Isolation of human T-cell lymphotropic virus type 2 from Guaymi Indians in Panama

Michael D. Lairmore§, Steven Jacobson‡, Fernando Gracia§, Barun K. De*, Luis Castillo§, Mario Larreategui§, Beverly D. Roberts*, Paul H. Levine†, William A. Blattner*, and Jonathan E. Kaplan*

*Retrovirus Diseases Branch, Centers for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333; ‡Neuroimmunology Branch, National Institutes of Health, Bethesda, MD 20892; §Division of Epidemiology, Gorgas Memorial Laboratory, Panama City, Panama; and ¶Environmental Epidemiology Branch, National Cancer Institute, Rockville, MD 20852

Communicated by Robert H. Purcell, August 23, 1990

ABSTRACT Human T-lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia/lymphoma and with a chronic degenerative myelopathy. However, another major type of HTLV, HTLV-II, has been isolated only sporadically, and little is known of disease associations, transmission routes, and risk factors for HTLV-II infection. Recent studies have found an elevated rate of seroreactivity to HTLV among Guaymi Indians from Bocas del Toro Province, Panama. To identify the cause of seroreactivity among this unique population we used HTLV-II-specific polymerase chain reaction techniques to detect HTLV genetic sequences from blood leukocytes of three seropositive Guaymi Indians. The HTLV-II primer-amplified polymerase chain reaction products from two of these subjects were partially sequenced and matched published HTLV-II nucleotide sequences in both gag (94% of 107 bases) and pol (98% of 112 bases) regions. A CD4+ T-lymphocyte line established from one of these same subjects produced HTLV-II-specific proteins when tested in antigen-capture and immunoblot assays, as well as mature HTLV particles. The demonstration of HTLV-II infection in this geographically and culturally isolated Central American Indian population without typical risk factors of HTLV infection suggests that HTLV-II infection is endemic in this population and provides an important clue to a potential natural reservoir for this virus.

Human T-cell lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia/lymphoma and with a chronic degenerative neurologic disease, HTLV-I-associated myelopathy/tropical spastic paraparesis (1). A second type of HTLV, HTLV-II, was initially isolated from a patient with hairy cell leukemia (2) but has subsequently been isolated only sporadically (3); detailed studies regarding disease associations, transmission routes, and risk factors for HTLV-II infection have not been reported. Recent studies using the polymerase chain reaction (PCR) technique have indicated that a high percentage of HTLV seroreactivity among i.v. drug users and blood donors in certain regions of the United States may be from HTLV-II (4, 5).

HTLV-I and HTLV-II are distinguished by restriction endonuclease cleavage sites, nucleotide sequence, major core protein size, and immunogenic properties (6–8). The two viruses appear to share ~60% overall nucleotide sequence. Despite nucleotide differences, the two virus types have a number of similar biological properties, including an ability to transform lymphocytes, predominant CD4 lymphocyte tropism, and an ability to elicit cytokine production from transformed cell lines (9–11).

Recent population-based seroepidemiologic studies revealed that 8% of 337 Guaymi Indians residing in Bocas del Toro Province, Republic of Panama, had antibody against HTLV (12, 13). Antibody was found almost exclusively in subjects 15 yr old and older (16% seropositivity), there was no evidence for household clustering of infection, and neither hematologic nor neurologic diseases usually associated with HTLV infection were identified (13). Furthermore, serum specimens from these HTLV-seropositive persons demonstrated weak immunoreactivity to envelope antigens of HTLV-I compared with the seroreactivity of HTLV-I seropositive controls (13). The Guaymi are descendents of Indian groups who have lived in relative isolation since the arrival of the Spanish in the 16th century and are still largely unadmixed with other racial or ethnic groups (14). Traditionally, the Guaymi practice a subsistence economy, although in recent years some families have migrated to Changuinola on the Caribbean coast to work on banana plantations. The Guaymi do not practice i.v. drug use, tattooing, or scarification, and medical procedures requiring blood transfusion are rare. The unusually high prevalence of HTLV seroreactivity, atypical epidemiology, and weak antibody reactivity to HTLV-I envelope antigens suggested that variant HTLV strains might be endemic in this population. The present studies were initiated to identify the nature of the virus accounting for this seroreactivity.

