Sequence Variants of Human T-Cell Lymphotropic Virus Type I from Patients with Tropical Spastic Paraparesis and Adult T-Cell Leukemia Do Not Distinguish Neurological from Leukemic Isolates

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Received 17 August 1989/Accepted 4 December 1989

We have amplified and sequenced DNA in the envelope (*env*) and long terminal repeat (LTR) regions of human T-cell lymphotropic virus type I (HTLV-I) proviruses from the peripheral blood of 10 HTLV-I-seropositive patients with tropical spastic paraparesis (TSP) and two patients with adult T-cell leukemia. The aim was to examine variation in these regions and to test the hypothesis that the sequences of leukemogenic HTLV-I isolates differ from those causing the neurological disease TSP. In 5 of the 12 HTLV-I-seropositive patients, more than one HTLV-I sequence variant was identified in the same individual. No two individuals shared identical sequences in either *env* or LTR U3. Sequence variations were found at 73 positions in 1,416 bases amplified in *env*. Sequence variability was found throughout the LTR-U3 region, including the sequences of two transcriptional enhancers. Several nucleotide changes common to both Caribbean and Japanese HTLV-I isolates allowed us to identify a consensus sequence that differs from the HTLV-I prototype sequence (M. Seiki, S. Hattori, Y. Hirayama, and M. Yoshida, *Proc. Natl. Acad. Sci. USA* 80:3618–3622, 1983). No sequence in the *env* or LTR U3 region was found to be characteristic of isolates from TSP patients. Although each isolate was distinct at the nucleotide level, the predicted protein sequence of HTLV-I *env* is less variable than that of human immunodeficiency virus *env*, suggesting that these lymphotropic retroviruses use different strategies to evade host immune responses.

Human T-cell lymphotropic virus type I (HTLV-I) has now been unequivocally associated with adult T-cell leukemia/lymphoma (ATL) (30) and with the chronic neurological disorder tropical spastic paraparesis (TSP) (2) and its Japanese equivalent, HTLV-I-associated myelopathy (HAM) (29). We have previously identified two minor variants of HTLV-I reverse transcriptase sequences from TSP patients and a third variant sequence from an HTLV-I-seropositive, asymptomatic individual (2). Reverse transcriptase sequences identical to that derived from an ATL patient (26) were found in two other TSP patients. These data suggest that no one variant of HTLV-I is associated with TSP.

The envelope (*env*) gene of retroviruses influences the tissue tropism of the virus. For example, mutations in the envelope genes of murine leukemia viruses modify tissue specificity and growth characteristics of these viruses in vivo and in vitro (20, 22, 27). Szurek et al. (27) identified amino acid mutations within the *env* gene of a temperature-sensitive mutant of Moloney murine leukemia virus which allowed the virus to replicate preferentially in neural tissue and caused hindlimb paralysis in infected animals. The mutations were positioned at the 3' end of gp70 and within the p15E hydrophobic transmembrane domain. Similarly, induction of the degenerative myeloencephalopathy in mice by the Cas-Br-E murine leukemia virus has been mapped to the amino-terminal portion of the *env* gp70 (20).

The U3 region of the retroviral long terminal repeat (LTR) is also involved in determining tissue tropism and tumorigenicity (10, 12). The results of genome fragment exchanges between murine leukemia virus strains with different tissue tropisms have localized the genetic elements for T-cell

lymphomagenesis to the enhancer region of the LTR extending 3' to the TATA box (10, 12, 13) and may also include the 3' end of the *env* coding sequence (8).

This evidence suggests that sequence differences within the *env* region and/or the LTR of HTLV-I contribute to the onset of neurological disease (HAM or TSP). We therefore amplified and sequenced these regions of HTLV-I provirus from patients with TSP and compared them with the equivalent sequences derived from ATL patients. Sequence analysis of the regions amplified in HTLV-I *env* and LTR from each of the TSP and ATL patients showed limited variation between sequences, none of which were characteristic of TSP. Eighteen distinct *env* sequences and six distinct LTR sequences were identified in 12 seropositive patients. Although no two *env* sequences were identical, the extent of sequence variation was very small compared with that of human immunodeficiency virus (HIV) *env*. These data suggest that HTLV-I isolates causing TSP do not carry determinants of neurotropism in *env* or LTR U3 that distinguish them from isolates causing leukemia.

