Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1446–1450, February 1991
Medical Sciences

Characterization of a variant of human T-lymphotropic virus type I isolated from a healthy member of a remote, recently contacted group in Papua New Guinea

(Melanesia/retrovirus/polymerase chain reaction)

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Contributed by D. Carleton Gajdusek, December 4, 1990

ABSTRACT **We report the characterization of a variant of human T-lymphotropic virus type I (HTLV-I) isolated from an interleukin 2-dependent, CD8$^+$ T-cell line derived from peripheral blood mononuclear cells of a healthy member of a remote, recently contacted hunter-horticulturalist group (Hagahai) in Madang province of Papua New Guinea. Antigenic characterization of this variant, designated PNG-1, by immunofluorescence, indicated no expression of *gag*-encoded proteins p19 and p24 (even after incubation with 5-bromo-2′-deoxyuridine), using monoclonal and polyclonal antibodies against HTLV-I *gag* gene products. Virus-specific proteins of 15, 19, 46, 53, and 61/68 kDa were demonstrated by Western blot analysis, using sera from patients with serologically and/or virologically confirmed HTLV-I myeloneuropathy, sera from HTLV-I-infected rabbits, and antibodies prepared against the C terminus of the major envelope glycoprotein gp46. Restriction endonuclease maps of PNG-1 proviral DNA differed from that of a prototype strain of HTLV-I (MT-2), but, as verified by polymerase chain reaction, PNG-1 was definitely HTLV-I, not HTLV-II. Nucleotide sequencing and further molecular genetic studies of this variant may provide insights into the origin and evolution of HTLV-I.**

Moderately to extraordinarily high prevalences of antibodies against human T-lymphotropic virus type I (HTLV-I) have been found in several remote population groups in Melanesia (Papua New Guinea, West New Guinea, Solomon Islands, Vanuatu, and New Caledonia) by using screening tests such as enzyme-linked immunosorbent assay and gelatin particle agglutination (1–9). However, the failure to confirm HTLV-I seropositivity in many Melanesian sera by Western blot analysis (2, 7, 8), the inability of such sera to neutralize a prototype strain of HTLV-I (2, 10), and the absence of diseases caused by HTLV-I have called into question the veracity of these high prevalences, leading some investigators to dismiss the claims of HTLV-I hyperendemicity in Melanesia as artifactual and clinically unimportant (4, 10). In support of the existence of HTLV-I in Melanesia, we have recently identified a case of HTLV-I myeloneuropathy in a lifelong resident of the Solomon Islands (11, 12) and have detected HTLV-I genomic sequences by polymerase chain reaction (PCR) in lymphocytes from this patient (13). Moreover, we have demonstrated a HTLV-I seroprevalence of 14%, as verified by strict Western blot criteria, among the Hagahai, a small group of hunter-horticulturalists living in the fringe highlands of Papua New Guinea (14), and have isolated a variant of HTLV-I from a healthy, asymptomatic carrier (15). We now report on the further characterization of this variant.

MATERIALS AND METHODS

Study Population. The Hagahai, a 260-member group occupying an area totaling 750 km^2, live at altitudes of 200–1800 m along the northern banks of the Yuat River Gorge in Madang province of Papua New Guinea. Like the highland and Sepik groups, the Hagahai, who first made contact with government and missionary workers in December 1983, lack the HLA-A2 antigen associated with recent Austronesian admixture, suggesting that they predate the last Austronesian migration into Papua New Guinea, currently dated to 5400 B.P. (16, 17). There are no nonhuman primates in Papua New Guinea or elsewhere in Melanesia. A survey conducted among 120 Hagahai, bled between February 1985 and January 1988, indicated a HTLV-I seroprevalence of 14% with a high frequency of indeterminate Western immunoblots (14).