MATERIALS AND METHODS

Guaymi Indian Subjects. Demographic, cultural, and HTLV serologic information regarding the Guaymi Indians have been reported (12–14). Briefly, the Guaymi subjects studied were individuals who migrated to Changuinola, Bocas del Toro Province, Panama to seek employment at a banana plantation. During the seroepidemiologic studies (12, 13) blood samples were collected (after obtaining oral informed consent from each subject or guardian), and peripheral blood mononuclear cells (PBMC) were cryopreserved after Ficoll separation. In addition, a study physician and staff carried out interviews, physical examinations, and clinical

Abbreviations: HTLV, human T-lymphotropic virus; PCR, polymerase chain reaction; IL-2, interleukin 2; gag, group-specific antigen; env, envelope; pol, polymerase; PBMC, peripheral blood mononuclear cells; MAb, monoclonal antibody.

†To whom reprint requests should be addressed at the present address: Ohio State University, Department of Veterinary Pathobiology, 1925 Coffey Road, Columbus, OH 43210.

The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M38253 for pol and M38254 for gag p24).
Table 1. Summary of family relationships, HTLV antibody status, and PCR results

<table>
<thead>
<tr>
<th>Family</th>
<th>Subject</th>
<th>Age, yr</th>
<th>Relation</th>
<th>HTLV antibody</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gag</td>
<td>pol</td>
</tr>
<tr>
<td>12</td>
<td>1*</td>
<td>26</td>
<td>Mother</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>30</td>
<td>Father</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>11</td>
<td>Son</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>4</td>
<td>Daughter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>11</td>
<td>33</td>
<td>Mother</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>10</td>
<td>29</td>
<td>Father</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>14</td>
<td>6</td>
<td>Son</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>15</td>
<td>8</td>
<td>Daughter</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>132</td>
<td>5</td>
<td>24</td>
<td>Mother</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>132</td>
<td>4</td>
<td>23</td>
<td>Father</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>132</td>
<td>7</td>
<td>5</td>
<td>Son</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

A study physician and staff carried out interviews, examinations, and collection of specimens in the subject’s home. Serum samples were tested for HTLV antibodies by using commercial enzyme immunoabsorbant assay (DuPont), according to the manufacturer’s recommendations. Seropositivity was confirmed by immunoblot assays as described (15). ND, not determined.

*T-cell line (Fig. 5) was derived from subject 12.1.

Polymerase Chain Reaction and Nucleotide Sequence. PCR was performed using total genomic DNA as described (16, 17). Primers were used to amplify 1 μg of total genomic DNA (equivalent to ≈150,000 PBMC) for each PCR amplification in 100-μl reaction volumes of 5 mM KCl/10 mM Tris, pH 8.5/10 mM MgCl2/0.2 mM of each dNTP/each primer at 100 ng/μl/2 units of Thermus aquaticus (Taq) polymerase (Perkin-Elmer/Cetus) (18). The amplification consisted of 34 repetitive three-step cycles under the following conditions: 25°-95°C and then 2-min periods of incubation at 95°C, 55°C, and 72°C per cycle in a thermal cycler (Perkin-Elmer/Cetus). PBMC DNA was amplified by using both HTLV-I- and HTLV-II-specific gag and pol primers (16, 19). Samples that were positive when amplified with either HTLV-I gag or pol primers were also PCR-amplified with env primers. HTLV-I primers were derived from gag sequence positions 1423-1444 in the sense strand and 1558-1537 in the antisense strand, from pol positions, 3015-3034 in the sense strand and 3154-3134 in the antisense strand, and from env positions 5627-5648 in the sense strand and 5792-5771 in the antisense strand. HTLV-II primers were derived from gag sequence positions 1424-1445 in the sense strand and 1561-1540 in the antisense strand, from pol positions 2989-3010 in the sense strand and 3131-3110 in the antisense strand, and from env positions 5602-5620 in the sense strand and 5804-5787 in the antisense strand**. The amplified products were separated in 1.8% agarose gels and probe by Southern hybridization by using specific 32P-labeled probes for HTLV-I—gag 1489-1513, pol 3050-3074, and env 5713-5737—and for HTLV-II—gag 1490-1514, pol 3025-3049, and env 5758-5779**. After blotting of blotted membranes, labeled probes were hybridized overnight at 42°C in prehybridization solution. Membranes were subsequently washed under high-stringency conditions [twice with 2% SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 56°C for 30 min, once with 1% SSC at 56°C for 15 min]. A sample was considered positive if amplified with primers from each of two separate viral regions. To obtain nucleotide sequence data the PCR products were isolated with 1.8% agarose, denatured by dimethyldisulfide, and sequenced dideoxynucleotide chain termination with Sequenase version 2.0, according to the manufacturer’s recommendations (United States Biochemical).

