No Detection of HTLV-I DNA in Punch Skin Biopsies from Patients with Cutaneous T-Cell Lymphoma by the Polymerase Chain Reaction

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In this study we have tried to take advantage of the high genetic homology between conserved regions of human T-cell lymphotropic virus (HTLV) types I and II, hoping that a possible retrovirus in patients with cutaneous T-cell lymphoma (CTCL) would have regions with homology to types I/II. DNA was extracted from punch skin biopsies from 21 patients and subjected to the polymerase chain reaction (PCR), using primer sets designed to match conserved regions in the HTLV-I/II genome. The PCR products were subjected to agarose gel electrophoresis with subsequent Southern blotting and hybridization to an HTLV-I probe. No bands of exogenous origin were seen on the agarose gel or by hybridization.

If a retrovirus is present in the skin in CTCL patients, it is either not related to HTLV-I/II, present at a copy number below the PCR detection limit, or has been cleared from the skin before the clinical symptoms appear. *J Invest Dermatol 98:417–420, 1992*

Infiltration of neoplastic T cells in apparent primary skin lesions is clinically encompassed by the term cutaneous T-cell lymphoma (CTCL). This term can be subdivided according to the clinical presentation into parapsoriasis en plaque, mycosis fungoides, and Sézary syndrome. The etiology of this disease entity still remains unresolved, but a human retrovirus has been a candidate ever since the discovery of the first human retrovirus [1]. This retrovirus—human T-cell lymphotropic virus type I (HTLV-I)—was first isolated from a patient thought to have CTCL. This disease was later rediagnosed as adult T-cell leukemia lymphoma (ATLL), stressing the clinical similarity between late-stage CTCL and ATLL. Subsequently, it was shown that 11% of CTCL patients in Scandinavia and the northern parts of West Germany had low-titered antibodies against HTLV-I antigens [2]. Later, a human retrovirus, distinguishable from the known human retroviruses and designated HTLV-V, has been isolated from several Italian patients with mycosis fungoides [3]. This virus has not yet been characterized, and its association with CTCL has so far only been shown by this group. However, the data taken as a whole suggests the presence of a so-far uncharacterized human retrovirus in at least some patients with CTCL.

Detection of retrovirus by electron microscopy requires active virus production, and detection of an integrated unknown retrovirus by in situ hybridization or low stringency cross-hybridization with a known retroviral DNA probe (e.g., HTLV-I) requires a relatively rich source of proviral DNA.

A DNA amplification technique, the polymerase chain reaction (PCR), as described under *Materials and Methods,* may be able to overcome the problems mentioned above. The hallmark of this technique is a thermostable DNA polymerase, the TAQ polymerase, which allows automatic cycling between DNA-denaturing conditions and DNA polymerization of the denatured single-stranded templates directed by specific primers [4,5]. This powerful technique has been used to show the HLA-type of single human hairs [6], to show as few as five leukemic cells in residual leukemia [7], to amplify specific DNA sequences from paraffin-embedded tissue [8], or to detect single-template HTLV-I DNA [9].

MATERIALS AND METHODS

DNA Skin punch biopsies were obtained from skin lesions from 21 patients with CTCL (four patients with parapsoriasis en plaque, 11 patients with mycosis fungoides, and six patients with Sézary syndrome). Genomic DNA was extracted by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation as described by Davies [10], and subsequently resuspended in water or low salt buffer. One to three micrograms of this genomic DNA was subjected to PCR reactions.

Primers The primers were designed to match highly HTLV-I/II conserved regions in the HTLV-I genome, based on the idea due to the evidence so far accumulated that an unknown, but related, CTCL retrovirus might be genetically homologous to those HTLV-I regions, which are highly conserved between HTLV-I and HTLV-II. In each primer set, the two primers were designed to

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Abbreviations:

ATLL: adult T-cell leukemia-lymphoma
CTCL: cutaneous T-cell lymphoma
dNTP: deoxyribonucleotide triphosphate
ELISA: enzyme-linked immunosorbent assay
HLA: human leukocyte antigen
HTLV: human T-cell lymphotropic virus
Kb: kilobase
PCR: polymerase chain reaction
SDS: sodium dodecyl sulfate
SSC: standard saline citrate
TAQ: thermus aquaticus