Virus Isolation. In May 1989, heparinized blood specimens, drawn with prior informed consent from each of 24 Hagahai men and women (of whom 7 had confirmatory and 17 had indeterminate HTLV-I Western immunoblots), were rushed by helicopter to the Papua New Guinea Institute of Medical Research in Goroka, where they were processed in a laboratory in which HTLV-I and other human retroviruses had not previously been handled. Mononuclear cells were separated with Sepracell (Supratech Corporation, Oklahoma City, OK) and stimulated with phytohemagglutinin (Wellcome); they were then maintained at 37°C in a humidified 5% CO_2/95% air atmosphere with biweekly changes of RPMI 1640 medium (Whittaker Bioproducts) supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, 10% (vol/vol) interleukin 2 (Advanced Biotechnologies, Columbia, MD), 2 mM L-glutamine, and 50 μg of gentamicin per ml. Isolation attempts were also conducted on cryopreserved peripheral blood mononuclear cells from 14 of the 24 Hagahai (5 with positive and 9 with indeterminate Western blot) by cocultivating their cells with an equal number of phytohemagglutinin-stimulated umbilical cord blood mononuclear cells obtained from healthy Caucasian neonates (Advanced Biotechnologies), who lacked evidence of HTLV-I infection as determined by PCR. Cultures were examined periodically for HTLV-I antigens by immunofluorescence, for reverse transcriptase activity, and for viral particles by electron microscopy.

Indirect Immunofluorescence Test. Cultured cells were examined for the expression of HTLV-I antigens by the

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Abbreviations: HTLV-I, human T-lymphotropic virus type I; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

indirect immunofluorescent antibody technique, initially by using sera from rabbits experimentally infected with HTLV-I (18) and sera from Colombian and Chilean patients with virologically confirmed HTLV-I myeloneuropathy (19, 20). In addition to these antibodies, PNG-1 cells were tested, before and after incubation with 5-bromo-2′-deoxyuridine (50 μg/ml) (Sigma) (21, 22), with monoclonal antibodies against HTLV-I p19 and p24 (Pan-Data Systems, and Cambridge Biotech, Rockville, MD) and with rabbit antisera prepared against native p24 protein and against 13- and 18-residue synthetic peptides of the C termini of p19 and gp46, respectively (23). Virus-specific antibodies were then detected by using either rhodamine-labeled goat antibodies against mouse or rabbit IgG $F(ab')_2$ (Accurate Chemicals, Westbury, NY) or fluorescein isothiocyanate-labeled goat antibodies against human IgG (Cappel Laboratories). Appropriate dilutions of HTLV-I antibody-negative mouse, rabbit, and human sera and HTLV-I-infected (MT-2) (24) and uninfected T cells (MOLT-3) (American Type Culture Collection) were included as controls.

Analysis of Viral Proteins. PNG-1 cells were lysed in 0.1 M Tris·HCl (pH 7.4) containing 0.5% sodium deoxycholate (Sigma), 0.5% Triton X-100, and 0.05% $NaDodSO_4$ at 4°C for 30 min, then centrifuged at 35,000 rpm (100,000 × *g*) in a Beckman 50.2 Ti rotor for 1 hr. Viral proteins were separated by electrophoresis on $NaDodSO_4$/polyacrylamide gels (25), and proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell), which underwent reaction overnight with the above-mentioned antibodies. Membranes were then incubated with alkaline phosphatase-labeled goat antibodies against human, rabbit, or mouse IgG $F(ab')_2$. Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

PCR. DNA was isolated from PNG-1, MT-2, and MOLT-3 cells by a nonorganic method (Oncor, Gaithersburg, MD). To minimize sample carryover, specimens were handled in an externally vented hood and all reagents were dispensed with positive-displacement pipettes (Rainin, Woburn, MA). Oligonucleotide primer pairs, which were specific for *gag* (p19, bases 863–886 and 1375–1397; p24, bases 1423–1444 and 1537–1560) (26, 27), *pol* (SK110, bases 4757–4778; SK111, bases 4919–4942) (28), *env* (bases 5684–5707 and 6128–6151) (26), and *tax/rex* (bases 7358–7374 and 7496–7516) (29) sequences of ATK-1, a prototype strain of HTLV-I (30), were synthesized on a PCR-Mate DNA synthesizer (Applied Biosystems). The reaction mixture consisted of 1 μM each oligonucleotide primer, 1 μg of DNA, 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer/Cetus), 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.05% Nonidet P-40, and 0.2 mM each dATP, dCTP, dGTP, and dTTP. After denaturation at 93°C for 4 min, the reaction mixtures were cycled 35 times at 93°C for 1 min, at 55°C for 2 min, and at 72°C for 3 min, with a DNA thermal cycler (Perkin–Elmer/Cetus). After one round of PCR using the *env* primers, products were further amplified by using "nested" primers (bases 5725–5745 and 6097–6117), which resulted in three additional products of 393, 427, and 434 bp. Amplified DNA was size-fractionated by agarose gel electrophoresis. After alkaline denaturation and neutralization, DNA was transferred to nylon membranes (Nytran, Schleicher & Schuell) and then hybridized with a ^{32}P-labeled full-length HTLV-I probe (as described below) or with oligonucleotide probes (for the *env* products, bases 5899–5919) end-labeled with [α-^{32}P]dCTP in a solution containing 6× SSPE, 1% $NaDodSO_4$, and 50 μg of heat-denatured salmon sperm DNA per ml at 62°C for 18–20 hr (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA). After successive washing with 6× SSPE/1% $NaDodSO_4$ for 30 min, and 1× SSPE/1% $NaDodSO_4$ for 3 min at 62°C, membranes were exposed to x-ray film at −80°C. For amplification of the *tax/rex* sequence, one of the primers was end-labeled with [γ-^{32}P]-dATP, and 10^6 counts were added to each reaction mixture. Amplified DNA was then resolved on an 8% polyacrylamide gel, after digestion with *Taq* I for 1 hr at 65°C, and visualized by autoradiography (29).

