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Neither Molecular Diversity of the Envelope, Immunosuppression Status, Nor Proviral Load Causes Indeterminate HTLV Western Blot Profiles in Samples From Human T-Cell Lymphotropic Virus Type 2 (HTLV-2)-Infected Individuals

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Although human T-cell lymphotropic virus type 2 (HTLV-2) is considered of low pathogenicity, serological diagnosis is important for counseling and monitoring. The confirmatory tests most used are Western blot (WB) and PCR. However, in high-risk populations, about 50% of the indeterminate WB were HTLV-2 positives by PCR. The insensitivity of the WB might be due to the use of recombinant proteins of strains that do not circulate in our country. Another possibility may be a high level of immunosuppression, which could lead to low production of virus, resulting in low stimulation of antibody. We found one mutation, proline to serine in the envelope region in the position 184, presented at least 1/3 of the samples, independent the indeterminate WB profile. In conclusion, we found no correlation of immune state, HTLV-2 proviral load, or env diversity in the K55 region and WB indeterminate results. We believe that the only WB kit available in the market is probably more accurate to detect HTLV-1 antibodies, and some improvement for HTLV-2 detection should be done in the future, especially among high-risk population. ***J. Med. Virol. 82:837–842, 2010.***

KEY WORDS: HTLV-2; Western blot; PCR in real time; proviral load of HTLV-2

INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2) are complex retroviruses that have the same genomic organization and 65–70% of nucleotide similarities [Poiesz et al., 1980; Kalaynaraman et al., 1982; Lowis et al., 2002]. Although the role of HTLV-2 infection in human disease has yet to be clearly defined, there is accumulating evidence that it may be associated with a spectrum of neurological and bacterial susceptibilities [Hall et al., 1996; Orland et al., 2003]. HTLV-2 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., 1998] and in a number of Amerindian populations [Biglione et al., 1993; Duenas-Barajas et al., 1993; Ferrer et al., 1993; Black et al., 1994]. In Brazil, a strain of HTLV-2 different than the standard 2a and 2b strains was found in IVDU in Sao Paulo and among Kaiapos Indians [Hall et al., 1994; Ishak et al., 1995; Eiraku et al., 1996; De-Araujo et al., 1998].

Despite high homology and serological cross-reactivity between HTLV-1 and HTLV-2, studies indicate the necessity of developing reagents that enable type-specific identification [Brodine et al., 1993; Rudolph and Lal, 1993]. Tests that used only lysate of HTLV-1 virus did not show sufficient sensitivity for detecting antibodies against HTLV-2 [Zehender et al., 1997; Waziri et al., 2000]. A recent study in our laboratory found that 50% of samples from a high-risk population identified by WB as indeterminate were HTLV-2 by PCR [Novoa et al., 2007].

Genetic diversity can lead to changes in the antigenic immunodominant epitope, which may affect the binding

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of antibodies and the sensitivity of diagnostic tests [Ishak et al., 2006]. For this reason, we studied the degree of homology of the protein K55 in a commercially available WB kit used worldwide as a confirmatory test.

SUBJECTS AND METHODS

All patients in the study attended the Institute of Infectious Diseases "Emílio Ribas" (IIER). The HTLV unit is made up of a multidisciplinary team, including infectious disease specialists, neurologists, a physical therapist and dentists. A total of 615 individuals had been seen in the HTLV out-clinic at Emilio Ribas from July 1997 to March 2009. Using our algorithm published previously [Novoa et al., 2007], 104 HTLV-2-infected subjects were disclosed in the last 12 years. For this study, we randomly chose 29 samples that filled the full criteria for HTLV-2 infection and 21 samples from individuals who were indeterminate WB. All the subjects were also tested by HCV (Abbott AxSYM® Antibody to Hepatitis C Virus, Abbott Laboratories, Inc., Abbott Park, IL) and HIV serostatus using routine testing from IIER (Abbott AxSYM® Antibody to HIV, Abbott Laboratories, Inc.) following by IFA (Biomanguinhos, Fiocruz Foundation, Rio de Janeiro, RJ, Brazil) to HIV confirmation or from the blank blood when they referred to the HTLV out-clinic. 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 (protocol number 51/07).

