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Endogenous retroviral elements, but not exogenous retroviruses, are detected in CD30-positive lymphoproliferative disorders of the skin

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Introduction

Primary cutaneous CD30-positive lymphoproliferative disorders (LPD) comprise a spectrum of disorders including lymphomatoid papulosis (LyP) and CD30+ pleomorphic or anaplastic large T-cell lymphomas (1–3). LyP is a chronic, recurrent lymphoproliferative disorder which manifests clinically as papulonodular, spontaneously regressing skin lesions (4). Histologically, large pleomorphic and anaplastic tumor cells are found which are activated T-lymphocytes expressing the CD30 antigen (5,6).

Viruses are known etiologic factors in the pathogenesis of non-Hodgkin's lymphomas (NHL) (7). In particular, retroviruses such as human T-lymphotropic virus 1 and 2 (HTLV-1 and 2) have been linked to adult T-cell leukemia/lymphoma (ATLL) and other forms of NHL (for review see refs 8 and 9). Primary cutaneous lymphomas, especially cutaneous T-cell lymphomas, have been considered to be caused by viruses. Several reports of the detection of HTLV-1 or 2 sequences in cutaneous T-cell lymphomas (CTCL) (10–13) have not been confirmed in other studies (8,9,14,15).

Ultrastructural and clinical features suggest that a virus may be involved in the etiology of LyP (Kadin, unpublished data). Viral-like particles (VLP) have been detected in LyP lesions by electron microscopy (16). In addition, the immunophenotype of LyP tumor cells is identical to the lymphoid tumor cells in ATLL associated with HTLV-1. Moreover, CD30 antigen expression is known to be triggered by viruses such as HTLV-1 and Epstein–Barr virus (17). HTLV-1 proviral sequences have been found in CD30+ large-cell cutaneous T-cell lymphoma (18). Nevertheless, neither retroviruses nor other oncogenic viruses have been detected in LyP (5,19–21). In up to 20% of cases, LyP is preceded or followed by other lymphoproliferative disorders such as Hodgkin's lymphoma, mycosis fungoides or systemic CD30+ anaplastic large-cell lymphoma (22–24). These tumors have been shown to be clonally related to LyP occurring in the same individual (25,26). Therefore, it is of importance to identify a putative viral etiology underlying these lymphomas, if one exists.

The aim of the present study was to investigate whether retroviruses are involved in the pathogenesis of LyP. To accomplish this goal, various methods were chosen to detect cytopathic effects in cocultivation experiments, reverse transcriptase activity in supernatants of cultured tumor cells, nucleic acid sequences of retroviruses in lesional tissue and cell lines established from CD30+ LPD and electron microscopy of tissue biopsies and cell lines to detect viral particles. To identify possible unknown retroviruses putatively associated with CD30+ LPD, consensus PCR for retroviral polymerase genes was used since these genes are generally the most conserved sequences among retroviruses (27). Here, we report the presence of viral-like particles, transcripts of endogenous retroviral elements (ERV), and reverse transcriptase (RT) activity, as well as the absence of exogenous retroviruses, in LyP.

Abbreviations: ATLL, adult T-cell leukemia/lymphoma; CTCL, cutaneous T-cell lymphomas; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HTLV-1 and 2, human T-lymphotropic virus 1 and 2; IUDR, 5′-iodo-2′ deoxyuridine; LPD, lymphoproliferative disorders; LyP, lymphomatoid papulosis; NHL, non-Hodgkin's lymphomas; RT–PCR, reverse transcription–polymerase chain reaction; VLP, viral-like particles.

Material and methods

LyP biopsies and cell lines

Twenty-seven glutaraldehyde-fixed biopsies from 13 patients with LyP were investigated by EM for the presence of viral-like particles. Eleven snap-frozen skin biopsies of LyP lesions from a total of 10 patients were available for extraction of nucleic acids. The tumor cell lines JK, Mac 1 and Mac 2A, had been established from progressive lesions of two LyP patients ('JK' and 'Mac') who showed development of CD30-positive anaplastic large-cell lymphoma during the course of LyP. Studies of T-cell receptor gene rearrangement showed that JK and Mac cells were each clonally related to all prior LyP skin lesions (for details see refs 25 and 28).

