Sequence Analysis of an Immunogenic and Neutralizing Domain of the Human T-Cell Lymphoma/Leukemia Virus Type I gp46 Surface Membrane Protein among Various Primate T-Cell Lymphoma/Leukemia Virus Isolates Including Those from a Patient with Both HTLV-I-associated Myelopathy and Adult T-Cell Leukemia


ABSTRACT

Human T-cell lymphoma/leukemia virus type I (HTLV-I) causes adult T-cell leukemia/lymphoma and HTLV-I-associated myelopathy. Specific regions within the outer envelope proteins of other retroviruses, e.g., human immunodeficiency virus type 1, are highly immunogenic and, because of the selective pressure of the host immune system, quite variable. Mutations in the external envelope protein gene of murine retroviruses and human immunodeficiency virus type 1 influence cellular tropism and disease pathogenesis. By contrast, no disease-specific viral mutations have been identified in HTLV-I-infected patients. However, all isolates studied thus far have originated from leukemic cell lines, peripheral blood mononuclear cells, or cerebrospinal fluid lymphocytes from patients with HTLV-I-associated myelopathy and adult T-cell leukemia/lymphoma and, therefore, may not truly reflect tissue-associated variation. The midregion of the HTLV-I gp46 external envelope glycoprotein (amino acids 190-209) induces an antibody response in 90% of infected individuals, and a heptadecapeptide in this region (amino acids 191-196) elicits its antibodies in rabbits which inhibit syncytia formation and infection of target lymphocytes. Because of the above, we expected the neutralizing domain of the gp46 env gene of HTLV-I to possess disease or organ-associated mutations selected by the infected host’s immune system. Hence, we amplified, cloned, and sequenced HTLV-I DNA directly from in vivo central nervous system, spleen, and kidney specimens, and a leukemic cell line from a patient (M. J.) with both HTLV-I-associated myelopathy and adult T-cell leukemia/lymphoma to discern the possibility of tissue- and/or disease-specific variants. In addition, we sequenced several HTLV-I isolates from different regions of the world, including Papua New Guinea, Bellona, and Liberia, and compared them to other previously published HTLV-I and related retroviral sequences. The 239-base pair sequence corresponding to amino acids 178 to 256 in gp46 displayed minor tissue-specific variation in clones derived from central nervous system tissues from patient M. J., but overall was highly conserved at both the DNA and amino acid levels. Variation was observed in this region among the other HTLV-I, simian T-cell lymphoma virus type I, and HTLV-II isolates in a pattern that was consistent with their known phylogenetic relationship. No consistent disease-related changes were observed. Although the neutralizing domains of HTLV-I and simian T-cell lymphoma virus type I differ considerably from that of HTLV-II, almost absolute conservation of these six amino acid residues was observed among the substrains of these two major strains of the primate T-cell lymphoma/leukemia viruses.

INTRODUCTION

The HTLV-I and HTLV-II are pathogenic retroviruses of humans (1, 2). While both viruses can transform both CD4 + and CD8 + T-lymphocytes in vitro, HTLV-I is associated with CD4 + and HTLV-II with CD8 + lymphocytosis in vivo (3, 4). HTLV-I is the etiological agent of ATL (1, 5) and HAM (6-9). Epidemiological studies indicate that ATL and HAM occur as rare, independent, and nonexclusive diseases, suggesting that the pathogenesis of ATL and HAM are different. HTLV-II is associated with T-cell neoplasms, some of which, when phenotyped, are CD8 + (2, 10, 11). It is also now felt to be the cause of a neurological disorder very similar to HAM (12-14). There are now known to be two major substrains of HTLV-I, Austronesian and African, with the African substrate having been disseminated throughout the world (15-19). Likewise, there are also two major substrains of HTLV-II in the New World, one of which, at least, has been shown to be endemic in Paleo-Amerindians of Central and South America (20, 21). It is not known whether HTLV sequence variation determines target cell tropism or alters viral expression or, conversely, whether the genetic predisposition of an infected individual influences disease manifestation (22). Studies performed thus far have been unable to associate any HTLV-I variant with a particular disease (23-25). However, these investigations examined HTLV-I strains from peripheral blood lymphocytes or, rarely, cerebrospinal fluid lymphocytes of infected patients, which may not accurately reflect tissue variation as is seen with HIV when direct CNS tissues are examined from patients with acquired immunodeficiency syndrome encephalopathy (26).

