Human T-cell lymphotropic virus type I (HTLV-I), human T-cell lymphotropic virus type II (HTLV-II), simian T-cell lymphotropic virus type I (STLV-I), and bovine leukemia virus (BLV) are exogenous oncogenic retroviruses. The HTLVs are associated with human lymphoid malignancies: HTLV-I with an aggressive form of leukemia/lymphoma, adult T-cell leukemia/lymphoma (ATL), and HTLV-II with a rare T-cell variant of hairy cell leukemia. STLV-I has been shown to be associated with malignant lymphomas in macaques. Further, reports of STLV-I in baboons, chimpanzees, and African Green monkeys have begun to document the extent of this viral group in primates. BLV is a causative agent of enzootic leukosis in cattle and sheep. Similarities in the genomic organization and biological properties of HTLV-I, HTLV-II, STLV-I, and BLV suggest that these viruses evolved from a common ancestral virus.

Because HTLV-I, HTLV-II, STLV-I, and BLV lack a typical oncogene of cellular origin, the X region, located 3' to the env gene and extending into the 3'-long terminal repeat (LTR) has come under particular scrutiny as potentially involved in transformation. Examination of the sequences in the X region, shared by all four viruses, reveals the presence of multiple open reading frames. Investigators have found that 1) antibodies to a polypeptide encoded from the longest of the open reading frames (Ior), can be detected in a fraction of infected individuals, 2) polyclonal sera generated to bacterially-synthesized determinants that correspond to the transactivating protein, TAT, encoded by the Ior gene have been successfully used to stain the nuclei of infected cells as detected by immunofluorescence, and 3) in co-transfection experiments the Ior-encoded protein enhanced expression of the convenient marker gene chloramphenicol acetyltransferase when fused to the viral LTR. Recent studies have shown that the TAT protein was capable of increasing the expression of the IL-2 and IL-2 receptor genes of the host genome. Neerenberg et al have described a transgenic mouse model in which the TAT protein of HTLV-I is strongly implicated in the development of mesenchymal tumors. Because of these characteristics, the Ior gene has been hypothesized to be involved in immortalization of infected cells.

A unique feature of the malignant diseases associated with HTLV-I and HTLV-II and the leukemia caused by BLV, is the apparent transcriptional dormancy of the integrated viral genomes in peripheral blood lymphocytes (PBLs). In addition to this transcriptional dormancy, the detection of viral genetic information in carriers and individuals with chronic lymphocytosis and smoldering leukemia is complicated by the generally small number of proviral genomic copies per cell and the small number of infected cells.

The investigators sought to develop a procedure that would facilitate the rapid detection of HTLV-I and HTLV-II sequences either in cultured or circulating PBLs. The nucleic acid amplification procedure, termed PCR, to the detection of these human oncoviruses. Judicious selection of specific oligonucleotides for primers and probes provides type-specific and simultaneous detection of these two retroviruses. The ability to amplify and detect highly conserved regions of these medically relevant viruses may facilitate the identification of, as yet, uncharacterized retroviruses.

**MATERIALS AND METHODS**

Enzymatic amplification. DNA was isolated from peripheral blood mononuclear cells (PBMCs) and tissues as previously described. One microgram of DNA was subjected to 30 cycles of successful application of the DNA amplification procedure, termed PCR, to the detection of these human oncoviruses. The nucleic acid amplification procedure, termed PCR, to the detection of these human oncoviruses. Judicious selection of specific oligonucleotides for primers and probes provides type-specific and simultaneous detection of these two retroviruses. The ability to amplify and detect highly conserved regions of these medically relevant viruses may facilitate the identification of, as yet, uncharacterized retroviruses.

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PCR amplification. PCR uses two primers that are complementary to the plus and minus strands of target DNA. The repeated denaturation, renaturation, and elongation of the primers provide an exponential increase of copies of the region flanked by the primers. Primers were selected to specifically direct amplification of either HTLV-I or HTLV-II and for regions that are common to both viruses. The location of the primers and probes and the length of the amplified products are summarized in Table 1.

