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Molecular Characterization of Human T-Cell Lymphotropic Virus Type 2 (HTLV-II) From People Living in Urban Areas of Sao Paulo City: Evidence of Multiple Subtypes Circulation

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Background: In Brazil, human T-cell lymphotropic virus type I and type II (HTLV-I and HTLV-II) are co-circulating and possess approximately 65% homology, which results in high cross-reactivity in serological tests. Based on the detection of EIA and Western blot (WB) tests, HTLV serodiagnosis yields indeterminate results in high-risk population, with the true determination of HTLV-II prevalence requiring a combined serological and molecular analysis. Molecular analysis of HTLV-II isolates has shown the existence of four distinct subtypes: IIa, IIb, IIc, and IId. The aim of this study was to evaluate the routine EIA and WB used in Sao Paulo city, as well as molecular methods for confirmation of infection and HTLV-II subtype distribution. Results: Two hundred ninety-three individuals, who were enrolled in the HTLV out-clinic in Sao Paulo city, Brazil, between July 1997 and May 2003, were tested by EIAs, and positive sera 232 (79%) reactive by one of the tests. When these sera were tested by WB revealed 134 were HTLV-I, 28 HTLV-II, 4 HTLV-I/II, and 48 were indeterminate. Polymerase chain reaction (PCR) on the indeterminate group showed that 20 (42%) were HTLV-II and 28 were negative. From a total of 48 HTLV-II subjects with DNA available, restriction fragment length polymorphism (RFLP) of the env region revealed 47 HTLV-IIa and 1 HTLV-IIb. The phylogenetic analysis was performed on 23 samples, which identified 19 as subtype a, Brazilian subcluster, and 4 as subtype b. This is the first time HTLV-II subtype b has been described in Brazil. However, further studies, such as a complete nucleotide DNA sequencing, need to be done to confirm these findings. ***J. Med. Virol. 79:182–187, 2007.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: human T-cell lymphotropic virus type I and II; Brazil; seroprevalence; Western blot; intravenous drug users

INTRODUCTION

Human T-cell lymphotropic virus Type 1 (HTLV-I) and Type 2 (HTLV-II) are closely related mammalian retroviruses which share a number of biological properties and have a tropism for T lymphocytes [Hollsberg and Hafler, 1993; Hall et al., 1994]. HTLV-I infection is endemic in certain geographical areas, including Japan [Yamaguchi, 1994], Melanesia [Nerurkar et al., 1992], the Caribbean Islands [Cesarie et al., 1999], and parts of Africa [Dumas et al., 1991] and Brazil [Cortes et al., 1989; Ferreira Junior et al., 1995; Bellei et al., 1996; Casseb et al., 1997; Dourado and Galvao-Castro, 2001; Shindo et al., 2002] and has been recognized as the etiologic agent of a mature T-cell leukemia/lymphoma (ATL) and a chronic myelopathy known as HTLV-I-associated myelopathy (HAM) or tropical spastic paraparesis (TSP) [Gessain et al., 1985].

On the other hand, HTLV-II infection has been shown to be endemic among intravenous drug users (IVDU) in parts of North America, Europe, and Southeast Asia [Zela et al., 1993; Fukushima et al., 1995] and in a number of Amerindian populations [Biglione et al., 1993; Duenas-Barajas et al., 1993; Ferrer et al., 1993; Black et al., 1994]. Although the role of HTLV-II infection in human disease has yet to be clearly defined, there is accumulating evidence that it may be associated

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with a spectrum of neurological, and possibly lymphoproliferative disorders [Hall et al., 1996].

Both HTLV-I and HTLV-II are transmitted by cell-to-cell contact with infected T cells and may occur via sexual contact (predominately male to female), mother to child, particularly through protracted breastfeeding, and parenteral injection of infected blood products (blood transfusion or intravenous drug users) [Ehrlich et al., 1989; Hjelle et al., 1990; Saji et al., 1990; Sullivan et al., 1991]. Shortly after transmission, antibodies can be detected by serological approaches [Andersson et al., 1999]. Due to the overall structural similarity between these two viruses, cross-reactivity in serological procedures is seen. The current screening assays, including EIA and particle agglutination, detect antibodies to both viruses and supplementary serological tests [Brodine et al., 1993; Busch et al., 2000], such as Western blot (WB) and immunofluorescent assays [Dana, 1988; Varma et al., 1995; Zrein et al., 1998], are used to confirm the results and to differentiate between HTLV-I and HTLV-II. The ability to make accurate and reliable diagnoses of HTLV-I and HTLV-II infections is important for ensuring the safety of blood products. Serological discrimination is needed for epidemiological and public health studies and counseling purposes, because morbidity associated with the two differs significantly.