Restriction Endonuclease Analysis. High molecular weight DNA, extracted from PNG-1, MT-2, and MOLT-3 cells and from fresh, uncultured mononuclear cells of the individual giving rise to the PNG-1 cell line, as well as cells of his seropositive mother and brother, was digested with *Eco*RI, *Pst* I, and *Sac* I (Boehringer Mannheim); separated by electrophoresis on 0.8% agarose gels; and then transferred onto nylon membranes (Schleicher & Schuell). Membranes were hybridized with a ^{32}P-labeled full-length HTLV-I probe (Oncor) in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 50% formamide, 0.5% $NaDodSO_4$, 10% dextran sulfate, and 50 μg of heat-denatured salmon sperm DNA per ml for 18 hr at 42°C. Membranes were then washed successively with 2× SSC/0.5% $NaDodSO_4$ for 10 min at ambient temperature, 0.1× SSC/0.1% $NaDodSO_4$ for 40 min at 55°C, and 0.1× SSC for 5 min at room temperature, and then exposed at −80°C to x-ray film (Kodak X-Omat AR) with intensifying screens.

Cytofluorographic Analysis and Histocompatibility Antigen (HLA) Typing. The surface phenotype of PNG-1 cells was determined by cytofluorographic analysis (Becton Dickinson) using monoclonal antibodies directed against T-cell-restricted (CD2, CD3, CD4, CD7, and CD8) and B-cell-restricted (CD19 and CD20) antigens and an antigen expressed on myeloid cells, monocytes, and macrophages (CD33). In addition, a monoclonal antibody against the major histocompatibility complex class II DR antigen (HLA-DR) was used. The standard microlymphocytotoxicity test was used for HLA typing of class I antigens.

RESULTS

Virus Isolation. Beginning at 2 weeks after cocultivation with umbilical cord blood mononuclear cells, virus-specific, intracytoplasmic fluorescence was observed in cells of a culture derived from a 20-year-old healthy Hagahai man, but the percentage of viral antigen-bearing cells remained low (<1%), and the cells grew sluggishly for 5 months. With only a few cell clumps remaining, MOLT-3 cells, newly acquired from the American Type Culture Collection, were added. This resulted, after ≈3 weeks, in the establishment of a long-term T-cell line, designated PNG-1, which expressed CD2 (99%), CD3 (99%), CD7 (97%), CD8 (93%), and HLA-DR (95%) surface markers. Expression of CD4 was detected at a low level (14%), as was expression of CD33 (14%), CD19 (1%), and CD20 (3%). PNG-1 cells required exogenous interleukin 2 for optimal growth and periodically exhibited widespread cell death. As determined by HLA typing, the PNG-1 cell line (A11, B56, B62, C4, DR2, DQ1) did not have antigens in common with either the umbilical cord blood cells (A2, B8, B57) or MOLT-3 cells (A1, A25, B18).

Rare virus particles resembling HTLV-I were observed only after PNG-1 cells were incubated with 5-bromo-2′-deoxyuridine (Fig. 1). The virus particles, measuring 100–120 nm in diameter, exhibited an electron-dense central nucleoid surrounded by an outer membrane. Multivesicular bodies, occasionally seen in cell lines infected with HTLV-I, were also evident (Fig. 1). Budding particles were not seen.

