Molecular cloning of human T-cell lymphotropic virus type I-like proviral genome from the peripheral lymphocyte DNA of a patient with chronic neurologic disorders

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ABSTRACT  Human T-cell lymphotropic virus type I (HTLV-I), the etiologic agent of human T-cell leukemia, has recently been shown to be associated with neurologic disorders such as tropical spastic paraparesis, HTLV-associated myelopathy, and possibly with multiple sclerosis. In this communication, we have examined one specific case of neurologic disorder that can be classified as multiple sclerosis or tropical spastic paraparesis. The patient suffering from chronic neurologic disorder was found to contain antibodies to HTLV-I envelope and gag proteins in his serum and cerebrospinal fluid. Lymphocytes from peripheral blood and cerebrospinal fluid of the patient were shown to express viral RNA sequences by in situ hybridization. Southern blot analysis of the patient lymphocyte DNA revealed the presence of HTLV-I-related sequences. Blot-hybridization analysis of the RNA from fresh peripheral lymphocytes stimulated with interleukin 2 revealed the presence of abundant amounts of genomic viral RNA with little or no subgenomic RNA. We have cloned the proviral genome from the DNA of the peripheral lymphocytes and determined its restriction map. This analysis shows that this proviral genome is very similar if not identical to that of the prototype HTLV-I genome.

Human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of a type of acute T-cell leukemia whose epidemiology shows clustering of the disease in southwestern Japan and the Caribbean (1, 2). Patients with this disease are characterized by the presence of antibodies to the structural proteins (gag and envelope) of HTLV-I as determined by ELISA and immunoblotting techniques (1, 2). Several lines of recent studies have indicated that in addition to its ability to induce leukemia, HTLV-I may also play an important role in certain disorders of the central nervous system. The studies that link HTLV-I with neurological disorders are: (i) occurrence of antibodies that crossreact with purified gag proteins in multiple sclerosis (MS) patients (3); (ii) presence of HTLV-I antibodies in sera obtained from 67–93% (depending on the geographical location) of patients with chronic neurologic disorders in tropical regions (4, 5) (referred to as “tropical spastic paraparesis”); and (iii) identification of chronic neurologic disorders among the Japanese population characterized by the presence of HTLV-I antibodies in high titer (6). These various forms of chronic neurologic disorder may be due to infection of HTLV-I or a related virus that exhibits crossreactivity to HTLV-I proteins.

We report here the study of one patient with chronic "progressive" neurologic disease, living in southwestern Florida, whose cerebrospinal mononuclear cells contain HTLV-I viral RNA sequences and intracellular HTLV-I-encoded antigens and whose peripheral lymphocytes show the presence of an integrated proviral genome in the DNA and expression of viral RNA. Molecular cloning of this proviral genome and restriction enzyme analysis indicates that it is virtually identical to that of HTLV-I.

MATERIALS AND METHODS

Patient Material. A 24-yr-old Haitian-born male living in the United States since 1980 developed increasing urinary frequency and urgency followed by pyelonephritis in 1979. Within 6 mo he developed severe low back pain accompanied by leg weakness and stiffness. Laboratory examination revealed a positive serum VDRL (Venereal Disease Research Laboratory) test for syphilis and FTA (fluorescent treponemal antibody adsorption) test. Cerebrospinal fluid (CSF) examination revealed 25 mononuclear cells per mm³, a total protein content of 87 mg/dl, 26% gamma globulin determined by electrophoresis, and a negative VDRL. He progressed to spastic paraplegia and hyperreflexic neurogenic bladder within 2 yr. Magnetic resonance imaging (MRI) revealed T2 (Tesla magnet and spin echo technique)-weighted multiple high-intensity lesions in the periventricular white matter in brain and in spinal cord, which are indistinguishable from those observed in MS. Visual evoked responses were bilaterally abnormal. Peripheral blood T4 antigen-carrying lymphocytes were reduced in number, as determined by flow cytometry. The patient serum was negative for HIV antibodies. Bone marrow biopsy showed no evidence of lymphoproliferative disease. This patient under study is referred to as 3-19-3 (7).

Radioimmunoprecipitation of HTLV-I Viral Proteins. Cells of MT-2, the HTLV-I-infected cell line of Japanese origin (8), and uninfected H9 cell line were metabolically labeled overnight with [35S]methionine described (7), and cell lysates were prepared. After clarification of the labeled cell lysates, they were treated with anti-HTLV-I antibodies or with serum or CSF samples of the patient under study and were incubated for 2 hr at 4°C. The antigen–antibody complexes were then precipitated by addition of a 10% (vol/vol) suspension of formalin-fixed Staphylococcus aureus Cowan (9). After three washings of the suspension, the samples were boiled in 2% NaDodSO4 containing 5% 2-mercaptoethanol and analyzed on NaDodSO4/10% acrylamide gels as described by Laemmli (10).

