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Multiple Isolates and Characteristics of Human T-Cell Leukemia Virus Type II

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Human T-cell leukemia (or lymphotropic) virus type II (HTLV-II) was isolated from eight HTLV-seropositive patients, six of whom were also infected with human immunodeficiency virus, by cocultivation of peripheral blood mononuclear cells (PBMCs) with BJAB, a continuous B-cell line. Restriction endonuclease mapping of the proviruses demonstrated consistent differences among isolates, and two distinct physical map patterns were observed. The results suggest the existence of two closely related molecular subtypes of HTLV-II, which are tentatively designated HTLV-IIa and HTLV-IIb. This finding was supported by preliminary nucleotide sequence analysis of the *env* gene region encoding the transmembrane glycoprotein gp21, which showed consistent differences between the two proposed virus subtypes. Exploitation of differences in restriction endonuclease sites allowed polymerase chain reaction amplification to detect and differentiate the two subtypes in fresh PBMCs of HTLV-seropositive intravenous drug abusers (IVDAs). The results of these studies confirm that HTLV-II infection is the prominent HTLV infection in seropositive IVDAs and also show that infection with both subtypes occurs. The finding of genetic heterogeneity in the HTLV-II group of viruses may have important implications for studies on its role in human disease and will be useful in characterizing the viruses present in newly discovered endemic foci in New World indigenous populations.

Human T-cell leukemia (or lymphotropic) virus type I (HTLV-I) and HTLV-II are genetically related members of a group of retroviruses sharing a tropism for T lymphocytes and an association with rare lymphoproliferative diseases (34, 42). HTLV-I infection is endemic in well-defined geographic areas and is associated with adult T-cell leukemia, a malignancy of mature T lymphocytes (10, 24, 27, 28, 32, 43), and with a chronic encephalomyelopathy known both as tropical spastic paraparesis and HTLV-I-associated myelopathy (6, 25). In contrast, HTLV-II infection has not been definitely associated with any human disease. However, isolation of the virus from two patients with very rare T-cell variants of hairy cell leukemia (5, 12, 15, 36), one of whom also had a coexisting CD8+ proliferative process (35), does suggest a potential role for the virus in lymphoproliferative disorders.

Although the true extent of infection by HTLV-II remains unknown, serological studies (33, 41), subsequently confirmed by DNA amplification using the polymerase chain reaction (PCR) (4, 9, 16, 20), have shown that intravenous drug abusers (IVDAs) are at increased risk for infection. More recently, similar studies have suggested that the virus may be endemic in New World indigenous populations (11, 12, 19, 31). Although the methods employed in these studies have allowed the identification of HTLV-II infection, relatively few attempts have been made to isolate and characterize the viruses involved. Such studies have long been needed to obtain a better understanding of the biology and to investigate possible genetic variation in this group of viruses. Recently we have described the use of cocultivation of peripheral blood mononuclear cells (PBMCs) with BJAB, a continuous Epstein-Barr virus-negative B-cell line to selectively isolate HTLV-II from IVDAs who were concomitantly infected with human immunodeficiency virus (HIV) (9). Preliminary studies using Southern hybridization analysis on two isolates suggested that there may be some genetic heterogeneity in the HTLV-II group of viruses. In this report we extend these findings and describe the isolation and characterization of HTLV-II from eight individuals both with and without HIV infection.

MATERIALS AND METHODS

Patient population and serological studies. All patients enrolled in this study were from the New York City area and were evaluated at North Shore University Hospital between 1984 and 1990. For virus isolation studies, six patients who were seropositive for both HIV and HTLV-I-HTLV-II and two who were seropositive for HTLV-I-HTLV-II alone were selected. All patients were shown to be HTLV-II infected by using PCR amplification methods previously described (9). The demographic characteristics of these patients are described in the Results section (Table 1). Twelve additional and randomly chosen HTLV-I-HTLV-II-seropositive patients, all of whom had a history of intravenous drug abuse (IVDA) and who were also HIV infected were selected for additional PCR amplification studies. Sera were screened for HIV and HTLV-I-HTLV-II antibodies by using commercially available enzyme-linked immunosorbent assays (Dupont and Cellular Products, respectively), with positive immunoreactivities being confirmed by Western immunoblot analysis. Written informed consent was obtained from each patient prior to study entry.

