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Detection of HTLV-I Genome in Seronegative Infants Born to HTLV-I Seropositive Mothers by Polymerase Chain Reaction

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We applied the polymerase chain reaction (PCR) method to detect *gag*, *env* and *pX* sequences of human T cell leukemia virus type I (HTLV-I) provirus in peripheral blood lymphocytes of seronegative infants born to HTLV-I seropositive mothers. Out of 22, five subjects were found to contain the HTLV-I provirus genome. Two of the five cases were judged to be negative for not only anti-HTLV-I antibodies but also the viral antigens on cultivated lymphocytes by the conventional antibody/antigen detection methods. These results indicate that PCR is of great use as a simple and highly sensitive method detect HTLV-I infection.

Key words: Polymerase chain reaction — HTLV-I — Vertical infection

Human T cell leukemia virus type I (HTLV-I) is an etiological agent of adult T cell leukemia (ATL),[1-3] and is thought to be mainly transmitted from mother to child via HTLV-I-infected lymphocytes present in the mother's milk. Consequently, interruption of breast feeding has been recommended in order to prevent vertical infection.[1-7] It is inadequate to evaluate vertical infection by measuring the HTLV-I-associated antibodies in infants, because maternally transmitted IgG exists in the newborn child for 6–9 months.[5] Furthermore when maternally transmitted IgG disappeared, antibodies specific for HTLV-I were detected in only half of the infants who showed the presence of the HTLV-I-associated antigen.[5] Thus, in infants, detection of the HTLV-I-associated antigens in peripheral blood lymphocytes (PBL) has usually been used as a method to confirm the HTLV-I infection. The conventional method to detect the HTLV-I antigens in PBL, however requires 4 weeks' culture period[5,7-9] and skillful technique, and can give a false-negative result in HTLV-I-infected cases in which lymphocytes show no detectable expression of the viral antigens. The most reliable method to confirm the HTLV-I infection is to detect the provirus genome of HTLV-I. We therefore applied a combination of the polymerase chain reaction (PCR) and Southern blot hybridization to detect the HTLV-I provirus genome as a simple and confirmatory method. The PCR is particularly useful in amplification of target sequences which are present in samples at a very low percentage, as in healthy carriers of HTLV-I.

We examined 22 seronegative cases born to seropositive mothers, of which 9 had received breast milk feeding and 13 bottle feeding, and detected HTLV-I sequence in 5 cases (Table I), including 2 cases that could not be judged to be positive by the conventional antigen staining method.

After isolation of mononuclear cells from heparinized peripheral blood by using the Ficoll-Conray method, high-molecular-weight cellular DNA was extracted as described.[10] To detect sequences of *gag*, *env* and *pX* in the HTLV-I genome, those sequences were first amplified by the PCR with the chemically synthesized oligonucleotide primers shown in Table II. The PCR was carried out according to the method of Saiki *et al.*[11] and Fujii *et al.*[12] PCR products were subjected to dot blot hybridization or Southern blot hybridization to detect specific target sequences amplified. The sizes of target sequences of *gag*, *env* and *pX* are 183 bp, 180 bp and 179 bp, respectively. MT-1 cells, an HTLV-I-infected T cell line, were used as a positive control for the HTLV-I genome.

The *gag* sequence was detected by dot blot assay after 25 cycles of the PCR with DNA samples not only from the HTLV-I antigen-positive cases (Nos. 1, 2 and 3) but also from the HTLV-I antigen-negative cases (Nos. 4 and 5), whereas genomic DNA samples from another 17 cases (Fig. 1A, lanes 6–22) and an HTLV-I-unrelated case (Fig. 1, lane 24) did not show any hybridization signal specific for the *gag* sequence. These results were confirmed by the combination of the PCR and the oligomer restriction method[12] in which the amplified *gag*

