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Abstract

Newly designed oligonucleotide primers, KI-7 and KI-8 for the human T cell lymphotropic virus type I (HTLV-I) pX gene were synthesized using an automated DNA synthesizer. Previously known HTLV-I-infected cell lines, MT-1 and MT-2, were used as positive controls and HTLV-I-uninfected cell lines, Molt-4, SBC-3, ABC-1, and EBC-1, as negative controls. Peripheral blood mononuclear cells from 17 patients with anti-HTLV-I antibody and 10 healthy individuals without anti-HTLV-I antibody were studied by polymerase chain reaction (PCR) with KI-7 and KI-8. All DNA samples from HTLV-I-infected cell lines and 17 patients with anti-HTLV-I antibodies showed positive signals of the HTLV-I pX gene. None of the DNA samples from HTLV-I-uninfected cell lines or 10 healthy individuals showed positive signals. When serially diluted DNA of MT-2 cells were amplified by 35 cycles of PCR, the detection limit of the pX gene by using the primer pairs was DNA from about 1.5 MT-2 cells. Specificity and detectable capacity of primer pairs, KI-7 and KI-8 were confirmed to be enough to use for the diagnosis of HTLV-I infection.

KEYWORDS: HTLV-1, polymerase chain reaction, oligonucleotide primer, DNA synthesis

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Newly designed oligonucleotide primers, KI-7 and KI-8 for the human T cell lymphotropic virus type I (HTLV-I) pX gene were synthesized using an automated DNA synthesizer. Previously known HTLV-I-infected cell lines, MT-1 and MT-2, were used as positive controls and HTLV-I-uninfected cell lines, Molt-4, SBC-3, ABC-1, and EBC-1, as negative controls. Peripheral blood mononuclear cells from 17 patients with anti-HTLV-I antibody and 10 healthy individuals without anti-HTLV-I antibody were studied by polymerase chain reaction (PCR) with KI-7 and KI-8. All DNA samples from HTLV-I-infected cell lines and 17 patients with anti-HTLV-I antibodies showed positive signals of the HTLV-I pX gene. None of the DNA samples from HTLV-I-uninfected cell lines or 10 healthy individuals showed positive signals. When serially diluted DNA of MT-2 cells were amplified by 35 cycles of PCR, the detection limit of the pX gene by using the primer pairs was DNA from about 1.5 MT-2 cells. Specificity and detectable capacity of primer pairs, KI-7 and KI-8 were confirmed to be enough to use for the diagnosis of HTLV-I infection.

Key words : **HTLV-I, polymerase chain reaction, oligonucleotide primer, DNA synthesis**

Adult T-cell leukemia (ATL) was first described in 1977 by Takatsuki *et al.* in Japan (1). ATL was initially thought to be a distinct entity localized in certain regions of southern Japan. Subsequently ATL has been found in many other areas including the Caribbean basin, northeastern South America, Central Africa and Taiwan (2-7). The epidemiology demonstrated a clear relationship to a type C retrovirus now known as human T-cell lymphotropic virus type I (HTLV-I). HTLV-I was first identified by Gallo *et al.* in T-lymphoblastoid cell line, known as HUT 102, that had been established from a patient with cutaneous T-cell lymphoma (8). In 1980, Miyoshi *et al.* established another cell line, known as MT-1, from a patient with ATL (9). Hinuma *et al.* also found a type C retrovirus in MT-1 (10). These viruses were subsequently shown to be identical (11). Seiki *et al.* isolated the first molecular clone of HTLV-I (12) and reported the complete nucleotide sequence of the proviral genome (13). The indirect immunofluorescent assay (IF) was established in 1981 as the serological test for the diagnosis of HTLV-I infection (14). Thereafter, the particle agglutination test (PA) (15), enzyme linked immunosorbent assay (ELISA) (16), radioimmunoprecipitation assay (RIPA) (17) and Western blot analysis (WB) (18) have been used for the same purpose. The development of suitably rapid and sensitive serological assays for the relatively low titers in individuals infected with HTLV-I has been complicated because of false-positives and false-negatives. Another complication in antibody screening methods is an age-dependent increase in seropositivity (19), suggesting that some individuals may not produce antibodies for a certain period following infection. Since only a small proportion of cells in peripheral blood and lymph nodes are infected with HTLV-I in asymptomatic carriers, the southern blot analysis with high specificity, but low sensitivity is not suitable as a

