Molecular Epidemiology of Simian T-Cell Lymphotropic Virus Type 1 in Wild and Captive Sooty Mangabeys

Vicki L. Traina-Dorge,1* Rebecca Lorino, Bobby J. Gormus,1 Michael Metzger,1 Paul Telfer,1 David Richardson,2 David L. Robertson,2 Preston A. Marx,1,3 and Cristian Apetrei1,3

Division of Microbiology and Immunology, Tulane National Primate Research Center, Tulane University Health Sciences Center, Covington,1 and Department of Tropical Medicine, Tulane University School of Public Health and Tropical Medicine, New Orleans,3 Louisiana, and School of Biological Sciences, University of Manchester, Manchester,2 United Kingdom

Received 27 April 2004/Accepted 20 September 2004

A study was conducted to evaluate the prevalence and diversity of simian T-cell lymphotropic virus (STLV) isolates within the long-established Tulane National Primate Research Center (TNPRC) colony of sooty mangabeys (Sm; Cercocebus atys). Serological analysis determined that 22 of 39 animals (56%) were positive for STLV type 1 (STLV-1). A second group of thirteen SM bush meat samples from Sierra Leone in Africa was also included and tested only by PCR. Twenty-two of 39 captive animals (56%) and 3 of 13 bush meat samples (23%) were positive for STLV-1, as shown by testing with PCR. Nucleotide sequencing and phylogenetic analysis of viral strains obtained demonstrated that STLV-1 strains from SMs (STLV-1sm strains) from the TNPRC colony and Sierra Leone formed a single cluster together with the previously reported STLV-1sm strain from the Yerkes National Primate Research Center. These data confirm that Africa is the origin for TNPRC STLV-1sm and suggest that Sierra Leone is the origin for the SM colonies in the United States. The TNPRC STLV-1sm strains further divided into two subclusters, suggesting STLV-1sm infection of two original founder SMs at the time of their importation into the United States. STLV-1sm diversity in the TNPRC colony matches the high diversity of SIVsm in the already reported colony. The lack of correlation between the lineage of the simian immunodeficiency virus from SMs (SIVsm) and the STLV-1sm subcluster distribution of the TNPRC strains suggests that intracolony transmissions of both viruses were independent events.

Primate T-lymphotropic viruses (PTLV) belong to the genus Deltaretrovirus (12). They include human T-cell lymphotropic virus type 1 (HTLV-1) (41, 55), simian T-cell lymphotropic virus type 1 (STLV-1) (31), HTLV-2 (16), STLV-2 (13, 51), STLV type L (STLV-L) (14), and the recently described virus type 1 (STLV-1) (31), HTLV-2 (16), STLV-2 (13, 51), STLV type L (STLV-L) (14), and the recently described

---

* Corresponding author. Mailing address: Division of Microbiology and Immunology, Tulane National Primate Research Center, 18703 Three Rivers Rd., Covington, LA 70433. Phone: (985) 871-6290. Fax: (985) 871-6248. E-mail: vtraina@tulane.edu.
by a geographical correlation between HTLV subtypes and the habitat range of the original nonhuman primate species (5, 21, 23, 46). Therefore, characterizing STLV-1 diversity in different species of nonhuman primates and correlating this characterization with HTLV diversity may provide significant information on the mechanism of HTLV emergence. To date, studies of STLV-1 diversity in different species of monkeys and apes in Central Africa have demonstrated the presence of specific STLV-1 variants in several subspecies of baboons (Papio hamadryas, Papio anubis, and Papio doguera) (25, 28, 43), gelada baboons (Theropithecus gelada) (53), swamp monkeys (Allenopithecus nigroviridis) (28), grivet monkeys (Cercopithecus aethiops aethiops) (28), talus monkeys (Cercopithecus aethiops talusculus), crested monkeys (Cercopithecus mona pogonias) (28), Syke’s monkeys (Cercopithecus albogularis) (43), patas monkeys (Erythrocebus patas) (43), mandrills (Mandrillus sphinx) (26, 36), SMs (Cercopithecus aethiops) (8, 43), agile mangabeys (Cercocebus agilis) (37), chimpanzees (Pan troglodytes) (19, 38, 39), and gorillas (Gorilla gorilla) (38). Interestingly, although SMs are the documented source of HIV-2 (4), information concerning STLV diversity and prevalence in SMs in their natural habitats has not been reported. Only a single STLV strain originating from a captive SM in the Yerkes National Primate Research Center (YNPRC) is known to date (8). Moreover, no diversity studies have been carried out with naturally occurring STLVsm.