MATERIALS AND METHODS

Patient groups. Ten HTLV-I-seropositive adult females of Jamaican origin were diagnosed as having TSP (17). These patients have been resident in the United Kingdom for between 10 and 29 years, and in all cases, onset of disease occurred after the age of 35 years. Two Jamaican-born males had HTLV-I-seropositive ATL. Control DNA samples were obtained from four healthy seronegative adult volunteers.

Oligonucleotide preparation. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer, using β -cyanophosphoramidite derivatives. Purified and desalted oligonucleotides were used as amplification primers

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TABLE 1. Oligonucleotide primers used for amplification of and hybridization to HTLV-I *env* and LTR sequences

Primer type and sequence	EMBL reference no. ^a
<i>env</i> (coding)	
5'-ttattcttccagttctgcc-3'.....	5229-5246
5'-gctgtactctcacaattgga-3'.....	5278-5297
5'-cagttaaccctacaagcac-3'.....	5844-5863
5'-ccatcgcttagcgcttcagc-3'.....	5973-5992
5'-cctcgccctcaggaaagagcc-3'.....	6218-6237
<i>env</i> (anticoding)	
5'-ggcatatcctggagcgctcg-3'.....	5696-5715
5'-gtgctttgtagggttaactg-3'.....	5844-5863
5'-gaaatattcttgcacacctc-3'.....	6246-6265
5'-gcttggtttacagggatgac-3'.....	6656-6675
LTR (coding)	
5'-tgacaatgaccatgagccc-3'.....	23-41
5'-cataagctcagacctccggg-3'.....	203-222
LTR (anticoding)	
5'-cccggagggtctgagcttatg-3'.....	203-222
5'-ggatggcggcctcaggtagg-3'.....	407-426

^a Base numbering is as in the EMBL data base.

and internal probes as shown in Table 1. For probes, 100 ng of an oligonucleotide was end labeled with [³²P]dATP (150 mCi/ml; Amersham International) by using T4 polynucleotide kinase.

Polymerase chain reaction. Genomic DNA was extracted from 5×10^6 to 10×10^6 fresh peripheral blood lymphocytes by the proteinase K technique (16). One microgram of DNA was amplified by using the polymerase chain reaction (25) in buffer containing 50 mM KCl, 10 mM Tris hydrochloride, pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, 0.05% Nonidet P-40, 0.2 mM each dATP, cDTP, dTTP, and dGTP (Pharmacia), 1 μ M each oligonucleotide primer, and 2 to 4 U of Taq DNA polymerase (Cetus Corp.). Samples were initially denatured at 93°C for 5 min, followed by 35 cycles of 93°C for 1 min, 55°C for 2 min, and 72°C for 3 min (for polymerase chain reaction products smaller than 500 nucleotides, 30-s extension times [72°C] were found to be efficient). The last extension reaction was prolonged to 10 min before cooling to 10°C.

³²P-end-labeled oligoprobe (2 to 5 ng) in buffer containing 0.9 mM NaCl, 90 mM Tris hydrochloride, pH 8.0, 6 mM EDTA, 0.5% (vol/vol) Nonidet P-40, 10% (wt/vol) dextran sulfate, and 100 μ g of salmon sperm DNA (Sigma) per ml was hybridized to the membrane at 37°C for 1 h. The membrane was washed at 40°C in 6 $\times$ SSC (0.9 M NaCl-0.09 M sodium citrate) before exposure to Fuji RX film at -70°C for 2 to 6 h.

Cloning and sequencing of amplified DNA. Isolated, amplified DNA was cloned into the *EcoRV* site of a modified version of M13K8.2 (28) by blunt-end ligation as previously described (2). The ligation product was transformed into *E. coli* JM101 by the method of Hanahan (7). Recombinant clones were screened by hybridization to an internal oligoprobe after transfer onto a nylon filter (Amersham International). ³²P-end-labeled oligoprobe (2 to 5 ng) in buffer containing 0.9 mM NaCl, 90 mM Tris hydrochloride, pH 8.0, 6 mM EDTA, 0.5% (vol/vol) Nonidet P-40, 10% (wt/vol) dextran sulfate, and 100 μ g of salmon sperm DNA (Sigma) per ml was hybridized to the membrane at 37°C for 1 h. The membrane was washed at 40°C in 6 $\times$ SSC (0.9 M NaCl-0.09 M sodium citrate) before exposure to Fuji RX film at -70°C

for 2 to 6 h. The DNA sequences of cloned fragments were determined by dideoxy chain termination, using Klenow fragment, T7 DNA polymerase (Pharmacia), or Taq DNA polymerase (Cetus Corp.).