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clinical genetic screening assay for HTLV-I infection. Thus, the development of appropriately sensitive diagnostic tools is urgently needed. Recently, a nucleic acid amplification method, polymerase chain reaction (PCR), has been used for the diagnosis of genetic materials (20). PCR technique was introduced into the detection of HTLV-I infection (21-23). But, only a few reports described specificity and detection capability of primer pairs specific for the HTLV-I pX gene. We synthesized many sets of primers, but most of them were not suitable for detecting the integrated HTLV-I proviral DNA. Only a set of KI-7 and KI-8 was suitable for use. In this study, the newly designed primer pairs specific for the HTLV-I pX gene were synthesized and evaluated for detection capability and specificity to HTLV-I proviral DNA.

Materials and Methods

Cells. The previously described HTLV-I-infected T cell lines, MT-1 and MT-2, were used as positive controls. The MT-1 (9) and the MT-2 cell lines (24) were established from cord lymphocytes that had been cocultivated with leukemia cells from patients with ATL. Molt-4 which is well-known human T cell line and human lung cancer cell lines, SBC-3 (25), ABC-1, and EBC-1 (26, 27), were used as negative controls. SBC-3, ABC-1, and EBC-1 were established from patients with small cell carcinoma, adenocarcinoma, and squamous cell carcinoma of the lung, respectively. All of these cell lines have been cultured in RPMI-1640 medium (Gibco Laboratories, NY, USA) supplemented with 10 % fetal calf serum (Gibco).

Positive and negative reference samples. Venous blood samples were obtained from 17 patients known to have positive anti-HTLV-I antibodies, and 10 healthy individuals at Okayama University Medical School and other cooperative hospitals in Japan. Sera were subjected to screening by indirect immunofluorescent assay using MT-1 and MT-2 cells. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation (Histopaque; Sigma Chemical Company, St Louis, MO, USA) at $360 \times g$ for 20 min from 20 ml heparinized venous blood.

Extraction of cellular DNA. PBMC and previously described cell lines were washed twice with phosphate buffer saline and collected after centrifugation at 700 and $270 \times g$ for 10 min, respectively. Each cell pellet was suspended in 1 ml of 10 mM Tris · HCl, pH 7.5/10 mM EDTA. After adjusting the cell number, 1.0×10^7 cells were treated for 15 min at 70 °C with 1 ml of cell lysis buffer, consisting of 10 mM Tris · HCl, pH 7.5/10 mM EDTA, 1 % sodium dodecyl sulfate (SDS) and protease K (Sigma) at 500 μg/ml. Protease K was added once more to a final concentration of 500 μg/ml and the solution was incubated at 37 °C overnight. Total cellular DNA was extracted according to the phenol-chloroform method (28). The DNA was precipitated by adding cold (−20 °C) ethanol. The strings of DNA were hooked out and washed with cold 70 % and 100 % ethanol. The purified DNA was dissolved at 1 μg/μl in 10 mM Tris · HCl (pH 8.0)/1 mM EDTA (TE) and stocked at 4 °C.

Southern blot analysis. The DNA equivalent to 1.5×10^6 cells (10 μg) was digested at 37 °C for 8 h or more with EcoRI (Nippon Gene Co., Toyama, Japan). The digested DNA was separated by 0.8 % agarose gel electrophoresis in Tris-borate (TBE) buffer (100 mM Tris · base/121 mM boric acid/2 mM disodium EDTA, pH 8.0). After soaking in 0.25 M HCl alkaline denaturation with gentle agitation in 0.5 M NaOH/1.5 M NaCl for 45 min and neutralization with gentle agitation in 0.5 M Tris · HCl/0.5 mM disodium EDTA/1.5 M NaCl, pH 7.2 for 1 h, DNA was transferred to nylon membranes, Hybond N (Amersham Corporation, Arlington Heights, IL, USA) using a transfer buffer, 3.0 M NaCl/0.3 M sodium citrate (20 × standard saline citrate ; SSC) by a capillary transfer method (29). After baking at 80 °C for 2 h, membranes were hybridized with ^{32}P-labeled 8.25 Kb HTLV-I DNA probe (30) (Oncor, Gaithersburg, MD, USA).