All attempts to isolate HTLV-I from six other Western blot-positive Hagahai (including the mother and brother of the young man from whom PNG-1 was derived) and from 17 Western immunoblot-indeterminate individuals were unsuccessful, but supernatant fluids from two other cultures contained low levels of reverse transcriptase activity and rare

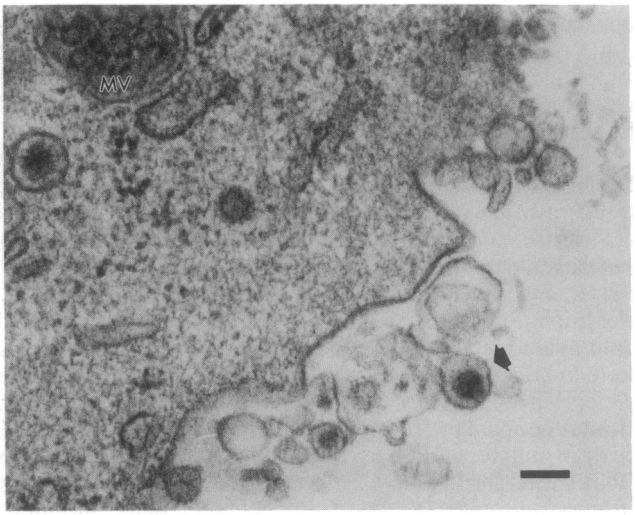

FIG. 1. Thin-section electron micrograph of PNG-1 cells incubated for 72 hr with 5-bromo-2'-deoxyuridine, depicting a solitary mature virus particle (arrow) and multivesicular bodies (MV). (Bar = 100 nm.)

HTLV-I-like particles were observed by thin-section electron microscopy. However, no long-term cell lines could be established.

Antigenic Characterization. By double-label immunofluorescence, the same cells were stained by using sera from patients with HTLV-I myeloneuropathy and sera from rabbits experimentally infected with HTLV-I (Fig. 2). Unlike MT-2 cells, which exhibited robust fluorescence with monoclonal and polyclonal antibodies to native and synthetic peptides of p19 and p24, PNG-1 cells exhibited no staining (data not shown). However, like MT-2 cells, PNG-1 cells were immunoreactive with polyclonal antibodies directed against a synthetic peptide spanning the extreme C terminus of the major envelope glycoprotein gp46.

Immunofluorescence data were corroborated by Western blot analysis: lysates of PNG-1 cells exhibited virus-specific bands at 15, 19, 46, 53, and 61/68 kDa when sera from patients with HTLV-I myeloneuropathy and from HTLV-I-infected rabbits and polyclonal antibodies against gp46 were used. By contrast, monoclonal antibodies directed against HTLV-I *gag*-encoded proteins p19 and p24 failed to label these proteins in PNG-1 cell lysates (Fig. 3). No reactivity was found by immunofluorescence or Western immunoblot with sera from HTLV-I-seronegative humans and rabbits.

Gene Amplification and Restriction Maps. HTLV-I genomic sequences were detected by PCR in DNA extracted from PNG-1 cells, using oligonucleotide primers specific for *gag*, *pol*, *env*, and *tax/rex* sequences of ATK-1. HTLV-I *env* sequences were evident only after nested PCR (Fig. 4). *Taq* I digestion of the 159-base-pair (bp) *tax/rex* amplified product from PNG-1 DNA resulted in a 138-bp rather than an 85-bp fragment, indicating that PNG-1 was HTLV-I, not HTLV-II (Fig. 5). No HTLV-I sequences were found by PCR in MOLT-3 DNA.

Restriction enzyme maps of PNG-1 proviral DNA were similar to but distinct from that of a prototype strain of HTLV-I (MT-2). A single band >9 kilobases (kb) was found after *Eco*RI digestion, indicating that PNG-1 DNA, like MT-2 DNA, did not contain an *Eco*RI cleavage site. Analysis of *Pst* I digests of PNG-1 proviral DNA indicated two cleavage sites, instead of the five or six typically found in prototype strains of HTLV-I. Thus, the three moderate-sized fragments of approximately 2.4, 1.6, and 1.3 kb generated by *Pst* I digestion of prototype strains of HTLV-I were not evident in PNG-1 DNA (Fig. 6). Instead, fragments of 5.0 and 4.4 kb

