	NA	+	+	+	+	Not done	+	+
11	NA	+	+	+	-	Borderline	+	+
44	NA	+	+	+	+	+	+	+
63	NA	+	+	+	+	+	-	-
95	184	+	-	+	-	-	-	+

complained of headache at the time of sample collection (Table 3).

Earlier studies principally in HIV and VL co-infected patients produced contradictory results. The dynamics of the humoral immune response studied in 20 VL patients co-infected with HIV-I in Spain defined the possible role of serology in the diagnosis and prognosis of VL. In this group, 46% of the patients were positive by IFAT or ELISA on at least one occasion prior to VL infection.

Results confirmed the limited usefulness of IFAT and ELISA in the diagnosis of VL in co-infected patients, and, although a useful tool for studying the natural course of leishmaniasis, immunoblotting also has limited value in diagnosis or treatment.[6] Furthermore, in contrast to the observations made in Europe, direct agglutination tests in Ethiopia have shown reasonable sensitivity in the diagnosis of HIV-VL co-infection.[32]

Detection of antigen may prove useful in co-infected patients. In an earlier study, using polyclonal anti-leishmania promastigote antisera, circulating leishmania antigen were detected in 8/20 (40%) early kala-azar patients and in 4/21 (19%) late kala-azar patients.[7] In the present study, 5/100 and 4/100 HIV-seropositive patients showed detectable levels of amastigote and promastigote *L. donovani* antigen, respectively, although antibody response to promastigote antigen could not be detected in two of these, nor response to rk39 antigen in another.

Furthermore, it has been suggested that detection of amastigote antigen rather than promastigote antigen may prove a better diagnostic marker in VL patients.[5] In one HIV-seropositive patient in the present study, amastigote antigen and antibody response to it proved positive while neither promastigote/rk39 antigen nor antibody response to them were detected. On retrospective analysis, the CD4+ cell count in this patient, who had a fever at the time of sampling, was found to be 184/μL.

Detection of anti-rk39 antibody has shown high sensitivity in HIV-VL co-infected patients.[22] In a recent report, 13/104 apparently healthy controls showed positive strip-test results and 93% specificity was reported for the diagnosis of Indian VL patients.[33] In the present study, using an rk39 antigen-based ELISA for diagnosis of KA and PKDL patients, we found this approach to be highly sensitive (95.7%) and specific (95.6%). One out of 18 (5.5%) malaria and 3/21 (14.5%) endemic controls were positive and none of the samples from tuberculosis patients and healthy controls was positive for antibody response to rk39 antigen.[5] Furthermore, 94.5% sensitivity and 93.7% specificity were reported for diagnosis of post kala-azar dermal leishmaniasis using rk39 antigen.[34]

In the present study, 9/88 HIV-seropositive patients were positive for anti-rk39 response; however, six out of these nine patients did not have detectable levels of antigenaemia or antibody response to promastigote and amastigote *L. donovani* antigens.

It remains to be seen whether such reactions represent false positives or may be the result of the detection of previously unsuspected subclinical infection.[22] However, irrespective of the presence of subclinical infection or false-positive cross-reactions, such HIV-seropositive patients should be followed up.

In the present study, immunoblot analysis of humoral immune responses to promastigote and amastigote *L. donovani* antigens indicated that in VL-seropositive, HIV-I-infected patients (group I) and VL-confirmed, HIV-negative cases (group II) the majority of the samples were immunoreactive with 45, 55–60 and 60–70 kDa promastigotes and 9, 31, 62–66, 116, 138, 200–205 amastigote antigenic fractions.

There are no previous reports on immunoblot analysis of HIV and VL co-infected Indian patients; however, the findings on VL-positive, HIV-negative patients support earlier work[35] in which VL sera recognised multiple promastigote antigenic fractions (40, 55, 56, 70 and 82 kDa). In that study, the 70-kDa antigen was immunoreactive in 94% patients and gave 10% false-positive reactions.

Kaul *et al*.[11] reported a 200-kDa *L. donovani* amastigote antigen that showed specificity for VL diagnosis, and in the present study five HIV/VL seropositive samples and 10 HIV-negative and VL-postive samples recognised a 200–205 kDa amastigote antigenic fraction.

Results from the present study also accord with an earlier one[36] of a series of 11 AIDS cases in which a similar pattern of reactivity between HIV and non-HIV patients with VL was reported, and reactivity differed only in the variable presence of a 14-kDa band in the HIV group. The 16-kDa component was found to be more sensitive and specific, while 14, 16, 30, 46 and 68 kDa *L. infantum* promastigote antigen fractions were recognised by >80% VL patients.

No obvious immunodominant antigen was found in HIV-VL co-infected patients in Spain, yet five groups of *L. infantum* promastigote antigens (14, 42–43, 57, 76 and 94 kDa) appeared to have potential for diagnostic use in HIV-infected individuals. In view of the high variability in immunoblot patterns, and the fact that not all patients will respond immunologically to the same antigen(s), it has been suggested that a combination of several antigens should be used.[24]

In conclusion, detection of anti-rk39 antibody response, amastigote antigenaemia, antibody response to amastigote

antigen, promastigote antigenaemia and antibody response to promastigote *L. donovani* antigen (in order of sensitivity) may prove useful in the early diagnosis of VL in HIV patients, and for prompt follow-up and prophylaxis. However, further studies are needed to define more precisely the immunoblot criteria for early diagnosis of VL in HIV-infected individuals.

The authors thank Mrs Gurjeet Bhatti and Ms Ritu Biala for technical assistance with the Western blots.

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