The sequence of primers selected for the amplification of human immunodeficiency virus (HIV-1) is from a region of the viral nucleocapsid gene (gag) that is highly conserved among the sequenced HIV-1 isolates. The primers, designated SK38 and SK39, are similar to the previously reported SK17 and SK18 primer pair25 but are extended at the 5' end by 14 and 13 bases, respectively. The amplifications were carried out using a thermostable DNA polymerase and an automated temperature cycling device (Cetus-Perkin Elmer, Norwalk, CT) as described by Saiki et al.26

Detection analysis. For dot-blot analysis, aliquots representing one third of the amplified DNA product were denatured in NaOH and spotted onto Genetran nylon membrane (Plasco, Woburn, MA) as described.27 After pre-hybridization and hybridization with an end-labeled probe, the filters were washed and autoradiographed overnight at −70°C with double intensifying screens (Cronex, DuPont, Wilmington, DE). The specific activities of the kinased oligonucleotide probes.28 After hybridization, the reconstitution and cleavage of a specific endonuclease site provides the generation of a "diagnostic" oligonucleotide of defined size. The investigators decided to use both OR and dot-blot detection procedures to compare their relative sensitivities in this system.

RESULTS

Strategy. The PCR has been used to facilitate the identification of alleles in the β-globin,22,27,30 HLA-DQα loci,22,24 ras,31 and chromosomal 14,18 translocation36 as well as the sensitive detection of HIV-125 and HTLV-1 nucleic acid sequences in cultured and tumor cells respectively.37 The amplification procedure has been successfully coupled to Southern blot,21,23-24 dot-blot,23-23-1 and oligomer restriction (OR) detection analyses.25,28 OR is a recently introduced detection procedure that exploits the rapid solution-based hybridization kinetics of target DNA and radioactively end-labeled synthetic oligonucleotide probes.26 After hybridization, the reconstituted and cleavage of a specific endonuclease site provides the generation of a "diagnostic" oligonucleotide of defined size. The investigators decided to use both OR and dot-blot detection procedures to compare their relative sensitivities in this system.

For HTLV detection, primers were selected to specifically direct amplification of regions of either HTLV-I or HTLV-II and for regions that are common for both viruses. Several criteria were used for the selection of virus-specific primers. First, although substantial genomic heterogeneity has not been shown for either of these viruses, only sequences from a gene that was expected to be conserved among viral isolates were selected. The pol gene was chosen because it encodes the reverse transcriptase that is required for replication. Second, the sequences chosen had to be dissimilar enough between HTLV-I and HTLV-II so as to preclude amplification of the other human lymphotropic virus. The HTLV-I-specific primers and probe designated SK54, SK55, and SK56 share 60%, 74%, and 40% homology, respectively, with the corresponding pol region of HTLV-II. The HTLV-II-specific primers and probe designated SK58, SK59, and SK60 share 70%, 35%, and 63% homology, respectively, with the corresponding pol region of HTLV-I.

In order to provide HTLV "group-specific" amplification, a region of conserved sequence between HTLV-I and HTLV-II was selected; primers with this characteristic will

Table 1. Primer Pairs and Probes for Amplification and Detection of the Characterized Human Retroviruses