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Table I. Amplification Primers Used in the Polymerase Chain Reaction

Primer	Nucleotide Sequence (5′ → 3′)	
1:	TGT AAC TAC GGC CCC GGC AC	
1R:	GTG CCG GGG CCG TAG TTA CA	
2:	GTT CCT TTG GAG ACC CAC TG	
3:	CAG TGG GTC TCC AAA GGA AC	
4:	CCT TGC CAG ATG TGG TTA GG	
5:	CCA GGG AAT AAC CCA GTA TTC CC	
353:	GAC GCC TTT TTC CAA ATC CCC	
104R:	CGA GGC ATC TGA TGT TGT G	
108:	CCC AAA GAG CCC ATC TTA CGT TCC	
108R:	CAG TCC CGA CTC CAG TGT CCT	
109:	GGC TTG TCT CCG CCC TAG C	
109R:	TCC CAG TTT AGT CCC CAG CC	
110R:	GCG AGC CAC CTC CTG AAC TGT C	
OL3:	CAC CTG GGA CCC CAT CGA TGG ACG CG	
OL4:	GTA AGG ACC TTG AGG GTC	
PC04:	CAA CTT CAT CCA CGT TCA CC	(human β-globin gene)
GH20:	GAA GAG CCA AGG ACA GGT AC	(human β-globin gene)

Table II. Expected Size of DNA Fragments Amplified with Primer Sets, and Position in the HTLV-I Genome

Primer Set	Position in HTLV-I Genome	Expected Size in Base HTLV-I/HTLV-II
108–108R	1703–1932 (GAG)	229
5–1R	2693–2955 (POL)	262
5–2	2696–3345 (POL)	649
353–2	2867–3345 (POL)	478
1–2	2936–3345 (POL)	409
3–104R	3326–4461 (POL)	1135/1138
3–4	3326–4506 (POL)	1180/1183
109–109R	6157–6496 (ENV)	339
OL3–OL4	7480–7591 (TAX)	111
OL3–110R	7480–8659 (TAX/LTR)	1179/1141

match opposite strands of the integrated HTLV-I provirus genome, and were designed to point (5′→3′) towards each other. The primer sets were constructed as shown in Table I.

PCR The PCR was performed in a reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (w/v) gelatine, 1.0–4.0 mM $MgCl_2$ (optimized individually for each primer set), 200 μM each dNTP, 0.8 μM each of the two primers, 1–3 μg target DNA, and 2 units TAQ polymerase from Perkin Elmer Cetus. The amplification consisted of 40 cycles of a) denaturing all DNA at 94°C for 1 min; b) annealing of primers to target DNA at 37°C (low stringency) or 55°C (high stringency) for 1–2 min; and c) synthesis of second-strand target DNA by the TAQ polymerase at 72°C for 1–2 min. The PCR was conducted on a Perkin Elmer Cetus DNA Thermal Cycler.

Analysis Fifteen microliters of the reaction mix was subjected to agarose gel electrophoresis on a 4% NuSieve/SeaKem (3 : 1) agarose gel. Subsequently, the gel was blotted onto nitrocellulose ad modum Southern [11] with the exception that the set-up was inverted (buffer on top), and the blot was thereafter hybridized to an 8.5-kb HTLV-I probe (derived from the chronically infected MT-2 cell line and labeled with ^{32}P or biotin by nick translation). The filters were washed twice at 2 × SSC, 0.1% SDS for 30 min at room temperature, and twice at 0.5 × SSC, 0.1% SDS for 30 min at room temperature. The blot was then exposed overnight at −70°C with Kodac XAR-5 film in a cassette with a Dupont Cronex intensifying screen. When a biotin-labeled probe was used, the PhotoGene nucleic acid detection system (BRL) was applied according to the manufactors instructions, and the blot was exposed for 2 to 15 min with AGFA curix RP2 film in a cassette.

Sensitivity The sensitivity of the PCR was established by making serial dilutions of DNA from a HTLV-I infected cell line (MT-2) in normal control DNA. These serial dilutions were subjected to PCR using the same primers and conditions as for the patient sample DNA. As few as 2–3 proviral copies could readily be detected (data not shown).