Table I. List of Infants Providing Genomic DNA

Case number	Age (yr)	Feeding	HTLV-I associated antigen[a]		Anti-HTLV-I antibody[b]
			Gin 14 (anti p19)	F 10 (anti gp21)	
1	3	breast	+	+	−
2	3	breast	+	+	−
3	3	breast	+	+	−
4	3	breast	−	−	−
5	2	bottle	−	−	−
6	3	breast	−	−	−
7	3	breast	−	−	−
8	3	breast	−	−	−
9	4	breast	−	−	−
10	4	breast	−	−	−
11	1	bottle	−	−	−
12	1	bottle	−	−	−
13	1	bottle	−	−	−
14	1	bottle	−	−	−
15	1	bottle	−	−	−
16	2	bottle	−	−	−
17	2	bottle	−	−	−
18	2	bottle	−	−	−
19	2	bottle	−	−	−
20	2	bottle	−	−	−
21	3	bottle	−	−	−
22	8	bottle	−	−	−

a) Mononuclear cells separated by Ficoll-Conray gradient centrifugation from peripheral blood were suspended in RPMI-1640 medium supplemented with 20% fetal calf serum and 10% delectinized TCGF and cultured for 4 weeks. The cells were then harvested, smeared on slides, fixed in acetone, and treated with mouse monoclonal antibodies to p19 and gp21 of HTLV-I.[8,9] After incubation at 37°C for 30 min, the slides were washed with phosphate-buffered saline and treated with FITC-conjugated anti-mouse IgG. Samples were considered to be positive when more than 0.1% of cells were positive for fluorescence under microscopy.
b) Anti-HTLV-I antibody titer was determined by an indirect immunofluorescence technique as described.[1] Sera diluted at 1 to 5 were assayed with acetone-fixed MT-1 cells.

Table II. Sequences of Synthetic Oligonucleotide Primers and Probes[a]

Primer or probe	Sequence	Length of amplified product (bp)
primer *gag*	AGCAGTTTGACCCCACTGCCAAAGACCTCCAAGACCTCCTGCAGTACCTTT	
primer *gag'*	GTTGTTGTGGATTGTTGGCT	183
probe *gag*	GAAACCCGAGGTATTACAGGTTATAACCC *Ava* I	
primer *env*	CTGCCCCCTCATCTTCGGTGATTACAGCCC	
primer *env'*	GAGTAACTTACTAGGTTAGGGCAGGGGGGC	180
probe *env*	ATTGGAGTCTCCTCATACCACTCTAAACCC	(post *Hinf* I digestion 126)
primer *pX*	ATGCGCAAATACTCCCCCTTCCGAAATGG	
primer *pX'*	AGGAGGGGCCAGGTGATGGGGGGGGAAAG	179
probe *pX*	CCCGGACTCCGGCCCCAAAACCTGTACACC	(post *Hinf* I digestion 93)

a) All oligodeoxynucleotides were synthesized by the solid-phase method with the use of an automatic DNA synthesizer (Applied Biosystems). The restriction endonuclease *Ava* I site used for the PCR oligomer restriction assay is indicated.

