

Original Article

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Pre- & post-treatment evaluation of immunological features in Indian visceral leishmaniasis (VL) patients with HIV co-infection

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Background & objectives: The risk of human immunodeficiency virus (HIV) co-infection in patients with visceral leishmaniasis (VL) or kala-azar in endemic areas has posed a major challenge in control programmes. We undertook this study to identify the high risk groups vulnerable to *Leishmania*-HIV co-infection in VL endemic State of Bihar, India. Further, immunological responses were also evaluated in these patients before and after treatment for VL to see the immune impairment associated with CD4 T cell count.

Methods: A total of 1511 subjects attending Voluntary Counselling and Testing Centre (VCTC) at Patna, Bihar were included in this study. VL was confirmed by splenic or bone marrow aspirates testing for parasite. HIV states was confirmed by two kits. Immunological parameters (CD4, CD8, IFN- γ , IL-4) were studied in co-infection patients.

Results: Of the 280 (18.53%) HIV-positive individuals, eight were diagnosed serologically and pathologically as VL patients co-infected with HIV. The humoral and cellular immune responses were evaluated in 18 Indian VL patients with (n=8) or without HIV (n=10) and 10 HIV seropositive subjects. Among the eight confirmed cases of VL, false negative direct agglutination test (DAT) result was observed in two who had HIV co-infection (sensitivity 80%), while none in 10 other VL cases who were HIV negative (sensitivity 100%). A very low CD4 cell count was observed in VL cases that had HIV co-infection compared to HIV negative VL or controls. All VL cases with or without HIV infection had lower Th1/Th2 ratio compared to controls. VL patients with or without HIV infection responded well to anti-leishmanial/anti-retroviral therapy with considerable degree of immunological reconstitution.

Interpretation & conclusion: A different immune response was noticed in patients with co-infection of HIV and *Leishmania*. Anti-leishmanial drug treatment led to improvement in immunological response in co-infected patients. Further studies need to be done to see the effect of combined therapy for VL and HIV on immunological parameters in these patients.

Key words Direct agglutination test - gama-interferon - HIV - interleukin 4 - visceral leishmaniasis

The spread of human immunodeficiency virus (HIV) infection to areas where visceral leishmaniasis (VL) is endemic has become a major challenge to the control of VL infection¹. Berhe *et al*² in 1995 first reported seven cases of HIV and *Leishmania* co-infection in Ethiopia (Africa) that increased to 72 by 1998. Up to 70 per cent of VL cases with HIV infection have been reported in Southern Europe³. The first case of VL-HIV co-infection was identified from the State of Bihar in India in the year 2000⁴. Subsequent studies diagnosed six more cases of HIV-VL co-infection from this State⁵. Since AIDS epidemic is looming large on the horizon of new millennium in India⁶, the State of Bihar which is endemic for VL, needs to be looked seriously for HIV-VL co-infection. It is anticipated that the co-infection by HIV and *Leishmania donovani* might cause reciprocal enhanced immunological disturbances. However, data on prospective distribution on CD4 cell count and cytokine pattern elicited by Th1 and Th2 functional subpopulation of T cells in HIV cases and cases co-infected with VL are relatively scarce. In order to identify the high risk groups vulnerable for HIV-*Leishmania* co-infection or HIV/AIDS, a large number of subjects attending a voluntary testing centre in Panta, Bihar were evaluated epidemiologically for magnitude of infection and risk load over a period of one year. A representative number of these subjects were later investigated for immune impairment associated with CD4+T cell count before and after treatment for VL. Type 1 and type 2 cytokine pattern in VL cases with or without HIV infection were also explored by the peripheral blood mononuclear cells of these patients compared to HIV cases and healthy controls.

Material & Methods

Screening for HIV: A total number of 1511 consecutive subjects (1127 males, 384 females, age range 1- >45 yr) were studied at Voluntary Counselling and Testing Center (VCTC) at Rajendra Memorial Research Institute of Medical Sciences, (RMRIMS), Patna, India during a period of one year (2004-2005). Their demographic details and risk behavior if any, were recorded. Blood samples (7.5 ml) were collected from these subjects. Most of the blood samples were tested immediately while a

few samples were stored at 2-8°C in the refrigerator and tested within 48 h. These samples were tested by ELISA (Lab System Kit) and Rapid test following the instructions given by the manufacturers. A representative number of serologically reactive HIV positive subjects were immunophenotyped for CD4+ and CD8+ T-cell count.

