

Highly Endemic Human T-Lymphotropic Virus Type II (HTLV-II) Infection in a Venezuelan Guahibo Amerindian Group

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Summary: Sera from 166 Guahibo Indians (55% of the population) living in southwest Venezuela were screened by enzyme-linked immunoassay for antibodies to human T-cell lymphotropic virus (HTLV) I and II. Positive samples were confirmed by immunofluorescence and Western blot. Forty-one Guahibos (24.8%) were found to be seropositive. Polymerase chain reaction (PCR) analysis of proviral DNA in mononuclear cell lysates revealed the virus to be HTLV-II. Prevalence increased with age, and sexual contact with HTLV-II-seropositive partners was identified as a risk factor for infection. PCR amplification of a region of the *pol* gene, utilizing the primer pair SK110/SK111, with subsequent digestion of the 140-base-pair amplification products with *Hinfl* and *MseI* restriction enzymes, showed an HTLV-II subtype-b restriction pattern in all cases. These data suggest that the substrain infecting this Guahibo community belongs to the b subtype, the most frequent among Paleo-Amerindian populations. **Key Words:** HTLV-II—Amerindians—Polymerase chain reaction—Venezuela.

The human T-cell lymphotropic virus type II (HTLV-II) was originally isolated from the transformed cells of a patient with hairy-cell leukemia (1) and, subsequently, from a second patient with a T-cell lymphoproliferative disease and B-cell hairy-cell leukemia (2). HTLV-II has been found in patients with cutaneous T-cell lymphomas (3), large granular lymphocytic leukemias (4,5), and a spastic neurodegenerative disorder similar to HTLV-I-associated myelopathy (6,7). However, there is no conclusive evidence that HTLV-II is etiologically involved in any of these diseases.

HTLV-II, endemic among intravenous drug users

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in the United States (8), has been shown to be naturally endemic in some native Amerindian populations, including the Pume in Venezuela (9-12). The mode of transmission of HTLV-II virus seems to be predominantly through sexual intercourse, intravenous drug use, and blood transfusions (8,13). Mother-to-child transmission of this retrovirus has also been suggested to occur mainly through breast-feeding (14-16) although at least one case of vertical infection in the absence of breast-feeding has been documented (17).

Sequence analysis and endonuclease restriction mapping studies of the transmembrane envelope protein gene of various HTLV-II isolates have revealed two genetically different subtypes of this virus, designated HTLV-IIa and HTLV-IIb (18,19). HTLV-IIb, whose prototype is the NRA cell line (19), is endemic in numerous Paleo-Amerindian tribes (12,18,20). HTLV-IIa, whose prototype is the

MoT cell line, has been detected mostly in intravenous drug users and in at least one isolated Brazilian tribe (21).

Previous reports from our laboratory showed the absence of HTLV-I/II infection among 305 Amerindians from five different Venezuelan tribes (22), and these data have been confirmed in at least two ethnic groups by others (11). More recently, we found serologic evidence of HTLV-II infection in another Amerindian group: the Pume, or Yaruro, with a seroprevalence of 8.7% (9). In the present report, we present serologic evidence, confirmed by polymerase chain reaction analysis, of a high prevalence of HTLV-IIb infection in a small community of Guahibo Indians in southwest Venezuela. These two groups, the Pume and the Guahibo, are to date the only tribes in Venezuela found to be endemically infected with HTLV-II.

MATERIALS AND METHODS

Guahibo Indian Subjects

The Guahibo subjects studied inhabit the tropical territory of Las Majadas, in the southwestern Venezuelan state of Bolivar. This group has migrated in recent years from the Colombian Vichada region. The population has been estimated at ~300 individuals, living in relatively small communities made up of variable numbers of highly endogamous families. Although the members of these communities interact with people living in nearby villages, they rarely mix with other ethnic groups. No evidence of drug abuse and/or recent blood transfusions has been documented among these individuals.

After obtaining oral consent through an interpreter, peripheral blood samples were collected by venipuncture from a total of 166 randomly selected individuals (55% of the censused population). Specimens were processed within 12–24 h of collection. Basic data, including name, age, gender, race, birthplace and, in some cases, family relationships, were collected by interviewing competent adults.