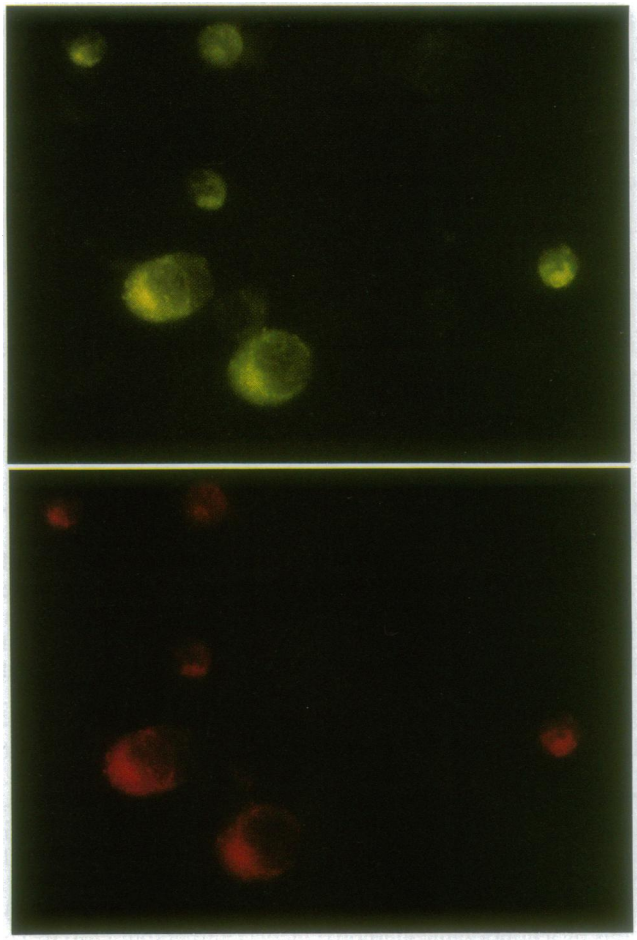

FIG. 2. Double-label immunofluorescence of PNG-1 cells, using serum from a Colombian patient with HTLV-I myeloneuropathy (*Upper*) and serum from a rabbit experimentally infected with HTLV-I (*Lower*). Antibodies to the species-specific IgG conjugated with fluorescein isothiocyanate (green) and rhodamine (red) are shown. Similar staining was observed with autologous serum and serum from a Chilean patient with HTLV-I myeloneuropathy. No staining was observed with sera from HTLV-I-seronegative humans and rabbits. (×500.)

were found. When digested with *Pst* I, DNA isolated from fresh, uncultured mononuclear cells of the Hagahai man from whom the PNG-1 cell line was derived, and from cells of his seropositive mother and brother, showed no hybridization with a full-length HTLV-I probe, probably due to the low copy number of the proviral genome.

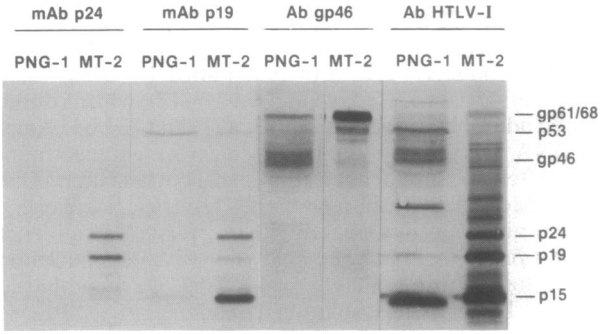

FIG. 3. Western analysis of PNG-1 and MT-2 viral proteins with monoclonal antibodies against HTLV-I p24 (mAb p24) and p19 (mAb p19), rabbit polyclonal antibodies prepared against a synthetic peptide of the C terminus of HTLV-I gp46 (Ab gp46), and serum from a rabbit experimentally infected with HTLV-I (Ab HTLV-I).

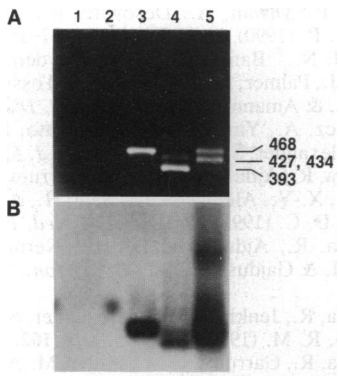

FIG. 4. (*A*) Ethidium bromide-stained agarose gel of HTLV-I *env* sequences amplified by PCR. (*B*) Southern analysis of amplified products hybridized with a ^{32}P-labeled HTLV-I *env*-specific 21-mer oligonucleotide probe (bases 5899–5919). PCR was performed on DNA isolated from MOLT-3 (lane 1), PNG-1 (lane 2), and MT-2 (lane 3) cells, and by nested PCR on DNA from PNG-1 (lane 4) and MT-2 (lane 5) cells. Numbers on right are bp.