In this study, we report a systematic characterization of STLVsm prevalence and diversity in a stable, well-established colony of SMs at the Tulane National Primate Research Center (TNPRC). Our recent studies of SIV isolates from SMs (SIVsm isolates) in this TNPRC colony revealed significant SIVsm lineage diversity, with six different genetic lineages co-circulating (20; C. Apeirei, unpublished data). These different SIVsm lineages show genetic divergence similar to that of HIV-1 subtypes and point to multiple introductions of SIVsm-infected SMs in the United States. Given the high prevalence of STLV infection in this colony, we also determined if the diversity of STLV isolates paralleled that of SIVsm isolates. These associations of viral diversity may establish infection timelines within the colony. Importantly, it would help to elucidate whether the modes of transmission of both viruses followed similar mechanisms. Finally, as a means to validate the diversity of STLV isolates in the primate centers of the United States, we characterized STLV strains from wild SMs.

MATERIALS AND METHODS

Collection of samples from the TNPRC colony and SMs living in the wild.

Thirty-nine SMs (Cercopithecus aethiops) from the colony at the TNPRC were studied. All animals were clinically evaluated, and blood samples were taken during the annual physical exam. Samples were centrifuged, and plasma and serum samples were aliquoted and frozen at −20°C for later testing. The buffy coat was removed and centrifuged on Ficoll-Hypaque, and peripheral blood mononuclear cells (PBMC) were isolated. PBMC were distributed in aliquots of 5 × 10^6 cells and cryopreserved at a ratio of 1:1 in cryoprotective medium (Cambrex BioScience, Walkersville, Md.) with 7% dimethyl sulfoxide in basal Eagle’s medium with Hanks’ balanced salt solution but without l-glutamine. Thirteen wild-SM bush meat samples were also obtained from fresh-meat markets in different regions of Sierra Leone during a survey in 1993. Samples were quick-frozen and stored at −80°C prior to testing.

Serological assays.

For monkeys in the colony, sera were initially screened qualitatively for STLV antibody by indirect enzyme-linked immunosorbent assay (ELISA) using commercially available HTLV-1 viral lysate (Zeptometrix, Buf-

falo, N.Y.). Seropositive samples were confirmed with InnoLia (Innogenetics, Ghent, Belgium), and levels of antibody were quantitated with a limiting dilution ELISA. These same sera were screened for the presence of SIV antibody using a commercial ELISA (Zeptometrix) designed for the detection of HIV-1 and HIV-2 antibodies. Seroreactive samples were confirmed positive by Western blotting (Zeptometrix).