Virus isolation from PBMCs. Virus isolation was carried out by using methods similar to those previously described

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TABLE 1. Demographic features of patients with HTLV-II infection[a]

Patient	Sex,[b] age	Race	Risk	HIV seropositivity	Clinical status[c]
1	F, 40	Afro-American	IVDA	+	AIDS (A)
2	M, 34	Caucasian	HS[d]	+	AIDS (D)
3	F, 38	Afro-American/ Seminole Indian	IVDA	+	AIDS (A)
4	M, 34	Caucasian	IVDA	+	AIDS (D)
5	M, 40	Afro-American	IVDA	+	AIDS (D)
6	F, 34	Hispanic	IVDA	+	Asympt
7	F, 42	Caucasian	IVDA	−	Asympt
8	F, 47	Afro-American	None	−	Asympt

[a] All patients were seropositive for HTLV-I-HTLV-II on enzyme-linked immunosorbent assay and Western blot assays. Six patients were also seropositive for HIV, and five of these had AIDS. The remaining HIV-infected patient had no AIDS-defining illness.
[b] F, female; M, male.
[c] A, alive; D, dead; asympt, clinically asymptomatic.
[d] HS, homosexual.

(9). PBMCs were prepared from heparinized venous blood samples by centrifugation on Ficoll-Hypaque density gradients. Cells were washed in RPMI 1640 medium containing 20% heat-inactivated fetal calf serum and 0.001 M L-glutamine and were resuspended in the same medium containing phytohemagglutinin (0.005 mg/ml) at a final concentration of 10^6 cells per ml. Cultures were maintained at 37°C for 3 to 4 days, at which time the medium containing phytohemagglutinin was removed and replaced with medium containing 10% interleukin-2 (IL-2) (Cellular Products). Cells were maintained at 37°C, and fresh IL-2 was added every 3 to 4 days. At 10 to 14 days, surviving cells were cocultivated with an equal number of BJAB cells and maintained in RPMI with 10% heat-inactivated fetal calf serum without IL-2. Cocultures were monitored daily for cytopathic effects, supernatants were assayed for reverse transcriptase activity at regular intervals, and cells were examined by electron microscopy when cytopathic effects became evident. For electron microscopy, cells were collected by centrifugation and fixed in 2.5% glutaraldehyde in phosphate-buffered saline. Thin sections were stained with uranyl acetate and examined in a Zeiss electron microscope.

Southern hybridization analysis. DNA was isolated from the BJAB cocultures by using phenol-chloroform extraction, and it was digested with restriction endonucleases (United States Biochemical Corp. and Bethesda Research Laboratories) according to the manufacturers' instructions. Samples were electrophoresed on 0.8% agarose gels, transferred to nylon membranes (Schleicher & Schuell, Inc.), and hybridized with [γ-^{32}P]dCTP random-primed labelled probes as previously described (9). Two HTLV-II probes, 3′ (3.5 kb) and 5′ (4.7 kb), each defined by *Bam*HI sites and which together covered the entire HTLV-II-Mo provirus, were employed (5, 14). After hybridization, membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) for 15 min, and then they were washed with 0.1× SSC containing 0.1% SDS at room temperature for 20 min and at 60°C for 45 min. Membranes were exposed to X-Omat AR film with intensifying screens at −70°C.

PCR studies. DNA from both PBMC cocultures and from fresh uncultured PBMCs was amplified by using primers which flanked the gene region encoding the envelope gp21 transmembrane protein. Primer sequences were bp 6031 to 6051 (5′ CTGCAACAACTCCATTATCCT 3′) and bp 6641 to 6661 (5′ CTGCAGAAGCTAGCAGGTCTA 3′) and were derived from the published sequence of HTLV-II-Mo (32). Amplification of DNA (50 to 100 ng from cocultures and 1 μg from fresh PBMCs) was performed in a total volume of 100 μl of reaction mixture containing 225 μM each dATP, dCTP, dGTP, and dTTP; 100 pmol of each primer; 50 mM KCl; 2.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); and 1 U of *Taq* polymerase (Cetus). Thirty cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and elongation for 2 min at 72°C were carried out on a DNA thermal cycler (Perkin-Elmer Cetus). In analysis of fresh PBMC DNA, the primers were labelled at the 5′ end with [α-^{32}P]dATP before amplification. Samples of the amplified products were then digested with *Xho*I (United States Biochemical Corp.) according to the manufacturer's instructions. Digested and undigested samples were directly electrophoresed on 1.5% agarose or 8% polyacrylamide gels. Gels were dried under vacuum and exposed to XAR-2 Kodak X-ray film with intensifying screens at −70°C for 1 to 2 h.