Serologic Assays

Sera were aliquoted and stored at -20°C . Antibodies to HTLV-I/II were screened by two commercial enzyme-linked immunoassay (EIA) kits (a whole virus lysate spiked with rgp21e, HTLV-I/II EIA kit from Cambridge Biotech, Worcester, MA; and HTLV-I/II micro-ELISA system from Organon Teknika, Durham, NC, U.S.A.). Those sera that were repeatedly reactive by at least one EIA test were confirmed by HTLV-I/II Western blot (WB) incorporating rgp21e (Cambridge Biotech). All samples were tested by an indirect immunofluorescence assay (IF) against HTLV-I- and HTLV-II-transformed cell lines (MT2 and clone 19, respectively), as previously described (9).

Polymerase Chain Reaction (PCR) Assays

Peripheral blood mononuclear cells (PBMCs) from 21 seropositive and 20 seronegative Indians were isolated from 20 ml of

heparinized blood by Ficoll-Hypaque density-gradient centrifugation. PBMCs were adjusted to 6×10^6 cells/ml in lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl_2 , 0.5% Tween 20, and 0.5% Nonidet P-40] and digested with 60 μg of proteinase K (Sigma, St. Louis, MO, U.S.A.) per milliliter for 60 min at 56°C . The enzyme was inactivated by heating at 95°C for 10 min, and samples were stored at -70°C . PCR assays were performed in 100 μl final volume, containing 25- μl lysate aliquots (equivalent to 150,000 PBMCs) for the first round of amplification and 2 μl of samples for the inner primer-amplification round. To determine whether the DNA was suitable for amplification, and to exclude false viral negatives, samples were initially amplified with β -globin primers (PCO4 and GH20; Perkin Elmer, Branchburg, NJ, U.S.A.) specific for a conserved region of the human β -globin gene (23). Generation of an amplified β -globin 268-base-pair (bp) DNA fragment provided a measure of the reproducibility, quality, and yield of each DNA extraction.

All cell lysates were amplified by nested PCR using *tax/rex* gene primer sets (TR101–TR104), which amplify both HTLV-I and II proviral sequences, followed by discrimination by specific restriction endonuclease cleavage (*Mbo*I for HTLV-I, *Dde*I for HTLV-II), as previously described (11). Nested PCR *env/tax* genes primer sets (ET401–4), which amplify only HTLV-II, were also used (11).

For the oligomer restriction assay (OR), cell lysates were amplified using the HTLV-III *pol* gene generic primers SK110/111, and the amplification products were digested with *Hinf*I or *Mse*I as previously described (24). Amplification products were analyzed by electrophoresis on 2–4% agarose gels, with visualization of bands by ethidium-bromide staining. The contamination-prevention measures of Kwok (25) were strictly applied throughout the study.

Chi-squared analysis was used to determine the significance of trends in prevalence in five age categories and to assess the association of HTLV-II infection with gender.

RESULTS

A total of 166 individuals, including 92 females and 74 males, aged 2–85 years, were studied. When the 166 samples were screened by EIA with two commercially available reagents, 41 sera (24.7%, including sera from 22 females and 19 males) were repeatedly reactive with both reagents. These 41 positive samples were confirmed by WB and positivity was assigned following the ASTPHLD criteria, as previously described (26). As illustrated in Fig. 1, the Indian sera reacted with the major gag (p24) protein and the recombinant gp21e protein. Although core band p19 was present in some of the samples, p24 was invariable stronger, suggesting HTLV-II infection in all cases (27). All 41 specimens were positive when tested by IF with both MT2 and clone 19 cell lines. When IF end points were determined by fourfold serial dilution, all samples tested exhibited at least a fourfold higher titer against the clone 19 cell line, indicating a pattern