DISCUSSION

Our data, indicating that a variant of HTLV-I is present among a small, recently contacted group in Papua New Guinea, dispel all remaining doubts that HTLV-I exists in Melanesia. Moreover, the remoteness of the Hagahai, and their isolation from Japanese and Africans, are consistent with the long endemicity of HTLV-I in Melanesia (8, 12, 14). This variant, the first HTLV-I isolate from Papua New Guinea, differed in some notable respects from HTLV-I strains isolated from patients with adult T cell leukemia/lymphoma and tropical spastic paraparesis/HTLV-I-associated myelopathy and from asymptomatic carriers in the Caribbean basin, Colombia, and southwestern Japan. First, as judged by Southern blot analysis of *Pst* I digests, PNG-1 proviral DNA was atypical, indicating a variant provirus. Second, the failure of monoclonal and polyclonal antibodies directed against p19 and p24 to label PNG-1 cells by immunofluorescence or to label viral *gag*-encoded proteins by Western immunoblot indicates possible epitopic differences and/or low level expression of these proteins. Finally, the scant production of mature virus particles even after induction with the nucleoside analogue 5-bromo-2′-deoxyuridine suggests possible differences in regulatory control of replication or maturation.

Infection of umbilical cord mononuclear cells with HTLV-I usually results in cell transformation and abundant virus production (24, 31, 32). However, some HTLV-I-transformed T-cell lines, established by cocultivating cord blood lymphocytes with virus-infected cells, are nonproducers and exhibit restricted expression of viral structural proteins (33). Similarly, the PNG-1 cell line, which was established after cocultivation with umbilical cord blood lymphocytes and later with MOLT-3, produced few mature virus particles and exhibited markedly reduced expression of *gag*-encoded proteins. However, the HLA phenotype of PNG-1 cells was distinct from that of the umbilical cord blood cells and MOLT-3 cells used in cocultivation and was consistent with HLA antigens found among the Hagahai (17). Thus, the PNG-1 cell line originated from the asymptomatic Hagahai individual.

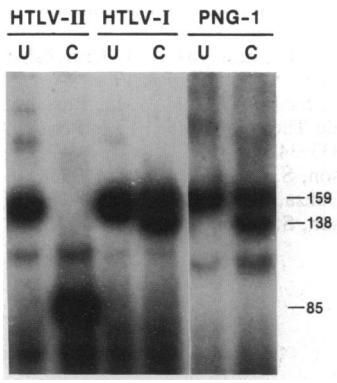

FIG. 5. PCR-amplified *tax/rex* gene product before (U) and after (C) *Taq* I digestion. After *Taq* I digestion, the 159-bp amplified product from PNG-1 and HTLV-I (MT-2) DNA was cleaved to a 138-bp fragment, while an 85-bp fragment resulted from HTLV-II (C3-44/Mo) DNA.

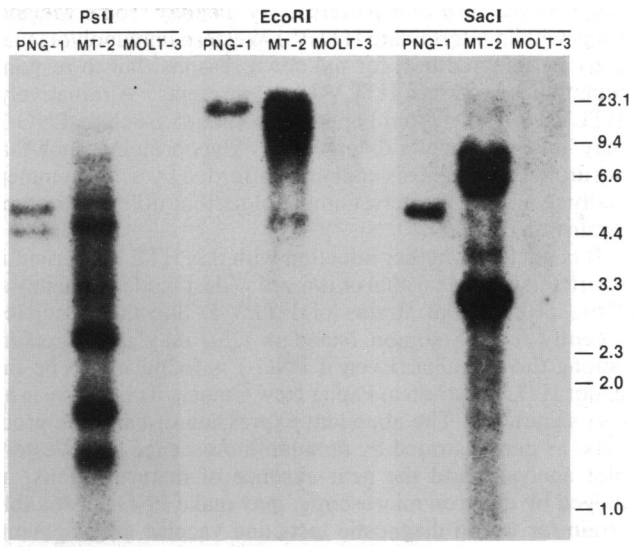

FIG. 6. Comparison of restriction endonuclease maps of DNA isolated from PNG-1 and MT-2 cells by using *Eco*RI, *Pst* I, and *Sac* I, hybridized with a ^{32}P-labeled full-length HTLV-I probe, under stringent conditions. DNA from MOLT-3 cells served as the negative control. Numbers on right are kb.

