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Detection of Epstein-Barr Virus in T-Cell Prolymphocytic Leukemia Cells *in vitro*

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Abstract

Background—Epstein Barr virus (EBV) is closely associated with the development of a number of tumors. During latent infection, EBV continuously expresses a number of viral genes which are essential for cell transformation and maintenance of the malignant phenotype of EBV-related tumors. There has been no previous link between EBV and T-cell prolymphocytic leukemia (T-PLL), a distinctive form of leukemia derived from T-cells at an intermediate stage of differentiation between a cortical thymocyte and a mature peripheral blood T-cell.

Objective—To determine if EBV was present in the T-PLL cells collected.

Study design—T-PLL cells were isolated from the peripheral blood of a patient diagnosed with T-PLL and continuously cultured for about one year. The existence of EBV in these cells was detected using multiple strategies including PCR, Western blotting, immunofluorescent assay and flow cytometry analysis.

Results—The EBV genome was present in these T-PLL cells by PCR analysis across multiple sites in the viral genome. In addition, these T-PLL cells expressed a number of EBV latent antigens. The EBV oncoproteins LMP1, EBNA1 and EBNA3C were expressed in the majority of the infected cells.

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Conclusion—This report suggests a potential link between EBV infection and T-PLL and provides new information about the potential contribution of EBV in the initiation or maintenance of T-PLL.

Keywords

Epstein Barr virus; T-cell prolymphocytic leukemia; infection

1. Introduction

Epstein-Barr virus (EBV) was first reported as the etiological agent of Burkitt's lymphoma in 1964 (Epstein, Achong, and Barr, 1964). Since then, EBV has been associated with many human pathologies including nasopharyngeal carcinoma which is a highly prevalent and aggressive cancer in Southeast Asia, infectious mononucleosis, and AIDS- and transplant-related B cell lymphomas (Pattle and Farrell, 2006). EBV has also been linked to T-cell lymphomas, Hodgkin's lymphoma, and NK leukemia or LGL leukemia (Young and Murray, 2003; Young and Rickinson, 2004), supporting the concept of a wide spectrum of EBV-associated disorders. The EBV genome encodes for a number of latent proteins such as EBNA1-3, LMP 1, 2 and others which are selectively expressed during different types of viral latency (Subramanian, Knight, and Robertson, 2002). Recent studies have demonstrated that EBNA2, EBNA-LP, EBNA3A, EBNA3C and LMP1 are important for cell transformation (Subramanian, Knight, and Robertson, 2002). Furthermore, different strategies are utilized by these latent antigens to drive virus-associated transformation or carcinogenesis among the EBV-associated disorders.

Although EBV is linked to a number of different types of tumors, the association between this virus and T-cell prolymphocytic leukemia (T-PLL) has not been previously explored. T-PLL, formerly categorized as T-cell chronic lymphocytic leukemia, is a rare and aggressive hematologic malignancy (Hoyer et al., 1995; Toyota, Nakamura, and Dan, 2002; Toyota, Nakamura, and Dan, 2005). It is a distinctive type of leukemia that is derived from T-cells at the intermediate stage of differentiation between a cortical thymocyte and a mature peripheral blood T-cell (Hoyer et al., 1995; Toyota, Nakamura, and Dan, 2002; Toyota, Nakamura, and Dan, 2005). Patients with T-PLL often have a complex karyotype with recurrent alterations that involve chromosomes 14, 11, and 8. Rearrangements of chromosome 14q32.1 through inversion [inv(14)(q11;q32)] and translocation [t(14;14)(q11;q32)] are the most common cytogenetic abnormalities reported in T-PLL (Croce, 1987; Croce et al., 1985). As a result of these rearrangements, TCL1, a gene that is physiologically expressed in CD4/CD8 double-negative thymocytes, is deregulated (Narducci et al., 1997; Narducci et al., 1995; Virgilio et al., 1994). Typically, the TCL1 locus rearranges with the T-cell receptor (TCR) α/δ chain locus in the above mentioned rearrangements, but it can also rearrange with the TCR- β chain locus on chromosome 7 in another translocation [t(7;14)(q35;q32.1)] (Narducci et al., 1997; Pekarsky, Hallas, and Croce, 2001a; Pekarsky, Hallas, and Croce, 2001b). Furthermore, several patients with mature T-cell leukemias have the translocation [t(X;14)(q28;q11)], which results in the rearrangement of the MTCP1 gene (a member of the TCL1 gene family) located at Xq28 (Madani et al., 1996; Stern et al., 1993). Therefore, chromosomal rearrangements in T-PLL juxtapose TCL1 and MTCP1 to

the TCR loci and lead to their activation. Recent genomic analyses of the 14q32.1 breakpoint region has revealed three additional genes, TCL1b, TNG1 (T-CL1 neighboring gene 1), and TNG2. These have an expression pattern similar to TCL1 in that they are not expressed in normal T-cells, but are expressed in T-PLL cell lines and cells from patients with T-PLL (Hallas et al., 1999; Pekarsky et al., 1999; Sugimoto et al., 1999). Activation of TCL1 through hypomethylation of its promoter has also been described (Yuille et al., 2001). These molecular events related to the above chromosomal aberrations are believed to play a casual role in the pathogenesis of T-PLL.