WB is most frequently used for confirmatory assays. The HTLV WB kits commonly use recombinant proteins specific for HTLV-I (MTA-1) and HTLV-II (K55) env glycoproteins. They are incorporated into the strips to increase sensitivity and distinguish antibody responses for each virus [Brodine et al., 1993; Varma et al., 1995]. The established HTLV-I/II WB interpretive criteria maintain that an infected individual must have an antibody response to p19 or p24 core bands and to the respective recombinant glycoproteins. Unfortunately, because of genetic heterogeneity among various isolates, no reactivity to MTA-1 and K55 were documented in certain geographic regions, especially in tropical areas [Nerurkar et al., 1992; Caterino-de-Araujo et al., 1994; Gessain and Mathieux, 1995; Segurado et al., 1997]. Furthermore, it has been reported that EIAs with HTLV-II recombinant antigens were unable to identify some HTLV-II-infected individuals if these samples had low antibody titer [Zehender et al., 1995; Eiraku et al., 1996; Caterino-de-Araujo et al., 1998]. Lack of antibody response might be due to either low proviral load or the proteins used in the assays did not match the infecting strain [Andersson et al., 1999].

Molecular studies based on nucleotide diversity of the long terminal repeat region (LTR) have shown that there are two HTLV-II genomic subtypes, named a and b, which differ by 3–6%. Subtype IIa was predominant in IVDU from USA, Sweden, and Ireland [Salemi et al., 1995; Switzer et al., 1995], but both IIa and IIb have been found in southern Italy and Spain [Vallejo et al., 1996], and IDUs from Vietnam have subtype IIb [Egan et al., 1999]. Brazilian Indians such as Kayapos [Fukushima et al., 1998] and IVDU from Sao Paulo were reported to be infected with a third variant called HTLV-IIc [Ishak et al., 1995] and HTLV-IId was identified in a Pygmy individual from Zaire [Goubau et al., 1993; Gessain et al., 1995]. Herein, we determined the specificity of the EIA assays in relation to the confirmatory WB 2.4 and used molecular methods to determine the HTLV-II strains circulating in Sao Paulo.

SUBJECTS AND METHODS

Study Population

Two hundred ninety-three adult patients, who were seen in the HTLV out-clinic at Institute of Infectious Diseases "Emilio Ribas," Sao Paulo city, Brazil between July 1997 and May 2003, were enrolled in this study. Written informed consent was obtained from each patient prior to collection of any blood specimens, and the study had the approval of the Ethical Committee Board.

Serological Assays

All serum samples were screened for the presence of antibodies to HTLV-I and HTLV-II using two commercially available EIA assays: HTLV-I/HTLV-II Ab Capture ELISA (Ortho Diagnostics, Raritan, NJ) which contains recombinant HTLV-I and HTLV-II envelope and core proteins for both coating and detection, and GE 80/81 Assay (Murex Diagnostics, Dartford, UK). Tests were performed according to the directions of the manufacturer. All specimens that were reactive for either EIA were confirmed by WB (HTLV Blot 2.4, Diagnostic Biotechnology, Singapore). Seropositivity was interpreted according to the stringent criteria issued by the manufacturer's instructions. A WB sample was scored as HTLV-I positive only if reactive to at least one gag protein (p19 or p24) and two env proteins (rgp46-I and GD21). It was scored as HTLV-II positive if p19 or p24 and rgp46-II and GD21 bands were identified, and was HTLV positive but untypeable if only p24, p19, and GD21 bands were observed. It was considered indeterminate if any other band patterns were present. Negative samples were those that did not exhibit any band.

Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) Analysis

The PBMCs were separated using the Ficoll-Hypaque gradient method and cryopreserved at 70°C. Genomic DNA was extracted using the GFX genomic blood DNA purification Kit (Amersham Pharmacia Biotech, Piscaway, NJ). Nested PCR was performed on positive and indeterminate WB samples to confirm the presence of HTLV.

Tax Region

To differentiate HTLV-I and HTLV-II infections, RFLP analysis of amplified product of a Tax region was carried out as previously described elsewhere

[Eiraku et al., 1996], using primers specific for this region of both HTLV-I and HTLV-II genomes. The beta-globin gene was studied to ensure that all extracted DNAs were amplifiable using primers PC04 and GH20 [Mahieux et al., 2000].

After the amplification of *tax* sequences, restriction enzyme digestion of the nested *tax* PCR product with endonucleases *Taq I* and *Sau 3A* was performed. Five microliters of second round products was digested in a 20 μl mix containing 10 units of the restriction enzyme and 2 μl of the 10× reaction buffer. *Sau 3A* digests were incubated for 90 min at 37°C and *Taq I* digests were incubated for 90 min at 65°C. The restriction site for the enzyme Taq I (T/CGA) is present in the amplified product of HTLV-II, generating two 69 bp and 53 bp bands (6 bp bands not visible), and it cuts the HTLV-I products to yield 122 bp bands (6 bp bands not visible). The endonuclease Sau 3A fails to cut the HTLV-II products, but cuts the HTLV-I products to generate distinct 104 bp and 24 bp bands and is visualized by electrophoresis on a 4% agarose gel [Tuke et al., 1992].

Env Region

When the samples were disclosed to be HTLV-II infections, amplification of the *env* region for RFLP was carried out using nested PCR to differentiate HTLV-IIa and HTLV-IIb [Hall et al., 1992].

Long Terminal Repeat Region

For nucleotide and phylogenetic studies, a 495-nucleotide region of the long terminal repeat (LTR) was amplified from nine randomly chosen PCR-positive samples, using nested PCR [Hall et al., 1992].

Nucleotide Sequencing and Phylogenetic Analysis

Direct DNA sequencing was performed on nine HTLV-II samples. PCR products amplified from the LTR region was purified using the Promega (Madison, WI) Wizard PCR prep system and sequenced in a Perkin-Elmer ABI Prism DNA 377 Sequencer using Taq FS dye terminator cycle sequencing (Perkin-Elmer Cetus, Norwalk, CA). The same PCR inner primers were used in the sequencing reactions. Multiple sequence alignment for the LTR region of the studied samples together with related sequences in the GeneBank/EMBL database was done with the dambe program [Xia, 2000] using the Clustal algorithm and further edited in the GeneDoc program [Nicholas et al., 1997]. The empirical transition: transversion ratio was determined in Puzzle 4.02 program. Aligned sequences were used in Philip v. 3.572 [Felsenstein, 1989]. Phylogenetic trees were constructed and evaluated by three different methods: neighbor-joining (NJ), parsimony (pars), and maximum-likelihood (ML). The reliability of the NJ trees was evaluated by analyzing 1,000 bootstraps replicates.

RESULTS

The study group consisted of 114 women and 179 men. The screening by EIA disclosed 232 (79%) reactive by one of the tests. These sera were tested by WB, which revealed 134 (46%) HTLV-I-infected subjects, 28 (10%) HTLV-II-infected individuals, 4 samples (1%) were classified as HTLV-I/II, and 48 (16%) were indeterminate. Twenty (45%) of 44 indeterminate reactors with available DNA were HTLV-II-positive by PCR. The remaining samples were negative. Two of the four HTLV-I/II reactors by WB were typed as HTLV-I and two as HTLV-II by PCR (Table I).

Therefore, 50 HTLV-II-infected subjects were found in this cohort. There were 34 men and 16 women, with a mean age of 40 (range 19–55 years old). Thirty-one were IVDU, 14 had heterosexual transmission, 2 women had an IVDU sexual partner, there was one case of vertical transmission, one was a blood transfusion receptor, and one was a homosexual man. Co-infection with HCV and HIV was observed in 24 (48%) patients, 12 (24%) were HIV only, and 2 were HCV only.