Synthesis of HTLV-I pX specific primer pairs. Oligonucleotide primer pairs were synthesized based on the published DNA sequence (13) corresponding to the pX region of HTLV-I by the phosphoramidite method (31) using an automated DNA synthesizer 380 B (Applied Biosystems Inc., Foster City, CA, USA). The 5'-sense primer sequence named KI-7 was located in bases 7053-7077 and the 3'-antisense primer sequence named KI-8 was in 7423-7399 (Fig. 1). After synthesis, oligonucleotide primers were deprotected with 28 % ammonium at 55 °C overnight and dried by vacuum centrifugation. Primers were dissolved in 10 mM Tris · HCl (pH 8.0)/1 mM EDTA at a final concentration of 20 μM.

Polymerase chain reaction. Specimens and all reagents were handled with disposable tips and different pipettes from those used only for the PCR product and treated in a room other than the one in which PCR was perfomed in order to prevent carryover of PCR product or contamination of HTLV-I positive DNA. The reaction mixture consisted of 1 μM each oligonucleotide primer, 1 μg of sample DNA, 2.5 units of Thermus aquaticus (Taq) DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA), 50 mM KCl, 10 mM Tris · HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001 % (w/v) gelatin, and 0.2 mM each deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). Sample DNA was denatured at 95 °C for 10 min before PCR. The reaction mixture was mixed in a sterile 0.5 ml microfuge tube and covered with heavy mineral oil (Sigma). The microfuges were incubated by step cycle at 95 °C for 1.5 min, at 37 °C for 1 min, and at 72 °C for 2 min (10 min in last cycle) for a total of 35 cycles, with a programmable heat block of PHC-1 (Techne Ltd, Cambridge, England). PCR products were extracted with 4 % isoamyl alcohol supplemented chloroform and precipitated with −20 °C ethanol. After dissolved in 10 mM Tris · HCl (pH 8.0)/1 mM EDTA,

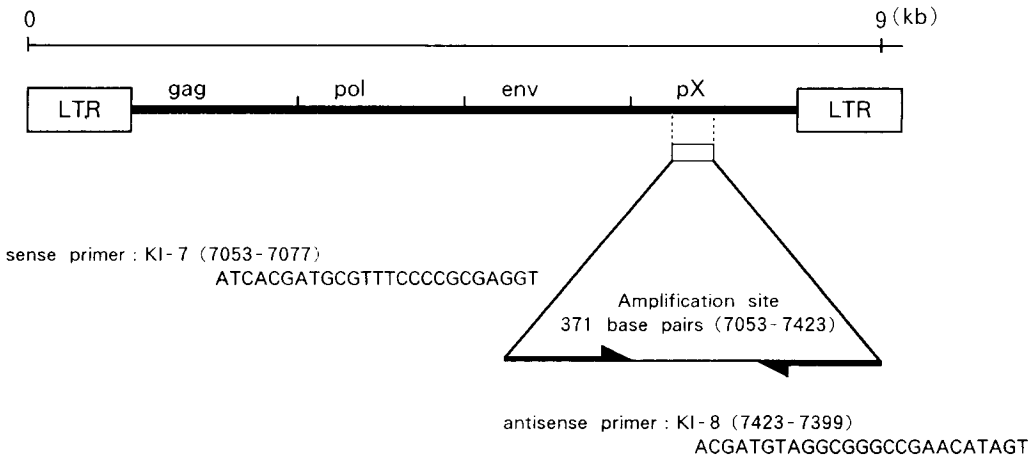

Fig. 1 Schematic diagram of oligonucleotide primers, KI-7 and KI-8. The 5' and 3'-primers, KI-7 and KI-8 are homologous to nucleotides 7053-7077 and 7423-7399, respectively, of HTLV-I pX gene.