Mutations in the env gene protein of murine retroviruses determine cellular tropism and specific disease outcome (27-29). Similarly, a variable portion of the HIV-1 extracellular membrane protein, the V3 loop, is the primary determinant of cell tropism (30) and is the principal neutralizing domain (31-33). Variations in this region are felt to be involved in the pathogenesis of HIV-1-associated diseases (34).

Neutralizing antibodies in the sera of HTLV-I-infected patients react against the gp46 outer envelope membrane protein. Specifically, there are regions in the HTLV-I gp46 protein, aa 166 to 229 and aa 190 to 209, which induce antibody reactions in 80 and 90%, respectively, of infected individuals (35, 36). A monoclonal antibody raised against an HTLV-I isolate from an ATL patient mapped to a smaller peptide including aa 186 to 195 (37) and was found to neutralize HTLV-I in vitro (38). Antibodies elicited in immunized rabbits to a subset of these peptides, aa 191 to 196, have also been shown to inhibit syncytia

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2 To whom requests for reprints should be addressed, at Regional Oncology Center, SUNY Health Science Center at Syracuse, Syracuse, NY 13210.

The abbreviations used are: HTLV, human T-cell lymphoma/leukemia virus; ATL, adult T-cell leukemia/lymphoma; HAM, HTLV-associated myelopathy; HIV, human immunodeficiency virus; CNS, central nervous system; aa, amino acids; PBM, peripheral blood mononuclear cells; PTLV, primate T-cell lymphoma/leukemia virus; STLV, simian T-cell lymphoma virus type I.

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Fig. 1. Alignment of sequenced clones with previously published HTLV-I isolates. The 239-base pair gp46 region amplified, cloned, and sequenced from patient M. J.'s various tissues. Enzymatically amplified proviral DNA clones sequenced for this study included those from tissues and the peripheral blood lymphocytic cell line [NIH-CTCL-11 (47)], of patient M. J., as well as the cell lines HSC-CTCL-11B (3), PNG-1 (48) and SI-3 (17). The HTLV-I isolates from the latter two cell lines are referred to as MELI and BELI, respectively. Other previously published primate T-cell lymphoma isolates and their countries of origin are shown. Amino acid changes are indicated below the standard line which is aligned with the Japanese prototype ATK sequence (EMBL numbering system). Fractions above a nucleotide indicate the number of clones with that change. The disease association for each isolate is indicated by ATL, HAM, and ASM, asymptomatic.

Although minor amino acid changes were found in clones from CNS tissue which were not present in clones from systemic organs, the portion of the envelope gene analyzed here from patient M. J. was highly conserved. When this gp46 envelope region was compared among HTLV-I isolates from asymptomatic, ATL and HAM patients from widely separated geographic locales, including the Melanesian HTLV-I strains MELI (previously referred to as PNG-1) (15, 43) and MEL5 (16), the amino acid changes and synonymous mutations observed correlated with geography rather than with disease, and except for 2 isolates, the neutralizing domain was conserved.

MATERIALS AND METHODS

Patient. Patient M. J., a 59-year-old Caucasian male, was a merchant seaman who traveled extensively in HTLV-I-endemic areas. M. J. was diagnosed in 1977 with mycosis fungoides which was reclassified as ATL in 1980 when it was shown that he was infected with HTLV-I (42, 44). In 1987, M. J. was diagnosed with HAM 6 months prior to his death and amplification of DNA from autopsy material revealed the presence of HTLV-I throughout his body (41). There were neuropathological changes consistent with HAM in the
CNS sections examined in this study, as well as leukemic involvement of the spleen. Further clinical details have been published elsewhere (40, 41).