In Situ Hybridization for HTLV-I RNA Sequences. In situ hybridization reactions were performed with [35S]-labeled RNA probe specific for the 3’ region of HTLV-I. A full-length HTLV-I clone [8.2 kilobases (kb)], pMT-2 (11), was inserted into the pSP64 plasmid at the Sst I site and used for in vitro transcription. The RNA probe averaged 1–2 kb in length and was used for in situ hybridization as described (3, 12). After

Abbreviations: HTLV-I and -II, human T-cell lymphotropic virus types I and II; CSF, cerebrospinal fluid; MS, multiple sclerosis.
autoradiography for 4–8 days, positive cells were scored. Control cells used were HTLV-I-infected HUT 102 B cells, HIV-infected H9 cells, uninfected H9 cells, and a CSF T-cell line from a healthy donor.

**Blot-Hybridization Analysis of DNA and RNA Sequences.** For blot-hybridization analysis, peripheral blood mononuclear cells were isolated from the patient’s blood by density gradient centrifugation on Lymphoprep (Nygaard; Accurate Scientific, Oslo, Norway). Cells were placed in culture medium [RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum] with 25 units of recombinant interleukin 2 per ml at a density of 2 × 10^5 per ml. After 5 days, when the cells were more than 92% T3 antigen-positive, cells were pelleted, washed, and used for the isolation of DNA and RNA. Approximately 10^8 cells were used for each preparation. High molecular weight DNA was isolated from the cells as described by Maniatis et al. (13). RNA was isolated by the guanidine thiocyanate method as described (14). Gel electrophoretic analysis and hybridization studies were carried out as described (13). All hybridization reactions were carried out under conditions of moderate stringency. Hybridization solutions contained 40% formamide, 5 × SSC (1 × = 0.15 M NaCl/0.015 M sodium chloride, pH 7), and 5 × Denhardt’s solution (1 × = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). The hybridization temperature was 42°C. Washings were performed three times at room temperature with 2 × SSC containing 0.1% NaDodSO₄ followed by one to three washings at 42°C in 0.1 × SSC containing 0.1% NaDodSO₄. In all of these experiments, a 32P-labeled probe of HTLV-I sequences was prepared by nick-translating the purified insert of the HTLV-I proviral genome derived from plasmid pMT-2 described previously (11).

**Molecular Cloning of the Proviral Genome from Patient DNA.** The genomic DNA of patient 3-19-3 was digested with SstI to completion and fractionated on a preparative agarose gel. DNA fragments migrating in the range of 6–12 kb were eluted from the gel by the electrophoresis technique and used for molecular cloning. λ Zap cleaved with SstI was used as the cloning vector, and the ligations and packaging reactions were performed according to the instructions of the manufacturer (Stratagene, San Diego, CA). Plaques containing HTLV-I-related sequences were identified by the plaque filter hybridization technique (15) with a nick-translated probe of HTLV-I proviral genome. Recombinant phage DNA was prepared as described (13), and DNA inserts containing proviral sequences were separated from phage arms by SstI digestion and preparative gel electrophoresis. Restriction endonuclease mapping was carried out with the purified insert.

**RESULTS**

**Presence of Antibody to HTLV-I in the CSF of Patient 3-19-3.** Earlier studies using ELISA assays had indicated that the CSF as well as the serum of patient 3-19-3 contained high-titered antibodies against HTLV-I antigens (8). To further confirm this, we performed radioimmunoprecipitation analysis in which MT-2 cells (infected with HTLV-I) were metabolically labeled with [35S]methionine, and the cell lysates were used to detect the presence of antibodies in the CSF of patient 3-19-3. As negative controls we used uninfected H9 cells metabolically labeled under identical conditions. Such an analysis showed that the patient’s CSF precipitated a number of proteins in HTLV-I-infected MT-2 cell lysates (Fig. 1, lane E) whereas there was little or no specific precipitation seen in uninfected H9 cell lysates (lane F). In MT-2 cell lysates (lane E), a major band of 66 kDa was precipitated, which corresponded to the envelope protein synthesized by these cells. An identical band in lane A was immunoprecipitated from these lysates by a monoclonal antibody to HTLV-I envelope antigen (16). In addition to the 66 kDa band, other prominent bands were seen in lane E—one of 57 kDa and the other of 24 kDa. These are likely to be the gag precursor protein and the processed p24 molecule. An identical band of 24 kDa was immunoprecipitated by a monoclonal antibody to HTLV-I gag protein (lane C), indicating that the 24-kDa band seen in lane E in fact represents the processed p24 antigen. These results conclusively show that the CSF of patient 3-19-3 contains antibodies that crossreact with HTLV-I envelope and gag proteins.