amplified DNA was size-fractionated by 1.2 % agarose gel electrophoresis. After alkaline denaturation for 20 min and neutralization for 20 min, amplified DNA was transferred to Hybond N by vacuum transfer method using VacuGene (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) for 1 h with 20 × SSC. After baking at 80 °C for 2 h, the membrane was hybridized with ^{32}P-labeled HTLV-I probe.

Hybridization with HTLV-I specific DNA probe. HTLV-I specific DNA probe (Oncor Inc., Gaithersburg, MD, USA) was ^{32}P-labeled by the primer extension method (32, 33) using a random primed DNA labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). One hundred ng of HTLV-I specific DNA was used according to the manufacturer's recommendation. Specific activity of α-^{32}P-dCTP (ICN Radiochemicals, Irvine, CA, USA) was 3,000 Ci/mmol. The ^{32}P-labeled probe was then ethanol-precipitated with 200 μg of denatured, fragmented salmon testis DNA and redissolved in 200 μl of 10 mM Tris · HCl (pH 8.0)/1 mM EDTA. After prehybridization for 2 h at 65 °C with 10 ml of hybri-buffer containing 6 × SSC, 0.5 % SDS, and 5 × Denhardt's reagent (50 ×; 5 μg of Ficoll, 5 μg of polyvinylpyrrolidone, 5 μg of bovine serum albumin, and H_2O to 500 ml), the membrane was hybridized over 12 h at 65 °C with ^{32}P-labeled probe and 10 ml of hybri-buffer. The filter was washed at 65 °C by shaking at 50 rpm 4 times with washing buffer containing 2 × SSC and 0.1 % SDS and twice with 0.1 × SSC and 0.1 % SDS.

Autoradiography. To increase the efficiency of autoradiography, intensifying screens, Cronex Lightening Plus (E. I. du Pont de Nemours & Company Inc., Wilmington, DE, USA) were used, 2 per film cassette. The filter was wrapped in Saranwrap to prevent contamination of intensifying screens and film holders and exposed to Fuji new RX film (Fuji Photo Film Co., Tokyo, Japan) for 3 to 7 days at −70 °C. The X-ray film was developed by hand for 2-5 min in X-ray developer, Rendor (Fuji Photo Film), and stopped in 3 % acetic acid bath for 1 min. The film was fixed for 5 min in Renfix (Fuji Photo Film), washed for 15 min in running water, and then evaluated.

Results

Southern blot analysis of cell lines. The result of Southern blot analysis for each cell is shown in Fig. 2.

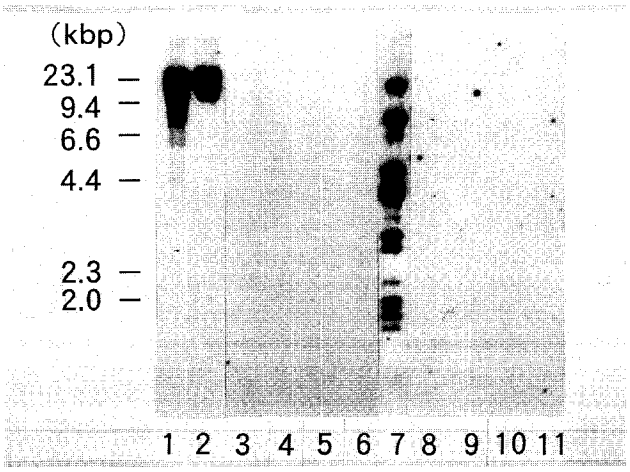

Fig. 2 Results of Southern blot analysis of six cell lines. Lanes 1 to 6 contain DNA fragments digested by restriction endonuclease EcoRI from MT-2, MT-1, Molt-4, SBC-3, ABC-1, and EBC-1, respectively. Lanes 7 to 11 contain DNA fragments digested by restriction endonuclease PstI from MT-2, Molt-4, SBC-3, ABC-1, and EBC-1, respectively.

