Co-Infection by HTLV-I/II is Associated With Increased Viral Load in PBMC of HIV-1 Infected Patients in Bahia, Brazil

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To evaluate the viral load in peripheral blood mononucler cells (PBMC) of patients infected by HIV-1 we performed 96 quantitative cultures in 74 patients infected by HIV-1, using the co-culture method. The viral load was expressed in tissue culture infectious doses (TCID), and the results were analyzed according to gender, age and clinical stage of patients, duration of previous antiretroviral therapy, detectable p24 antigenemia, CD_4^+/CD_8^+ cell counts, co-infection by HTLV-I/II and viral subtype. We detected a statistically significant association between co-infection by HTLV-I/II and viral load higher than >50 TCID (p=0.003). We also found a significant association between co-infection by HTLV-I/II and p24 antigenemia (p=0.028). CD_4^+ cell counts were significantly higher for patients presenting negative cultures, but there was no detectable association between lower CD_4^+ cell counts and higher TCID. The majority of patients were infected by subtype B virus. The observation in this study that co-infection with HTLV-I/II was significantly associated with higher viral load raises the possibility that these agents act as co-factors of AIDS progression, in doubly-infected patients.

Key Words: HIV-1 Co-infection, HTLV-I/II, HIV-1 viral load.

Several markers have been used to determine the stage of the acquired immunodeficiency syndrome (AIDS), and to predict the clinical evolution and outcome for HIV infected patients [1-3]. Serum measurement of neopterin, beta-2 microglobulin, p24 antigenemia, and CD_4^+ counts have been used extensively for that purpose, but all of these markers have limitations which have generated a search for a better predictor of HIV infection evolution [4-9].

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Viral load measurement was available several years after the recognition of AIDS, but its use was initially restricted to research laboratories [10-13]. However, the simplification of the viral culture method and, more recently, the development of commercially available kits for measuring plasma concentrations of viral RNA, have provided clinicians and researchers with a very useful tool and with a higher sensitivity than previously used methods [14-16].The new method is highly effective in predicting the clinical course of HIV infection, and it has been incorporated into the current strategies for monitoring and evaluating treatment. The measurement of cell-associated viral load is also useful for understanding viral behavior and biological characteristics including phenotypic differences among viruses and their association with a greater or less aggressive course of disease [17-20].

A number of factors are thought to be involved in HIV pathogenesis, including host genetic background, the viral subtype that caused the infection, the ability of the immune system to control the acute infection, and the presence of co-infections, such as HHV-6, and the Human Lymphotropic Virus type I and II (HTLV-I and II) [21-23].

HTLV-I is endemic in Bahia. Previous studies estimated its prevalence to be as high as 1.8%, among blood donors, and reached 11.9% among HIV infected patients [24, 25]. HTLV-II was detected with a lower frequency in the same studies. Its prevalence was 0.2% among blood donors and 4% among HIV-1 infected patients. Simultaneous detection of these two viruses was possible in 0.8% of HIV-infected patients.

Although some evidence has been presented to support the role of HTLV co-infection as a potential co-factor in disease progression, its real significance in AIDS pathogenesis remains to be determined [26-29].

Subtype analysis of HIV-1 identified in Bahia has shown that subtype B is involved in the vast majority of cases diagnosed in this region. However, other subtypes have been reported in the last few years, such as subtypes F and recombination of subtypes B and F [30-33].

We performed this study in order to evaluate the potential impact of HTLV-I/II co-infection and viral subtype on cell-associated HIV-1 viral load among patients with a confirmed diagnosis of HIV infection.

Material and Methods

Subjects

The study participants were patients followed at the AIDS clinic of Federal University of Bahia Hospital. They were invited to participate in the study when their blood was drawn for CD_4^+/ CD_8^+ determinations. The inclusion criteria was a Western blot (WB) confirmed HIV infection, agreement to give blood samples for viral cultures, and absence of active opportunistic infections. Clinical staging of HIV infection was performed by CDC 1993 revised criteria [34].

Cell-associated viral load measurement

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation, and subsequently co-cultivated with 3 days phytohemagluttinin-stimulated normal cells in 24 well microplates, using a concentration of 2 million cells for the first well, and then successive 10-fold dilutions in the subsequent wells (200,000; 2,000; 200; and 20 patient cells/ml). The results of positive cultures were expressed in Tissue Culture Infective Doses (TCID), which ranged from 0.5 (2,000,000 cells/ml) to 5,000 TCID (corresponding to 20 cells/ml). For each culture, 1 microplate well contained only donor's cells, without HIV infection, used as a negative control. Supernatants were assayed on day 14 of viral cultivation, for p24 antigen. Cultures were considered positive if they had an antigen concentration >40pg/ml. Some patients could have more than 1 culture performed during the study period if the second specimen was collected after a time interval greater than 3 months. For analysis purposes, we stratified the results of viral load as "high" (>50 TCID), and "low" (<49 TCID).