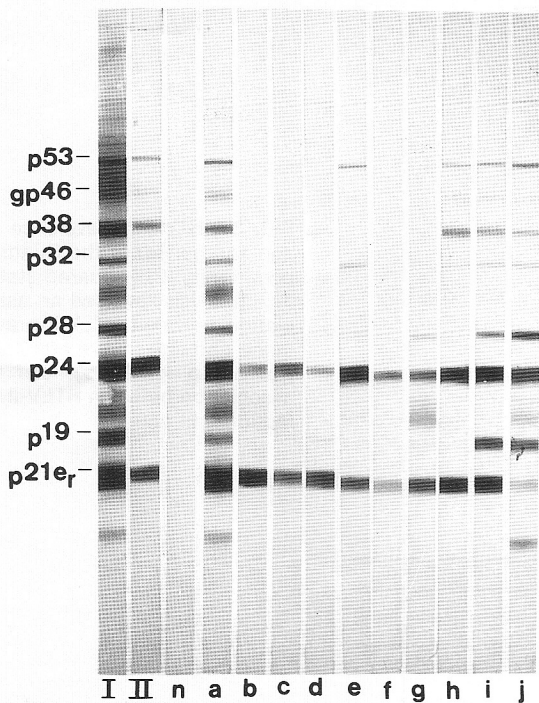


FIG. 1. Western-blot of representative samples from enzyme-linked immunoassay-positive Indian sera (strips a to j) versus negative control (strip n) and HTLV-I (strip I) and HTLV-II (strip II) positive controls. HTLV-I/II infection was confirmed by reactivity to p24 and to gp46 or recombinant p21e. The more intense reactivity at p24 than p19 in most blots strongly suggests infection by HTLV-II (27).

consistent with HTLV-II infection (data not shown).

When the cell lysates obtained from 21 seropositive individuals were amplified by nested PCR for the *env/tax* region, 67% (14 of 21) of the samples yielded the expected 829-bp HTLV-II-specific band. All 21 samples tested were successfully amplified with the HTLV-I/II generic primer pairs for the *tax/rex* region, yielding the expected 159-bp band. When this amplification product was subjected to restriction endonuclease cleavage, all Guahibo samples tested showed a unique *DdeI* restriction site, resulting in an HTLV-II-restriction cleavage identification (Fig. 2). Of 20 serologically negative samples, 18 were confirmed as negative by nested PCR for both *env/tax* and *tax/rex* regions. Two samples were inconclusive because they showed proviral sequences only when the *tax/rex* region was tested but not for the *env/tax* and *pol* regions (data not shown). These subjects were considered as seronegative for further data analysis.

Once the HTLV-II prevalence was established by

serology and PCR, analysis by age and gender was performed, as shown in Fig. 3. HTLV-II prevalence increased with age among both males and females ($p < 0.005$), but no association with gender was found.

When information regarding marital and mother-children relationships was gathered among 80 of the subjects sampled, five of six women whose husbands were seropositive were also found seropositive. Only two of 15 children born to seropositive mothers were also seropositive.

Finally, OR subtyping of 10 seropositive samples resulted in an HTLV-II subtype-b pattern in all cases, with a unique *MseI* restriction endonuclease site that generates two fragments of 88 bp and 52 bp, and no cleavage with *HinfI* (Fig. 4). Only the enzymatically amplified MoT cell line lysate showed the expected HTLV-II subtype-a pattern,