HTLV-I-infected T cells *in vivo* (34) and persistently infected T-cell lines (31, 35) are typically CD4$^+$. By contrast, the PNG-1 cell line was predominantly CD8$^+$. As has been suggested for isolates of human immunodeficiency virus (HIV), virus-infected CD4$^+$-activated T cells may be recognized and killed by CD8$^+$ cells *in vitro*, leading to the isolation of variants (36). The expansion of CD4$^+$ and CD8$^+$ T cells in culture, with subsequent killing of virus-infected cells by CD8$^+$ cells, may also explain the spontaneous cell death periodically observed during the maintanence of PNG-1 cells, as well as the difficulty encountered in isolating HTLV-I from the other Western immunoblot-positive Hagahai.

The Hagahai, like several other Melanesian populations, exhibit high frequencies of indeterminate HTLV-I Western immunoblots, characterized by strong reactivity to multiple *gag*-encoded proteins without antibodies to *env* gene products (2, 8, 12, 14). Although our attempts to isolate HTLV-I from such individuals in this study were unsuccessful, HTLV-I genomic sequences have recently been detected by PCR in some Melanesians with indeterminate HTLV-I Western immunoblots (unpublished observations). Renewed virus isolation attempts are necessary to determine whether these individuals are infected with HTLV-I variants, in light of the recent isolation of a HIV variant from a healthy individual with an indeterminate HIV Western blot from Gabon, Africa (37). The Gabon HIV isolate did not possess particularly divergent *env*-encoded proteins, but sera from HIV Western blot-indeterminate individuals failed to immunoprecipitate these proteins. In Gabon, then, an abnormal host immune response may be responsible at least for a portion of the

atypical Western blot patterns. By analogy, some Melanesians with indeterminate HTLV-I Western immunoblots may truly be infected but, for unknown reasons, fail to respond immunologically to HTLV-I *env* proteins. Alternatively, HTLV-I variants from Papua New Guinea, such as PNG-1, may have sufficiently divergent *env* glycoproteins, such that accurate serodiagnosis cannot be afforded by using commercially available Western immunoblots that utilize prototype strains of HTLV-I.

It is unclear whether infection with this HTLV-I variant is restricted to the Hagahai or is more widespread in Melanesia. More prototypical strains of HTLV-I, like those isolated recently from Solomon Islanders (13), may also circulate among the Hagahai. Even if PNG-1 is found not to be the major HTLV-I strain in Papua New Guinea, its isolation is no less significant. The abundant expression of *env* gene products, as demonstrated by immunofluorescence and Western blot analysis, and the near absence of mature virions, as judged by electron microscopy, may make PNG-1 a suitable strain for use in diagnostic tests and vaccine development. Moreover, nucleotide sequencing of PNG-1 proviral DNA, as well as studies of the biology and virology of this variant, will help to elucidate how this virus is maintained and disseminated in isolated population groups and provide insights into the origin and evolution of HTLV-I.

We thank Mr. George Anian for assistance in the field; Dr. Hiroko Minagawa for sera from HTLV-I-infected rabbits; Dr. Carlos A. Mora for sera from Colombian and Chilean patients with HTLV-I myeloneuropathy; Drs. Steve S. Alexander and Erik P. Lillehoj for antibodies against HTLV-I *gag* and *env* gene products; Dr. Michael W. Baseler for fluorescence-activated cytometric analysis; Dr. Dean Mann and Ms. Mary O'Donnell for HLA typing; Drs. Bernard J. Poiesz, Shu-yuan Xiao, and Mark S. Godec for assistance with PCR; and Mr. Kunio Nagashima for electron microscopy. Dr. Mark A. Miller was a fellow of the Howard Hughes Medical Institute during his tenure in the Laboratory of Central Nervous System Studies. This work was supported in part by grants from the National Geographic Society Committee for Research and Exploration and the L. S. B. Leakey Foundation.

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