HTLV-I proviral DNA was monoclonally integrated in MT-1 and MT-2 cell lines and not detected in Molt-4, SBC-3, ABC-1, or EBC-1 cell lines. Therefore, MT-1 and MT-2 can be used as true positive controls, and other cell lines used as true negative controls.

PCR of DNA from cell lines. The result of PCR for each cell line is shown in Fig. 3. In PCR of DNA from MT-1 and MT-2 cell lines, markedly amplified and hybridized products of expected size are shown. In DNA from Molt-4, SBC-3, ABC-1, and EBC-1, no hybridized signal was observed.

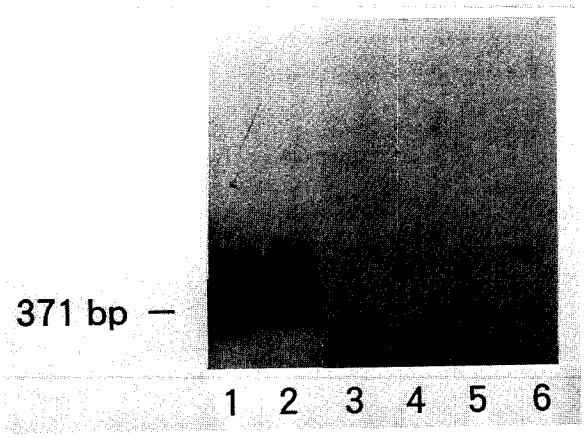

Fig. 3 Results of polymerase chain reaction (PCR) of six cell lines. Lanes 1 to 6 contain DNA from MT-1, MT-2, Molt-4, SBC-3, ABC-1 and EBC-1, respectively.

PCR of DNA from PBMC in anti-HTLV-I antibody positive patients. The DNA from 17 patients with anti-HTLV-I antibodies were examined for HTLV-I integration by PCR targeted to the pX region. In all of the DNA samples from 17 patients, positive signals were shown in 371 base pairs at various intensities (Fig. 4).

PCR of DNA from PBMC in anti-HTLV-I antibody negative individuals. The DNA from 10 healthy adults without anti-HTLV-I antibodies were tested by PCR, none of which showed positive results (Fig. 5).

Detectable capacity of PCR using KI-7 and KI-8 primer pairs. MT-2 cells were used as the source of HTLV-I proviral DNA to estimate the detectable capacity of PCR. The DNA extracted from MT-2 cell was serially diluted and adjusted to 1 μg (equivalent to 1.5×10^5 cells), 100 ng (1.5×10^4), 10 ng (1.5×10^3), 1 ng (1.5×10^2), 100 pg (1.5×10), and 10 pg (1.5). Each volume of DNA was applied to 35 cycles of PCR with KI-7 and KI-8 primer pairs. Amplified DNA was analysed according to