**Tissue Preparation.** Sections of frontal, parietal/occipital, temporal/parietal and occipital cortex, kidney, and spleen were stored at -70°C since the time of autopsy in April 1987 until the time of DNA extraction. Each tissue was minced and lysed with a solution (150 mM NaCl, 25 mM disodium EDTA, pH 7.5, 1.0% lauryl sulfate) containing 200 μg of proteinase K by shaking over 7.5, 1.0% lauryl sulfate) containing 200 μg of proteinase K by shaking overnight at 37°C. DNA was then extracted with chloroform:phenol and precipitated with ethanol. DNA was resuspended in 250 μl of Gene-Screen (NEN Research Products). Transfer, prehybridization, and hybridization were performed according to manufacturer's specifications. Plaques with the correct insert were detected with the 32P-end-labeled internal probe HTIE (5901-5940) and electrophoresis on an 8% polyacrylamide gel followed by autoradiography.

**Cloning and Sequencing.** To facilitate subsequent cloning of the amplified product, the primers were designed to contain DNA linkers with restriction sites (15). Thirty μl of the final polymerase chain reaction mixture was digested in a total volume of 50 μl with 20 units of both KpnI and SacI in the presence of the recommended buffer (New England Biolabs, Beverly, MA). Amplified fragments were ligated into pBR322 (18). Plaques with the correct insert were detected with the 32P-end-labeled internal probe HTIE (5901-5940) and electrophoresis on an 8% polyacrylamide gel followed by autoradiography. The primers were HTIE (5708-5731) + and HTIE (5993-5971)- (see Sherman et al., Ref. 15, for nomenclature). Amplified DNA for HTLV-I was analyzed by liquid hybridization of 30 μl of the final reaction mixture with the 32P-end-labeled internal probe HTIE (5901-5940) and electrophoresis on an 8% polyacrylamide gel followed by autoradiography.

**DNA Amplification.** Each sample was amplified in a final volume of 100 μl for 45 cycles in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) by being heated to 94°C for 15 s, 55°C for 1 s, and finally 68°C for 1 s before being cycled back to 94°C. All oligonucleotides were synthesized by using a DNA synthesizer (Applied Biosystems, Foster City, CA) and purified by reverse-phase chromatography. The primers were HTIE (5708-5731) + and HTIE (5993-5971)- (see Sherman et al., Ref. 15, for nomenclature). Amplified DNA for HTLV-I was analyzed by liquid hybridization of 30 μl of the final reaction mixture with the 32P-end-labeled internal probe HTIE (5901-5940) and electrophoresis on an 8% polyacrylamide gel followed by autoradiography.

**Sequence Analyses.** Sequences were aligned by using the GCG software package (49) and the GAP alignment program (50). The phylogenetic analysis was performed using the GCG software package (49).

Fig. 1. Continued.
Using Parsimony Program (51) was used to compare the HTLV-I clones sequenced here and all other published envelope sequences of the PTLV including HTLV-II and STLV-I (16, 52-64).

RESULTS

Sequence Analysis. Fig. 1 depicts the sequences of a component of the gp46 envelope protein gene from base 5732 to base 5970 (aa 178-256) of HTLV-I strains from patient M. J. and the cell lines PNG-1, SI-3, and HSC-CTCL-11B. Also shown is a comparison of this region with other published corresponding PTLV sequences. The divergence in these 239 base pairs among all of the HTLV-I isolates ranged from 0 to 10.9% at the nucleic acid level and from 0 to 7.6% at the amino acid level. However, when compared to the prototype ATK sequences more than one-half of the HTLV-I isolates had absolutely no changes in the 79 aa studied.