RESULTS

In order to test if the primer sets would detect both HTLV-I and HTLV-II DNA as expected, PCR was conducted after initial establishment of optimal $MgCl_2$ and TAQ polymerase concentrations for each primer set. All primer sets produced bands of the expected size (Table II) as seen by agarose gel electrophoresis (Fig 1*a*) as well

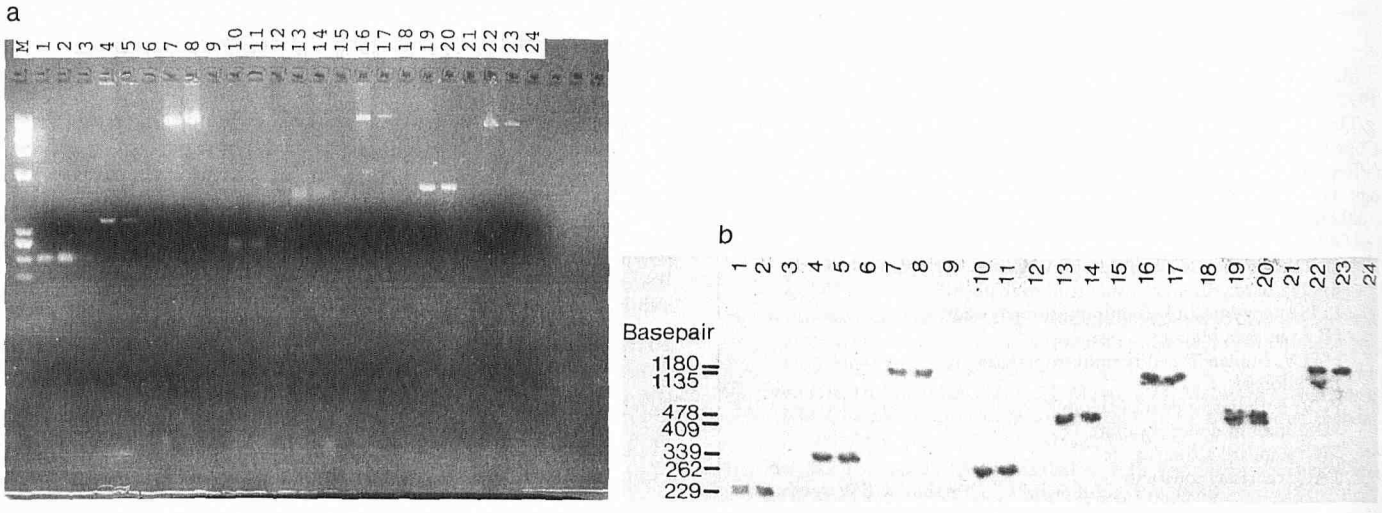

Figure 1. *a*: Agarose gel electrophoresis on a 4% NuSieve/SeaKem (3 : 1) gel of 15 μl amplification product following amplification of HTLV-I and HTLV-II control DNA. All primer sets were tested with 1 ng genomic DNA from a patient infected with HTLV-II *(lanes 1, 4, 7, 10, 13, 16, 19, and 22)*, 1 ng DNA from the HTLV-I–infected cell line MT-2 *(lanes 2, 5, 8, 11, 14, 17, 20, and 23)* and 1 μg genomic DNA from a normal blood donor *(lanes 3, 6, 9, 12, 15, 18, 21, and 24)*. M, phiX174 DNA marker; *lanes 1–3*, primers 108-108R; *lanes 4–6*, primers 109-109R; *lanes 7–9*, primers OL3-110R; *lanes 10–12*, primers 5-1R; *lanes 13–15*, primers 1–2; *lanes 16–18*, primers 3–4; *lanes 19–21*, primers 353-2; *lanes 22–24*, primers 3-104R. *b*: Exposure of a Southern blot of *a*, hybridized with a biotin labeled (nick translation) 8.5 kb HTLV-I probe and washed twice at 0.5 × SSC, 0.1% SDS for 30 min at room temperature.