Serological Assays

Serum samples were screened for the presence of antibodies to HTLV-1 and HTLV-2 using two commercially available EIA assays: HTLV-1/HTLV-2 Ab Capture ELISA (Ortho Diagnostics, Raritan, NJ) which contains recombinant HTLV-1 and HTLV-2 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—DBL, Singapore). Seropositivity was interpreted according to the stringent criteria in the manufacturer's instructions. A WB sample was scored as HTLV-1 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-2 positive if p19 or p24 and rgp46-II and GD 21 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.

Peripheral Blood Mononuclear Cell (PBMC) Separation

PBMCs were collected in heparinized tubes and isolated using Ficoll-Hypaque density gradients (Amersham Pharmacia Biotech, Piscataway, NJ). The cells were washed, adjusted to 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, and grown with or without 2.5 μg/ml of phytohemagglutinin (PHA) at 37°C, 5% CO_2 for 24 hr. The supernatant fluids were harvested and stored at −70°C for the performance of the assays.

Polymerase chain reaction 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). Nested PCR was performed on positive and indeterminate WB samples to confirm the presence of HTLV.

Tax region. To differentiate HTLV-1 and HTLV-2 infections, RFLP analysis of amplified product of a *Tax* region was carried out as previously described [Eiraku et al., 1996], using primers specific for this region of both HTLV-1 and HTLV-2 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 U 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-2, generating two 69 and 53 bp bands (6 bp bands not visible), and it cuts the HTLV-1 products to yield 122 bp bands (6 bp bands are not visible). The endonuclease Sau 3A fails to cut the HTLV-2 products but cuts the HTLV-1 products to generate distinct 104 and 24 bp bands and are visualized by electrophoresis on a 4% agarose gel.

Env region. When the samples were disclosed to be positive for HTLV-2, HTLV or indeterminate by WB, additional amplification from 2 millions of PBMC to the *env* region was done using the following primers shown in Table I. These primers were designed from a consensus strain using the Primer 3 Program (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/), which provides a list of possible primers, and after the alignment, we chose

TABLE I. Genomic Sequence of Primers for PCR and Used for Sequencing in the HTLV-2 Samples

Primers	Sequences
E_FR_S	GCTACAATGCCCCTACTTGG
E_FR_AS	CCTATGGGAGGAATGTGAGC
E_SR_S	GTCTCCAGTCCATCCTGGAA
E_SR_AS	CAGGGGCCAAACAATATGAC
E_seq1_as	ATGGAGATGTTGGGGGTGTA
E_seq1_s	TACACCCCCAACATCTCCAT
E_seq2_as	TGACTATGGCCTGGGTAAGG
E_seq2_s	CCTTACCCAGGCCATAGTCA

those with better amplication and expect fragment size. A product with 1,000 bp was amplified and used for direct sequencing [Novoa et al., 2008].

Nucleotide sequencing and amino acid analysis. Direct DNA sequencing was performed on 50 HTLV-2 samples. PCR products amplified from the *env* region were purified using the Promega (Madison, WI) Wizard PCR prep system and sequenced in a Perkin-Elmer ABI Prism DNA 3100 Sequencer using Taq FS dye terminator cycle sequencing (Perkin-Elmer Cetus, Norwalk, CA). The same PCR inner primers were used in the sequencing reactions. DNA sequences were translated form the DNA sequence and then analyzed. Multiple sequence alignment for the *env* region of the studied samples together with related sequences in the GenBank/EMBL database were further edited in the DNA Star program. Aligned sequences were used in Bioedit software (Ibis Biosciences, Carlsbad, CA) and the amino acid (AA) sequences were compared to the K55 protein.