Cloning and nucleotide sequence analysis of amplified products. PCR-amplified products, using unlabelled primers, were electrophoresed on low-melting-temperature agarose gels; after ethidium bromide staining, DNA bands were excised and the agarose was melted by heating it at 70°C for 15 min. After centrifugation, DNA in supernatants was ligated to plasmid pCR1000 (Invitrogen) and used to transfect *Escherichia coli* (INVα F′), using conditions recommended by the manufacturer. Positive colonies were selected and grown in LB medium, and plasmids were purified by standard methods (38). DNA sequencing with M13 reverse and forward primers was carried out by using the dideoxy chain termination method (Sequenase; United States Biochemical Corp.). At least two clones from each isolate were sequenced to ensure the reliability of the method.

Nucleotide sequence accession number. DNA sequences have been deposited in GenBank under accession number MA5226.

RESULTS

Virus isolation. In a recent study we have shown that HTLV-II could be isolated from PBMCs of IVDAs who were seropositive for both HIV and HTLV by cocultivation with BJAB, a continuous B-cell line (9). In the present study we extend these findings and describe the isolation of HTLV-II from individuals with and without concomitant HIV infection. Eight patients, all of whom were seropositive for HTLV-I-HTLV-II, were selected; and the ages, sex, race, and possible risk factors for infection are shown in Table 1. Six patients, five of whom had a history of IVDA, had concomitant HIV infection. Two were HIV negative; of these one had a history of IVDA, with the other having no known risk factors for HTLV infection. Cocultivation of phytohemagglutinin- and IL-2-stimulated PBMCs with BJAB cells resulted in prominent syncytium formation within 12 to 48 h (Fig. 1). Although cell death would eventually occur, upon continued cocultivation with fresh BJAB cells the cell lines could be maintained for several months. Supernatants of cocultures had Mg^{2+}-dependent reverse transcriptase activity (data not shown), and electron microscopy demonstrated the presence of type C particles (Fig. 1). Thus, it appears that HTLV-II replication in BJAB cells is cytopathic, with cell-free virus production, which contrasts to the situation in vivo, in which the infection