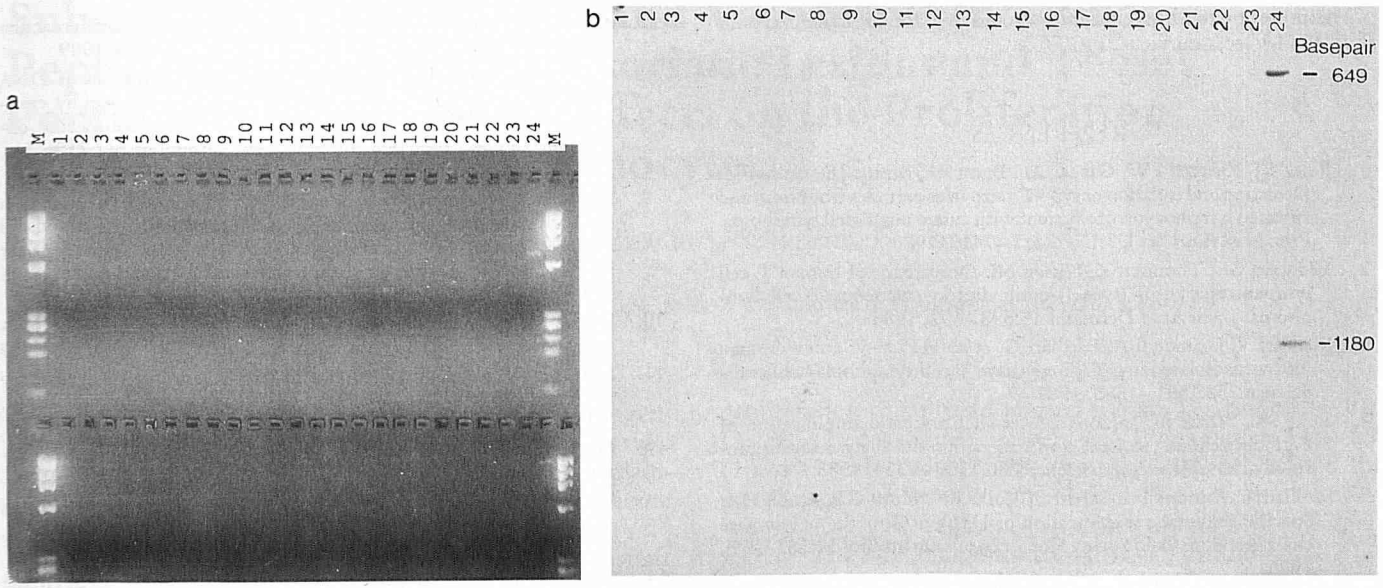

Figure 2. *a*: Agarose gel electrophoresis on a 4% NuSieve/SeaKem (3 : 1) gel of 15 μl amplification product following amplification of CTCL patient DNA with two primer sets. One microgram genomic DNA from the 21 included CTCL patients were amplified with two primer sets (*top lanes 1 – 21* and *bottom lanes 1 – 21*). Controls: 1 μg genomic DNA from a normal blood donor (*top lane 22* and *bottom lane 22*), no DNA (*top lane 23* and *bottom lane 23*), and 1 ng DNA from the HTLV-I – infected MT-2 cell line (*top lane 24* and *bottom lane 24*). *Top lane M,* phiX174 DNA marker; *top lanes 1 – 24,* primers 5-2; *bottom lane M,* phiX174 DNA marker; *bottom lanes 1 – 24,* primers 3-4. *b*: Exposure of a Southern blot of *a,* hybridized with a biotin-labeled (nick translation) 8.5-kb HTLV-I probe and washed twice at 0.5 × SSC, 0.1% SDS for 30 min at room temperature.

as Southern blot hybridization to an 8.5-kb HTLV-I probe (Fig 1*b*), following PCR with 1 ng DNA from the HTLV-I – infected MT-2 cell line as well as with 1 ng genomic DNA from a patient infected with HTLV-II.

Both high and low specificity amplification produced a band of the expected size in the positive control (1 ng total DNA from the MT-2 cell line), but never in the negative controls (1 μg genomic DNA from normal blood donors and samples without DNA), and never in a patient sample, as seen by agarose gel electrophoresis (Fig 2*a*), although two of the 21 patients included in a previous enzyme-linked immunosorbent assay (ELISA) study were found to have antibodies reacting with detergent-disrupted HTLV-I [2].