RESULTS

Sequence diversity in HTLV-I *env* from TSP and ATL. HTLV-I *env* sequences were cloned from all 10 HTLV-I-seropositive TSP patients and two ATL patients but not from four normal individuals. In all, 172 HTLV-I partial *env* clones were sequenced.

Nucleotide changes (differences from the λ ATK-1 sequence described by Seiki et al. [26]) were seen at a total of 73 positions over the 1,416 bases of HTLV-I *env* that were sequenced (Fig. 1). A total of 44 nucleotide substitutions were found in single individuals only, whereas 29 were shared in isolates from several individuals. Twelve base changes common to all TSP isolates were found, and nine of these are also seen in HAM isolates (29). Only five of the nucleotide base changes found in all TSP and HAM isolates altered the amino acid code of the *env* polypeptide. All of the base changes common to all TSP and HAM isolates were also seen in some ATL isolates (Table 2). These data indicate that the amino acid sequence of λ ATK-1 is unusual at these five positions in the present study can be considered part of a consensus sequence of HTLV-I *env*.

In four patients, two variant sequences were identified in the same individual, and a fifth had three variant sequences. In all cases, a dominant species was found in the clones sequenced from each patient. None of the isolates from patient DNA had a sequence identical to that of the HTLV-I isolate described by Seiki et al. (λ ATK-1) (26). Similarly, none of the variant sequences isolated from different individuals were identical along the entire length of *env*. We have previously estimated the net error rate of Taq DNA polymerase in polymerase chain reaction to be 1 in 1,890 nucleotides after 40 cycles of amplification with the concentrations of Mg²⁺ and deoxynucleoside triphosphates specified (2). The frequency of base changes seen in *env* greatly exceeded 1 in 1,890; also, each base change was frequently observed in more than one isolate (although each isolate had a unique combination of such changes). We are therefore confident that the great majority of these base changes are not the result of errors made by Taq polymerase.

The twelve nucleotide changes shared by all isolates are clustered at the 5' end of gp46 (between positions 5257 and 5612) and in the transmembrane protein p21 (between positions 6269 and 6650). Four of the five coding changes lie in the p21 protein, and three lie either within or adjacent to the 3' end of the putative immunosuppressive peptide sequence described by Cianciolo et al. (3) (Fig. 1), which is highly conserved between retroviruses (21). One amino acid substitution (resulting from a base change at position 6650) was found within the 3' hydrophobic region of p21, which probably spans the host cell membrane (4).

Nucleotide changes in HTLV-I LTR U3 region in TSP and ATL. The base changes identified within the 5' LTR U3 region of HTLV-I isolated from all patients are shown in Fig. 2. Nucleotide changes were found throughout the LTR region amplified. Of these, six were found in all TSP and ATL isolates (Fig. 2). At positions 231 and 232, eight patients yielded sequences with A-to-G transitions as seen in HAM (29), whereas four other sequences had deletions at both positions. In no case was the reference λ ATK-1 sequence of LTR U3 found in any of the patient isolates.

FIG. 1. Sequence variations seen in isolates from TSP and ATL samples are shown above the relevant position in the λ ATK-1 reference sequence. In all isolates, the same base change was seen at each position of variation. Base changes causing amino acid coding changes are boxed. Changes common to all TSP samples are indicated (*). The gp46/p21 cleavage site is marked, and the immunosuppressive peptide sequence in p21 is underlined.

DISCUSSION

Models of neurological disease in mice induced by murine leukemia viruses indicate that single amino acid changes in the envelope gene and LTR confer neurotropism on otherwise leukemogenic viruses (see Introduction). We tested this hypothesis in HTLV-I isolated from patients with TSP or ATL. We have identified HTLV-I proviral sequences from 10 HTLV-1-seropositive patients with TSP and from two patients with ATL. The sequences identified lie in the envelope gene and the LTR U3 region of HTLV-I. These Caribbean isolates show limited diversity with reference to

the Japanese sequences λ ATK-1 (26) and HAM (29). The nucleotide changes seen in all of the isolates sequenced in the present study and which are also found in HAM (Table 2) indicate that these nucleotides are part of a consensus sequence of HTLV-I; more than one minor sequence variant of this consensus sequence may be present in seropositive individuals. The cloning of more than one HTLV-I sequence variant in each of five individuals recalls the recent finding of extensive diversity between HIV sequences isolated from single patients (6, 24). These data also support our previous finding that no one sequence variant of HTLV-I is specific to TSP (2). They suggest that even if certain base changes are characteristic of HTLV-I isolates that cause neurological disease, such changes do not abolish the leukemogenic potential of the virus.