Parasitological and immunological investigations: Among the HIV seropositives, 8 were observed to be co-infected with *Leishmania*. These cases presented with typical signs of kala-azar including fever, splenomegaly, pancytopenia, and weight loss. Confirmation of VL infection was done by diagnostic splenic or bone marrow aspirates. Slides after Giemsa staining were examined microscopically for parasite density by logarithmic scale from zero (zero parasite per one thousand oil immersion field) to +6 (>100 parasite/field) following the methods of Chulay & Bryceson⁷. A total of 10 HIV seropositive subjects along with 5 HIV cases co-infected with *Leishmania* were selected for the immunological investigations before and after completion of treatment for VL. Ten cases of clinically (fever, hepatosplenomegaly, pallor *etc.*), parasitologically (splenic aspiration) and serologically direct agglutination test (DAT) confirmed cases of VL and 10 apparently healthy controls were also studied to compare the level of immune-competence in co-infection. Immunological investigations included demonstration of anti-leishmanial antibody titre by DAT⁸. Subsequent experiments were performed using immunofluorescent staining for CD4 and CD8 count and detection of level of interferon- γ (IFN γ) and interleukin-4 (IL4) cytokines in culture supernatants. For CD4 and CD8 count, whole blood was collected in EDTA-containing vacutainer tubes (Becton Dickinson, Rutherford, USA). The blood was stained with 20 μ l fluorescent BD Tri Test monoclonal antibodies specific for CD4/CD8/CD3 conjugated to (FITC), (PE) and per (CP) respectively and incubated for 30 min at room temperature in a dark area. After staining, cells were suspended in 450 μ l of 1 x Lysing solution and incubated at room temperature for 10 min in dark. Cells were examined for fluorescence in a FACS-calibur (BD, San Diego, USA). Flowcytometry data were evaluated on Cell Quest software. For cytokine detection, whole blood was

collected in vacutainer tubes containing heparin. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation, washed in phosphate buffered saline, pH 7.2-7.4 and resuspended in (RPMI) 1640 medium with 10 per cent foetal calf serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. These cells were cultured in 24-well plate at a concentration of 1×10^6 cells/ml and incubated at 37°C in a water saturated air atmosphere with 95 per cent humidity, 5 per cent CO₂ (CO₂ incubator). The supernatants after 48 h of infection were tested for the cytokines. (IFN γ and IL4 level) by ELISA. The ELISA kits used were purchased from Quantikine R & D (Minneapolis, USA) for quantitative estimation of cytokine levels of IL4 and IFN γ .

Treatment: VL patients were treated with amphotericin B intravenously in the dose of 1 mg/kg body weight in 5 per cent dextrose slowly over 4-6 h for 15 injections on alternate days. HIV patients were treated with anti-retroviral therapy (combination of zidovudine 200 mg, lamivudine 150 mg and nevirapine 200 mg tablets twice daily). HIV-VL co-infection patients were treated with a combination of the above-mentioned drugs.

Results

HIV testing: The common symptoms among the HIV-positive individuals at the time of screening were gastrointestinal problems associated with genital/venereal diseases infections, weakness and loss of appetite.

Of the 1511 samples, 280 (18.53%) were detected positive with ELISA and/or rapid test. Among the seropositives, 91 (24%) were females and 189 (17%) were males. The maximum positivity (17%) was observed in the age group 15-30 yr followed by 11 per cent in 1-14 yr age group. The characteristic pattern of CD4+ T cell counts in different categories of HIV-positive subjects (n=58) was studied by flowcytometry. These included 35 commercial sex workers (CSW), 18 spouses, 2 cases of mother-to child transmission (MTCT) and 1 each of intravenous drug user (IVDU), sexually transmitted disease (STD) patient, and homosexual CSW group recorded

a more declined CD4 cell count (<200 per µl) as compared to other groups. The immunophenotyping, done for CD4+ cell count in these groups, was part of an initial screening to support serology.