Cell cultures and cocultivation

All cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C. Peripheral blood mononuclear cells (PBMC) from a healthy individual were separated by Ficoll gradient and maintained in culture without stimulation. JK and Mac 2a were cocultivated with lymphoid cell lines SupT1, CemX, MT2 and H9 as well as with peripheral blood mononuclear cells of a healthy subject according to the protocols described by Miyoshi *et al.* (29).

Induction

In addition, JK cells were treated for 48 h with 5′-iodo-2′ deoxyuridine (IUDR) to enhance transcription of putative retroviruses. Alternatively, cells were treated with sodium butyrate (1 μM) added to the medium containing RPMI 1640 and interleukin-2 (IL-2).

Electron microscopy

Twenty-seven skin biopsies of LyP lesions from a total of 13 patients, and cells from one tumor cell line were prepared and studied by electron microscopy (30). The controls included various inflammatory and neoplastic disorders such as eczema ($n = 3$), lichen planus ($n = 3$), psoriasis ($n = 3$), and other cell T-cell lines ($n = 2$).

RT assay

The supernatants of tumor cell lines were tested for reverse transcriptase (RT) activity with colorimetric highly sensitive manganese-based and magnesium-based RT assays (Cavidi HS-kit Mn^{2+} RT, Cavidi Tech, Uppsala, Sweden), respectively. Supernatants were filtered through a 0.45 micrometer Nalgene filter, added to a Beckman centrifuge tube and centrifuged at 50 000 r.p.m. for 1 h in a 90 Ti Beckman rotor under vacuum at 4°C. HIV-1 infected cells (HIV B3 cells) and HTLV-1 infected cells (LCL-Mok and TCL-Kan cells) served as positive controls for the RT assays. Negative controls consisted of supernatants of cultured PBMC of three healthy, HIV-seronegative individuals.

Table I. Oligonucleotides and length of amplification products used in the described PCR protocols

Primers	Sequence	Product length
GAPDH		480 bp
GAP.F1	5′-AGG CTG GGG CTC ATT TGC-3′	
GAP.R1	5′-GTG CTC AGT GTA GCC CAG GAT G-3′	
HTLV		135 bp
HTLV S	5′-CTA TGG AGA GTA CTA CCC CAA GG-3′	
HTLV AS	5′-CTC GTC ATC CAT GTA CTG AAG-3′	
HTLV Probe	5′ TTT AAA AAT AGT CCC ACC CTG TTC-3′	
ERV		82 bp
ERV 1S	5′-CAA CTC TCT GCT TTG TAT CAT AAT C-3′	
ERV 1AS	5′-ATA GAC CAG TGT GAT ATC TTG CG-3′	
CONSENSUS (CODEHOP)-Primers		120 bp
LPQGM	5′-TAC CAG TGG AAT GTT CTA CCN CAR GGN ATG-3′	
LPQGF	5′-TAC CAG TGG AAT GTT CTA CCN CAR GGN TT-3′	
LPQGW	5′-ATC AGA TCC TAC TAA CAD RTC RTC CAT RTA-3′	
	Y = C / T; K = G / T; R = A / G; N = A / C / G / T	

The Mn-based assay has been shown to detect RT from ERV with higher sensitivity than the Mg-based assay or conventional RT assays using radioactive substrates (31).

RNA extraction

RNA from tissue samples was extracted using the Atlas Pure RNS Isolation kit (Clontech, Bo. K1038-1, Palo Alto CA, USA). RNA was extracted from cells using the Qiagen RNAEasy Kit (Qiagen, Valencia, CA). RNA was reverse transcribed using a cDNA synthesis kit for RT–PCR (Reverse Transcription system, No. A 3500, Promega Corp., Madison, WI, USA) using oligo-dT primers and degenerate retroviral primers, respectively. RNA was extracted from ultracentrifuged supernatants by QIAamp viral RNA mini kit (Qiagen, Valencia, CA).