Only once was a band near the expected size amplified from the CTCL patient samples. This DNA was cut from the gel, and was by conventional subcloning and sequence analysis ad modum Sanger [12] found to have 70% nucleotide identity over a 150-basepair part of the human β-globin gene (data not shown). An endogenous human retrovirus with a similar sequence relatedness has previously been described [13].

After Southern blot and hybridization to an 8.5-kb HTLV-I probe, only the positive control produced a signal (Fig 2*b*), always to a band at the expected size (Table II). Even at low stringency washing (exposure after the second 2 × SSC, 0.1% SDS wash) the HTLV-I probe did not hybridize to any of the patient samples. In order to show that these negative results are not caused by total absence of DNA in the PCR samples, 1 μg genomic DNA from each patient was subjected to PCR using primers PC04 and GH20, which amplifies a 268-bp fragment of the human β-globin gene [14]. All patient samples showed a band of the expected size (Fig 3).

DISCUSSION

The search for a retroviral presence in cutaneous T-cell lymphoma has now entered its second decade. Despite the discovery of HTLV-I or HTLV-I – like antibodies in patients with CTCL [2], and the report on the isolation of a new human retrovirus from seven Italian patients with mycosis fungoides [3], reports on further progress have failed to emerge.

A retroviral infection might still play a role in the pathogenesis of CTCL. Such a hypothetical virus could — either after integration or via expression of some viral product during its replication (maybe without integration) — trigger the events leading to CTCL. By the time the clinical symptoms finally appear, the virus may have disappeared, either due to a replication defect developed during infection, or due to disappearance of the target cells by immune clearance, or from a cytopathic effect of the virus. This would explain the negative PCR results in the two patients found HTLV-I antibody positive in a previous study [2]. However, because this study was conducted with detergent-disrupted HTLV-I virus particles and not with purified HTLV-I antigen, the possibility remains that the antibodies reacting in the previous study were in fact cross-reacting antibodies not of viral origin.

The negative findings in this study do not rule out an unknown retrovirus as a possible etiologic agent in CTCL. A virus genetically related to HTLV-I could be present in patient DNA, but in extremely low copy numbers (e.g., under 1 proviral copy per 25,000 cells) demanding a sensitivity that would exceed the sensitivity of the PCR in our study.

The possibility also remains that despite the implications of the cross-reacting antibodies, the hypothetic CTCL-virus is not closely related to HTLV-I. This could be true for the whole genome, or (least likely) just for the regions that we have investigated. Dissimilarity throughout the genome would render the PCR useless in the search.

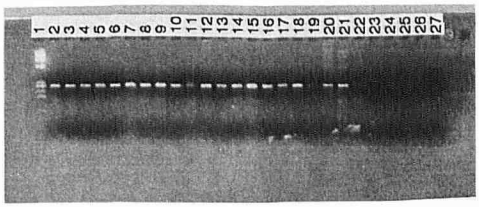

Figure 3. Amplification of CTCL patient DNA and non-human DNA with human β-globin primers PC04 and GH20 (amplifies a 268 base-pair fragment of the human β-globin gene). *Lane 1,* phiX174 DNA marker; *lanes 2 – 22,* 1 μg CTCL patient DNA; *lanes 23 – 26,* negative controls, 100 ng legionella pneumophilia serogroup 1 DNA; *lane 27,* no DNA.

The present data, however, provide no evidence for the presence of an HTLV-related virus in CTCL.

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ERRATUM

An abstract by Koch et al entitled "Measurement of Flurbiprofen Absorption In Vivo Through Hairless Rat Skin Using ^{19}Fluorine-Nuclear Magnetic Resonance," presented at the 1991 SID annual meeting (JID 96:576, 1991; Abstract 270), contains an error:

The flux of flurbiprofen in vivo was reported as $40 \pm 6\ \mu g/cm^2/h$ ($n = 3$). The correct flux for this group of three animals should have been $90 \pm 8\ \mu g/cm^2/h$. Since this publication, we have added more animals to this group and have conducted additional trials on some of these five rats and find the current flux value for $n = 5$ to be $93 \pm 22\ \mu g/cm^2/h$.

We anticipate that this value will change as more experiments (more trails or additional rats) are conducted. The flux is approximately four times greater rather than two times greater and really does not change the overall importance of the finding compared to in vitro methods.