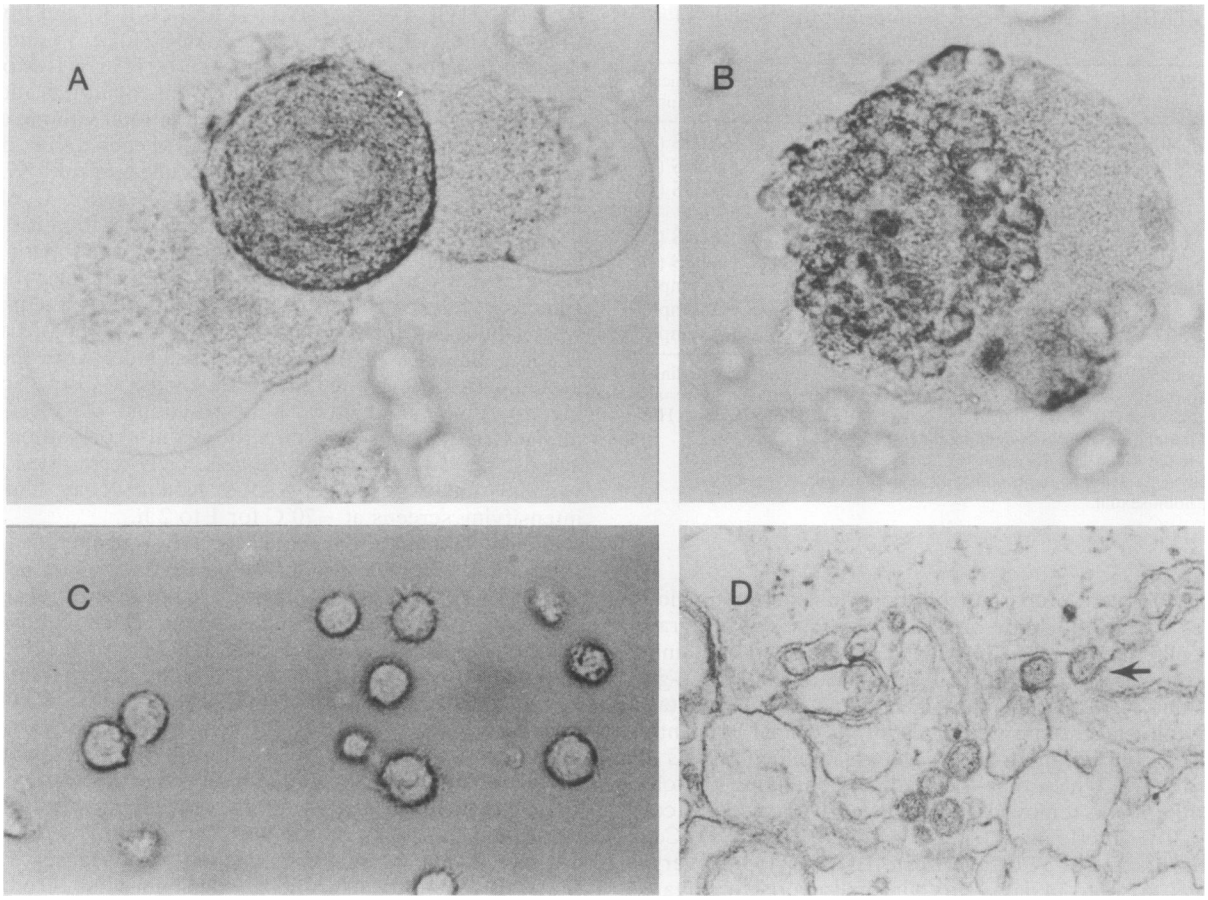

FIG. 1. Light and electron microscopic examination of cocultures of BJAB cells and PBMCs from a patient seropositive for HIV and HTLV-I-HTLV-II. (A and B) Typical giant and syncytial cell formation seen at 2 days after cocultivation; (C) uninfected BJAB cells. Magnifications, ×400. (D) Electron microscopy of the cocultured cells. Particles with typical type C morphology are evident. The arrow shows a virus particle budding from the cell membrane. Magnification, ×80,000.

remains cell associated and presumably there is little or no virus replication.

Provirus mapping studies. Southern hybridization analysis of DNA from the cultures confirmed the presence of the HTLV-II provirus (Fig. 2). Hybridization of chromosomal DNA digested with *Bam*HI with the 3′ probe gave the expected signal of 3.5 kb in all eight isolates. However when the 5′ probe was employed, only four DNAs (isolates 1 to 4) produced the expected 4.7-kb signal defined by the two *Bam*HI sites which have been described for HTLV-II-Mo (5, 14). The remaining four proviruses (samples 5 to 8) had an additional *Bam*HI site in the 5′ end, with signals at 3.2 and 1.5 kb being observed. Physical maps of the eight proviruses were prepared after partial and complete digestion with single and combinations of restriction enzymes and Southern hybridization analysis. The results are shown in Fig. 3. It could be demonstrated that isolates 1 to 4 and 5 to 8, respectively, had identical physical maps and that there were consistent differences between these isolates. In summary, isolates 5 to 8 differed from isolates 1 to 4 in that the former were all found to have an additional *Bam*HI site in the 5′ end of the provirus and did not have the expected *Xho*I, *Sac*I, *Pst*I, and *Bgl*II sites in the 5′ end nor a *Xho*I site in the 3′ end (see Fig. 5). Comparison of the physical maps of the isolates with those available for isolates Mo and NRA (Fig. 3) showed that isolates 1 to 4 had identical maps to that of isolate Mo. Although a detailed physical map of the NRA isolate is not available, it would appear from the published data (36) that it is similar to that of isolates 5 to 8 in that it was reported to have an additional *Bam*HI site in the 5′ end and did not contain the two *Xho*I sites described in the Mo isolate. The findings of consistent differences in the physical maps suggest that there may be two closely related molecular subtypes of HTLV-II, and for the purposes of further studies these were tentatively designated HTLV-IIa and HTLV-IIb.

PCR amplification. PCR amplification methods were established to allow both the identification of HTLV-II and to differentiate the two proposed subtypes with the aim of screening PBMC DNA of HTLV-I-HTLV-II-seropositive individuals. Primers were selected to amplify the gene region encoding the $gp21^{env}$ transmembrane protein, because the physical maps show that type a isolates have a *Xho*I site in this region, whereas type b isolates do not. Thus, *Xho*I digestion of amplified DNA should allow a differentiation of the two subtypes. In these experiments both primers were labelled at the 5′ ends prior to amplification and the products were directly analyzed by electrophoresis and autoradiography after *Xho*I digestion. The results obtained are shown in Fig. 4. Lanes 1 and 2 were DNA samples from PBMCs of