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Virus Region</th>
<th>Position</th>
<th>Size of Amplified Product</th>
<th>Probe Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK43</td>
<td>HTLV-I, II X</td>
<td>7,358-7,377 (HTLV-I)</td>
<td>159 bp</td>
<td>SK45 7,447-7,486 (HTLV-I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7,248-7,267 (HTLV-II)</td>
<td></td>
<td>7,337-7,376 (HTLV-II)</td>
</tr>
<tr>
<td>SK44</td>
<td>HTLV-I, II X</td>
<td>7,386-7,406 (HTLV-II)</td>
<td>119 bp</td>
<td>SK56 3,446-3,460</td>
</tr>
<tr>
<td>SK54</td>
<td>HTLV-I pol</td>
<td>3,365-3,384</td>
<td>103 bp</td>
<td>SK60 4,237-4,276</td>
</tr>
<tr>
<td>SK55</td>
<td>HTLV-I pol</td>
<td>3,466-3,483</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK58</td>
<td>HTLV-I pol</td>
<td>4,198-4,217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK59</td>
<td>HTLV-I pol</td>
<td>4,281-4,300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK38</td>
<td>HIV-1 gag</td>
<td>1,541-1,578</td>
<td>155 bp</td>
<td>SK19 1,595-1,635</td>
</tr>
<tr>
<td>SK39</td>
<td>HIV-1 gag</td>
<td>1,638-1,665</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The sequence of each oligonucleotide uses the numbering system provided in the initial reports of the total viral genomic sequences. The oligonucleotides designated SK39, SK44, SK55, SK56, SK59, SK60 are complementary to the viral plus strand; SK19, SK38, SK43, SK45, SK54, and SK58 are complementary to the viral minus strand.

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permit the simultaneous detection of both viruses. The substantial homology of the X regions of HTLV-I and HTLV-II\(^{18}\) served as the region of choice. An alignment of the sequences from HTLV-I, HTLV-II, and STLV-I that correspond to the region selected for primers (SK43, SK44) and probe (SK45) is shown in Fig 1. Given the essential role of the TAT protein in viral transcription and replication and the similarity between the two characterized human and simian lymphotropic viruses in the Oncovirinae subfamily, this primer pair may also facilitate the identification of other viruses that are as related as HTLV-I and HTLV-II. Demonstration of the successful use of primers with this characteristic, therefore, would serve as a model system for such an approach.

**Sensitivity of PCR in a reconstituted model system.** The investigators wished to initially establish the efficiency and sensitivity of PCR amplification of the HTLV-I viral genome. Previously, it had been shown, using the β-globin locus as a model system, that these procedures allowed the detection of 600 copies of a specific sequence in the DNA from approximately 150,000 cells (1 μg) with an efficiency of approximately 70%.\(^{32}\) DNA extracted from a cell line established from PBLS of a patient who was positive for antibodies to HTLV-I by ELISA, immunoblotting, and radioimmunoprecipitation was used as a positive control. Southern blot analysis using a recombinant plasmid containing a portion of the gag and pol genes of HTLV-I (pATK32), indicated the presence of approximately one viral genome copy per cell.\(^{39}\)

To determine the sensitivity of detection, cellular DNA containing HTLV-I genomic sequences were serially diluted into DNA extracted from an uninfected cell line, amplified with the HTLV-I pol gene-specific primers (SK54/55) and analyzed by both OR (Fig 2, panel A) or dot-blot hybridization (Fig 2, panel B). The requirement of a PvuII site for the amplification of regions of the pol and tat genes of HTLV-I and HTL V-II. DNAs extracted from uninfected cells and cells infected with HTLV-I, HTLV-II, and HIV-1 were amplified with the various primer pairs and analyzed by dot-blot with the respective probes. The specificity of the primers and probes are shown in Fig 3.

**Specificity of primers and probes for human lymphotropic viruses.** Given the increasing reports of co-infection with multiple lymphotropic viruses,\(^{42}\) the investigators were interested in the specificity of the primer pairs selected for the amplification of regions of the pol and tat genes of HTLV-I and HTLV-II. DNAs extracted from uninfected cells and cells infected with HTLV-I, HTLV-II, and HIV-1 were amplified with the various primer pairs and analyzed by dot-blot with the respective probes. The specificity of the primers and probes are shown in Fig 3.

