Development of Adult T-Cell Leukemia-like Disease in African Green Monkey Associated with Clonal Integration of Simian T-Cell Leukemia Virus Type I

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ABSTRACT

Proviral integration of a simian retrovirus highly homologous to human T-cell leukemia virus type I was examined in cellular DNAs extracted from primary peripheral blood lymphocytes of 31 adult African green monkeys (Cercopithecus aethiops) that were seropositive for simian T-cell leukemia virus type I (STLV-I). Among these monkeys, one case with overt leukemia, showing plasmacytoid leukemia cells similar to those in human adult T-cell leukemia (ATL), and five cases in a preleukemic state of ATL-like disease were found. Judging from the integration site of the provirus genome, primary lymphocytes of these leukemic or preleukemic cases contained monoclonally proliferated STLV-I-infected cells, whereas lymphocytes of other seropositive monkeys without hematological abnormalities were polyclonal, and those of seronegative monkeys did not contain the provirus. The restriction patterns with PstI and SstI of most STLV-I proviruses were identical to those of the previous isolate from this species, but in three monkeys there was a deletion of one PstI site. From the correlation of the development of simian ATL-like disease with the monoclonal integration of the STLV-I provirus genome, it should be indicated that STLV-I has similar leukemogenicity to human T-cell leukemia virus type I, and so STLV-I infection in African green monkeys will be useful as an animal model of human ATL.

INTRODUCTION

HTLV-I1 is an exogenous retrovirus isolated from mature T-cell malignancies (1–4), and the virus is known to be the etiological agent of ATL (5–8). HTLV-I does not contain any known typical oncogenes (9) and is not integrated into a specific site in chromosomal DNA (10). Therefore, the mechanism of leukemogenesis by HTLV-I infection is unknown, although it has been suggested that it is due to a virus-coded trans-acting factor (11, 12). Establishment of an animal model system should be helpful in studies on leukemogenesis by HTLV-I infection.

After the discovery of HTLV-I in humans, Old World monkeys and apes were found to have antibodies cross-reacting with HTLV-I antigens (13–18), and several strains of HTLV-I-related simian retroviruses, STLV-I, were isolated from seropositive primates such as the chimpanzee (Pan troglodytes), African green monkey (Cercopithecus aethiops), and several macaque species (19–21). STLV-I has the same genomic arrangement as HTLV-I, LTR-gag-pol-env-pX-LTR, and each corresponding region is highly homologous to that of HTLV-I (20–23). However, restriction mapping indicated that STLV-I could be distinguished from HTLV-I and that there were several subtypes of STLV-I (22, 23). Sequence analysis of the STLV-I genomes revealed 90–95% sequence homology with HTLV-I (24, 25). Furthermore, STLV-I was shown to be able to immortalize cultured T-cells (20, 21, 26). These structural and biological similarities between STLV-I and HTLV-I suggest the usefulness of STLV-I infection in monkeys as an animal model of HTLV-I infection in man.

Recently, a high proportion of the macaques that developed spontaneous lymphoma were found to have antibodies cross-reacting with HTLV-I antigens (27). However, there is no distinct evidence for the leukemogenic potential of STLV-I. Hematological abnormalities have been examined in several monkey species, and a typical ATL-like disease (28) and its preleukemic state were found in African green monkeys naturally infected with STLV-I. This communication demonstrates the close association of the development of ATL-like disease with clonal integration of the STLV-I provirus genome, and indicates the possible leukemogenic potential of STLV-I.

MATERIALS AND METHODS

Monkeys. Forty-three adult African green monkeys, which had been imported from several countries in Africa and kept in Tsukuba Primate Center for Medical Science for 4–6 years were examined. They were kept under suitable conditions in individual cages and fed appropriately for breeding.

Examination of STLV-I Infection. Serum antibodies against STLV-I, which cross-react with HTLV-I, were examined by indirect immunofluorescence with MT-1 cells (29). Expression of STLV-I antigens was examined in primary uncultured and short-term cultured lymphoid cells by immunofluorescence procedures as described previously (21).

Hematological Examination. Fresh primary peripheral blood samples collected from African green monkeys were taken for examination of morphology of the blood cells in Giemsa-stained preparations, and erythrocyte and leukocyte counts. Counts of atypical cells were made of lymphocytes with an incised or lobulate nucleus among more than 400 lymphoid cells in each case.