EBV infects the vast majority (>90%) of healthy adults and can infect T cells *in vivo* (Young and Rickinson, 2004). In addition, the infection of T-cells appears to convey an increased risk of malignancy such as lymphoma. This raised the possibility that EBV might also contribute to the development of another hematologic malignancy, T-PLL. A 70 years old male T-PLL patient was observed with an increased titer of EBV antigens, suggesting that there was a high level of EBV viremia and that EBV infection might be associated with this rare malignancy. In this report, we sought to determine if EBV was present in the tumor cells obtained from this EBV-positive patient with T-PLL. We found that EBV DNA can be continuously detected in the cultured tumor cells and that the majority of the essential EBV latent genes were expressed in these cells suggesting a latency pattern that is geared toward growth of the latently infected cells.

2. Methods

2.1. Patient

The study patient was a 70 years old male diagnosed at the Hospital of the University of Pennsylvania with T-cell prolymphocytic leukemia (T-PLL) (IRB#706586). He had an increase in the EBV titer which was determined by detection of the antibody against viral capsid antigen (VCA). The diagnosis was primarily based on FACS analysis of the bone marrow aspirate as well as a peripheral blood sample. The combined clinical, morphologic (absence of flower cells) and immunophenotypic features were also consistent with T-cell prolymphocytic leukemia.

2.2. Lymphocyte isolation and cell culture

Primary lymphocytes were harvested from peripheral blood mononuclear cells using a Lymphoprep gradient as described previously (Lee et al., 2001). The enriched lymphocyte population was washed and plated at 50,000 cells in 150 μ l of complete medium per well in a 96-well tissue culture plate. The cells were grown and maintained in RPMI 1640 medium (Hyclone Inc., Logan, UT) supplemented with 2 mM L-glutamine, 5 μ g of gentamicin per ml, and 10% heat-inactivated fetal bovine serum. 96-well plates were incubated at 37°C with 5% CO₂. The plates were treated once every 8 days with a fresh 100 μ l of complete medium.

2.3. DNA lysate preparation and PCR analysis

Approximately 50,000 T-PLL cells derived from the patient's peripheral blood cells were collected and were centrifuged at 2,000 rpm for 5 min. The cell pellet was washed with phosphate-buffered saline (PBS) twice and resuspended in 50 μ l of 0.2 $\times$ PBS, heated to

95°C for 15 min, and then switched to 56°C for 1 h with proteinase K treatment (10 mg/ml). The enzyme was then inactivated at 95°C for 30 min. A 5- μ l portion of the lysate was used for PCR amplification of EBV-specific regions for 40 cycles, and standard protocols were followed using primers indicated in table 1. DNA was also prepared from the same number of control BJAB cells. PCR was performed by heating at 95°C for 5 min and subsequently for 40 cycles, each cycle including 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by further incubation at 72°C for 10 min, in a PTC-100 programmable thermal cycler (MJ Research, Inc., Waltham, MA). Five μ l aliquots of PCR products were then assayed by electrophoresis on a 2.0% ME-agarose gel and visualized by ethidium bromide.

2.4. Immunofluorescence assay

Immunofluorescence analyses were performed as described previously (Lan et al., 2004). One million T-PLL cells were collected and briefly fixed using a 1:1 methanol and acetone mix ice cold at -20°C, blocked in the appropriate serum, and then incubated with the pre-cleared patient serum or specific primary antibody for EBNA3C and LMP1 for 1 h. Cells were washed and then further incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate at 1:1,000 dilutions in PBS for 1 h. Slides were washed and visualized with an Olympus XI70 inverted fluorescence microscope (Olympus Inc., Melville, NY) and photographed using a digital PixelFly camera and software (Cooke, Inc., Warren, MI).