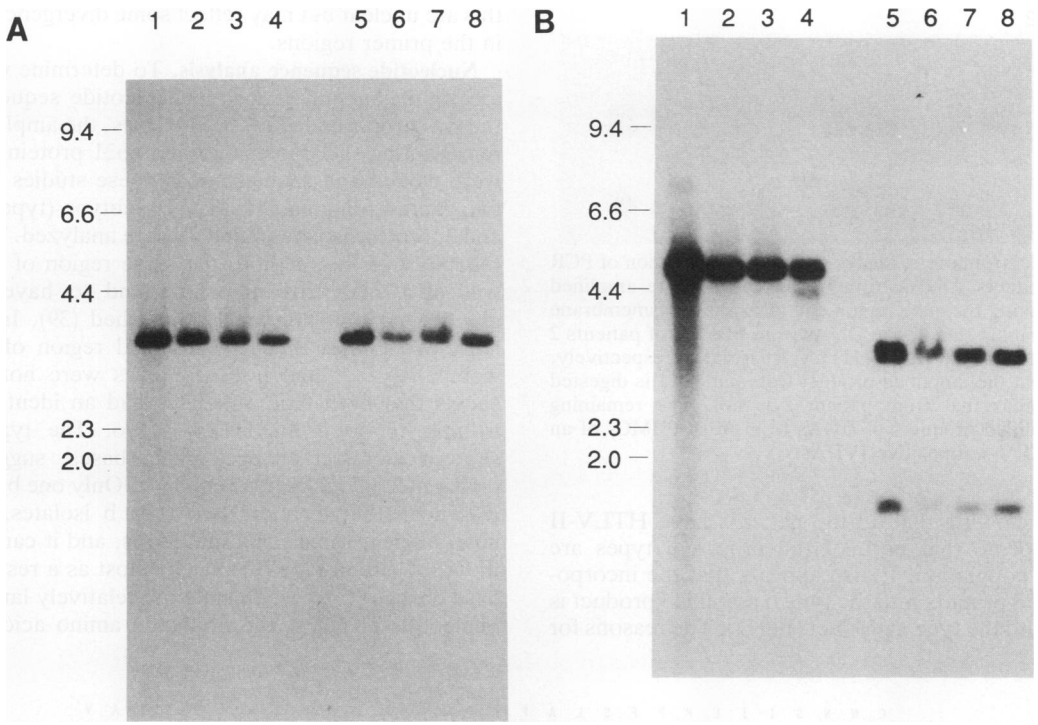

FIG. 2. Southern hybridization analysis of DNA from BJAB-PBMC cocultures established from eight patients (lanes 1 to 8). DNAs were digested with *Bam*HI and were analyzed with HTLV-II 3′ (3.5 kb) (A) and HTLV-II 5′ (4.7 kb) (B) probes.

patients known to be infected with types a and b, respectively (isolates 2 and 6). It could be seen that after amplification of type a DNA, *Xho*I digestion produced signals at 450 and 180 bp. As expected, the 630-bp product resulting from amplification of the type b DNA was not digested by *Xho*I. Analysis of the 12 PBMC DNAs showed that in 8, digestion of the amplified product gave the two fragments of the expected size, whereas four remained undigested. Thus,

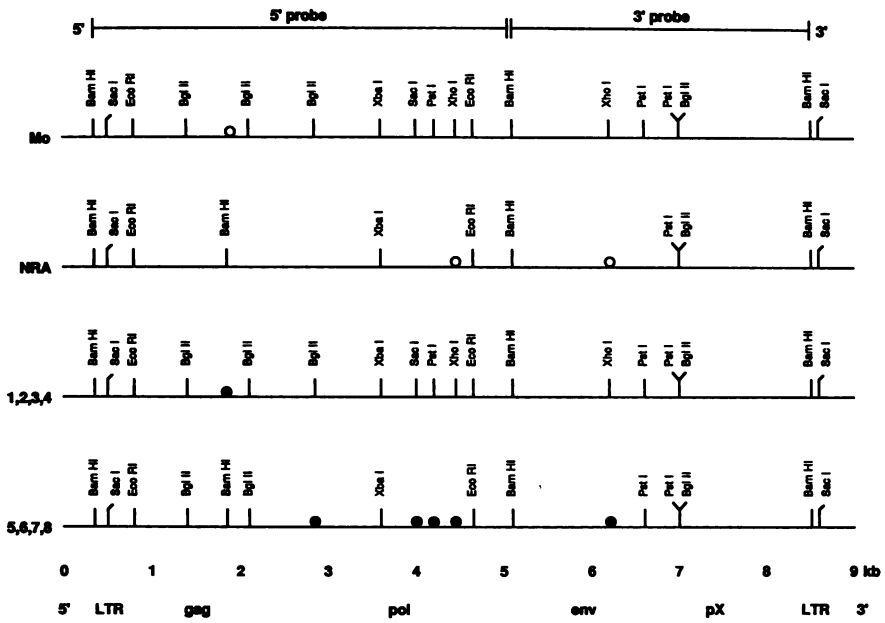

FIG. 3. Restriction enzyme maps of the proviruses of the eight HTLV-II isolates and comparison with maps of the two original virus isolates (Mo and NRA) from patients with atypical hairy cell leukemia. Maps were constructed from Southern blot hybridization analyses after digestion of the DNAs with the indicated restriction endonucleases. Differences between isolates 1 to 4 and 5 to 8, respectively, are noted by the solid circles. The physical map of Mo was prepared by computer analysis of the published sequence (39). The map of NRA was as previously published (36) and is adapted and used with permission of the publisher. Differences between the Mo and NRA isolates are indicated by the open circles.