Detection and Analysis of Provirus DNA. Southern blotting (20–23) and nucleotide sequence (24, 25) analyses have shown the availability of cloned HTLV-I DNAs for detection of the proviral genome of STLV-I. Therefore, HTLV-I probes were used in this hybridization assay. Primary lymphoid cell preparations were isolated from fresh heparinized peripheral blood taken from African green monkeys. High molecular weight cellular DNAs extracted from these cells were analyzed by Southern blot hybridization (30). Samples of cellular DNAs (3 μg/lane in Case 1 and 8 μg/lane in all other cases) were digested with restriction endonuclease, EcoRI, SstI, or PstI, and subjected to 0.8 or 1.0% agarose gel electrophoresis. The DNAs were then transferred to a nylon membrane filter and hybridized with 32P-labeled cloned DNA fragments of the HTLV-I genome under relatively stringent conditions in buffer containing 900 mM sodium chloride, 90 mM trisodium citrate, 0.1% sodium dodecyl sulfate, and 10 mM EDTA at 65°C for 24–48 h. As shown in Fig. 1, pATK-32 (gag-pol) (kindly provided by Dr. M. Yoshida, Cancer Institute, Tokyo, Japan), pHT-39.1 (env-pX), and pHT-39.2 (LTR) (kindly supplied by Dr. K. Shimotohno, National Cancer Center Research Institute, Tokyo, Japan) were used as probes. After hybridization, the filter was washed three times in a solution containing 75 mM sodium chloride, 7.5 mM trisodium citrate, 0.1% sodium dodecyl sulfate, and 1 mM EDTA at 55°C and then exposed to X-ray film at −70°C.
RESULTS

STLV-I Infection. Serum antibodies against STLV-I were detected in 31 of 43 African green monkeys examined. STLV-I antigens could be also detected in cultured lymphoid cells from 28 of 31 seropositive monkeys, but not in all of their primary uncultured lymphocytes. The antigen-positive cells constituted approximately 1–20% of the total cells after culture for 1 week. None of the cultured lymphocytes collected from 12 seronegative monkeys expressed viral antigens.

Hematological Abnormalities. During observation for 2 years, one female African green monkey infected with STLV-I (Case 1) died of overt leukemia with enlargement of the lymph nodes and spleen (28). In the peripheral blood of this case, 88.0% of the total lymphocytes were atypical lymphocytes with a lobulate or incised nucleus (Table 1; Fig. 2A) and were morphologically similar to the leukemic cells in human ATL patients. All the other seropositive and seronegative monkeys were clinically healthy and had no apparent leukocytosis or anemia. However, a small number of atypical lymphocytes (Fig. 2, B–E) were found in 5 cases (Cases 2–6) that gave positive reactions for STLV-I antibody and antigen. In these cases, 3.5–22.0% of the lymphocytes were found to be atypical cells with a lobulate or incised nucleus (Table 1), which were also very similar to human ATL cells, and so the monkeys were tentatively diagnosed as being in the preleukemic state. The other 25 seropositive monkeys (Cases 7–31) had no apparent atypical lymphocytosis and they were considered to be healthy viral carriers. None of the 12 seronegative cases (Cases 32–43) showed any hematological abnormalities.

Integration of Proviral DNA. For detection of integration of STLV-I provirus in primary lymphocytes, the cellular DNA samples were first digested with EcoRI, which does not cut the proviral genome, and the digests were examined by Southern blot analysis with a HTLV-I representative probe consisting of pATK-32, pHT-39.1, and pHT-39.2. In this analysis, provirus genome integrated into a certain site of chromosomal DNA should be detected as a single discrete band, while genomes integrated into random sites should be observed as a smear. In the typical case of leukemia (Case 1), the EcoRI digest gave a discrete band of high molecular weight DNA in this hybridization assay indicating the monoclonality of the leukemic cells with respect to the integration site (Fig. 3A). Of the 5 clinically healthy seropositive monkeys with atypical lymphocytosis which were considered to be in a preleukemic state, 3 cases (Cases 3–5) gave a discrete band similar to that in Case 1, while 2 cases (Cases 2 and 6) gave a single band accompanied by a smear (Fig. 3A). The single prominent band with or without a smear indicated clonal proliferation of lymphocytes infected with STLV-I in the preleukemic monkeys. Proviral DNA was also detected in 17 of the 25 seropositive monkeys without hematological abnormalities (Cases 7–23). In the DNA samples from these healthy carriers, various intensities of hybridization were observed as a smear or a smear with several faint bands by this hybridization assay (Fig. 3A). These patterns in healthy carriers indicated that various numbers of lymphocytes in each monkey had integrated provirus DNA and that most of these.