TABLE I. Reactivity in the WB, PCR, and RFLP Findings Among 214 HTLV-Seropositive or Indeterminate Individuals

		PCR + RFLP				
WB 2.4 profile		HTLV-I	HTLV-II	Neg.	NR	Total
HTLV-I	p19, p24, GD21, rgp46-I	134	0	0	0	134
HTLV-II	p19, p24, GD21, rgp46-II	0	28	0	0	28
HTLV-I/II	p19, p24, GD21	02	02	0	0	04
HTLV indeterminate	GD21, gp21, p19	0	01	0	0	01
	GD21, p19	0	01	2	0	03
	gp46, p36, p33, p28, p24,	0	0	1	0	01
	rgp46-II, gp21fr, p28, p24	0	01	0	0	01
	rgp46-II, p19	0	01	0	0	01
	rgp46-II, GD21	0	02	1	0	03
	rgp46-II, Gp21, p24	0	02	0	0	02
	rgp46-II, p24	0	04	0	0	04
	rgp46-II, p24, p19fr	0	01	0	0	01
	rgp46-IIfr, p24	0	01	0	0	01
	Other profiles	0	06	0	04	10
Total		136	50	24	04	214

From a total of 48 HTLV-II subjects with DNA available, RFLP of env region revealed 47 samples as HTLV-IIa and 1 as HTLV-IIb. In order to study the HTLV-II infection diversity in this cohort, 16 samples from IVDU co-infected with HIV, 5 samples from the heterosexual transmission group (one co-infected with HIV) and one pair (mother–son) (Table II) who were urban Brazilian Indians were sequenced following the previously described protocol [Eiraku et al., 1996]. All of the methods resulted in trees of similar topology with well-documented separation of the HTLV-IIa and HTLV-IIb subtypes.

The phylogenetic analysis of the LTR region revealed that 19 samples (1, 4, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and 23) aligned as HTLV-II subtype a, belonging to the Brazilian subcluster. Four samples showed alignment to HTLV-II subtype b (2, 3, 5, and 9).

DISCUSSION

High WB sensitivity for HTLV-I but not for HTLV-II antibodies was seen in this cohort. Almost 50% of the WB indeterminates were typed as HTLV-II by molecular methods. Previous studies have shown WB sensitivity ranging from 70% to 84% for HTLV-II antibodies among South American Indians and higher risk populations in United States [Varma et al., 1995; Poiesz et al., 2000]. In fact, WB assay has been shown to be inadequate as a supplemental assay for the confirmation of specific reactivities for HTLV-II antibodies among higher risk population such as IVDU [Zehender et al., 1995; Liu et al., 1999; Poiesz et al., 2000].

In this study, molecular approaches were very helpful in resolving WB indeterminate results in samples from HTLV-II-infected individuals. Other Brazilian investigators demonstrated that HTLV-I infection is also a cause of indeterminate WB results in blood donors [Morimoto et al., 2005]. This high frequency of indeterminate results clearly emphasizes the difficulty in assessing true HTLV-II seroprevalence. Some HTLV-II-infected subjects may have a low proviral load, eliciting partial or even absence of detectable circulating antibodies [Miyata et al., 1995; Manns et al., 1999]. It has been suggested that individuals who are co-infected with HIV-1 more often lack detectable HTLV-II antibodies because of the immunosuppression [Zehender et al., 1995; Medrano et al., 1997], but this state may be not the cause [Bassani et al., 2006]. We were unable to confirm this hypothesis, but it cannot be excluded that the sensitivity in WB and PCR was affected by HIV-1 infection in some cases. In many cases, despite high EIA titers, sera only reacted to one antigen in the WB.