This consensus sequence of *env* appears to be present worldwide, since our sequence data show features in common with both Caribbean (15) and Japanese (26, 29) isolates of HTLV-I. This supports early evidence of limited variation in HTLV-I from restriction mapping (31). Ratner et al. (23) have described a distinct strain, called HTLV-Ib, which is present in Africa and differs by restriction mapping from other HTLV-I strains in the loss of five restriction sites within *env*. Only one of these sites, *Hpa*I at position 6269, is missing in all of our isolates.

The amount of variation in HTLV-I sequences is much less than that in HIV (Fig. 3), probably reflecting the different strategies adopted by the viruses in evading the host immune response. In the HTLV-I *env* gene, the 5' region of gp46 and the 3' region of p21 contain most of the sequence changes common to all of our isolates. The region of *env* spanning the gp46/p21 cleavage site is relatively invariant between isolates. The majority of clones (45 out of

TABLE 2. Nucleotide changes in HTLV-I *env* in all TSP isolates

EMBL no.	Nucleotide substitution	Identity with substitution in isolate from ^a :		Amino acid change
		HAM ^b	ATL	
5257	t→c	+		
5408	c→t		+	
5423	c→t		+	
5471	g→a	+	+	
5609	a→g	+	+	
5612	c→a	+	+	H→Q
6269	g→a		+	
6402	c→t	+	+	R→C
6409	c→t	+	+	P→L
6419	c→t	+	+	
6432	c→t	+	+	P→S
6551	t→c	+	+	
6650	a→c	+	+	K→N

^a +, Same substitution.

^b From Tsujimoto et al. (29).

FIG. 2. Sequence variations in HTLV-I LTR from TSP and ATL are indicated above the reference λ ATK-1 sequence. Changes common to all TSP samples are indicated (*). Insertions (v) were seen at positions 145 (cc), 267 (c), 304 (aa [in HAM]), and 379 (ct [in HAM]). Positions 231 and 232 show either a base transition (a→g) or a deletion (—) in all isolates; in all other cases, the same base change was seen for each variant position. The poly(A) signal and the TATA box are boxed, and the 21-base-pair enhancer repeats are underlined.

63) sequenced between primers 5973 to 5992 and 6246 to 6265 showed identity with the λ ATK-1 sequence. The homologous region spanning the protease cleavage site is also relatively conserved between retroviruses (see the legend to Fig. 3).

Proteins derived from infected host cells have been shown to bind the imperfect 21-nucleotide repeats in the LTR U3 region (1, 19); this binding is influenced by the presence of the HTLV-I *tax* gene product, which functions as a transactivator (19). The cell-specific DNA-binding factors may determine the cell type in which the virus is preferentially

expressed and therefore the type of disease resulting from infection (9, 14).

We have found sequence variation within and around the 21-base-pair enhancer sequences in the LTR region amplified from all patients. As DNA-binding proteins in neural tissue extracts have not been identified, it is not known whether these show a preference for certain sequences in the LTR; hence, the significance of these sequence changes is not known. It remains possible that determinants of neurotropism lie elsewhere in the HTLV-I genome, for example, in the *tax* gene. Indeed, identification of specific isolates

FIG. 3. HTLV-I *env* amino acid sequence is much less variable than that of HIV-I. The sequences of isolates from 15 HIV-seropositive patients (18) and 15 HTLV-I-seropositive patients (from this study and references 15 and 29) were used to calculate the Kabat-Wu variability index (11), defined as (number of different residues)/(frequency of the commonest residue). The region spanning the protease cleavage sites (–100 to +70 amino acids) is relatively well conserved between HTLV-I and other retroviruses. This was shown by using the ALIGN program of Dayhoff et al. (5) to compare this region in HTLV-I and HIV (score, 4.0), Moloney murine leukemia virus (score, 7.2), and bovine leukemia virus (score, 7.8).

from central nervous system tissue may indicate those sequences which are selected by or adapted to infection in neural tissue.

ACKNOWLEDGMENTS

We are grateful to M. Yoshida for providing the HTLV-I-containing plasmids and J. I. Bell for constant help and advice. We thank S. Nightingale, J. K. Cruickshank, W. Rosenberg, and D. Catovsky for providing the blood samples.

S.D. and C.R.M.B. were supported by the Wellcome Trust.

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