CD_4^+ and CD_8^+ cells count

CD_4^+/ CD_4^+ determinations were performed by indirect immunofluorescence assay, using monoclonal antibodies (OKT4, OKT8, and OKT11, Ortho, Raritan, NJ). The last 15 CD_4/ CD_8 counts were performed by flow cytometry, using an EPICS II Profile analyzer (Coulter Corporation), according to the manufacturer's recommendations.

HTLV-I/II serology

Serum samples were screened by a commercially available enzyme immunoassay (Coulter Detect, Coulter Corp.). Reactive samples were then

confirmed by WB (Fujirebio, Tokyo, Japan). Discrimination between HTLV-I and II was attempted by a synthetic-peptide based assay (Coulter Select, Coulter Corp.) and by a WB capable of identify in the 2 viruses (Cellular Products, Buffalo, NY).

Viral subtyping

The genetic characterization of the HIV-1 subtypes was performed in 46 samples: The first 19 patients already had their viral subtyping done by PCR of a V3 loop region, while the viral genotyping of the remaining 27 subjects was performed by heteroduplex mobility assay - HMA [30, 35].

Statistical analysis

All statistical calculations were done using the computer software EPI-Info 6.01 and SPSS for Windows, version 6.1.

Results

Number of cultures and patient characteristics
We performed 96 viral cultures, 85 of them (88.5%) originated from male patients. Sexual contact was the predominant means of transmission of the retroviral infection. Table 1 shows the mean age, gender distribution and likely route of acquisition of HIV infection among the patients participating in the study, as recorded at the time of each blood collection. At that time, 63.5% of men and 45.4% of women fulfilled the CDC criteria for the diagnosis of AIDS. At the time of blood culture, 53% of patients were being treated with AZT.

Detection of p24 antigen in serum samples

Antigenemia was detected in serum samples of 38.8% of male patients and in 50% of women (p=0.5, Fisher exact test). There was no significant difference in p24 antigen detection, in serum samples of patients who were receiving AZT compared with those without any previous antiretroviral therapy (p=0.3, Yates corrected). The mean time of AZT use was 8.3±6.9 months.

CD_4^+/ CD_4^+ cell count

The mean CD_4^+ and CD_8^+ cell count was 310±299 cells/ml, and 390±288, respectively. Women had a higher mean of CD_4^+ cell counts compared with men: 504±401 cells/ml versus 285±276 cells/ml, (p=0.02, Kruskal-Wallis test), but CD_8^+ counts were similar (p=0.3).

Serology for HTLV-I/II

Antibodies against HTLV-I were detected in 4 (4.2%) samples. No patient was positive for HTLV-II alone, but simultaneous infection by HTLV-I and II was detected in 4 additional samples. These samples had a significantly higher probability of having detectable serum p24 antigenemia (RR=2.13; 95% CI:1.3-3.38 - p=0.028, Fisher exact test).

Viral genotyping

Viral characterization revealed a vast majority (43/46) of subtype B strains. One sample showed reactivity for sequences of subtypes B and C, suggesting a likely recombination of these 2 subtypes. Two samples showed a divergent pattern and could not be conclusively characterized. Complete genetic sequencing is in progress.

Viral load quantitation

Twelve (12.5%) patients had negative cultures. The highest viral load (5,000 TCID) was detected in 4 (4.5%) patients. Women had a higher probability of a result greater than 50 TCID (p=0.05, Fisher exact test), but the culture's positivity rate was similar for men and women (p=0.1). Age was not associated with significant variation in viral load, as displayed by Figure1.

Table 1. Characteristics of the patients at the time of sample collection for viral cultivation

		Likely route of HIV acquisition (%)				
Gender* (±sd)	**Mean age**	**Homosexual**	**Bisexual**	**Heterosexual**	**IVDU**	**Total**
Male	37.7±8.6	64 (75.3)	10 (11.8)	3 (3.5)	8 (9.4)	85(88.5)
Female	32.8±10.1	-	-	9 (81.8)	2 (18.2)	11 (11.5)
Total	37.1±8.8	64 (66.7)	10 (10.4)	12 (12.5)	10 (10.4)	96 (100)

* Men were significantly older than women (p=0.014, Kruskal-Wallis test).