We found one HTLV-II infection in an HIV-1-uninfected IVDU. This prevalence seems to be similar to previous studies in Europe and in Sao Paulo City [Zehender et al., 1995; Caterino-de-Araujo et al., 1998]. In addition, we noted HTLV-II infection in two urban Indian descendents, showing that this infection is also co-circulating in non-IVDU populations or among people who were infected by sexual heterosexual contact with IVDU, especially women. Although HIV-infected IVDU were more likely to be infected with HTLV-II, 12% of HIV-negative IVDU cases were positive for HTLV-II infection. Therefore, HIV-infection and IVDU may have a strong predictive value for HTLV infection in Sao Paulo, when compared to the HIV-1-infected with sexual risk population [Casseb et al., 1997]. We also noted a

TABLE II. Demographic Data, Risk Factors, and HTLV-II Subtyping by LTR Region

Id	Sex	Risk	HIV-1 status	WB	HTLV-II subtype
1	M	IVDU	POS	HTLV-II	a
2	F	IVDU	POS	HTLV-II	b
3	F	IVDU	POS	HTLV-II	b
4	M	IVDU	POS	HTLV-II	a
5	M	IVDU	POS	HTLV-II	b
6	M	IVDU	POS	Ind (rgp46-II, p24)	a
7	M	IVDU	POS	Ind (rgp46-II, p19)	a
8	M	IVDU	POS	Ind (rgp46-II, gd21)	a
9	M	IVDU	POS	Ind (gd21, p24)	b
10	M	IVDU	POS	Ind(rgp46-Ii, gp21fr, p28, p24, p19)	a
11	F	IVDU	POS	Ind (rgp46-II, gd21)	a
12	F	IVDU	POS	Ind (gd21fr, p53fr, p28fr)	a
13	F	IVDU	POS	Ind (rgp46-II, p24)	a
14	M	IVDU	POS	Ind (rgp46-II, p28, p24)	a
15	M	IVDU	POS	I/II	a
16	M	IVDU	POS	I/II	a
17	F	Hetero	NEG	HTLV-II	a
18	F	Hetero	NEG	HTLV-II	a
19	F	Hetero	NEG	Ind (rgp46-II, gp21, p24)	a
20	M	Hetero	NEG	Ind (rgp46-II, gp21, p24)	a
21	F	Hetero	NEG	Ind (rgp46-II, gp21, p24)	a
22	F	Hetero	POS	HTLV-II	a
23	M	Vertical	NEG	Ind (rgp46-II, p24)	a

Id, identification; M, male; F, female; POS, positive; NEG, negative; IND, indeterminate; WB, Western blot; LTR, long terminal repeat; Hetero, heterosexual; IVDU, intravenous drug users.

high frequency of HCV/HIV co-infection in this population. This is to be expected because these viruses share similar transmission routes and are endemic in our country. Sharing IV apparatus during drug addition leads to a high risk for transmission.

Study of the genetic diversity of HTLV-II is important for several reasons. First, it provides the necessary phylogenetic information for understanding the origin of the virus and for tracing the movements of ancient HTLV-II-infected populations. Second, it provides a laboratory tool for subtyping the virus for epidemiologic studies tracking maternal, blood borne or sexual transmission. Third, it allows monitoring for the appearance of unusual viral variants. We focused on the study of the long terminal repeat (LTR) region because it is more divergent than the pol or the gp21 region. The LTR is a non-coding region and may not be subjected to the same strict environmental forces of the structural genes of HTLV-II. A full-length sequence of HTLV-II from a Brazilian IVDU has indicated that instead of a new subtype HTLV-IIc, this sequence represents a subcluster [Lewis et al., 2000]. In addition, we found four cases with high homology (97%) to sequences representative of HTLV-IIb subtype. This is the first time HTLV-II subtype IIb has been described in Brazil. This subtype may have been introduced either by sexual contact and/or intravenous drug use with foreigners from USA or Europe, as happened in Asia during the Vietnam War [Fukushima et al., 1998]. However, we only had 400–500 base pairs for each sequence and direct DNA sequencing in contrast to the work elsewhere which used around 600 pair bases and DNA cloning [Egan et al., 1999].

This high genetic diversity level may indicate multiple entrances of HTLV-II infection into the Brazilian IDU population. Similar diversity is observed in HIV-1 infection, with four subtypes and a subtype B variant, found to be co-circulating in Brazil [Morgado et al., 1998]. We suggest that prevalence studies of HTLV-II infection among IVDU should be done with a combination of molecular and serological approaches.

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