PCR and Southern blot hybridization

Successful amplification of a sequence of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (for sequences see Table I) mRNA indicated that the samples were adequate for RT–PCR analysis and that no PCR inhibitors were present. To avoid contamination and product carryover, DNA extraction, PCR and gel electrophoresis were done in separate laboratories.

Specific sequences of HTLV-1 and 2 were detected by means of a PCR protocol with virus-specific primers followed by Southern blot hybridization with specific radioactive labelled probes for HTLV-1 and 2 (for sequences of oligonucleotides and probe see Table I). HTLV-1 infected cells (LCL-Mok and TCL-Kan cells) served as positive controls for the amplification of corresponding retroviral sequences.

Amplification of ERV sequences

For amplification of ERV 52–123-related sequences, specific primers were designed which amplify a 82 bp long region (for sequences of oligonucleotides see Table I). The PCR conditions were as follows: 35 cycles consisting of 30 s denaturation at 96°C, 30 s annealing of oligonucleotides at 55°C and 30 s extension at 72°C.

Degenerate PCR

Degenerate primers with a 5′ end clamp and variable nucleotide positions at the 3′ end, recently described as Consensus-Degenerate Hybrid Oligonucleotide Primers (CODEHOP)-PCR (32), were used to amplify a highly conserved region of the reverse transcriptase genes of various retroviruses by a PCR protocol. The primers LPQGF, LPQGM, LPQGW and YMDD have been described in detail by Rose and coworkers (32). LPQGF have been designed to amplify preferentially oncogenic retroviruses, whereas LPQGW amplify predominantly lentiviruses. The cDNA templates were first amplified using 35 PCR cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min). The amplification products were electrophoresed in a 2% agarose gel and visualized by UV transillumination after staining with ethidium bromide. The primers were shown to amplify HTLV-1 and HIV-1 sequences from infected cells. Carry over of trace amounts of genomic DNA was prevented by digestion of nucleic acid extracts from tissues by DNase. For each sample, replicate RT–PCR was performed (data not shown). The same amount of RNA was used for all samples analyzed by RT–PCR.

Cloning and sequencing

The RT–PCR products of expected size were cloned into a pCR2.1 vector (TOPO TA Cloning kit, No. K4500-01, Invitrogen, Carlsbad, CA), and the resulting recombinant plasmids were sequenced using the BigDye Terminator Ready Reaction kit (Perkin Elmer Cat. No. 4303151, Foster City, CA) on a ABI Prism 377 automated DNA sequencer (Perkin Elmer, Foster City, CA).

Sequence analysis

Homology searches against various databases were performed using BLASTN and BLASTX programs (33,34).

Results

Electron microscopy

Nine of 27 biopsies from 13 patients with LyP showed multiple large cytoplasmic membrane-bound vacuoles of variable sizes up to 10 μm, either filled with or lined on their inner surfaces with small viral-like particles (VLP) (Figure 1). Many of these structures with poorly visible or non-electron dense centers were morphologically similar to immature retrovirus particles (10,35,36). A few particles contained irregularly shaped and located electron dense core material. Budding of these particles from the plasma membrane was not seen in biopsies. Nor were visible free particles present in the nucleus or cytoplasm. The diameter of the particles in cytoplasmic vacuoles was 60–80 nm. No clinical and/or histologic differences between virus-positive and negative lesions could be identified.

The tumor cell line JK, derived from a patient with LyP, and cultured in the presence of IL-2 and sodium butyrate was examined by electron microscopy. VLP analogous to those present in biopsies were present in similar cytoplasmic vacuoles and as clusters adjacent to tumor cell surfaces (Figure 2). Classical images of budding were not evident in the EM sample.

Cocultivation

No cytopathic effect could be observed after cocultivation of tumor cell lines JK and Mac 1 or Mac 2A with lymphoid cell lines SupT1, CemX, MT2 and H9 as well as with peripheral blood mononuclear cells of a healthy subject after 48 h, 7 days or 14 days. Furthermore, no syncytia formation of JK cells was observed after induction with IUDR for 48 h.

