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

(retrovirus)

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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 indicate that a high percentage of certain groups of i.v. drug users and blood donors are infected with HTLV-II. Seroepidemiologic 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 p24 *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 clin-

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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.

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‖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

Family	Subject	Age, yr	Relation	HTLV antibody	PCR results					
					HTLV-I			HTLV-II		
					gag	pol	env	gag	pol	env
12	1*	26	Mother	+	−	−	−	+	+	−
12	3	30	Father	−	−	−	ND	−	−	ND
12	2	11	Son	−	−	−	ND	−	−	ND
12	6	4	Daughter	−	−	−	ND	−	−	ND
66	11	33	Mother	+	−	−	−	+	+	+
66	10	29	Father	ND	−	−	ND	−	+	−
66	14	6	Son	−	−	−	ND	−	+	−
66	15	8	Daughter	−	−	−	ND	−	−	ND
132	5	24	Mother	+	−	−	−	+	+	+
132	4	23	Father	−	−	−	ND	−	−	ND
132	7	5	Son	ND	−	−	ND	−	−	ND

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.

ical evaluations. Family relationships, HTLV antibody status, and HTLV PCR (below) results are summarized in Table 1. Serum samples were tested for HTLV antibody by using commercial enzyme immunoabsorbant assay (DuPont) according to the manufacturer's recommendations. Seropositivity (reactivity to *gag* p24 and *env* gp46 or gp 61/68) was confirmed by immunoblot assays as described (8, 15). Due to extensive cross-reactivity among viral proteins, our serologic methods did not distinguish antibody reactivity to HTLV-I from reactivity to HTLV-II (8).

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 $MgCl_2$/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 *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* 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 ^{32}P-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 blocking 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 dimethylsulfoxide, 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 2), 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 12-1 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 12-1 cell line (10^8 cells per 5 ml of lysing buffer, ref. 15). HTLV-I (MT-2, ref. 21) and HTLV-II (Mo-T, ref. 22) lysate antigens were obtained from a commercial source (Hillcrest Biologicals, 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 4 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 avidin–biotin–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 $\approx 1 \times 10^7$ 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).

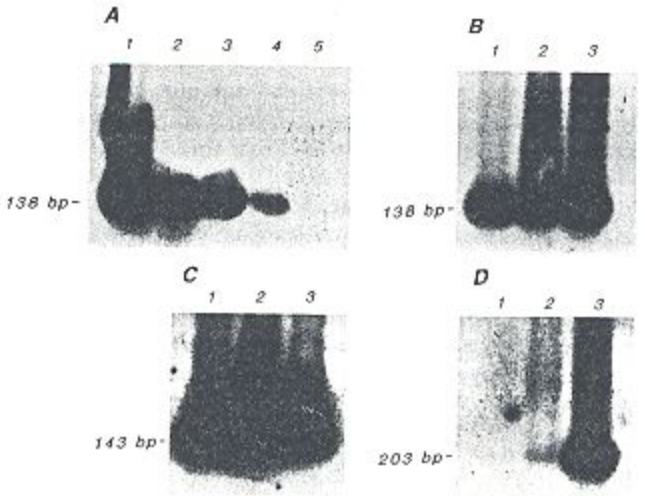

FIG. 1. Southern analysis of PCR-amplified products using ^{32}P-labeled oligonucleotide probes. The results with HTLV-I primers indicated no specific amplified products, whereas the HTLV-II primers amplified the specific products under the same conditions. (*A*) Amplification and probing for HTLV-II *gag* sequences. Serial 10-fold dilutions (undiluted–10^{-4}) 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).

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, but 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-I 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