Table 2. Mean CD_4^+/ CD_8^+ Cell counts according to viral load

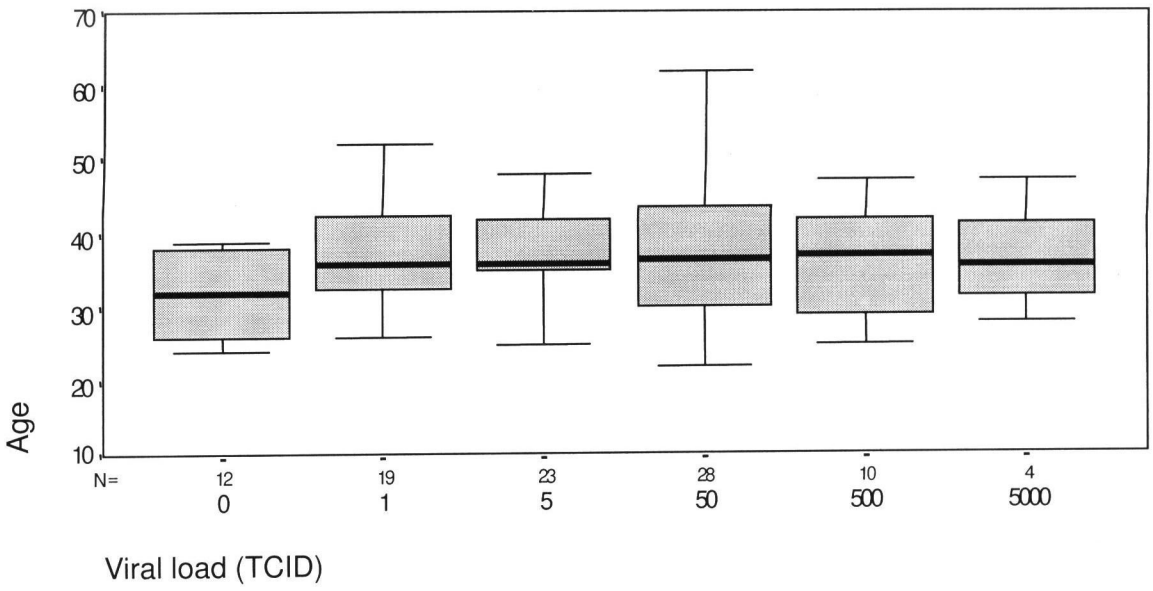
TCID	Number of cultures (%)	CD_4^+(mean±sd)	CD_8^+(mean±sd)
0.0	12 (12.5)	688 ± 473*	356 ± 291
0.5	19 (19.8	298 ± 284	393 ± 298
5	23 (23.9)	255 ± 259	395 ± 274
50	28 (29.9)	226 ± 164	386 ± 302
500	10 (10.4)	309 ± 174	445 ± 282
5.000	4 (4.2)	150 ± 83	352 ± 392

*p = 0.001 Kruskal-Wallis test, comparing CD_4^+ mean for negative and positive cultures. There was no significant difference between CD_4^+ means corresponding to different TCID, even considering the cultures with results above or below 50 TCID. CD_8^+ counts were similar for all culture results.

Table 3. Distribution of viral load according to the results of HTLV-I/II serology in the samples cultivated for HIV

		Viral Load (TCID)					
Serology	**N (%)**	**0.0**	**0.5**	**5**	**50**	**500**	**5000**
Negative	88	12	19	19	28	6	4
Positive	8	0	0	4	0	4	0
HTLV-I	4	0	0	3	0	1	0
HTLV-I e II	4	0	1	0	0	3	0
Total	**104 (100.0)**	**12 (12.5)**	**20 (22.4)**	**26 (24.0)**	**28 (29.2)**	**14 (10.4)**	**4 (4.2)**

Figure 1. Distribution of viral load in PBMC according to patients age



There was no difference between mean age and the different levels of TCID.

The mean CD_4^+ cell count was similar for different TCID. However, positive cultures had a significantly lower mean CD_4^+ cell count, when compared with those presenting with negative results (263±167 versus 318±316 cells/ml, respectively, p=0,001, Kruskal-Wallis test). There was no difference between the mean values of CD_8^+ counts for positive and negative cultures regardless of the resulting TCID. Table 2 shows the results of viral cultivation, according to CD_4^+/ CD_8^+ cell counts.

Previous use of AZT did not interfere with the positivity of cultures. However, patients who were not receiving AZT had a greater probability of a viral load >50 TCID (RR=1,67; 95% CI: 1,1 - 2,57; p=0.04, chi-square test).

Viral load >50 TCID was also marginally associated to p24 antigenemia (RR=3.5; 95% CI: 0.97-12.0; p=0.08, Fischer exact test), but p24 Ag was detected in a similar proportion of positive and negative viral cultures.