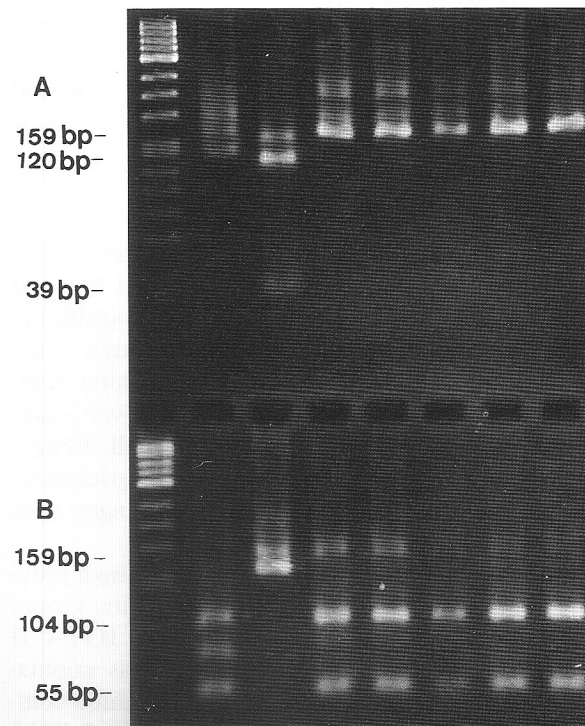


FIG. 2. Polymerase chain reaction detection of HTLV-II proviral sequences in peripheral blood mononuclear cell lysates from Guahibo Amerindians. The virus type was determined by restriction endonuclease cleavage of the 159-base-pair (bp) DNA fragment obtained after generic *tax/rex* amplification: *MboI* cleaves HTLV-I into 120- and 39-bp fragments, but does not cut HTLV-II (A); *DdeI* cleaves HTLV-II into 104- and 55-bp fragments, but does not cut HTLV-I (B). PCR products were run on 4% agarose gels and stained with ethidium bromide for visualization. M, molecular weight marker; lane 2, MoT cell line (HTLV-II-positive control); lane 3, MT2 cell line (HTLV-I-positive control); and lanes 4-8, Guahibo samples.

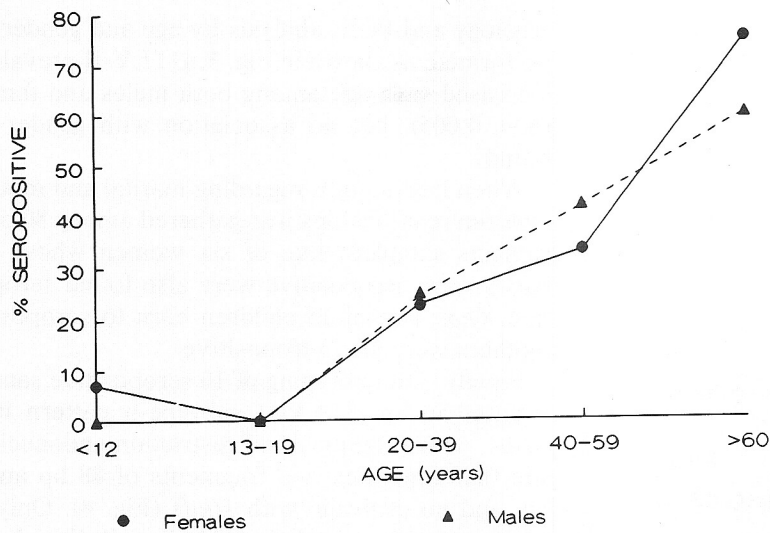


FIG. 3. HTLV-II prevalence by age and gender in the Guahibo community. Chi-squared statistical analysis revealed an association between HTLV-II prevalence and age in five age categories ($p < 0.005$), but no association between HTLV-II infection and gender.

Age group	< 12	13-19	20-39	40-59	> 60	Total
Males (n)	3	15	32	19	5	74
Females (n)	14	15	31	24	8	92

with a *HinfI* cleavage of the 140-bp DNA fragment into 104-bp and 36-bp fragments (Fig. 4, lane c).

DISCUSSION

The Guahibo Indians inhabit a broad geographic area, which includes both Colombian and Venezuelan territory. Most of their population is located in the Vichada region in Colombia, although some groups live in Venezuela (28). The Guahibo community studied, from Las Majadas Bolivar state, comprises ~300 individuals in two small villages that support themselves by means of agriculture, hunting, fishing, and commercial exchanges with nearby towns.

In this report, we present evidence of a high prevalence of HTLV-II infection in this Guahibo group. Immunofluorescence analysis revealed HTLV-II rather than HTLV-I-type infection in this population. The presence of HTLV-II in the Guahibo community was also clearly demonstrated by PCR DNA amplification of mononuclear cell lysates. Two different approaches, including type-specific virus DNA amplification and type-specific restriction enzyme cleavage fragmentation, indicated HTLV-II infection in this group. Each successful amplification required adequate homology between four separate primers (the outer and inner pairs) and the target DNA sequence, and restriction cleavage identification required the proper positioning of a

specific 6-bp fragment in the target proviral sequence.