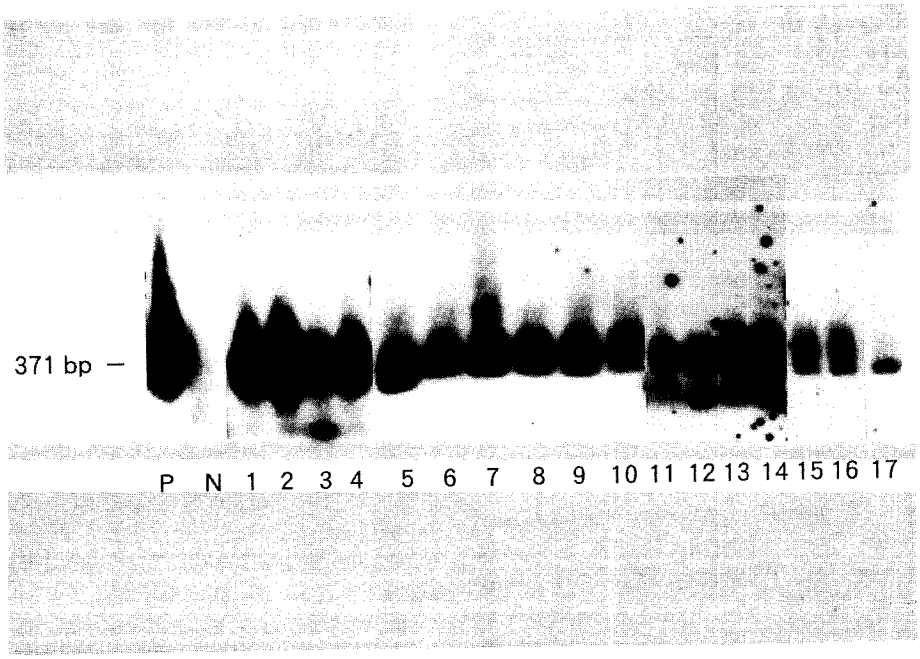

Fig. 4 Results of PCR in patients with anti-HTLV-I antibody. Lanes P and N contain amplified DNA from MT-2 as a positive control and Molt-4 as a negative control. Lanes 1 to 17 contain DNA from 17 patients with anti-HTLV-I antibody. PCR: See Fig. 3.

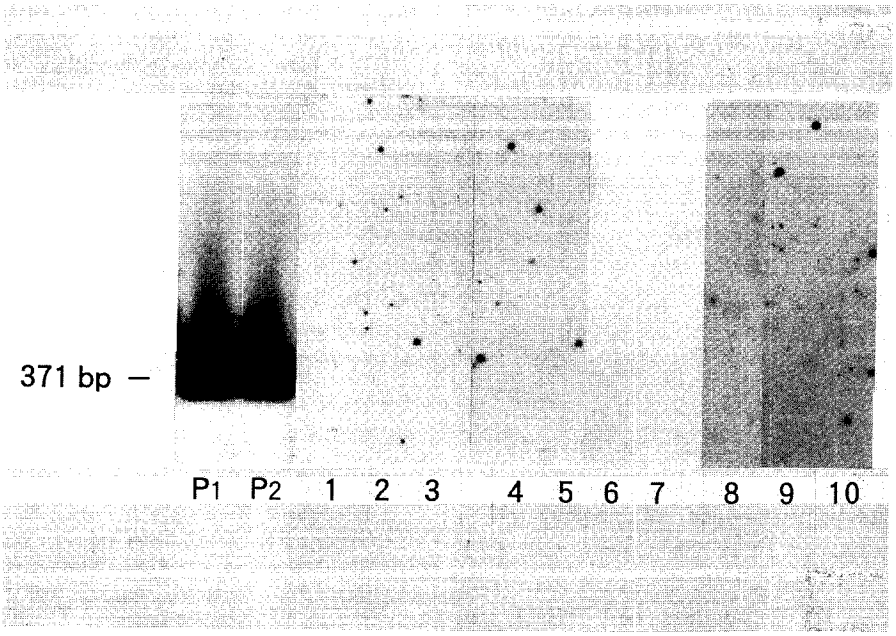

Fig. 5 Results of PCR in healthy individuals without anti -HTLV-I antibody. Lanes P_1 and P_2 contain DNA from MT-1 and MT-2 as a positive control. Lanes 1 to 10 contain DNA from healthy individuals. PCR: See Fig. 3.