Real-Time PCR for HTLV-2 Proviral Load Quantification

The forward and reverse primers used for HTLV-2 DNA quantification were selected using the Oligo (Version 4, National Biosciences, Plymouth, MN) and Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA) software programs and checked by a search on the GenBank. The probe carried a 5′ reporter dye FAM (6-carboxy fluorescein) and a 3′ quencher dye TAMRA (6-carboxy tetramethyl rhodamine). For quantitation of the human albumin gene, the primers Alb-S (5′-GCTGTCATCTCTTGTGGGCTGT-3′) and Alb-AS (5′-AAACTCATGGGAGCTGCT GGTT-3′) and the Alb TaqMan probe (5′-FAM-CCTGTCATGCCCACACAAA-TCTC TCC-TAMRA-3′) were used, as described previously [Dehee et al., 2002]. Albumin DNA quantification was performed in parallel on all samples in order to determine the amount of cellular DNA present and was used as an endogenous reference to normalize variations due to differences in PBMC count or DNA extraction. The protocol was done in accordance with previous published data, and the sensitivity was 10 copies/10^4 PBMC [Montanheiro et al., 2005].

Antibodies for Flow Cytometry Analysis

Flow cytometry analyses were performed with fresh blood samples, using a Coulter® EPICS® XL-MCL™ Flow Cytometer (Beckman Coulter, Fullerton, CA). To determine the $CD4^+$ and $CD8^+$ T cells expression, total lymphocytes were first identified by forward and side scatter, and the 10,000 cells were then gated for $CD4^+$ or $CD8^+$ cells using monoclonal antibodies (Beckman Coulter).

Statistical Analysis

Possible differences in patient characteristics or laboratory values among the four groups were evaluated with two-way Mann–Whitney's test or chi-square test when appropriate. In both cases P values <0.05 were considered statistically significant.

RESULTS

A total of 50 HTLV-2-infected subjects identified by PCR and RFLP were evaluated in this study, where 29 patients were HTLV-2 and 21 were indeterminate by WB. Therefore, 50 HTLV-2-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, two women had an IVDU sexual partner, there was one case of vertical transmission, one was a blood transfusion recipient, and one was one 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 II shows the demographical data of the subjects. The mean age was 40 years old (range 20–59 years of age), 23 were men, the median of $CD4^+$ and $CD8^+$ cell counts was similar, 443 versus 608 cells/mm^3 and 781 versus 746 cells/mm^3, for the positive WB and indeterminate WB, respectively. Demonstrable HTLV-2 proviral load was more often present in the WB inclusive group ($P = 0.02$). HCV infection was more common in the

TABLE II. Some Demographic and Immunological Characteristics of HTLV-2-Infected Individuals

Variable	HTLV-2-positive WB (n = 29)	HTLV indeterminate WB (n = 21)	Total (n = 50)	P-value
Gender				
Female/men	14/15	9/12	23/27	NS
Age				
Mean, years	41	38	40	NS
T $CD4^+$ cells				
Median, cells/mm^3 (percentile 25–75%)	443 (188–663)	608 (227–941)	476 (190–899)	NS
T $CD8^+$ cells				
Median, cells/mm^3 (percentile 25–75%)	781 (551–1,044)	746 (473–1,288)	781 (500–1,166)	NS
HCV (n, %)	8 (28)	12 (57)	20 (40)	0.04
HIV (n, %)	18 (62)	14 (67)	32 (64)	NS
DNA proviral load HTLV-2 (n, % >10 copies/10^6 PBMC)	0/13 (0)	6/17 (35)	6/30	0.02

NS, not statistically significant; WB, Western blot.

TABLE III. Reactivity in the Western Blot in 27 HTLV-2-Infected Subjects or Nontypeable HTLV

ID sample	WB 2.4 profile
321/334	GD 21, GP 21, p24wk
35	GD 21, p19, p24
82/158/307/317/335	GD 21, p24
193	GD 21wk, p53wk, P28wk
361	rgp 46-Iwk, p24, p19, GD21
415	rgp 46-Iwk, GD 21
472	rgp46-II, GD 21
116	rgp46-II, gp21, p24
101	rgp46-II, P28, P24
346/350	rgp46-IIwk
80/95/274/285/394/539	rgp46-II, P24
81/302/265	rgp46-II, p24wk, p19
Total	25

wk, weak.

indeterminate WB group ($P = 0.04$), but HIV status was similar in both groups.

Table III demonstrates the WB profile noted among the indeterminate WB profiles. The majority of the samples displayed the rgp46-IIweak, P24 bands, but there is no usual pattern for the indeterminate sera.