**In Situ Hybridization for HTLV-I RNA Sequences.** Peripheral lymphocytes derived from patient 3-19-3 that were propagated in the presence of interleukin 2 were tested for the presence of HTLV-I-related sequences by in situ hybridization with a 35S-labeled RNA probe specific for the 3’ region of HTLV-I. A full-length HTLV-I clone (8.2 kb), pMT-2 (11), in a phage SP6 vector was used for the preparation of the RNA probe. The RNA probe averaged 1–2 kb in length and represented the 3’ end of the HTLV-I genome. The results of these hybridization experiments are shown in Fig. 2. As negative controls, peripheral lymphocytes derived from a normal individual and propagated under identical conditions in the presence of interleukin 2 were used in these studies. More than 50% of the cells derived from patient 3-19-3 hybridized with the RNA probe, whereas no hybridization was seen with lymphocytes derived from a normal individual (Fig. 2). These results suggest that a large proportion of the peripheral lymphocytes of the patient under study expresses viral RNA sequences.

**Blot-Hybridization Analysis of the Patient DNA and RNA for HTLV-I-Related Sequences.** To further characterize the nature of the proviral genome present in the lymphocyte population and to understand the nature of the viral RNAs that are synthesized by these cells, we carried out blot-
hybridization analysis of DNA and RNA extracted from the patient's peripheral lymphocytes. For Southern analysis, the DNA was cleaved by a number of restriction enzymes and electrophoresed on a 1% agarose gel. After transfer to a nitrocellulose paper, they were hybridized to a nick-translated probe of HTLV-I proviral genome under conditions of moderate stringency. A representative pattern of hybridization is shown in Fig. 3. When the DNA was digested with Sst I, an 8.0-kb band was seen to hybridize with the HTLV-I probe, while a 4.0-kb band was seen when the DNA was cleaved with HindIII.

Blot-hybridization analysis of the RNA extracted from the cells also showed the presence of abundant quantities of viral RNA in the peripheral lymphocyte population of this patient (Fig. 4). For comparison, RNA extracted from a cell line infected with HTLV-II was used. The HTLV-II-infected cell line showed the presence of both genomic and subgenomic RNAs that cross-hybridize to the HTLV-I probe. Surprisingly, the RNA extracted from peripheral lymphocytes of patient 3-19-3 contained only the genomic RNA. Only trace amounts of subgenomic RNA could be detected in these cells. The minimal appearance of this species of RNA could be either due to a defect in the splicing reaction that leads to the generation of the smaller RNAs or due to lack of strong crossreactivity with the envelope region of the HTLV-I probe used in these studies. Of the two, the former possibility appears to be greater.

**Molecular Cloning and Restriction Enzyme Analysis of the Proviral Genome from Patient 3-19-3 Lymphocytes.** To further characterize the nature of the proviral DNA, we undertook molecular cloning of the integrated viral genome. For this, the DNA was cleaved with Sst I, and a DNA fraction enriched for viral sequences was inserted into λ Zap vector. Positive clones were identified by hybridization to nick-translated HTLV-I probe and plaque-purified. Four positive clones were identified from a library of 100,000 plaques. Preliminary analysis indicated that all of the four clones contained similar-size inserts; therefore, only one of these clones was used for further analysis. After a large-scale preparation of the recombinant phage clone, the purified insert was isolated and used for restriction enzyme analysis. The restriction map of the clone isolated from patient 3-19-3 is shown in Fig. 5. All of the enzymes that are known to cleave the proviral genome of HTLV-I also cleaved the molecular clone isolated from 3-19-3 lymphocyte DNA at approximately the same positions. A comparison of the restriction map of the 3-19-3 clone with that of HTLV-I (11, 17) showed a striking similarity through the entire length of the proviral genome. The present analysis does not preclude the presence of minor deletions, insertions, or mutations in the sequence of the viral genome. Such minor alterations might explain the lack of synthesis of subgenomic RNAs in the patient lymphocytes. Nucleotide sequence analysis should provide a better comparison of the two viral genes and a rational explanation for the lack of synthesis of subgenomic RNAs in these cells.