![Fig. 2. Abnormal lymphocytes with an incised or lobulate nucleus in the peripheral blood of leukemic and preleukemic African green monkeys infected with STLV-I. A, Case 1; B and C, Case 2; D and E, Case 6. Giemsa, × 1000.](image-url)

![Fig. 1. Regions of HTLV-I provirus covered by the cloned DNA fragments used as probes and the restriction endonuclease map of AG-STLV-I provirus (17).](image-url)

![Table 1. Hematological findings in African green monkeys in leukemic or preleukemic state of ATL-like disease](table-url)

<table>
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<th>Monkey</th>
<th>WBC (/µl)</th>
<th>Lymphocytes (/µl)</th>
<th>Atypical cells (%)</th>
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<td>Control</td>
<td>7,000 ± 1,900</td>
<td>3,900 ± 1,100</td>
<td>&lt;1.0</td>
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</tbody>
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* Percentage of lymphocytes with a lobulate or incised nucleus.

* Values obtained from 12 STLV-free African green monkeys.

* Mean ± SD.
Fig. 3. Integration of the provirus genome of STLV-I in the primary lymphocytes of African green monkeys. Cellular DNAs extracted from the primary lymphocytes of African green monkeys were digested with EcoRI (A), SstI (B), or PstI (C) and subjected to Southern blot analysis with a representative mixture probe. Numbers of lanes, case numbers of African green monkeys, i.e., Lane 1, DNA of the STLV-I-infected monkey with overt ATL-like leukemia (Case 1); Lanes 2-6, DNAs of the STLV-I-infected monkeys in the preleukemic state of ATL-like disease (Cases 2-6); Lanes 7-19, DNAs of the STLV-I-infected healthy carrier monkeys (Cases 7-19); Lanes 20 and 21, DNAs of STLV-I-free monkeys. kb, kilobase.

cells were polyclonal, but that a few clonally expanded cells were also present in some cases. Positive results were not obtained by this hybridization assay in the DNA samples from 8 of 25 seropositive monkeys (Cases 24-31) indicating that there were too few cells with integrated provirus DNA for detection in their peripheral blood. No hybridization was observed with the DNAs of 12 seronegative monkeys, which confirmed that STLV-I is exogenous in African green monkeys.

For characterization of the proviral DNA, the restriction pattern with SstI was examined in these African green monkey lymphocytes. The DNA samples were digested with SstI which cuts only in the 5' and 3' LTRs of one isolate of STLV-I of African green monkey origin, AG-STLV-I, in a cultured cell line reported previously (23), and the digests were analyzed by blot hybridization with the representative mixture probe. In all 14 seropositive monkeys examined, which were in leukemic, preleukemic, and hematologically normal states, a strong band of 8.5 kilobases was detected as in a previous isolate (23) indicating the presence of a common SstI site in the LTR of the proviral genome of AG-STLV-I and of the intact provirus genome in all of the infected lymphocytes (Fig. 3B).

For examination of the detailed restriction patterns and the clonalities of the infected cells by viral cellular junction bands, these DNAs of monkey lymphocytes were digested with PstI, which cuts the provirus genome of the previous isolate of AG-STLV-I at several sites (23) (Fig. 1), and the digests were hybridized with a representative mixture probe. In the monkey with ATL-like disease (Case 1), 4 small fragments of 1.0, 1.2, 1.3, and 1.6 kilobases corresponding to internal fragments of the previous AG-STLV-I isolate, and 2 larger fragments of 2.5 and 2.8 kilobases were found in the PstI digest (Fig. 3C). DNA samples from 5 preleukemic monkeys also contained the 4 small internal fragments and several faint bands of more than 1.6 kilobases. In most of the leukemic, preleukemic, and he-
matologically normal monkeys, the 4 internal fragments identical to those of the previous AG-STLV-I isolate (23) were commonly detected in the proviral genome by this assay, but in 3 monkeys (Cases 8, 13, and 14), no 1.3-kilobase band was detectable, and a discrete band of 2.2 kilobases was found instead.