Co-infection cases: Amongst the 280 HIV seropositives, eight cases were diagnosed as co-infection cases with VL. There was no significant difference with respect to duration of illness, spleen/liver size and Leishman Donovan (LD) grade among the VL and co-infection cases.

Anti-leishmanial antibody response: The sensitivity of the DAT was 80 per cent for HIV-positive VL cases and 100 per cent for the VL cases. Two co-infection cases were shown to have anti-leishmanial antibody titre below the cut-off level (1 : 800). All VL cases had antibody titre above the cut-off value.

Cellular immune responses: In blood from 10 healthy controls, CD4+ cell count was 1099 ± 429.1 (Table). CD4+ cell count in kala-azar patients was lower compared to that of controls. The CD4 cell count in HIV cases alone and a co-infection cases were somewhat different: a very low cell count was observed in co-infection cases compared to VL cases alone, although HIV cases also recorded a much declined CD4 count compared to both VL and control groups. Sufficient mononuclear cell samples were available in a total of 25 samples (5 from co-infection

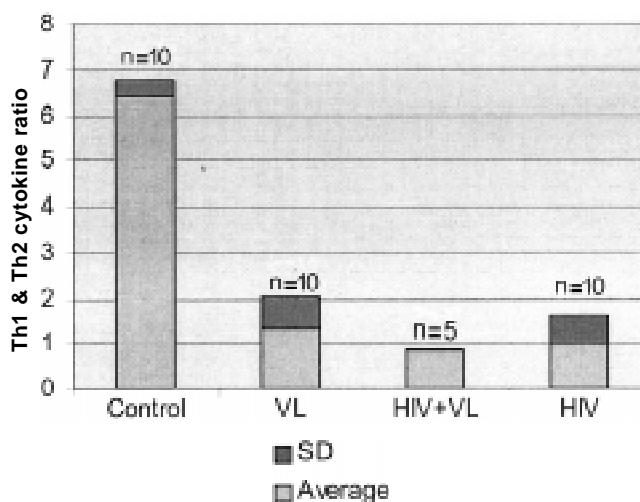


Fig. Pre-treatment Th1 and Th2 cytokine (IFN- γ /IL4) ratio in VL cases with or without HIV, compared to HIV cases and control.

Table. Detection of anti-leishmanial antibody and cytokine activities in VL cases with or without HIV

Parameters	Control (n=10)	VL (n=10)		VL with HIV (n=8)		HIV (n=10)	
		BT	AT	BT	AT	BT	AT
Anti <i>L. donovani</i> Ab (DAT) positive	0	10	0	6	6	0	0
CD4 μ / μ l	1099 \pm 429.1	453 \pm 265.8	723 \pm 190.4	162.33 \pm 144.6	180.66 \pm 140.9	325.1 \pm 254.1	524.2 \pm 359.3
CD8/ μ l	653 \pm 287.6	222 \pm 96.8	421 \pm 59.0	840.33 \pm 295.4	1009 \pm 212.1	1304.1 \pm 683.4	1433.1 \pm 830.1
CD4/CD8 ratio	2.046 \pm 1.7	2.153 \pm 1.1	1.650 \pm 0.5	0.298 \pm 0.2	0.202 \pm 0.2	0.230 \pm 0.2	0.379 \pm 0.2

Values are mean \pm SD

*CD4 and CD8 count (per ml of peripheral whole blood) was done by using Tri test antibodies with true count tubes (BD Bio Sciences, USA) and analysed through Cellquest-software on flowcytometry

BT, before treatment; AT, after treatment

group) for both IFN- γ and IL4. Th1/Th2 ratio become low in both categories of VL infection as well as in HIV infection when compared to controls (Fig.). This reduction was more prominent in co-infection cases indicative of enhanced immunological disturbances in this group.