Cell-Surface Antigen Expression and HTLV Antigen Assays. Expression of CD4 (Leu 3a), CD8 (Leu 2a), and CD25 [interleukin 2 (IL-2) receptor], and IgG1 control was detected with fluorescein isothiocyanate-conjugated murine monoclonal antibodies (mAbs; Becton Dickinson) and then analyzed by fluorescence-activated analysis (FACS-scan, Becton Dickinson). Soluble HTLV antigen from subject 12-I PBMC culture supernatant was determined by enzyme immunoabsorbant capture assays for HTLV-I p19 (Cellular Products) and for HTLV-I and -II p24 (Coulter). The assay specific for HTLV-I used polyvalent rabbit antiserum to HTLV-I coated onto microtiter plates to capture soluble HTLV-I antigen, which detects HTLV-I antigen by using a mAb specific for HTLV-I p19 (20). Bound mAb is detected with peroxidase-conjugated goat anti-mouse IgG, and color is developed with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The antigen-capture assay, which recognized both HTLV-I and HTLV-II p24 core antigen, uses a murine mAb specific for p24 of HTLV-I and -II coated onto microwell strips to capture soluble HTLV-I/II p24 antigen. Bound HTLV antigen is recognized by biotinylated human antibodies to HTLV-I/II. Streptavidin-horseradish peroxidase is then complexed with biotin-linked antibodies, and color develops from the reaction of the peroxidase with hydrogen peroxide substrate in the presence of 3,3',5,5'-tetramethylbenzidine chromagen. Resultant absorbance values of both tests were detected and compared with known standard curves of viral core antigens in the same trial. Immunoblotting was used to detect HTLV antigens from cell culture lysates as described (15). Cellular lysates were prepared from Guaymi 12-I cell line (106 cells per 5 ml of lysing buffer, ref. 15). HTLV-I (MT-2, ref. 22) and HTLV-II (Mo-T, ref. 22) lysate antigens were obtained from a commercial source (Hillcrest Biologics, Cypress, CA). Viral antigens were suspended in sample buffer [Tris buffer at 0.1 mol/liter, pH 6.8, containing 0.5% SDS, bromophenol blue at 0.10 μg/ml, 20% (vol/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol, heated at 95°C for 5 min and electrophoresed in precast gradient gels (4-20%)

**Nucleotide sequence positions were derived from The Los Alamos National Laboratory, Human Retroviruses and AIDS, 1989: HTLV-I, J02029; HTLV-II, M10060.
polyacrylamide, EmproTech, Bethesda, MD), and probed by using an aminobiotin-peroxidase procedure as described (15).

**Electron Microscopy.** Ultrastructural examination for HTLV particles was done by using a PBMC suspension culture (from subject 12-1, Table 1) containing ~1 × 10^6 cells, which was washed free of media and resuspended in 2.5% glutaraldehyde in phosphate buffer at pH 7.4 for 2 hr at 4°C. The cells were then pelleted, and the fixative solution was removed and replaced with phosphate buffer at pH 7.4. The pellets were postfixed by 1% osmium tetroxide and stained with uranium and lead acetate salts.

**RESULTS**

To identify HTLV we collected PBMC from three HTLV-seropositive Guaymi and eight of their seronegative family members. Genomic DNA was isolated from the PBMC samples and assayed by PCR to test for HTLV sequences. We used oligonucleotide primer pairs that would selectively amplify HTLV-I or HTLV-II nucleotide sequences. DNA from the three seropositive Guaymi were positive when using HTLV-II gag and pol sequence primers (Fig. 1). In addition, HTLV-II env primers consistently amplified two of the three PBMC samples from these seropositive subjects (Fig. 1). These same DNA samples were PCR negative when using HTLV-I-specific primers derived from HTLV-I gag, pol, and env sequences (Table 1).

Six of the eight PBMC DNA samples from family members were negative for HTLV-II amplification with both gag and pol primers, and two samples (from subjects 66-10 and 66-14, Table 1) were amplified with pol primers but were negative with gag primers. All eight family members were negative for HTLV-I PCR amplification when using conserved gag and pol primers.