RT assays

Conventional radioactive RT assays performed on supernatants of JK and Mac cell lines and cocultures did not reveal

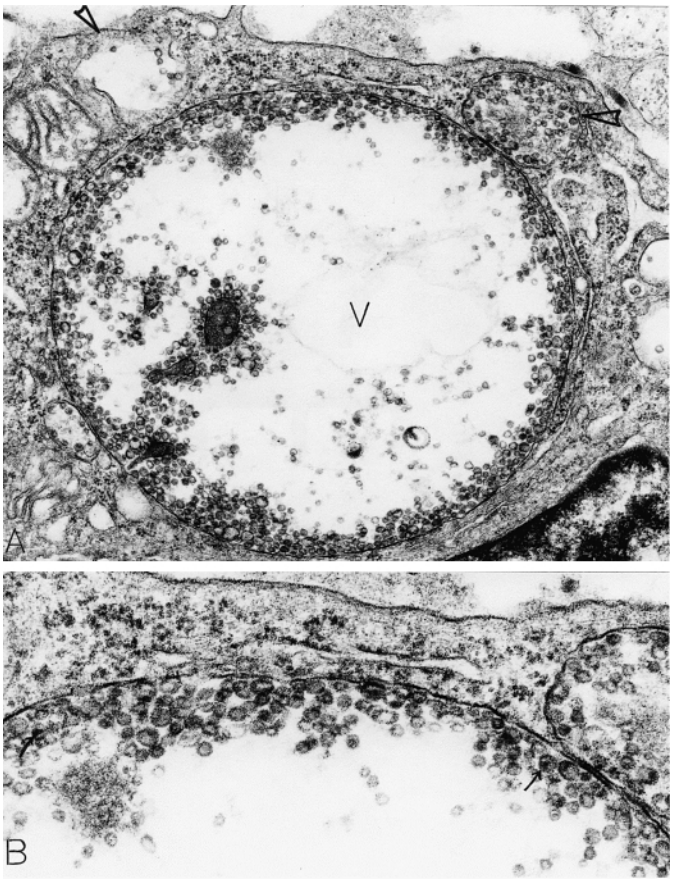

Fig. 1. Viral-like particles in a tumor cell in a skin biopsy of a patient with lymphomatoid papulosis. In (**A**), a giant-sized vacuole (V) in the cytoplasm contains numerous small structures resembling immature retroviral particles, most of which are attached to the inner vacuolar membrane. Two small vacuoles (arrowhead) close to the cell surface also contain viral-like structures. A portion of the inner vacuole membrane is enlarged in (**B**). This allows visualization of dense core-like structures (arrows) in some of the particles (**C**, indicated by arrow). (A) ×43 000; (B) ×80 000; (C) ×200 000.

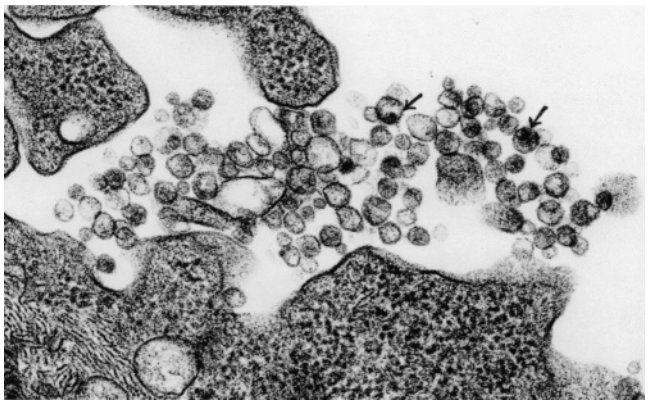

Fig. 2. Viral-like particles in the extracellular space adjacent to a tumor cell in the JK tumor cell line. Note that some of the particles contain dense cores (arrows). ×63 500.

detectable RT activity. Even after induction of cells with IUDR, which is a known inducer of retroviral transcription, no RT activity was detected by conventional RT assays. In contrast, the colorimetric manganese-based RT assay allowed detection of RT activity in the ultracentrifuged supernatant of JK cells after induction with IUDR for 48 h, with a median value of RT activity of 903 μU/μl compared with <200 μU/μl for uninduced JK cells. No RT was activity detected in the control cell lines.