For identification of the fragments other than the common internal fragments in the leukemic and preleukemic monkeys and for analysis of the restriction maps of the 3 provirus genomes showing different restriction patterns with PstI, these PstI digests were hybridized with the probes of pATK-32, pHT-39.1, and pHT-39.2, respectively. In the DNA sample from a typical ATL-like case, 2 discrete bands of 2.5 and 2.8 kilobases hybridized strongly with the LTR probe, pHT-39.2 (Fig. 4). As internal fragments, 3 bands of 1.0, 1.2, and 1.6 kilobases and 2 bands of 1.0 and 1.3 kilobases were detected with pATK-32 and pHT-39.1, respectively (Fig. 4). The pHT-39.1 probe, which covers env-pX and a small part of LTR, also hybridized with 2 cellular viral junction bands, and the 2.8-kilobase band, which was more intense than the 2.5-kilobases band, probably represented the 3' flanking sequence. These data on Case 1 clearly indicated that the tumor cells were monoclonal with respect to the integration site of the provirus and that the restriction map of the internal sequences of the provirus was the same as that of the previous AG-STLV-I isolate.

The proviral genome of Case 2 in the preleukemic state was also analyzed with each probe (Fig. 4). The restriction map of PstI in this case, deduced from the small-sized internal fragments that hybridized with each probe, was identical to that in Case 1 and the previous isolate. Two bands of 2.9 and 3.4 kilobases detected with the representative probe, which were less intense than the viral cellular junction bands in Case 1, also hybridized with the LTR probe, indicating the presence of monoclonally expanded cells infected with STLV-I. Furthermore, the faint bands that were larger than the internal ones detected by the representative probe in the PstI digests from other preleukemic monkeys (Cases 3–6) also hybridized with the LTR probe. These data indicating the clonality of cells were consistent with the results obtained by EcoRI digestion assay. The different restriction pattern with PstI detected with the representative probe in 3 cases (Cases 8, 13, and 14) was analyzed with each probe. In Case 13, the newly detected 2.2-kilobase band hybridized with both pATK-32 and pHT-39.1, and the 1.0- and 1.3-kilobase internal bands usually detected with pATK-32 and pHT-39.1, respectively, were not detectable (Fig. 4). The same results were obtained in Cases 8 and 14. These findings indicated deletion of one PstI site at the pol and env junction in these proviruses, suggesting that they were variants of AG-STLV-I.

**DISCUSSION**

In the present study, provirus integration of STLV-I was investigated in primary lymphocytes of African green monkeys infected with STLV-I. The primary lymphocytes obtained from seropositive monkeys did not express detectable viral antigens, but most of them contained the proviral genome in their chromosomal DNA. Therefore, the expression of viral antigens is considered to be suppressed in vivo by serum antibodies or some inhibitory factors, as is known to be the case in HTLV-I and bovine leukemia virus infections (31, 32). When the lymphocytes are transferred to culture, they express viral antigens and release extracellular virus particles which infect uninfected cells (19–21). Lymphoid cell lines producing STLV-I are known to have multiple copies of the integrated provirus (23). Therefore, it was necessary to examine the fresh uncultured lymphocytes to determine the correlation of the development of leukemia with the manner of provirus integration.