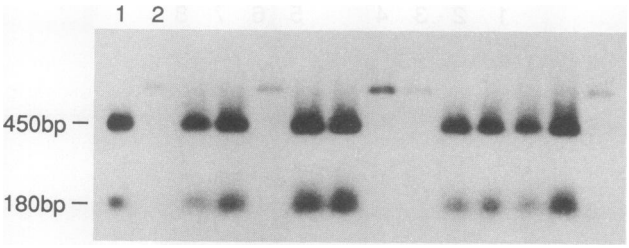

FIG. 4. Gel electrophoretic analysis after *Xho*I digestion of PCR amplification products. DNAs from fresh PBMCs were amplified with primers flanking the gene region encoding the transmembrane gp21 protein. Samples 1 and 2 are DNAs from PBMCs of patients 2 and 6 and represent HTLV-IIa and HTLV-IIb isolates, respectively. It can be seen that the amplified product from patient 1 is digested with *Xho*I, whereas that from patient 2 is not. The remaining samples are amplified products of DNAs from fresh PBMCs of an additional 12 HTLV-seropositive IVDAs.

in addition to showing that all the patients have HTLV-II infection it appears that both of the proposed types are present in this population. It also appears that the incorporation of labelled primers into the type b amplified product is less than that into the type a product (Fig. 4). The reasons for this are unclear but may reflect some divergence of sequence in the primer regions.

Nucleotide sequence analysis. To determine whether there are significant differences in nucleotide sequence between the two proposed HTLV-II subtypes, the amplified products representing the transmembrane gp21 protein gene regions were cloned and sequenced. In these studies four isolates, two representing each HTLV-II subtype (type a, isolates 1 and 3; type b, isolates 6 and 7) were analyzed. To ensure the reliability of the method, the same region of HTLV-II-Mo was also analyzed and was found to have a sequence identical to that previously published (39). In addition, at least two clones of each amplified region of each isolate were analyzed, and no differences were noted. Figure 5 shows that both type a isolates had an identical sequence to that reported for HTLV-II-Mo. The type b isolates showed identical changes in 25 bases, suggesting a 4% divergence in nucleotide sequence. Only one base difference was noted between the two type b isolates. All changes noted appear to be point mutations, and it can be seen that the *Xho*I site in type b isolates is lost as a result of a single base change (C to T). Despite the relatively large number of nucleotide changes, the predicted amino acid sequence of

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           C  N  N  S  I  I  L  P  P  F  S  L  A  P  V  P  P  P  A  T  R  R  R  R  A  V  P  I  A  V
HTLV-IIA   CTGCAACAACTCCATTATCCTCCCCCCTTTTTCCCTCGCTCCCGTACCTCCTCCGGCGACAAGACGCCGCCGTGCCGTTCCAATAGCAGT
HTLV-IIB   (ISOLATE 6)                                C
           (ISOLATE 7)                                C

             (F)                                                                                    (F)
           W  L  V  S  A  L  A  A  G  T  G  I  A  G  G  V  T  G  S  L  S  L  A  S  S  K  S  L  L  L
HTLV-IIA   GTGGCTTGTCTCCGCCCTAGCGGCCGGAACAGGTATCGCTGGTGGAGTAACAGGCTCCCTATCTCTGGCTTCCAGTAAAAGCCTTCTCCT
HTLV-IIB     T        T        G        C                 A                 T
             T        T        G        C                 A                 T

           E  V  D  K  D  I  S  H  L  T  Q  A  I  V  K  N  H  Q  N  I  L  R  V  A  Q  Y  A  A  Q  N
HTLV-IIA   CGAGGTTGACAAAGACATCTCCCACCTTACCCAGGCCATAGTCAAAAATCATCAAAACATCCTCCGGGTTGCACAGTATGCAGCCCAAAA
HTLV-IIB (XHO I)      T                                                          A        G
                      T                                                          A        G