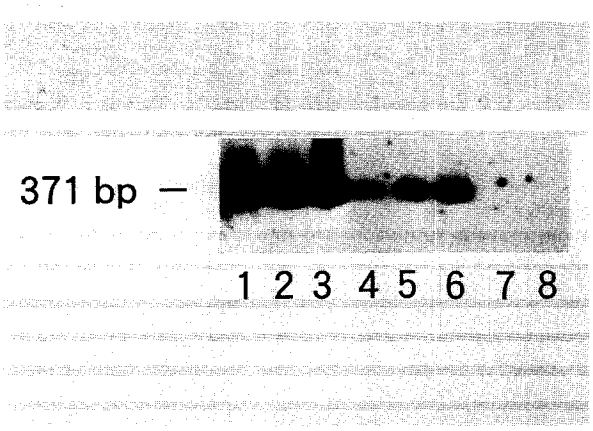

Fig. 6 Results of PCR of serially diluted MT-2 DNA. Lanes 1 to 6 contain 1 μg (equivalent to 1.5×10^5 cells), 100 ng (1.5×10^4), 10 ng (1.5×10^3), 1 ng (1.5×10^2), 100 pg (1.5×10), and 10 pg (1.5), respectively, of MT-2 cells. Lane 7 contains DNA from Molt-4. Lane 8 contain all of reaction mixture without sample DNA. PCR: See Fig. 3.

Southern hybridization (Fig. 6). Positive signal was shown in 10 pg of template DNA. The detectable capacity of PCR using KI-7 and KI-8 primer pairs was equivalent to 1.5 cells of MT-2.

Discussion

HTLV-I has been shown to be associated with ATL (8, 14) and HTLV-I associated myelopathy (HAM) (34). Direct detection of HTLV-I virus particles in infected individuals, particularly in healthy carriers and those with chronic lymphocytosis and smoldering ATL, is difficult because of transcriptional dormancy in PBMCs and the small number of infected circulating cells. Since Saiki *et al.* (20) reported the gene amplification method using PCR, only small volumes of the target DNA have been able to be amplified to the detectable level by PCR. Since this method of PCR was introduced into the diagnosis of HTLV-I infection (21, 22), HTLV-I infection has been diagnosed directly in patients with undetectable levels of anti-HTLV-I antibody by proving the existence of the HTLV-I proviral genome in PBMCs by DNA amplification procedure (35). In this report, newly designed HTLV-I pX primer pairs were synthesized and successfully applied to a DNA amplification method to

facilitate the detection of HTLV-I genomic sequences.

In experiments using established cell lines, HTLV-I infected cell lines, MT-1 and MT-2, were clearly distinguished from other cell lines as SBC-3, ABC-1, and EBC-1 by classical Southern blot analysis. PCR with newly synthesized primers, KI-7 and KI-8, proved to be sensitive and could specifically amplify the HTLV-I proviral DNA.

Examination of samples from patients with anti-HTLV-I antibodies and healthy individuals without anti-HTLV-I antibodies showed good correlation between serological positivity and PCR result. All 17 seropositive patients were found to be positive by genetic diagnosis using PCR, while all of 10 seronegative individuals were negative. PCR results accord perfectly with the results of serological study.

The serial dilution experiment of positive DNA showed the sensitivity of PCR using KI-7 and KI-8 primer pairs to have a sensitivity to detect pX gene from DNA of 1.5 cells of MT-2. Southern blot analysis can detect between 1% and 10% of the cells in a mixed population if they contain a rearranged (36) or novel sequence (37). Since 10μg of DNA (the equivalent of approximately 1.5×10^6 cells) is typically used for Southern blot analysis, a specific DNA sequence derived from 1.5×10^4 to 1.5×10^5 cells in 1.5×10^6 cells can be discerned. Since PCR in this study needs one tenth as much total DNA as Southern blot analysis and can detect specific DNA sequence from 1.5 cells, it is concluded that the PCR described here is 10^3 to 10^4 times more sensitive than Southern blot analysis. In conclusion, the newly designed HTLV-I pX primers designated as KI-7 and KI-8 were specific and sensitive enough to detect the presence of HTLV-I viral sequences. PCR using these primers can directly detect the minimal infection of HTLV-I and identify the presence of viral sequences in PBMCs, lymph nodes, broncho-alveolar lavage cells and other materials.

As even minimal contamination in the assay process will cause a severe problem due to the extremely high sensitivity, caution must be taken in setting up such experiments and in interpreting the results. However, the dramatic increase in sensitivity provided by PCR may contribute to determining the precise HTLV-I infection in ATL and other HTLV-I related diseases.

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