HTLV-I was shown to interact directly with target cells which later grew as malignant transformed cells, because the primary leukemic cells of all ATL cases tested were found to be monoclonal with respect to the integration site of HTLV-I (5–8).
Furthermore, preleukemic ATL cases showing no typical clinical symptoms were found that had abnormal lymphocytes with an incised or lobulated nucleus in the peripheral blood (33, 34), and monoclonal integration of the provirus genome was also detected in these cases (34). Lymphocytes from healthy carriers of HTLV-I had multiple integration sites (6). In the present study on the development of ATL-like disease in the African green monkey, one case of typical ATL-like disease and 5 cases of the preleukemic state were found among the STLV-I-infected monkeys. Southern blot analysis showed that leukemic cells derived from the typical leukemia case were monoclonally expanded cells in which the provirus genome was integrated into one site and that the lymphoid cells of the preleukemic monkeys also contained clonally provirus-integrated cells. However, the STLV-I-infected lymphocytes derived from healthy virus carrier monkeys that had no abnormal lymphocytes were polyclonal with respect to the integration site of the provirus genome, and the lymphocytes of seronegative monkeys did not contain the STLV-I provirus genome. From these observations, successive events are considered to occur as follows: (a) the proviral genome is randomly integrated into virus-sensitive lymphocytes; (b) then some infected lymphocyte clones proliferate among the infected cells; (c) one selected clone transformed by STLV-I begins to grow; and (d) finally the transformed cell clone proliferates infinitely. It is conceivable that the lymphocytes of healthy virus carrier monkeys (Cases 7–31) are in stage I or II, those of preleukemic cases (Cases 2–6) in stage III, and those of the overt leukemia case (Case 1) in stage IV. Observations on the clinical course of human pre-ATL patients showed that overt ATL developed in some cases after 1–5 years of the pre-ATL stage (34). We reexamined the clinical and hematological features and proviral integration in African green monkeys in the preleukemic state at 6 months after their initial examination, but in all cases findings were similar to those at the initial examination (data not shown). Thus a fairly long time seems necessary for development of overt leukemia from the preleukemic stage in both humans after HTLV-I infection and monkeys after STLV-I infection.

Analysis of the proviral genome in leukemic cells showed that HTLV-I is the causative agent of human ATL (5–8). The AG-STLV-I genome in a virus-producing lymphoid cell line was found to have a genomic construction containing pX, and each gene was shown to be highly homologous to that of HTLV-I (23). Furthermore the nucleotide sequence homology of AG-STLV-I LTR with HTLV-I LTR was 94.7% (25). The clinical and hematological features observed in cases of overt leukemia and preleukemia in African green monkeys were very similar to those in human cases of ATL. Moreover, in this work we found that development of the ATL-like disease in the monkey was clearly correlated with clonal proliferation of lymphocytes with integrated provirus, as is shown in human ATL (5–8). From these virological analyses of the association of STLV-I with the development of leukemia, it should be indicated that AG-STLV-I has a leukemogenic potential similar to that of HTLV-I.

The abnormal lymphocytes with incised or lobulated nuclei observed in the leukemic and preleukemic monkeys were morphologically very similar to those in human ATL patients. Furthermore, they were shown to have T-cell surface markers and interleukin 2 receptor (28). However, these lymphocytes in African green monkeys were positive for Leu 2a and negative for Leu 3a (28), although those in human ATL patients are known to have helper/inducer T-cell markers (35). Recently, we found that this AG-STLV-I could immortalize cultured normal lymphocytes collected from African green monkeys that expressed Leu 2a antigen. Thus, the Leu 2a-positive lymphocytes may be the target of AG-STLV-I in this monkey species.

Previously an STLV-I-producing cell line, GM0650 (21), was established from a seropositive African green monkey and the provirus genome in this cell line was analyzed (21, 23). To determine whether this isolate in GM0650 was a prototype of species-specific STLV-I in African green monkeys, the restriction sites of SphI and Psrl in the provirus genome were examined in primary lymphocytes of 14 infected African green monkeys in this study. In 11 of these monkeys the provirus genome gave the same restriction pattern as that of the previous isolate in GM0650. However, 3 monkeys were infected with a variant virus with the minor difference that one Psrl site was not present. From these observations, the virus in GM0650 cells can be regarded as the species-specific STLV-I that is commonly transmitted among African green monkeys. The African green monkeys kept in Tsukuba Primate Center seemed to have been infected with STLV-I in their native countries in Africa, since they were seropositive from the time of their import into Japan (data not shown). Although we have no data on the exact places where they had lived in the wild, it is conceivable that the 3 monkeys infected with the variant AG-STLV-I were from a part of Africa different from that of the other monkeys, or that they belong to a subspecies different from that of the other African green monkeys.

The present study demonstrated the development of an ATL-like disease and the possible leukemogenic potential of species-specific STLV-I in African green monkeys. This natural infection system should be a useful animal model for studies on the infection and leukemogenicity of HTLV-I in humans.

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REFERENCES


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