To further characterize the identity of the PCR-amplified products, we directly sequenced the HTLV-II gag and pol PCR products from two of the seropositive persons (Fig. 2).

![Fig. 1. Southern analysis of PCR-amplified products using 32p-labeled oligonucleotide probes. The results with HTLV-I primers indicated no specific amplified products, whereas the HTLV-II primers amplified the specific products under the same conditions. (A) Amplification and probing for HTLV-II gag sequences. Serial 10-fold dilutions (undiluted–10^-10) of control HTLV-II cell line Mo-T (lanes 1–4) and normal donor PBMC (lane 5). (B) Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3) amplified and probed for HTLV-II gag sequences. (C) Amplification and probing for HTLV-II pol sequences: Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3). (D) Amplification and probing for HTLV-II env sequences: Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3).](image1)

![Fig. 2. Comparison of genomic sequence of HTLV Guaymi Indian (GI) PBMC PCR-amplified gag and pol products. (A) Nucleotide sequence alignment of genomic DNA product of gag region from PBMC sample of Guaymi subject 12-1, Table 1. Nucleotide differences are noted and compared with HTLV-II gag sequence (positions 1451–1558) and HTLV-I gag sequence (positions 1450–1557)**. (B) Nucleotide sequence alignment of genomic DNA product of pol region from PBMC sample from Guaymi subject 132-5 (GI), Table 1. Nucleotide differences are noted compared with HTLV-II pol (positions 3012–3124) and HTLV-I sequences (positions 3037–3149)**. The DNA sequence of the gag p24 region (from subject 12-1, Table 1) matched the corresponding HTLV-II gag sequence in 101 (94%) of 107 nucleotide bases and only 66 (62%) of 107 HTLV-I gag nucleotide bases. The pol PCR product (from subject 132-5, Table 1) was sequenced and was identical to the corresponding HTLV-II pol sequence in 110 (98%) of 112 nucleotides and only 73 (65%) of 112 HTLV-I pol nucleotides.

To further define the HTLV-II infection in this population, we cultured Ficoll-separated PBMC from subject 12-1. We established a primary IL-2-dependent T-lymphocyte line, which at 14 weeks in culture had a surface phenotype characterized by CD2^+ (99%, T-cell-sheep erythrocyte receptor), CD3^+ (99%, T-cell receptor complex), CD4^+/CD8^− (80%, helper/inducer, T lymphocyte), CD25^+ (60%, IL-2 receptor) reactivity (Fig. 3). This primary cell line was maintained independent of normal donor feeder cells through 24 weeks, remained IL-2 dependent, and slowed in replicative capacity and was cryopreserved. The infectious capacity of the HTLV culture was established by cocultivation and infection of normal donor PBMC and by infection of rabbits with irradiated (5000 rads; 1 rad = 0.01 Gy) Guaymi PBMC 12-1 culture (23). Supernatants of both primary and cocultured cells were positive in an antigen-capture assay capable of detecting the major core antigen gag p24 of both HTLV-1 and HTLV-II; these same supernatants were negative when using an HTLV-I-specific antigen-capture assay for HTLV-I gag p19 (Fig. 3).

HTLV proteins in cell culture lysates were identified by immunoblot analysis (Fig. 4). Polyvalent serum from an HTLV-I-infected patient and mAbs specific for either HTLV-I (env gp46) or recognizing both HTLV-I and
Fig. 3. Cell-surface antigen expression and HTLV antigen production of HTLV-II-producing cell line derived from Guaymi subject 12-1 of Table 1. Expression of CD4 (Leu 3a), CD8 (Leu 2), and IL-2 (Leu 2) receptor, and IgG1 control was determined with fluorescein isothiocyanate-conjugated murine mAbs (Becton Dickinson) and then analyzed by fluorescence-activated analysis (FACSscan, Becton Dickinson). (A) Relative staining of cell-surface antigens is indicated for the primary PBMC culture established from Guaymi subject 12-1 at 14 weeks after culture initiation. (B and C) Comparison of viral antigen from Guaymi (G 12-1) PBMC culture supernatants versus known HTLV-I (MT-2) and HTLV-II (Mo-T) culture supernatants. Guaymi PBMC 12-1 culture supernatant antigen was detected by an antigen-capture assay that used a mAb to common epitopes of p24 of both HTLV-I and -II (B) and is not detected by HTLV-I p19-specific antigen-capture assay (C). Supernatants from HuT 78 (HTLV-negative cell line) and PBL (normal cultured peripheral blood lymphocyte) used for negative controls.