To circumvent the need to use multiple primer pairs for the simultaneous detection of the HTLV viruses, the investigators used the primer pair designated SK43/44. As highlighted in Fig 1, the oligonucleotides chosen are complementary to the plus and minus strands of HTLV-I but contain a minimal number of mismatches with HTLV-II. Primer pair SK43/44 coupled with the probe designated SK45, provide for the simultaneous detection of the HTLV-I and HTLV-II...
viral genomes (Fig 3). Because of the homology of STLV-I in this region, the investigators would expect that STLV-I could also be amplified and detected using this primer pair-probe combination. These data demonstrate that both HTLV-I and HTLV-II–specific primer pairs, and primer pairs that recognize conserved sequences in both viruses can be designed and successfully used.

Application of PCR to DNA extracted directly from patient specimen. To validate the PCR procedure, DNA from cultures established from seropositive and Southern blot positive adult T-cell leukemia/lymphoma (ATL) patients as well as seronegative, non-risk group individuals were analyzed. All DNAs to date from cultures established from ATL patients were positive (two representative cultures are noted in Table 2); all 20 seronegative samples (from non-risk groups) tested were negative by PCR. All four DNAs isolated directly from PBMCs of seropositive ATL patients scored positive for proviral DNA by PCR. Three of the four samples were also positive by Southern blot, and p19 immunofluorescent assay (IFA) procedures. Having established the sensitivity and specificity of the PCR-based assay, the investigators examined two samples collected from seronegative ATL patients that were negative for HTLV-I by Southern blot analysis. Both samples were positive by PCR analysis and p19 IFA. The reasons for and explanation of this observation are discussed in a previous report. Further, DNA extracted from three seropositive members of a high risk group (one from an endemic area and two intravenous drug abusers) were all positive by PCR thereby demonstrating persistent infection with this retrovirus.

The investigators next examined DNA extracted from multiple tissues of a patient seropositive for HTLV-I and HIV-I. Initially, this patient was diagnosed as suffering from Hodgkin’s disease, but was subsequently found to have a CD4+ lymphocytosis which progressed to a pulmonary lymphoma.39 DNAs isolated from PBLs and various tissues at autopsy were analyzed by PCR initially with HTLV-I specific primers. HTLV-I viral sequences were detected in the lymph nodes (ie, cervical, mediastinal, hylar, and portal nodes), spleen, kidney and brain but not in the liver, testes, or heart (Fig 4, row 1, lanes 1 through 10). In addition, DNA extracted from PBLs and cell lines propagated from PBLs either 2 years or one day before death were positive by the PCR dot-blot procedures (Fig 4, row 1, lanes 11 and 12, respectively). While cell lines and fresh blood samples obtained 2 years before death were positive by Southern blotting and cell culturing, those obtained just before death were negative by both. Furthermore, all tissues obtained postmortem were negative by Southern blot.

Because this patient was seropositive for HIV-I and suffered from toxoplasmosis, DNAs from these tissues were further analyzed with the HIV-1 gag–specific primers and probe. PCR dot-blot analyses indicated the presence of HIV-1 viral sequences in all the organs and tissues tested, except the heart (Fig 4, row 2). DNA extracted from PBMCs of this patient was positive for HIV-1 at the time of death but weakly positive 2 years before death. In separate experiments, if the HTLV-I and HIV-1–specific primer pairs were incorporated into a single PCR reaction, sensitive and specific detection of both viruses was achieved (data not shown). These data begin to define the organs infected by HTLV-I and HIV-1 in a co-infected individual and support persistent infection for extended periods of time with both viruses. Due to the nature of the amplification procedure, the investigators can only conclude that both viral genomes were present in the tissues examined; coincident infection of a single cell could not be delineated.