The sequences of 3 Japanese HTLV-I strains, 2 from ATL patients (ATK, MT-2) and 1 from a HAM patient (HS-35), were identical to each other, except for the synonymous change from C to A at position 5902 in MT-2, while the Japanese HAM isolate, TSP-1, had the same change, but also possessed 2 aa substitutions from alanine to threonine and leucine to histidine at aa 187 and 255, respectively. ATL isolates from a Romanian (H990), a North American (SP), a Liberean (HSC-CTCL-11B), and a Caribbean patient (Pt1) all had the same alanine to threonine substitution at aa 187 compared to ATK. All Brazilian isolates were from ATL patients and their amino acid sequences were identical to each other and to the Japanese prototype ATK, except for a valine to isoleucine substitution at aa 248 in Pt8.

Two Caribbean ATL isolates, HS-35 and 1010/3, possessed a serine to proline substitution at aa 192. This finding is interesting because the neutralizing domain from aa 191 to 196 is conserved in the highly variant Zairian, Melanesian, and Australian HTLV-I isolates, as well as the STLV-I isolates PtM3 and su. The mutations in the MEL1 isolate were almost all in wobble positions such that only 2 amino acid substitutions, both from valine to isoleucine, were noted in the entire region from aa 178 to 256. The MEL5 and MSHR-1 isolates had the same 2 amino acid substitutions as MEL1 plus an additional cystine to serine change in MEL5. Only 3 amino acid changes for the STLV-I isolate PtM3 were noted, including the proline for serine substitution also seen in HTLV-I strains from patient M. J. and the cell lines NIH-CTCL-11, from patient M. J. were examined for tissue-

tropic determinants that might be used to predict disease development for the infected patient.

We examined a 79-amino acid region from aa 178 to 256, which has been shown in many studies to give rise to neutralizing antibodies, and found considerable conservation among HTLV-I variants from Africa and Asia. The African EL and Z17 strains possessed no amino acid changes from base 5732 to 5970, although they displayed 2.5% DNA sequence heterogeneity from ATK in this region, which is reflective of the overall divergence of this strain for the entire genome (54). The Melanesian and Australian HTLV-I strains MEL1, MEL5, and MSHR-1 possessed 7.5 to 7.9% nucleic acid heterogeneity from
prototype HTLV-I ATK, which is approximately the diversity found for other regions of their genomes (15, 16, 19). In fact, the variation in the env gene region sequenced here reflected the evolutionary relationships of the PTLV described previously (15, 18, 21). Relative to ATK, only 2 amino acid changes were noted in this 239-base pair region for MEL1, which is in contrast to the 5 amino acid substitutions seen in a 173-base pair region of the transmembrane protein gene or the 4 amino acid substitutions detected previously in a 140-base pair region of the pol gene for MEL-1 (15). Further, when the Indonesian STLV-I isolate PtM3 was compared to HTLV-I ATK, the long terminal repeat displayed 10% heterogeneity (58), while the envelope region analyzed here was 11.7% different. Again, however, only 3 amino acid changes in the cognate gp46 env protein were noted. The relative conservation of the extracellular membrane protein of HTLV-I and STLV-I isolates analyzed here implies that it performs a crucial function in the life cycle of the virus.

No specific mutations in the HTLV-I env gene have been shown to correlate with ATL or HAM (23, 25). However, these studies evaluated peripheral blood HTLV-I or, rarely, cerebrospinal fluid isolates from the HAM patients and may not truly reflect tissue variation as is seen with HIV (26). This study is the first to compare in vivo samples from CNS tissues and visceral organs, including spleen, kidney, and a leukemic cell line from a patient (M. J.) who had both ATL and HAM. A few distinct changes were found among the strains from M. J.’s tissues. The clones derived from visceral organs and peripheral lymphocytes were identical. However, there was a statistically significant increase in the number of quasispecies in the CNS tissues of patient M. J. compared to the strains from visceral organs or PBMs (3 of 12 versus 0 of 18).