The lack of evidence supporting transmission by transfusion or the use of needles among this population, together with the nonmixing with other ethnic groups, makes it highly likely that HTLV-II is endemic in this community, as has been described in many native American tribes.

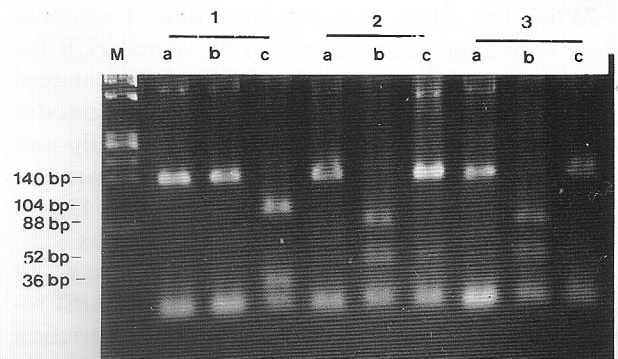


FIG. 4. Oligomer restriction assay for the determination of the HTLV-II substrain present in the Guahibo Indians. HTLV-II-positive cell lysates were amplified with the primer pair SK110/SK111, and 140-bp amplification products were undigested (lane a) or digested with the restriction endonuclease *MseI* (lane b), which digests HTLV-IIb sequences only into 88- and 52-bp fragments, or *HinfI* (lane c), which digests HTLV-IIa substrain only into 104- and 36-bp fragments. Polymerase chain reaction products were run on 4% agarose gels and stained with ethidium bromide for visualization. M, molecular weight marker; 1, MoT cell line amplification products; and 2-3, representative Guahibo amplification products.

The seroprevalence observed in this Guahibo community increased with age, as has been previously reported in other HTLV-II-endemic Amerindian tribes (11,16,29). No significant association was found between seroprevalence and gender, suggesting sexual transmission of the virus, but not indicating greater male-to-female efficiency of infection as has been reported by others (13). Data on marital relationships among the individuals studied also support possible sexual transmission in some cases: five of six wives of seropositive husbands included in the study were also seropositive, and all of the husbands of seropositive women were also infected. Vertical or milk-borne transmission of HTLV-II virus does not seem to be the primary means of transmission, since only 13% of sampled Guahibo born to seropositive mothers were also seropositive. This vertical transmission rate is similar to that found by others for HTLV-II with breast-feeding (16,17).

Total concordance between sequenced and OR-based subtyping of HTLV-II proviral DNA amplified by PCR has been reported among 22 different HTLV-II isolates (24); this method has therefore been proven to be a rapid and specific assay for the definition of HTLV-II substrains, facilitating molecular epidemiologic studies of this retrovirus. The OR assay involves PCR amplification of a 140-bp region of the *pol* gene, flanked by the primers SK110/SK111. Sequence analyses of this proviral region have revealed a mutation in the HTLV-IIb isolates that creates a unique *Mse*I restriction endonuclease site, while a C/T mutation present in HTLV-IIa isolates creates a unique *Hinf*I site. Based on the restriction pattern, it is possible to differentiate both HTLV-II substrains (24). The isolate from the Guahibo group evaluated belongs to subtype b, as shown by the OR subtyping assay (Fig. 4). HTLV-IIb infection has also been reported among the Guaymi Indians of Panama, the Seminoles of Florida, and the Tobas and Maticos of Argentina (18,24).

Numerous reports, including ours, on HTLV-II infection of Paleo-Amerindians have failed to demonstrate a pathogenic role for this virus. The lack of evidence of lymphoid malignancies or myelopathies in these communities suggests the possibility of a benign virus-host interaction as the result of a long interaction period that has taken place over thousands of years of common evolution.

We conclude, from these results and previous reports, that HTLV-II infection is present in the Gua-

hibo and Pume groups, but not in other Amerindians studied who reside in Venezuela (9,11,22). Ongoing molecular characterization studies, including cloning and sequencing of the SK110/111 140-bp *pol* region and other proviral regions, will further characterize the HTLV-IIb substrain present in the Guahibo Amerindian group. These results may provide important clues as to the origin of this isolate and aid in comparative phylogenetic studies with other substrains of this family of retroviruses.

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