HTLV-II (gag p24) were used to demonstrate that the cell lysate reactivity was consistent with HTLV-II and not HTLV-I (Fig. 4). The lysates failed to react with mAbs directed against a specific epitope of HTLV-I env p46/gp65 (24). However, cell lysates did react with HTLV-I patient serum that contained cross-reactive antibodies for HTLV-I and -II and with a mAb reactive to common epitopes shared in gag p24 of both HTLV-I and -II (25) (Fig. 4).

Ultrastructural analysis of the cell culture revealed 80- to 120-nm mature retroviral particles consistent with HTLV (Fig. 5). Mature intact viral particles were primarily adjacent to the surface of plasma membranes or between cell processes and were less frequently found budding from the plasmalemma (Fig. 5). Viral particles consisted of irregular nucleocapsids with fine granular core material surrounded by an envelope. Some viral particles assumed hexagonal to polyhedral shapes.

DISCUSSION

We report HTLV-II infection from a defined non-i.v.-drug-using population, a finding that has important implications for understanding the phylogeny of human retroviruses. HTLV-II genomic DNA was amplified by PCR in all three PBMC samples from seropositive Guaymi, each from separate family units; PCR products were sequenced and matched known HTLV-II nucleotide sequences in two conserved viral genomic regions (gag and pol). In addition, we isolated HTLV-II from cultured PBMC from one of these same subjects. These data together with the generally weak serologic reactivity to HTLV-I envelope antigens in the Guaymi population (13) suggest that our HTLV-II isolate is primarily
responsible for the HTLV seroreactivity in this Central American Indian population.

The Guaymi are descendants of Indian groups who have lived in relative isolation since the arrival of the Spanish in the 16th century and are still largely unadmixed with other racial or ethnic groups (14). Typical risk factors for HTLV infection could not be identified among the population. Guaymi Indians do not practice ritual scarification or tattooing; i.v. drug use does not occur, disposable needles and syringes are almost universally used in Panama by physicians and in all hospitals and health centers, and transfusion of blood is rare (12).

The mode of transmission of HTLV-II among the Guaymi Indian population was not investigated in our study. Mother-child transmission and sexual transmission may be important in maintaining endemicity of the virus infection in the population, but seropositivity is rare in Guaymi children, and familial clustering is inconsistently demonstrated (13). Our finding of HTLV-II pol PCR amplification among two of eight family members suggests that the virus infection may be latent in certain individuals as suggested (1) for HTLV-I infection. However, this PCR reactivity was demonstrated for only one set of PCR primers in a conserved region (pol), and we cannot exclude the possibility of nonspecific amplification of similar host or closely related viral sequences. The absence of known HTLV-associated disease, either adult T-cell leukemia or spastic paraparesis, may suggest that the HTLV-II infection is less pathogenic than HTLV-I or has evolved a more benign relationship in the population. However, to determine the role of HTLV-II in disease among the Guaymi, further monitoring of infected persons is necessary because of the long latent period between infection and disease characteristic of this group of retroviruses and because of the relatively small population studied.

It is possible that the HTLV-II isolate we have identified in this study may differ from previous HTLV-II isolates. In the United States, HTLV-II infection has recently been found to be more prevalent than previously believed in certain groups of i.v. drug users and normal blood donors (5). It will be important to identify the role of HTLV-II in human disease (if any) and to identify transmission routes, risk factors, and pathogenetic mechanisms for this human retrovirus. Further molecular characterization, including complete sequencing, may provide important clues for the origin of the Guaymi HTLV-II isolate and for comparative phylogenetic studies of this family of retroviruses.

We thank Ms. Cynthia Goldsmith for electron microscopic examination of cell cultures; Drs. Rima Khabbaz, Dan Bednarik, Renu Lal, and Tom Folks for their discussions and constructive reviews of the manuscript; and Dr. Gary Toedter (Coulter Immunology) for supply of antigen capture assays. Our study was supported, in part, by the National Cancer Institute, National Institutes of Health, Contract NCI-CP-31015 with the Gorgas Memorial Institute.