PCR and sequencing. High-molecular-weight genomic DNA was extracted from the cryopreserved PBMC or frozen bush meat samples from all animals using the DynaEase tissue kit (QIAGEN, Valencia, Calif.). Concentrations were determined spectrophotometrically. One microgram of DNA from each animal was tested by PCR amplification for STLV-1 env and terminal repeat (LTR) sequences as well as for STLV-3 gag sequences. STLV-1-specific primers derived from highly conserved regions of the prototype HTLV-1 gag and env genes (45) (GenBank accession number J02029) were used as follows. Forward primer env-f (5′-TTTGGACGCGCGCTCAAGTATGCCTCCCCTG-3′) and reverse primer env-r (5′-ACTTAGAATTCGGAGGTCTGTAGTCGAGGGGAGG-3′) (11) produced a 522-bp partial envelope fragment mapping positions 6024 to 6592 on HTLV-1gag and spanning the cleavage site of gp66 and most of the p21 transmembrane protein. Forward primer LFR-5 (5′-AATGACATGACCACCTAACAGGTTTACATCC-3′) and reverse primer LTR-3 (5′-CAGGGTCAGCCCCAAAGCGTTGAGG-3′) produced the full-length 725-bp LTR fragment (positions 25 to 757 on HTLV-1LTR). Each 50-μl reaction mixture contained 1× AmpliTaq Gold PCR buffer II; 2.0 μM MgCl2; a 200 μM concentration each of dATP, dCTP, and dGTP; a 20 μM concentration each of the forward and reverse STLV-1-specific primers; and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, Calif.). Reaction mixtures were incubated at 95°C for 10 min and then subjected to 95, 58, and 72°C for 1 min each for a total of 40 cycles, followed by a final 10-min extension at 72°C. Near-full-length STLV envelope sequences were amplified for a selection of SM samples determined on the basis of short env-sequence results. Forward primers ORT-5 (5′-AGACTCTCCTAAAGCGAGCTGTCATG-3′) and env-f (5′-GCTTGGTTACGGAGTACTGAGG-3′) were used to amplify a fragment of 1,609 bp encompassing the 1,529 bp of the envelope gene (positions 5120 to 6735 on HTLV-1gag). Each 50-μl reaction mixture contained 1× AmpliTaq Gold PCR buffer II; 2.0 μM MgCl2; a 200 μM concentration each of dATP, dCTP, and dGTP; a 20 μM concentration each of the forward and reverse STLV-1-specific primers; and 2.5 U of AmpliTaq Gold polymerase. Reaction mixtures were incubated at 95°C for 10 min and then subjected to 95, 58, and 72°C for 1 min each for a total of 40 cycles, followed by a final 10-min extension at 72°C.

Since several recent studies have reported the extensive distribution of STLV-3 in sub-Saharan African primates from the Papionini tribe (30, 53), specific STLV-3 primers and conditions described previously (30) were used to screen all samples. SIV-specific primers were also used on the same samples in order to characterize gag (846 bp) and env transmembrane glycoprotein (436 bp), as described elsewhere (20).

PCR products were electrophoresed in 1% agarose; all positive samples were further processed, and amplified fragments were isolated by excision from agarose gel and purification with commercially available PCR cleanup reagents (QIA-GEN), sequencing, and phylogenetic analysis.

Sequencing.

All STLV-1-positive 522-bp env and 725-bp LTR PCR-amplified fragments isolated from both the TNPRC colony and SMs from Sierra Leone were purified as described above and sequenced directly, and the alignments were compared. Full-length envelope fragment sequences were amplified from animals from each of the two subclusters, purified, and sequenced as described above. The sequencing was carried out using the ABI BigDye Terminator sequencing reaction kit (version 3.1) and an automated DNA sequencer (model 377; Applied Biosystems).

Phylogenetic analysis.

Alignments were constructed for the partial and full-length HTLV-1 and STLV-1 env genes and LTR regions for each of the animals with the CLUSTAL W program (48). Nucleotide sequences for env and LTR nucleotide sequences representative of each HTLV-1 subtype and STLV-1 lineage originating from different species from and geographical regions of Africa were downloaded from GenBank. Newly derived STLV-1 sequences obtained from captive and wild SMs were aligned with those previously reported strains by using the CLUSTAL W (48) profile alignment option. The resulting alignments were adjusted manually where necessary. Regions of ambiguous alignment and all gap-containing sites were excluded. Phylogenetic trees were inferred from the nucleotide alignments by the neighbor-joining method by using the PHYLIP model of nucleotide substitution (15) implemented in the PHYLIP 3.60b10, with a transition/transversion ratio of 2. The reliability of the branching order was assessed by performing 100 bootstrap replications, again by using neighbor joining and the HK85 model. Phylogenetic trees were also inferred by the maximum likelihood method using PAUP*, with models inferred from the
alignments by use of Modeltest 3.06 (42). The neighbor-joining tree topology was used as the starting tree in a heuristic search using tree bisection reconnection branch swapping.

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers for the TNPRC STLVsm sequences submitted to GenBank are AY876016 through AY876044.

RESULTS

A serological and molecular survey to evaluate STLV prevalence and diversity within the TNPRC captive-SM colony was conducted. The majority of the colony animals were originally obtained from the YNPRC between 1980 and 1990. Two animals were imported from Africa, and one was purchased from the New Iberia Research Center (New Iberia, La.).