           R  R  G  L  D  L  L  F  W  E  Q  G  G  L  C  K  A  I  Q  E  Q  C  C  F  L  N  I  S  N  T
HTLV-IIA   TAGACGAGGATTAGACCTCCTATTCTGGGAACAAGGGGGTTTGTGCAAGGCCATACAGGAGCAATGTTGCTTCCTCAACATCAGTAACAC
HTLV-IIB                                          A                 T
                                                  A                 T

           H  V  S  V  L  Q  E  R  P  P  L  E  K  R  V  I  T  G  W  G  L  N  W  D  L  G  L  S  Q  W
HTLV-IIA   TCATGTATCCGTCCTCCAGGAACGGCCCCCTCTTGAAAAACGTGTCATCACCGGCTGGGGACTAAACTGGGATCTTGGACTGTCCCAATG
HTLV-IIB              A              G           T                 T        G
                      A              G           T                 T        G

           A  R  E  A  L  Q  T  G  I  T  I  L  A  L  L  L  L  V  I  L  F  G  P  C  I  L  R  Q  I  Q
HTLV-IIA   GGCACGAGAAGCCCTCCAGACAGGCATAACCATTCTCGCTCTACTCCTCCTCGTCATATTGTTTGGCCCCTGTATCCTCCGCCAAATCCA
HTLV-IIB                                    C           T                 C
                                            C           T                 C

                                      (S)      (A)
           A  L  P  Q  R  L  Q  N  R  H  N  Q  Y  S  L  I  N  P  E  T  M  L  *                 6660
HTLV-IIA   GGCCCTTCCACAGCGGTTACAAAACCGACATAACCAGTATTCCCTTATCAACCCAGAAACCATGCTATAATAGACCTGCTAGCTTCTGCAG
HTLV-IIB   A     C  G                 G        G              G
           A        G                 G        G              G
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FIG. 5. Nucleotide sequence of the gene region encoding the transmembrane glycoprotein of HTLV-IIa and HTLV-IIb. PCR amplification was carried out with DNA from PBMC-BJAB cocultures, and amplified products were cloned in plasmid pCR1000. Primers employed in PCR amplification are underlined. HTLV-IIa, represented by the isolates from patients 2 and 3, was found to have an identical nucleotide sequence to that previously reported for the Mo isolate (39). Base changes in the HTLV-IIb (patients 6 and 7) sequences are noted. It can be seen that the *Xho*I site in type IIa isolates is eliminated by a single base pair change (C to T) at position 6209. The predicted changes in amino acid sequence are noted in parentheses. The stop signal is denoted with an asterisk.

the gp21 of the type b isolates would have only four differences (Fig. 5).

DISCUSSION

In a recent report, we demonstrated that cocultivation of PBMCs from IVDAs who were dually infected with HTLV-II and HIV with BJAB cells, a continuous Epstein-Barr virus-negative B-cell line, allowed the selective cultivation of HTLV-II (9). In the present report we have extended these studies and describe the use of this method to isolate HTLV-II from eight individuals, including those with and without HIV infection. The observation that the virus can infect and replicate efficiently in these cells offers unique opportunities for further study of HTLV-II. The virus is clearly cytopathic in BJAB cells, and there is considerable production of cell-free virus as judged by reverse transcriptase activity and electron microscopic examination. Thus, in addition to allowing analysis of molecular events during HTLV-II infection, cultivation of the virus in the BJAB cell line should permit large-scale production of virus and/or virus antigens which may be useful for serological and immunological studies. It is presently unclear whether BJAB cells support only the replication of HTLV-II and not HTLV-I. However, all preliminary attempts to induce syncytium formation with HTLV-I using a similar method of cocultivation have been unsuccessful (8a). This result may suggest a preferential tropism of HTLV-II for these cells and/or that HTLV-II has a greater potential to cause cell fusion.

The isolation of HTLV-II from eight individuals has allowed for the first time a detailed comparison of this group of viruses. The literature contains only a small number of reports on the isolation and molecular analysis of HTLV-II. In addition to the two original isolates, both of which were from patients with atypical hairy cell leukemia (Mo and NRA, respectively), the virus was subsequently isolated from an IVDA with clinical features of AIDS (8) and from a patient with hemophilia A who had received multiple blood product transfusions (14). Although the latter isolates were not directly compared, the published physical maps of the proviruses would suggest that both were identical to the Mo isolate. Recently, we isolated and characterized HTLV-II from two IVDAs who were concomitantly infected with HIV (9). Restriction enzyme mapping showed that whereas one isolate had an identical map to that of Mo, the second showed similarities to NRA, and the findings suggested that there may be genetic heterogeneity in the HTLV-II group of viruses.