After treatment for VL and co-infection cases, response pattern as manifested by immunological parameters was entirely different. The kala-azar patients recorded a much higher CD4+ cell count along with more pronounced IFN- γ dominated T-helper cell response in these patients. The co-infection cases failed to demonstrate any significant change in the Th2 dominated CD4+ T-cell response as detected prior to start of anti-leishmanial therapy in them (Table).

Ten HIV-positive patients started anti-retroviral treatment and were further evaluated for CD4 count 6 months post-treatment. The CD4 cell count increased in the patients who started these treatment regimens. One patient voluntarily interrupted the treatment, which showed a decrease of CD4 cell count to <200/ μ l after 6 months.

Discussion

Morenso *et al*⁹ previously reported that HIV infection in VL patients usually depresses both components of the immune responses (humoral and cell-mediated) of the human host. This is unlike to VL patients where a high anti-leishmanial antibody titre is demonstrated^{8,10}. Our study is in agreement

with other reports which suggest that a sizeable proportion of VL patients with HIV co-infection fail to demonstrate anti-leishmanial antibodies by using sensitive methods such as DAT, ELISA, indirect immunofluorescent antibody test (IFAT)^{1,11,12}. We recommend that detection of anti-leishmanial antibodies in VL patients with HIV co-infection may be done by alternative approaches such as immunoblotting, which has been reported to have acceptable sensitivity in diagnosis of VL infection in these patients^{11,13,14}.

As apparent from the present study, the immunological competence with respect to CD4 cell count was low in HIV cases. The patients presenting with kala-azar also recorded a low CD4 count compared to the control. The cellular immune response was highly suppressed with more severely reduced CD4 cell count in co-infection cases. Several reports suggest that visceral infection with *Leishmania* is marked by profound immunosuppression and that preferential expansion of either one of the two subsets of CD4+ T cells, Th1 (IFN- γ and IL2 releasing cells) or Th2 (IL4, IL5 and IL10) dictate the outcome of leishmanial infection¹⁵⁻¹⁹. Among these cytokines IFN- γ usually produced by CD4 cells is key mediator given its ability to promote cellular immune activation in human VL patients²⁰. However, studies to examine the role of this cytokine are relatively scarce in co-infection cases. Our study showed that the disease in VL cases without HIV was associated with much profound expansion of IL4 mediated Th2 cytokine response. A similar IL4 dominated Th2 cytokine

response was also seen in HIV infected individuals and even in cases with *Leishmania* infection among HIV co-infected subjects. This shift from a Th1 to Th2 cytokine pattern was accompanied with CD4+ cells impairment. Interestingly, we recorded more progressive depletion of CD4+ cells in VL patients with co-infection. These co-infection cases released very high IL4 compared to VL cases. Thus, it appears that IL4 plays an important role not only in VL cases with or without HIV infection but also during the course of HIV infection. Mayanja-Kizza *et al.*²¹ reported increased rate of spontaneous and antigen induced apoptosis in T-cells. The inhibition of IFN- γ in HIV patients as shown in the present study might be because of tumour necrosis factor (TNF) that has shown to provide an important signal for the apoptosis of activated CD4 T-lymphocytes²². It is tempting to speculate that leishmanial infection was facilitated by limited immunological control by severe reduction of T-helper cells during the co-infection. These results imply that VL in HIV positive subjects may be the consequence of a re-activation of a latent infection. We hypothesize that *Leishmania* might be serving as an important co-factor in the progression of HIV related diseases in VL endemic areas.

Anti-leishmanial drug treatment led to significant rise in the CD4 count with IFN- γ dominated cell response in the VL cases. HIV cases after treatment for 6 months were also shown to have high CD4 cell count with some improvement in the cytokine pattern. These HIV cases if followed up further, might give information on the time period during which CD3, CD4 and CD8 cell count would suddenly drop down making a complete immunological collapse despite the best use of therapy. It was also shown that prolonged anti-leishmanial treatment in co-infection cases did not favour a shift from a Th2 to a Th1 response. We used sodium stibogluconate and amphotericin B for treating these co-infection cases. Possibly, some endogenous cytokine production might be mandatory for these drugs to function optimally in such cases. More advanced highly active anti-retroviral drugs, if given in combination can accelerate the efficacy of these drugs in the co-infection cases.

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