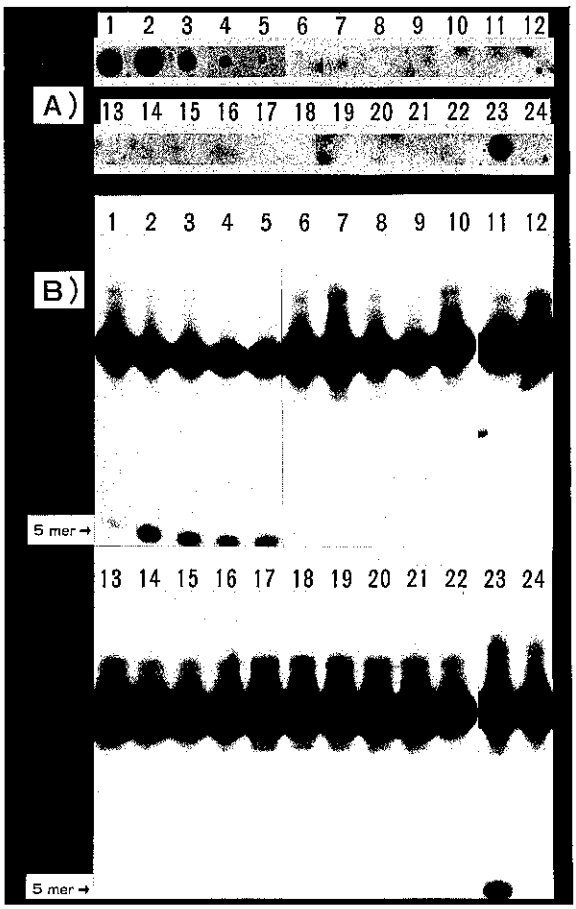

Fig. 1. Detection of the *gag* site of HTLV-I after PCR amplification. A) Dot blot hybridization of the *gag* site of HTLV-I. Genomic DNA was extracted from cells of the subjects (Nos. 1–22) listed in Table I, from MT-1 cells (No. 23) and from an HTLV-I non infected subject (No. 24). DNA (1 μg) was sujected to 25 cycles of PCR amplification in 100 μl of PCR buffer (10 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.2% gelatin, dNTPs at 200 μM, and *gag* and *gag'* primers at 1 μM). Primer extension was initiated by addition of 2.5 units of Taq polymerase (Stratagene, California) and repeatedly carried out by serial cycles of denaturation (2 min at 95°C), annealing (2 min at 40°C) and synthesis (2 min at 70°C). After 10 cycles of amplification, 2.5 units of Taq polymerase was newly added. DNA in aliquots (10 μl) of the amplified mixture was blotted onto nitrocellulose filters and hybridized with the ^{32}P end-labeled *gag* probe as described.[12] Numbers of spots correspond to those of the samples shown in Table I. B) Detection of the HTLV-I *gag* sequence by the PCR oligomer restriction method. DNA in an aliquot (10 μl) of the amplified mixture was heated at 95°C for 10 min, and then mixed with 5'-end-labeled *gag* probe, and digested with *Ava* I restriction endonuclease at 55°C for 1 h. After that, the sample DNA was electrophoresed in 20% polyacrylamide gel. If the *gag* site is amplified the labeled *gag* probe could hybridize to the site and be cleaved with the restriction enzyme *Ava* I, resulting in generation of a 5 mer oligonucleotide band on the autoradiogram. Lane numbers correspond to sample numbers as in Fig. 1A.

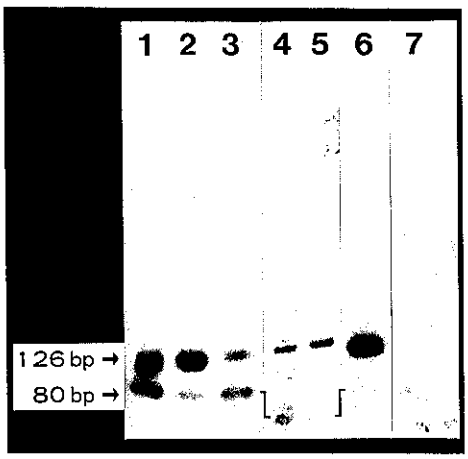

Fig. 2. Southern blot assay of the PCR-amplified HTLV-I *env* site. The DNA samples were amplified with the *env* and *env'* primers by the PCR method as described in Fig. 1. DNA samples in aliquots (10 μl) of the reaction mixture were treated with *Hinf* I, electrophoresed on a 6% polyacrylamide gel and transferred to nitrocellulose filters. The filters were hybridized with the ^{32}P 5'-end-labeled *env* probe. Lane numbers 1–5 are the same as in Fig. 1, lane number 6 is MT-1 cells and lane number 7 is the HTLV-I non infected subject.

sequences were hybridized with the 5'-end-labeled *gag* specific probe containing the *Ava* I site (Table II) and the labeled fragment (5 mer) was detected after *Ava* I digestion, electrophoresis and autoradiography. As shown in Fig. 1B, 5 mer ^{32}P-labeled fragments were seen with the amplified samples which were positive in the dot blot hybridization experiment, but no 5 mer band was seen from the samples which were negative in the dot blot hybridization experiment (Fig. 1B, lanes 6–22) or the HTLV-I-unrelated case (Fig. 1B, lane 24).

To examine the *env* specific sequence, the PCR products were digested with *Hinf* I and subjected to Southern blot hybridization (Fig. 2). If the *env* sequence between primers specific for *env* is amplified by the PCR, *Hinf* I digestion could generate 126-bp and 54-bp fragments from the 180-bp amplified fragment. The oligonucleotide probe specific for the *env* sequence detected the 126-bp band in lanes with samples Nos. 1, 2, 3, 4 and 5 (Fig. 2, lanes 1–5) as well as MT-1 (Fig. 2, lane 6). No *env* specific band was detected with a sample from another case (data not shown) and an HTLV-I-unrelated case (Fig. 2, lane 7), although a nonspecific band with a length of 80 bp was noted in all cases tested. As the nucleotide sequences at the 3'-ends of the two primers for the *env* sequence are complementary (5'-GCCC3'/ 3'CGGG-5'), the 80 bp product detected in Fig. 2 may be