The presence of multiple viral strains within a single individual, or quasispecies, has been reported previously for HTLV-I isolates derived from the PBMs of ATL and HAM patients (23, 25). It is important to note that quasispecies were seen only in CNS-derived strains of patient M. J. It is possible that HTLV-I-infected lymphocytes migrated into CNS tissues and that compartmentalization allowed for genetic drift or, alternatively, that strains from systemic sources mutated and then migrated into the CNS. The former possibility seems more likely, since only one strain was found in systemic tissues and the leukemic cell line while quasispecies of that strain were found in CNS tissues. Therefore, the demonstration here and elsewhere of quasispecies in HTLV-I-infected individuals in a manner similar to that for HIV isolates (26) suggests the possibility that genetic drift existed in vivo and allowed for tissue adaptation. Since the lentiviruses mutate at a comparatively faster rate, not as much variation exists for HTLV-I relative to HIV.

The fact that in a majority of HTLV-I-infected individuals neutralizing antibodies are generated against the portion of the outer membrane protein analyzed here (35, 36), while this region remains conserved, implies that it performs a crucial function in the viral life cycle. This observation has been partially confirmed by mutagenesis studies which indicate that certain amino acid insertions in and around this portion of the envelope protein of HTLV-I inhibited gp61 envelope precursor protein cleavage and/or syncytia formation (73). It is not known whether the presence of these neutralizing antibodies to HTLV-I env proteins is able to influence disease development or prognosis. However, neutralizing antibodies have been found against conserved regions of the HIV-1 envelope protein in patients suffering from acquired immunodeficiency syndrome (74). It may be that a combination of active cellular immunity and a primed humoral response is required for defense against an ongoing or even a primary viral infection. Recombinant vaccinia viruses that express HTLV-I envelope proteins, including the region analyzed here, have been shown to induce antibodies in rabbits that are protective against challenge with HTLV-I-producing cells (75). Similarly, cynomolgus monkeys (Macaca fascicularis) have been successfully immunized with recombinant HTLV-I envelope protein against subsequent challenge by HTLV-I-producing cells (76). The demonstration that aa 178 to 256 is conserved in CNS-derived strains and even variant HTLV-I strains

Fig. 2. Dendrogram analyses using the Phylogenetic Analysis Using Parsimony Program comparing DNA sequences (A) and amino acid sequences (B) shown in Fig. 1. The HTLV-II (MoT) and (Nra) sequences were considered to be the outgroup. The mutations unique to each branch are shown. The region analyzed was highly conserved and the majority of HTLV-I isolates could not be segregated by more than 1–2% at either the nucleic acid or amino acid level. The branching pattern shown here reflects the evolutionary pattern previously described for other genomic regions (15, 18, 21).
such as MEL1 and MELS implies that peptides from this region may be useful for human vaccine or passive immunization strategies. Such immunization might not be useful for protection against HTLV-II infection, however, since 4 amino acids differed in the hexapeptide from 191 to 196. The observation that rabbits immunized with HTLV-II fail to develop neutralizing antibodies to HTLV-II and vice versa supports the hypothesis that the neutralizing domains of these two PTLV strains must be different.4

In light of the fact that both HTLV-I and HTLV-II have been linked with a similar neurological disorder (6–9, 12–14), it is doubtful that their surface membrane-neutralizing domains are involved in the pathogenesis of this disease. However, the differences in the amino acid sequences of the neutralizing domains of HTLV-I and HTLV-II may explain why HTLV-I is associated with CD4 and HTLV-II with CD8 lymphocytes (1, 3–5, 10, 11).

Further sequence analyses of the gene encoding the extracellular membrane protein of HTLV-I and its predicted tertiary structure are necessary to ascertain if the high degree of sequence conservation reported here is widespread among isolates. It will be most important to compare HTLV-I strains from neural tissues of HAM patients with isolates from varied geographic locales since there are divergent results reported here is widespread among isolates. It will be most important to ascertain if the high degree of sequence conservation necessary to study its origin and the structure of its recombinant genomes in leukemic tissues. J. Virol., 66: 2456–2463, 1992.


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Michael P. Sherman, Syamalima Dube, Timothy P. Spicer, et al.