**DISCUSSION**

Results presented in this communication demonstrate the presence of a HTLV-I-like viral genome in the lymphocyte population of a patient with a chronic disease of the central nervous system without any discernable symptoms of leu-
revealed the presence of HTLV-I, the virus that causes adult T-cell leukemia. Since magnetic resonance imaging of this patient revealed periventricular and cerebellar white matter lesions that cannot be distinguished from defined cases of MS, the tentative diagnosis of the patient could fall into the category of the progressive type of MS. The patient’s CSF was found to contain antibodies that precipitate the envelope and gag proteins of HTLV-I, and cells from CSF and peripheral blood were found to contain sequences that cross-hybridize with the HTLV-I probe. Southern blot analysis indicated the presence of viral DNA sequences that cross-hybridized with the HTLV-I-specific probe, and RNA blot analysis showed the synthesis of viral RNA. Although these cells synthesized abundant quantities of genomic RNA, little subgenomic RNA could be detected in these cells. This could indicate the presence of a defect in the viral genome that allows little normal splicing. Alternatively, cellular regulatory mechanisms might control and minimize the levels of virus-derived subgenomic RNAs. Sequence analysis of the viral genome should provide an explanation for this abnormality seen in these cells.

Molecular cloning and restriction enzyme analysis of the proviral genome allowed us to compare the structure of this clone with that of prototype HTLV-I clone (11, 17). Such an analysis indicates that the two DNA clones were very similar if not identical in their restriction maps. It is, however, possible that minor variations occur between the two clones in the form of mutations, deletions, or insertions, which might account for the lack of synthesis of subgenomic RNAs. Irrespective of these possible differences, the present study firmly establishes a close relationship between the proviral genome present in patient 3-19-3 and the prototype HTLV-I. This result also explains the observation that the patient’s serum and CSF contained a high-titer of antibodies against HTLV-I proteins.

While the case, 3-19-3, belongs to the group of patients who show a high titer of HTLV-I antibodies, there is another group of patients with chronic neurologic disorders whose sera show a low level of antibodies, mostly detected through seroactivity with a purified preparation of HTLV-I p24 antigen (3, 18). We have even described a case of chronic neurologic disease where HTLV-I sequences were detected by in situ hybridization in CSF lymphocytes in the absence of serum antibodies (18). These results may indicate that, in contrast to the case 3-19-3, the virus harbored by these patients may be related only distantly to the HTLV-I prototype. Molecular cloning of the proviral genome by using crossreactive antibodies or DNA probes from these cases should provide important clues regarding the structural differences between the viral genes.

Alternatively, it is possible that in these cases the number of infected cells expressing HTLV-I antigen (8) or showing viral sequences by in situ hybridization is too low to be detected with these techniques. Amplification of viral DNA through the polymerase chain reaction with various oligonucleotide primers will considerably increase the chances of detecting cells harboring the HTLV-I agent regardless of the serological reaction of the patient.

Although until 1985 the possible role of HTLV-I in chronic neurologic disorders was unknown, there is a strong indication that the tat gene of HTLV-I, when introduced into the cells of the nervous system, may produce proliferative disorders. Evidence for this was obtained through the experiments of Hinrichs et al. (19) who developed transgenic mice containing the tat gene of HTLV-I under the control of its own long terminal repeat. Several of these transgenic mice were found to develop tumors that closely resemble human neurofibromatosis, the most common single gene disorder known to affect the nervous system. It is clear that not all cases of MS could be shown to have a retroviral etiology. Based on serological evidence, it seems that only a fraction of chronic neurologic disorders observed in either tropical or moderate climates can be linked at present with HTLV-I infection. Applying the polymerase chain reaction to detect HTLV-I DNA may increase the sensitivity of detection and might reveal a higher proportion of patients that harbor an HTLV-I or HTLV-I-related agent. If these data will be accompanied by a careful study of clinical symptoms of each patient, the accumulated data may permit us to gain an insight into the role of HTLV-I agents in the mechanism of lesions induced in the central nervous system. It is possible that HTLV-I and its related group of viruses exert their influence on a common set of cellular genes whose deregulation leads to disorders of the nervous system. If this were true, HTLV-I-associated nervous disorders provide excellent model systems to identify the set of cellular genes that act as targets for the action of viral genes such as the tat gene. Identification of cDNA clones for mRNAs that are overexpressed or underexpressed in HTLV-I-infected cells of the central nervous system (compared to their uninfected controls) by differential screening of cDNA libraries should provide an indication of the target genes that are involved in virus-induced nerve disorders. Study of the fate of these genes in cases with no apparent retroviral involvement should provide an exciting prospect of finding a common pathway that might be involved in the generation of a common disorder by HTLV-I and possibly other viruses that also may play a role in the etiology of chronic neurologic disorders.

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