In the present study using eight virus isolates we have confirmed that there is genetic heterogeneity, but this is restricted, and there appear to be two different but consistent physical provirus map patterns. Four of the proviruses were identical to isolate Mo, with the other four having identical differences in six restriction endonuclease sites. Interestingly, these isolates had physical maps which were similar to that reported for the NRA isolate. Although NRA was not extensively characterized and nucleotide sequence data is not available which would allow the generation of a physical map, it would appear that these four isolates are closely related to and perhaps identical to NRA.

The finding of two consistently different provirus map patterns among eight virus isolates is unlikely to be coincidental and suggests that there may be at least two closely related subtypes of HTLV-II, which we have tentatively designated HTLV-IIa and HTLV-IIb. On the basis of restriction endonuclease site differences, it could be expected that the nucleotide sequence of the proposed subtypes could vary by at least 5% (37) and this was supported in preliminary analysis of the gene region encoding the transmembrane protein gp21. Of 631 bases analyzed, the two type b isolates had 25 identical base changes. Importantly, no differences were observed between the two type a isolates and HTLV-II-Mo, and only one base difference was observed between the two type b isolates. These findings support the idea that there may be at least two subtypes of HTLV-II and demonstrate that there is strong sequence conservation in this gene region within each subtype.

The results of the present studies are too preliminary to allow a comparison of the degree of nucleotide sequence divergence occurring in HTLV-II with that in HTLV-I proviruses. In the areas of Japan, the Caribbean basin, and Africa in which HTLV-I is endemic, the upper limit of divergence between HTLV-I isolates has been estimated at 3.5%, but it is generally much lower than this (3, 13, 17, 18, 23, 29, 30). The majority of nucleotide differences are single point mutations, and no correlation has been observed between the changes and virus pathogenicity (3, 17, 18). In contrast, recent studies of HTLV-I isolates from newly discovered foci in Melanesia have shown up to 10% divergence in nucleotide sequence from those isolated in the historically established areas (7). It may well be that these new isolates represent distinct molecular subtypes of HTLV-I and may have unique biological properties. By analogy with HTLV-I it can be expected that some divergence in nucleotide sequence will also be found within the two proposed subtypes of HTLV-II. Further studies will determine whether this occurs more frequently in certain gene regions and, since many of the changes appear to be point mutations, whether this will also produce restriction enzyme site polymorphism(s). Furthermore, continued analysis of new HTLV-II isolates may lead to the identification of additional subtypes with perhaps differing biological properties. If further analysis confirms significant nucleotide differences among HTLV-II isolates, this may well explain some of the inconsistent results observed in a number of PCR studies in which certain gene regions of HTLV-II could be amplified whereas others could not (4, 16).

The present study also extends our previous observations that HTLV-II is the prominent HTLV infection in seropositive IVDAs who are concomitantly infected with HIV (9). Employing PCR to amplify gene regions which contained differences in restriction endonuclease sites, 12 seropositive IVDAs were all shown to have HTLV-II infection, with 8 and 4 being infected with subtypes IIa and IIb, respectively. Recently it has been reported that HTLV-II infection may be endemic in certain New World indigenous populations. Similar studies to those described here should determine whether one of the proposed subtypes occurs exclusively or predominates.

The present study may have important implications for investigations into the role of HTLV-II in human disease. At present no definite disease associations exist, but this may well be due to the small number of individuals identified with HTLV-II infection and to the relatively long latent periods expected between infection and the onset of disease. Future studies to better define this role should take into consideration potential genetic heterogeneity and possibly different biological properties of the viruses. It is also unknown whether HTLV-II influences the natural history of HIV infection and/or vice versa. We have recently shown that 12.5% of the HIV-infected IVDAs in our population are

HTLV seropositive (9) and this is predominantly due to HTLV-II infection (4, 9, 16, 20). Studies of homosexual males concomitantly infected with HTLV-I and HIV have suggested that they have significantly increased risk for the development of AIDS than individuals infected with HIV alone (1, 21). Similarly, it has been suggested that mortality is higher in IVDAs with AIDS if there is concomitant HTLV infection (26). This apparent increase in HIV pathogenicity is supported by in vitro studies that the HTLV *tax* gene products can activate HIV gene expression in infected T cells (2, 40), that there is increased production of infectious HIV in cell lines mitogenically stimulated with HTLV-I (44), and that pseudotype formation may occur which could possibly lead to an extended tropism for HIV (22). Prospective clinical and immunological investigations in this IVDA population will allow a better understanding of such interactions and of the role of HTLV-II in human disease.

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