**DISCUSSION**

HTLV-I has been shown to be associated with ATL; HTLV-II, since isolated from two patients with a rare leukemia, has been implicated in a T-cell variant of hairy cell leukemia. Direct detection of HTLV-I or HTLV-II virus particles in infected individuals, particularly those with chronic lymphocytosis and smoldering leukemia, is difficult because transcriptional dormancy in PBLs and the small
ENZYMATIC AMPLIFICATION OF HTLV-I

Fig 4. Analysis of PCR-amplified DNA extracted from multiple tissues and PBLs. PCR amplification and dot-blot detection were performed as described in Materials and Methods. Columns 1 through 10 represent DNA extracted from tissues collected at autopsy from testicles, TE; kidney, KI; cervical lymph node, CL; mediastinal lymph node, MN; liver, LI; brain, BR; heart, HE; spleen, SP; hylar lymph node, HN; and portal lymph node, PN. Columns 11 and 12 represent DNAs extracted from PBLs collected 2 years and one day, respectively, before death. The primer-probe combination used to provide HTLV-I specific amplification and detection was SK54/55 and SK56; HIV-1 specific amplification and detection was provided by SK38/39 and SK19.

The investigators report here the successful application of a DNA amplification procedure to facilitate the detection of HTLV-I and HTLV-II genomic sequences using portions of the pol gene and X region as targets.

In reconstitution experiments, the amplification procedure as described here has a sensitivity 25 to 250-fold greater than Southern blot analysis. After validating the PCR-based detection of proviral DNA using seropositive ATL patients and seronegative individuals not in a risk group, we demonstrated the presence of viral sequences in two seronegative ATL patients that were negative by Southern blot analysis. Although most ATL patients have antibodies to the proteins encoded by HTLV-I, Shimoyama et al have reported seronegative ATL patients. PCR-based detection of proviral DNA will contribute to determining the extent of seronegative ATLs associated with HTLV-I infection and perhaps assist in better understanding the reasons for seronegativity. In addition, HTLV-I nucleic acid sequences were detected in seropositive, asymptomatic individuals and probably represent those who are at higher risk for the development of HTLV-I-related diseases.

Examination of samples from a patient exposed to HTLV-I and HIV-I indicated the presence of HTLV-I viral sequences in PBMCs, lymph nodes, brain, kidney, and spleen while the testicles, liver, and heart appeared uninfected. These same tissues were all negative by Southern blot analysis. The incorporation of an HIV-I-specific primer into the above amplification reaction likewise indicated that all these tissues except the heart harbored this lentivirus. Through their studies, the investigators therefore suggest that multiple primer pairs can be used in a single DNA amplification reaction to identify multiple viruses. The inability to demonstrate the presence of HTLV-I and HIV-I viral sequences by Southern blot analysis demonstrates the increased sensitivity of the amplification process. The dramatic increase in sensitivity provided by PCR, however, makes even minimal contamination by blood a serious problem and, therefore, caution must be used in setting up such experiments and interpreting the results. Given the increasing reports of coincident infections with the HTLV-I and HIV-1, such as analysis will assist in determining the precise infection(s). In addition, detection of seropositive, virus-positive individuals is expected to play an important role in the identification of individuals at higher relative risk who would benefit most from therapeutic intervention before the onset of disease.

To simplify the detection of multiple viruses, the investigators demonstrated that amplification directed by conserved sequences of the X region of HTLV-I and HTLV-II allowed simultaneous detection. While not necessarily informative for specific patient evaluation, the concomitant detection of these two viruses could simplify the screening of blood donors or blood components for these agents by reducing the number of tests required.

The substantial homology of the HTLV-I and HTLV-II X regions has been postulated to be consistent with the critical role that the TAT protein, encoded by one of the open reading frames of this region, plays in the regulation of viral transcription and replication. An added advantage to the use of sequences conserved between HTLV-I and HTLV-II is the potential to detect a virus or viruses related to, but distinct from, these two viruses. Since the amplification procedure can be modified to facilitate rapid cloning and sequencing, a probe for an uncharacterized virus may be developed. Such a procedure would obviate the need to propagate such a virus initially in tissue culture. Diseases that represent distinct clinicopathologic entities such as tropical spastic paraparesis, Kawasaki syndrome, multiple sclerosis, as well as lymphoblastic and histiocytic lymphomas may be productive targets for such an approach.

ACKNOWLEDGMENT


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Enzymatic amplification of HTLV-I viral sequences from peripheral blood mononuclear cells and infected tissues

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