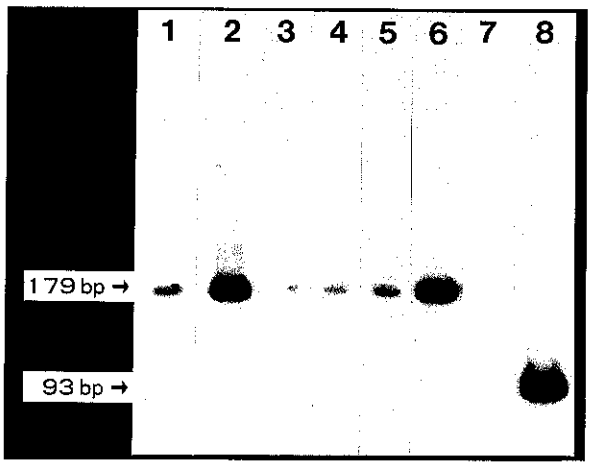

Fig. 3. Southern blot assay of the PCR-amplified HTLV-I *pX* site. The *pX* sites were amplified with the *pX* and *pX'* primers and analyzed by Southern blotting as described in Figs. 1 and 2. Lane numbers are the same as in Fig. 2. Lane 8 is MT-1 treated with *Hinf* I.

the so-called "primer-dimer." Although we re-examined the PCR amplification of *env* at a higher annealing temperature of 55°C in order to prevent primer-dimer formation, the 80 bp band did not disappear.

Similarly the *pX* sequence was amplified and detected (Fig. 3). In addition to MT-1 and the HTLV-I antigen-positive cases (Nos. 1, 2 and 3), the amplified samples from the HTLV-I antigen-negative cases (Nos. 4 and 5) gave a clear positive band hybridized with the *pX* specific probe. The specificity of the hybridized band was demonstrated by the change in size of the band to 93 bp from 179 bp after digestion with *Hinf* I, whose site is present in the amplified target sequence. No band was seen with the cases in Fig. 1A, B, lanes 6–22 (data not shown) and the HTLV-I-unrelated case (Fig. 3, lane 7). When the number of amplification cycles was increased from 25 to 40, the *pX* sequence was no longer detected from HTLV-I genome-negative cases (Table I, cases 6–22).

These results clearly demonstrate that there are cases where HTLV-I is transmitted, possibly vertically, although it is not detected by the conventional methods to identify the viral antigens and antibodies specific for HTLV-I. The lower sensitivity of the antigen staining method may be attributable to little or no expression of the HTLV-I-associated antigens in PBL of some infants, such as cases Nos. 4 and 5 presented here. The PCR method could be of great use in detection of the HTLV-I provirus genome, in particular in examination of infants born to HTLV-I-carrying mothers as shown here, and in examination of cases in which infection with HTLV-I is not confirmed by the conventional antigen and/or antibody detection methods. It is considered to be problematic whether cases with slight contamination of HTLV-I are likely to be pseudo-positive. We recollected peripheral blood lymphocytes from 5 cases in Table I, and detected HTLV-I provirus by the PCR in all 5 cases, so we judged these 5 cases to be HTLV-I provirus-positive. The reason why antibodies against HTLV-I could not be detected in the five cases is not known at present. The following possibilities can be considered: (1) tolerance in newborn, (2) existence of an immunosuppressive gene(s) against HTLV-I antigen related to HLA antigen, and (3) depressed antibody production caused by the immaturity of B cell response in the infantile stage. Loche and Mach reported HIV-infected seronegative cases using the PCR method,[13] so the mechanism by which antibodies specific for retroviruses fail to be induced is considered to be a worthwhile topic for further studies.

It has been reported that HTLV-I is mainly transmitted from mother to child via breast milk on the basis of the conventional antigen detection method.[4-7] We must reaffirm this phenomenon by using the PCR method, which has the advantage of higher sensitivity of detection and independency of expression and replication of viruses. There may be another transmission route(s) between mothers and neonates, e.g. intrauterine infection, because No. 5 case received bottled milk. We are now studying the incidence of vertical transmission of HTLV-I and intrauterine infection.

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