Figure 1 depicts the amino acids (AA) sequences of the K55 protein studied in 27 subjects. The serine was replaced by proline in the 184 position of the envelope protein in 20 samples (12 from indeterminate WB profiles and 8 among positive WB profiles). However, no replacement was also seen some subjects in seven samples (four positive and three indeterminate WB). Thus, the presence of this change was not significant regarding the indeterminate WB profile ($P = 0.7$).

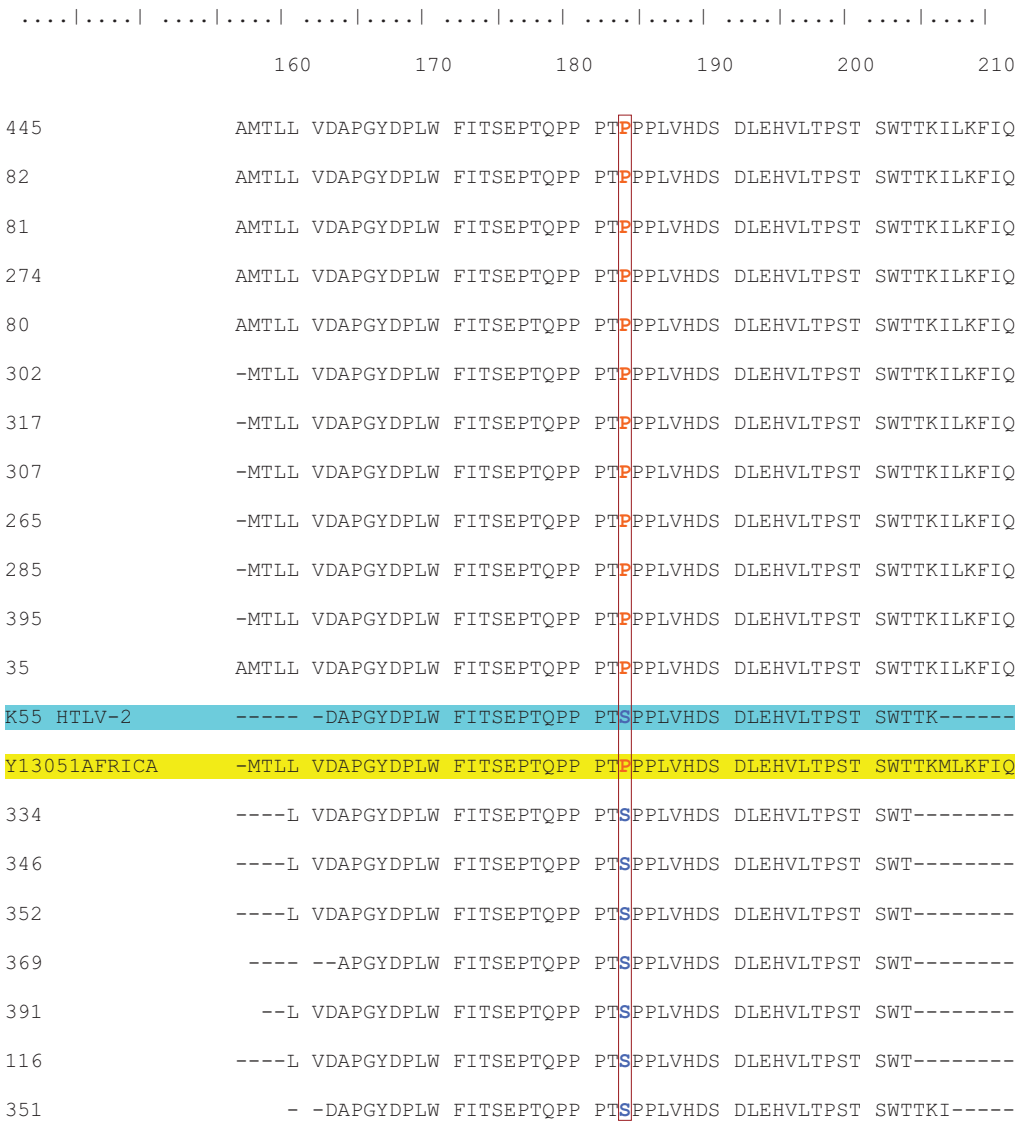

Fig. 1. Comparison of sequences of HTLV-2-positive samples and indeterminate by WB with K55 protein and one African HTLV-2 sequence of the envelope region (shown only 17 sequences).