PCR studies

HTLV 1 and 2 sequences could not be detected by RT–PCR with virus-specific oligonucleotides in lesional tissue of LyP and cell lines (data not shown).

Degenerate PCR with CODEHOP-primers LPQGF and YMDD revealed amplification products of expected size in two of six LyP cases, JK cells and the ultracentrifuged supernatant of JK cells induced for 48 h with IUDR (data not shown). The PCR products of 87 bp length were subsequently cloned and automatically sequenced. Homology searches against various databases were performed using BLASTN and BLASTX programs and demonstrated that nucleotides 1–72 of our 87 bp long amplified products showed similarity of 98% with nucleotides 16–87 of the pol region of ERV 52–123 (Genbank accession no. S71609) with one nucleotide change at position 20 (a → t) of our amplified product. The sequence of our fragment is: 5′-gct ttg tat cat aat ctt att cgg aga gaa ctt gat agc ttt tca cat ccg caa gat atc aca ctg gtc tat-3′ which does not contain any stop codons. One of the putative corresponding amino acid sequences is: A L Y H N L I R R E L D S F S H P Q D I T L V Y. In this open reading frame, this amino acid sequence has similarity of 66% to the pro-pol-dUTPase polyprotein (aa 83–106) of murine endogenous retrovirus ERV-L (Genbank accession no. T29097) and to the reverse transcriptases. No sequence similarity to known gag or env genes protein sequences was found by Genbank analysis.

Thus, oligonucleotides specific for ERV 52–123 were designed to assess expression levels of this ERV in LyP and cell lines. As shown in Figure 3, higher levels of mRNA transcripts were detected in all three tumor cell lines JK and Mac1 and 2A (lanes 1, 2, 5 and 6), supernatants of tumor cell lines (lanes 3 and 4) and the majority of LyP biopsies (lanes 7–10) compared with PBMC of healthy individuals (lanes 11–13) and T-cell lines CEM-X, HTLV-1 infected H9 and HTLV-1 negative Sup T1 (lanes 14–16).

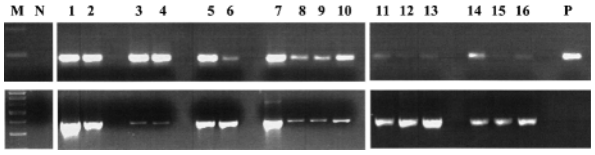

Fig. 3. RT–PCR for ERV 52–123: Higher levels of amplified mRNA sequences in CD30+ tumor cell lines (lanes 1, 2, 5 and 6), supernatants of tumor cell lines (lanes 3 and 4) and LyP lesional tissue (lanes 7–10) compared with PBMC of healthy individuals (lanes 11–13) and T-cell lines CEM-X, HTLV-1 infected H9 and HTLV-1 negative Sup T1 (lanes 14–16). The upper images represent amplified ERV transcripts, whereas the lower images the GAPDH levels from corresponding samples. N: negative control; P: positive control (cloned PCR product); M: molecular weight marker.

Discussion

In this study, we demonstrate the presence of intra- and extracellular viral-like particles in lesional tissue and tumor cell lines from primary cutaneous CD30-positive LPD. By electron microscopy, these particles exhibited the morphology of immature retroviral particles (10,35,36). Similar particles have been described in cultured peripheral blood cells and T-cell lines from patients with Sézary's syndrome, a disorder belonging to the group of CTCL (37,38), and we have seen similar particles in cutaneous lesions in a patient with erythrodermic CTCL (see Figs 279 and 284 in ref. 39). Similar VLP have not been present in a wide variety of diagnostic human skin biopsies including eczema, lichen ruber, and psoriasis (Dvorak, unpublished data).