A high prevalence of retroviral infection was observed in the TNPRC colony. Thus, of the tested SMs, 56% (22 of 39) were seropositive for STLV-1, 72% (28 of 39) were seropositive for SIV, and 39% (15 of 39) were dually infected (Table 1). All positive samples were confirmed by PCR amplification with STLV-1 env and SIV gag and env primers.

A study to evaluate STLV-1 prevalence in wild SMs was also conducted. Thirteen SM bush meat samples originating from Sierra Leone in Africa were obtained, cryopreserved, processed for nucleic acid, and tested for STLV-1 DNA by the same PCR strategy used for the colony animals. Three of the 13 samples (23%) were positive for STLV-1 sequences, showing a relatively low level of natural infection in the wild. However, testing for SIV infection showed that 7 of the 13 wild-SM samples (54%) were positive for SIVsm (1). One SM was infected with both STLV-1 and SIV.

Testing for the presence of STLV-3 in both captive and wild SMs failed to reveal evidence of natural infection with this virus in our study groups.

Nucleotide sequencing of STLV-1 from all infected animals was performed to characterize viral diversity in the TNPRC colony and wild SMs. Initial PCR amplifications were performed for two gene regions and resulted in a 522-bp fragment from the STLV-1 env gene and a 725-bp fragment from the env LTR region. These positive samples were confirmed by PCR amplification with STLV-1 env and SIV gag and env primers.

In order to confirm these results and to further investigate the extent of STLV-1 diversity in the TNPRC colony, we sequenced and analyzed near-full-length envelope sequences of a smaller group of samples selected to include representatives from subclusters 1 and 2. Figure 2 shows the amino acid alignment of those near-full-length envelope sequences. These analyses confirmed the subclustering pattern observed with the 522-bp partial envelope comparison (Fig. 1b). Note that the LTR phylogenetic analysis was based on the 513 bp left after the removal of gap-containing sites from the alignment.

Translations of the near-full-length sequenced STLV-1 env and amino acid alignments were produced to evaluate the extent of segregation of STLV strains in the TNPRC SM colony. This analysis included the following SM strains: A024, G077, and F105, representative of subcluster 1, and A023, M941, G072, and M938, representative of subcluster 2. Also included was 93SL121, a wild STLV-1sm strain. Amino acid alignments of the envelope sequences from each of the two STLV subclusters were generated, and N-glycosylation sites were identified (Fig. 2). The alignment anchored with the Yerkes SM sequence (STLVsm Yerkes) and showed very few functional amino acid changes among the lineages tested. The four glycosylation sites were marked with boxes showing complete conservation of two sites. Two glycosylation sites, however, showed variations from STLVsm Yerkes. The first variation was a synonymous mutation observed in subcluster 2 viruses at the second glycosylation site (position 222) (Fig. 2), whereas the second was a mutation nonsynonymous with STLVsm Yerkes at the third glycosylation site (position 244,
and was present in all tested strains irrespective of their subclustering patterns.

Table 2 summarizes and compares SIVsm lineage distributions according to STLV subclustering. This analysis was done to investigate if a correlation between STLV subclusters and SIVsm lineages could be established in the TNPRC animals. Although the lineages were assigned arbitrarily, multiple SIVsm lineages were present within the various animals documented for each STLV-1sm subcluster. This finding was more clearly observed for the STLV-1sm subcluster 2-infected SMs. While animals M945 and G080 were infected with lineage 2 SIVsm, animals G076, G078, M920, A038, and M938 were infected with lineage 1 SIVsm, and animals M941, A023, and M951 were infected with lineage 3 SIVsm. Interestingly, only half (4 of 9) of the subcluster 1 animals were coinfected with SIV, whereas most (11 of 12) of the subcluster 2 animals were coinfected with SIV. Comparisons of the diversity patterns of the two retroviruses showed no correlation and suggest that SIV and STLV transmissions were independent within the colony.