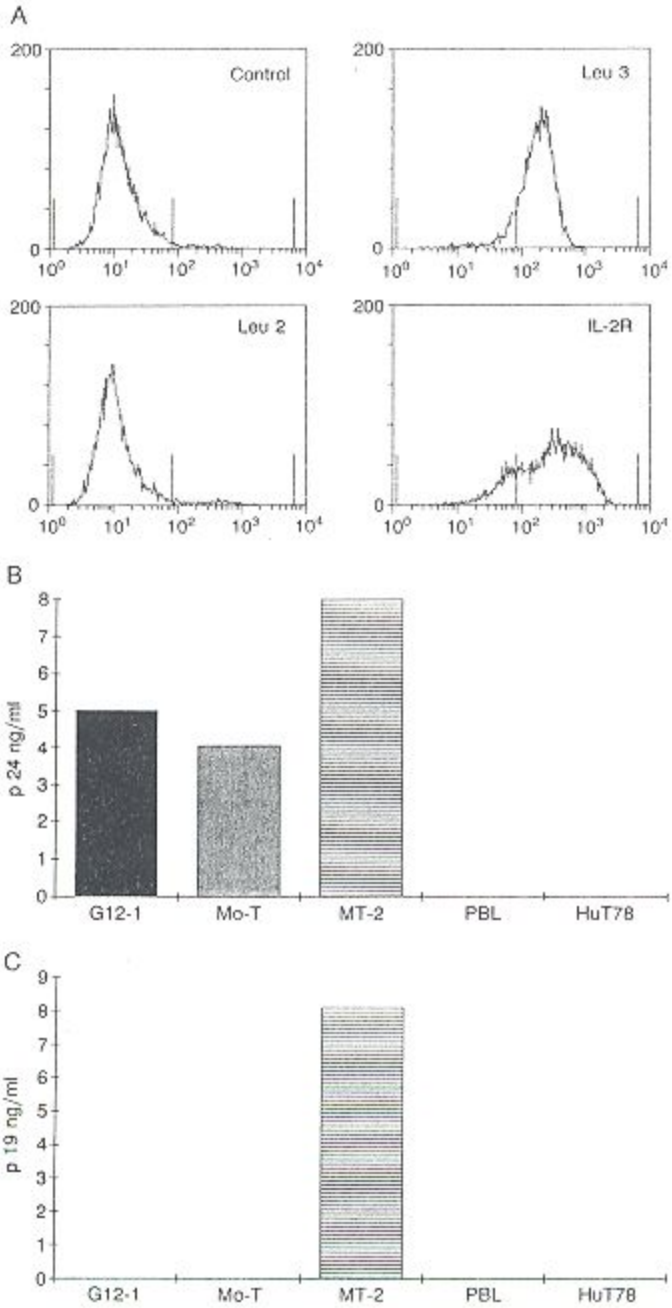

FIG. 3. Cell-surface antigen expression and HTLV antigen production of HTLV-II-producing cell line derived from Guaymi subject 12-1 of Table 1. Expression of CD4 (Leu 3a), CD8 (Leu 2), CD25 (IL-2 receptor), and IgG1 control was determined with fluorescein isothiocyanate-conjugated murine mAbs (Becton Dickinson) and then analyzed by fluorescence-activated analysis (FACScan, Becton Dickinson). (*A*) Relative staining of cell-surface antigens is indicated for the primary PBMC culture established from Guaymi 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

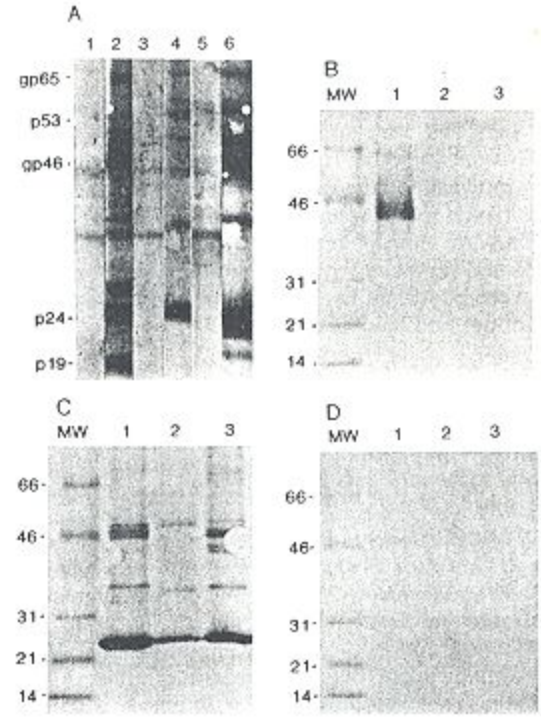

FIG. 4. Immunoblot cell lysate analysis of T-cell line from Guaymi subject 12-1. Reactivity to polyvalent and mAbs compared with HTLV-I and HTLV-II cell lysates. Guaymi 12-1 PBMC lysate reacted positive to polyclonal antiserum and mAb that recognize both HTLV-I and HTLV-II but failed to react to murine mAb specific for HTLV-I. (*A*) HTLV-I MT-2 lysate (lanes 1 and 2), Guaymi 12-1 lysate (lanes 3 and 4), and HTLV-II Mo-T lysate (lanes 5 and 6). Cell lysates reacted against polyvalent HTLV antiserum lanes (2, 4, and 6) or negative control serum (lanes 1, 3, and 5). (*B–D*) Lanes: MW, molecular weight markers; 1, HTLV-I MT-2 lysate; 2, Guaymi 12-1 lysate; 3, HTLV-II Mo-T lysate. (*B*) Cell lysates reacted with murine mAb to HTLV-I envelope gp46 and gp68 epitope (24). (*C*) Cell lysates reacted with murine mAb to HTLV-I and HTLV-II p24 and precursors (25). (*D*) Cell lysates reacted against normal mouse ascites.

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

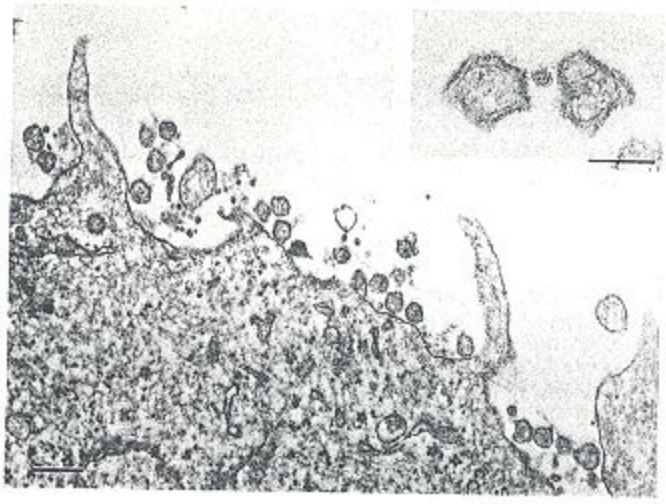

FIG. 5. The T-cell line derived from Guaymi subject 12-1 was used for ultrastructural analysis. Electron photomicrograph of HTLV particles from 12-1 cell line adjacent to cell membrane. (×26,000; bar = 200 nm.) (*Inset*) HTLV viral particles. (×48,000; bar = 100 nm.)

responsible for the HTLV seroreactivity in this Central American Indian population.

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). Typical risk factors for HTLV infection could not be identified among the population. Guaymi Indians do not practice ritual scarification or tatooing; 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 phylogenic studies of this family of retroviruses.

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