Patients with a previous diagnosis of AIDS were more likely to have a positive culture (RR=1.29; 95% CI: 1.07 – 1.57; p=0.002, for patients stage C, and RR=1.31; 95% CI:1.07-1.59; p=0.001, for patients stage 3, Fischer exact test), but the clinical stage was not associated with variations in viral load. Patients co-infected by HTLV-I/II had significantly higher TCID, when compared with those infected by HIV alone (p=0.003, chi-square test). However, this association disappeared when we compared only the patients co-infected by HTLV-I (p=0.3), but remained when the comparison was done with patients co-infected by HTLV-I and II (RR=19.09; 95% CI:2.18-167.56; p=0.007, Fisher exact test). Table 3 shows the results of viral load according to the serology for HTLV-I/II infection.

Viral subtype was not associated with TCID variation, antigenemia, or HTLV-I/II co-infection. Similarly, there was no detectable relationship between age and these factors.

Interestingly, CD_4^+/ CD_8^+ counts were not associated with the level of viral load, even when specimens were stratified into patients groups of "high" and "low" TCID.

Discussion

Viral quantitation in both plasma and cell compartments is currently considered a very predictive tool for HIV infection evolution [36-41]. Measurement of the amount of viral particles or genetic material in blood and other tissues provided clinicians and researchers with an accurate method to estimate the efficacy of antiretroviral therapy and onset of viral resistance to drugs [42, 43]. Although there is no doubt about the advances these methods have brought, some questions still remain concerning the factors associated with variation in viral load. In this study we evaluated the correlation between cell-associated viral load in HIV-infected subjects, characteristics of patients, CD_4^+/ CD_8^+ counts, viral subtype and co-infection by HTLV-I/II.

The patient sample studied was representative of our AIDS clinic population. Monotherapy with AZT was the current treatment recommendation when this work was done. We did not find any association between the level of viral load and prior use of AZT. The likely explanation for this is the relatively long time of therapy, since most of patients were using the drug for longer than 6 months, when the potential benefit of monotherapy was probably gone. Additional evidence favoring this interpretation is that p24 antigenemia rates in patients using AZT and those not using AZT were the same. We would expect at least a reduction in p24 detection rate in the group under therapy if the drug was still active [42-44].

Co-infection by HTLV-I, and by HTLV-I and II was detected in 8 (8.3%) patients (4 in each group). This rate is lower than we previously described in a larger sample, but it is still higher than the estimated prevalence in the general population (2%). We detected a strong correlation between simultaneous co-infection by HTLV-I and II and viral load higher than 49 TCID. There was also an association between p24 antigenemia and co-infection, which reinforces the possibility that these patients had an increased viral replication, in comparison with those singly infected, or co-infected by HTLV-I alone.

Previous *in vitro* experiments demonstrated a reciprocal activation of HIV-1 and HTLV-I when cell cultures were co-infected [45-47]. However, a recent cross-sectional study found no differences in RNA-viral load in the serum of patients, regardless of their serological status for HTLV co-infection, although some problems in methodology may have limited the potential for this study to conclusively define the issue [48, 49]. Our results suggest that triple retroviral infection may be associated with an increase in HIV replication. However, the small sample size and the cross-sectional design of our study do not allow us to make firm conclusions about the results of such an interaction.

A lower mean count of CD_4^+ cells was predictive for a positive culture, but we did not find any association between the level of TCID and CD_4^+ cell levels. CD_8^+ cell counts were not significantly associated with viral load or with culture positivity. There is evidence suggesting that co-infection by HTLV-I/II in HIV patients is associated with increased CD_4^+ counts, although without any apparent clinical benefit [28].

In this study, the relationship between higher CD_4^+ cell count and female gender may be explained by the rate of co-infection by HTLV-I/II among women. The small number of women in our sample, however, does not permit us to conclusively answer this question.

Patient demographics such as age, gender and clinical stage were not associated with a variation in viral load. Patients presenting with a previous diagnosis of AIDS were more likely to have a positive culture, but we did not detect an association between clinical stage and TCID results.

Viral characterization showed a large predominance of subtype B among the samples tested. However, the finding of a sample suggesting a recombinant strain of subtypes B and C, and a 2 samples with a non-typeable virus, suggest that other variants may be circulating in Bahia. This fact deserves additional investigation.

In conclusion, in our study, higher viral load in PBMC was associated with co-infection by HTLV-I and HTLV-II, but not HTLV-I alone. The same correlation was observed for p24 antigenemia. Mean CD_4^+ cell count and clinical stage of disease were predictive for positive cultures, but not for variation in viral load. The predominant viral subtype in our sample was subtype B.

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