**DISCUSSION**

The overall prevalence of retroviral infections from both STLV-1 and SIV in the captive-SM colony at the TNPRC was shown to be high, with 56 and 72%, respectively, of SMs being infected. These results are higher than the 33%-positive finding for STLV-1 and the 57%-positive finding for SIVsm previously reported for the captive-SM colony at the YNPRC (8); however, this fact may reflect the extended time that has elapsed since monitoring as well as the availability of more-sensitive tools for detection. For the first time, both a virus characterization and a prevalence evaluation of STLV-1sm in wild SMs are reported. The prevalences of both viruses in wild SMs from bush meat samples collected from Sierra Leone were shown to be lower, with 23 and 54% of samples being positive for STLV-1 and SIV, respectively. This could be due to the smaller sampling number or, potentially, to less physical contact between the animals in the wild. During the years since the formation of the colony, many animals were housed in pairs for breeding or socialization purposes, which created increased potential for the transmission of viruses among members of the colony. In addition, direct inoculation of several animals with primary biological material from others within the colony was performed to develop a nonhuman primate leprosy model. Retrospective studies have shown that these experiments resulted in SIVsm transmission (2, 34).

Unexpectedly, the STLVsm strains present in the TNPRC colony were shown to form two different subclusters in phylogenetic trees. Given the low evolutionary rates of STLV, these two subclusters are not very divergent; yet, as shown by our comparative analysis of the STLVsm strains originating from SMs living in the wild, the two subclusters show enough divergence to be considered as resulting from two independent introductions of STLV in the TNPRC colony by founder monkeys at the time of their importation from Africa. The STLVsm sequence previously reported from the YNPRC SMs (8) clustered within TNPRC subcluster 2. Interspersion of the wild STLVsm isolates within the colony STLVsm strain subclusters...
FIG. 2. Amino acid alignment of the near-full-length envelope proteins from the two newly identified STLV-1sm lineages 1 and 2, the YNPRC STLV-1sm, and the wild-SM strains show highly conserved sequences. The four N-glycosylation sites within the STLV-1 envelope are boxed for identification. Two sites at positions 140 and 403 are shown to be conserved, while two sites are not: one with a nonsynonymous mutation (A→V) at position 244 and the other with a mutation synonymous to the amino acid at position 222 of lineage 2 viruses. The nucleotide changes responsible for the position 222 changes are shown in the expanded box marked by the arrow.
confirms an African origin for the TNPRC viruses and suggests a Sierra Leone origin for the SM colonies in the United States. Our previous results also show the cocirculation of six different SIVsm lineages in the TNPRC colony (20), which points to the infection of at least six of the founders prior to their importation and corroborates the prevalence levels that we observed in wild SMs, in which the SIVsm prevalence was greater than that of STLV-1sm.

The lack of correlation between the distribution of SIVsm lineages and STLV subclusters suggests that the means of intracolony transmission of the two viruses were different. Both viruses are blood borne, have similar transmission routes, and preferentially infect lymphocytes; however, the requirements for cell-associated dissemination and clonal expansion of the PTLV-infected cells (1a, 9, 54) dramatically contrast with the efficient cell-free infections of the lentiviruses and may explain the discordance observed. Maintenance of viral replication by clonal expansion of PTLV-infected cells, rather than replication and propagation of new virions by reverse transcription of integrated proviral DNA, also explains the lower level of mutational change and overall genetic stability of the PTLV compared with those of the highly divergent lentiviruses. The low level of sequence divergence observed among PTLV strains allowed detailed evolutionary tracking and showed distinct geographical clustering of PTLV-I strains (21, 46). Geographical clustering, rather than host-related divergence, may explain the differences observed between the STLVsm from SMs and that from agile mangabeys, a related species (Fig. 1).

Given the geographical clustering of PTLV-I strains in Africa, study of HTLV-1 diversity in Sierra Leone will be necessary to test the potential of STLVsm cross-species transmission to humans, as has been reported for SIVsm and HIV-2.

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Serostatus for:</th>
<th>Lineage of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STLV-1sm</td>
<td>SIVsm</td>
</tr>
<tr>
<td>A024</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E1043</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F105</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G077</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M918</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M921</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M928</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M926</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M937</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G076</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G078</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M920</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M941</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M945</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A023</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A038</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F102</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G075</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G080</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M938</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M951</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS

This work was supported by NCCR, NIH, grants P51-RR00164-42 (V.L.T.-D.) and R01-AI44595-05 (P.A.M. and C.A.).

REFERENCES

that intracolony transmissions are predominantly the result of male-to-male}

**Mandrillus sphinx** analysis of SIV and STLV type I in mandrills (M. sphinx). 