DISCUSSION

We studied 50 samples from HTLV-2-infected subjects from our cohort in Sao Paulo city, Brazil. We noted no correlation between T cell counts and HTLV-2 proviral load among those with indeterminate WB profiles, although viral detection was only performed on 30 specimens. In fact, HTLV-2 infection produces a low replication rate, and in one study, no virus was detected in the PBMCs of the majority of patients [Montanheiro et al., 2008]. HTLV-2 is genetically more stable than other RNA viruses [Salemi et al., 1999], with a relatively low number of replication cycles and therefore, a frequency of mutations. Cimarelli et al. [1995] demonstrated the existence of a high variation in proviral load in PBMCs in individuals who used drugs. The high genetic stability of HTLV-2 could be explained considering that in some individuals the proviral load is very low, suggesting a slow viral replication, whereas in patients with high proviral load, replication could be, mainly, by clonal expansion of infected cells, via cell multiplication (mitosis) and especially during the reverse transcription [Wattel et al., 1995]. Murphy et al. [2004] showed that the proviral load was significantly higher in patients infected by HTLV-1 infected compared with HTLV-2. In individuals with chronic infection the proviral load may be related to the quantity of the infecting inoculum and the route of transmission. High proviral load of HTLV-2 was related to infection by blood transfusion and low proviral load was related to infection via sexual transmission. Despite these findings, there is a need to deepen the understanding of immunopathogenesis and molecular characteristics of HTLV-2, because of few studies related to proviral load of HTLV-2.

Another point seen in our study was the possibility of immunosuppression to be responsible for the indeterminate WB. One study, conducted in Spain, showed that the number of $CD4^+$ T cells affected the EIA sensitivity but did not affect the sensitivity of the WB [Bassani et al., 2006]. It is possible to consider that the immune system is an important factor and may result in indeterminate serum samples, possibly by the lower production of anti-HTLV-2 antibodies [Bassini et al., 2006]. However, our results indicated that T cells count did not influence the presence of indeterminate WB, even in HIV-1-infected subjects.

Gallo et al. found that the WB kit had a sensitivity of 68% with weak HTLV-2 positives and a specificity of 70% with negatives. Although all positives reacted with rgp21, the p24 antigen in the kit was not strong enough to react with all positives and 11 HTLV-2 specimens reacted nonspecifically with the HTLV-1env protein. Since all positives react with rgp21, which is the most sensitive antigen in the WB kit, they recommended that specimens not reacting with this protein be called negative, regardless of other bands. This would have increased the specificity of the HTLV WB 2.4 kit to 86%. They concluded that the indeterminated WB is caused by lack of sensitivity and specificity of the WB antigen and poor interpretation criteria, not by variances of the specimens [Gallo et al., 1994].

We also studied if the envelope diversity was the cause of the indeterminate WB. We found one mutation, proline to serine in the envelope region in the position 184, was described among African samples [GenBank]. However, this mutation was seen in patients with positive and indeterminated WB profile [Lal et al., 1994]. We did not perform phylogenetic analysis of these sequences, but the reference strain used here, from Africa, may indicate that mutation is present at least 1/3 of the samples. In fact, both sequences were found in our samples, taken together these findings, we may speculate that both strains are circulating in Sao Paulo: one from Africa and another from US/Europe origin. Therefore, since both sequences are present regardless of WB profile, additional studies should be done to find why indeterminate WB occurs. Finally, we found no correlation of immune state, HTLV-2 proviral load, or *env* diversity in the K55 region, and WB indeterminate results. We believe that the only WB kit available in the market is probably more accurate to detect HTLV-1 antibodies, and some improvement for HTLV-2 detection should be pursued in the next WB generation, especially among high-risk populations with a combination of molecular and serological approaches should be tested in the future.

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