To characterize these particles, PCR-based approaches were chosen to search for known, and for closely related, but yet unknown exogenous and endogenous retroviral sequences. By virus-specific PCR, no evidence of HTLV-1 and HTLV-2 presence could be found in lesional tissue and cell lines of CD30+ LPD of the skin. These results are in accordance with the data reported in another study (20). In addition, we did not identify sequences of closely related, yet unknown retroviruses by applying a consensus PCR method aimed at detecting a highly conserved retroviral transcriptase region. These data indicate that exogenous retroviruses closely related to HTLV-1 or -2 are probably not present in CD30+ LPD of the skin. Despite various features which may indicate the involvement of oncogenic exogenous retroviruses in the pathogenesis of CD30+ LPD, there is so far no evidence for a role of these viruses in the pathogenesis of CD30+ LPD based on our findings and the data reported in the literature. Furthermore, the absence of exogenous retroviruses is in accordance with the lack of well-documented cases suggesting horizontal or vertical transmission of CD30+ LPD.

However, using the same consensus PCR approach, we detected transcripts of human endogenous retroviral sequences (HERVs) in lesional tissue and cell lines of CD30+ LPD of the skin. ERV are inherited genomic elements with structural features of integrated retroviruses (40,41). Whereas ERV are mostly silent genomic elements, some groups can form retrovirus-like particles and be enzymatically active (42,43). Sequencing of PCR products found in LyP tissue and cell lines revealed that the amplification products belong to ERV 52–123. These ERV transcripts were originally isolated from PBMC of patients with systemic lupus erythematosus, a systemic autoimmune disorder (44).

These retroviral elements are closely related to human endogenous retroviral ERV 9 sequences which have been isolated from the T-lymphoma Peer cell line (45). So far, these ERV 52–123 have not been linked to human tumors. The pol fragment has a 65% similarity to the pol fragment of HTLV-1 and 2. Except for this fragment, other parts of the genomic structure of ERV 52–123 remain unknown. Thus it remains to be proven whether this sequence is part of a complete provirus. Similarly, further work needs to be done to identify the whole ERV sequence found in our samples. The detection of mRNA shows that ERV 52–123 is transcriptionally active. Moreover, reverse transcriptase activity was detected in the supernatant of tumor cells by a highly sensitive Mn-based RT assay developed to detect RT activity of ERVs. Although it remains unclear whether this RT activity originates from ERV particles, RT activity and high levels of ERV transcripts were both found in LyP lesional tissue and cell lines.

In addition, the same ERV sequences could be found in the supernatant of tumor cell lines harboring ERV 52–123 which might result from release of VLP in the extracellular space as we observed by electron microscopy. Interestingly, cocultivation of tumor cells with other potentially susceptible T-cells did not reveal detectable transmission. This is not surprising since most ERV have lost their infectivity due to deletions and mutations during evolution.

Since ERV are inherited, it is difficult to establish an association between these viral elements and a particular disease. To establish causality, one cannot only rely on the presence or absence of these elements as in the case for exogenous viruses. In this study, higher levels of transcription of ERV 52–123 sequences could be found in lesional tissue and cell lines of LyP, compared with controls. Similarly, a high level of ERV expression has been detected in mouse lymphoma and in human tumors such as germ cell tumors (GCT) (46). Furthermore, viral ERV gag protein is produced in large quantities by GCT and serum antibodies directed against ERV env protein can be found in 85% of patients with GCT, whereas healthy individuals and patients with inflammatory diseases or tumors other than GCT very rarely develop such antibodies (46). Recently, Boese and coworkers reported that the HERV protein cORF supports cell transformation as shown by tumor induction in nude mice (47).

Our data suggest that exogenous retroviruses related to HTLV-1 and HTLV-2 are not involved in the pathogenesis of primary cutaneous CD30+ LPD. Because of the high preferential expression of HERV 52–123 in lesional tissue and cell lines of LyP, and the detection of VLP by electron microscopy, we postulate that the activation of endogenous retroviral elements may be associated with the pathogenesis of primary cutaneous CD30+ lymphomas. Further studies are necessary to demonstrate that the particles found by EM represent an endogenous retrovirus as suggested by the data obtained by RT–PCR and RT assays.

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