2.5. Western blotting

T-PLL from the patient and an EBV-negative cell BJAB control were lysed with 300 μ l of reporter lysis buffer (Promega, Inc., Madison, WI) respectively, and 40 μ l of this lysate was used. Briefly, lysates were boiled in SDS-loading buffer, fractionated by SDS-PAGE and transferred to a 0.45 mm nitrocellulose membrane. The membranes were then probed with patient serum absorbed against B cell antigens or EBV-specific antibodies followed by incubation with appropriate infrared-tagged secondary antibodies and viewed on an Odyssey imager (LiCor Inc., Lincoln, NE).

2.6. FACS analysis

For the determination of the expression of CD3 positive cells, one million cells from patient samples, negative controls (B-cell) and antibody controls were incubated with rabbit polyclonal PE-tagged anti-CD3 antibody (2 mg/ml in PBS containing 1% BSA) for 30 min at room temperature. After thoroughly washing with 1X PBS the cells were incubated with FITC-conjugated anti LMP1 mouse monoclonal antibody for 30 min. Cells were then washed and analyzed on a flow cytometer (Becton Dickinson, San Diego, CA) equipped with 488 nm Argon laser light source and a 623 nm band pass filter for FITC fluorescence. For determination of LMP-1 signals, cells were fixed in 1% paraformaldehyde followed by permeabilization with 0.05% Triton X 100 at room temperature. The cells were washed with 1X PBS 3 times at 5 minute intervals. In this fixing procedure the cytoplasm remains intact and the LMP1 antibody was allowed to gain access to the intracellular compartment. A total of 10,000 events were acquired for analysis using CellQuest software (Becton Dickinson, San Diego, CA). Cells were gated as described earlier (Lan et al., 2006), and histogram plots

of PE fluorescence (*x* axis) versus counts (*y* axis) were shown as a fraction of logarithmic fluorescence intensity. A dot plot of PE fluorescence (*x* axis) versus FITC (*y* axis) was also determined as a fraction of logarithmic fluorescence intensity.

3. Results

3.1. Growth of T-cell prolymphocytic leukemia cells *in vitro*

The diagnosis was based on the FACS analysis of the bone marrow aspirate as well as the peripheral blood sample. The results of FACS on the bone marrow demonstrated an aberrant expanded population (70% of total events) of CD2, CD3, CD5, CD7, CD8, and CD25 T cells. These cells were CD1a, CD4, CD16, CD30, CD56, CD57, TdT and gamma-delta negative. In addition, B-cells were essentially absent (Table 2). The FACS performed on peripheral blood demonstrated an essentially identical immuno-profile and the markedly high CD7 intensity was not reduced in comparison to other pan-T antigens. The combined clinical, morphologic (absence of flower cells) and immuno-phenotypic (CD8 positivity, without loss of CD7 expression) features were consistent with T-PLL (Table 2).

Mononuclear cells preparations from the patient were cultured. These cultures initially contained the T-PLL tumor cells as well as other normal lymphocytic cells. The normal cells gradually died within a month and only large T-PLL tumor cells remained as large clumps suggesting a transformed phenotype (Fig. 1A). Microscopic findings of the hematoxylin and eosin (HE) stained slides of needle aspirate showed many mature lymphocytes (Fig. 1B). The surface of some of the lymphoid cells showed enhanced ruffling (middle panel arrow). These were identified as atypical lymphocytes that continued to proliferated in culture over time. The right panel displays cells with distorted nuclei (right panel arrow). These cells were slightly smaller but their morphology was consistent with PLL (Fig. 1B).

3.2. Confirmation of EBV sequence in the T-PLL cells

We performed PCR amplification of different regions of EBV genome to determine if there was EBV infection of the T-PLL cells derived from the patient's peripheral blood cells which were predominantly T cells with an almost absent B cell population. Genomic DNA was prepared from the equal numbers of T-PLL cells which were continuously grown for 6 months in culture; EBV positive cell line (BC1), an EBV-negative cell line (BJAB), and the peripheral blood cells of the same patient. These were subjected to PCR analysis. Figure 2 demonstrates strong amplification of EBV target sequence in the T-PLL sample (Fig. 2). There was also a relatively lower amplification in the peripheral blood sample, suggesting that only a fraction of the circulating T cells which made up the PBMC were infected with EBV in the context of the total PBMC population. Importantly, the size of the PCR products from the above two samples was the same as the one obtained from EBV positive BC1 cells (Fig. 2). The amplification was specific and without contamination, as shown by the absence of amplification in the PCR controls, DNA from EBV-negative BJAB cells, and the controls without DNA added (Fig. 2). We also obtained amplification from the DNA sample prepared from the T-PLL cells continuously cultured for 12 months *in vitro* (data not shown).

3.3. EBV antigens were expressed in T-PLL cells

These results indicate that the EBV genomic DNA was present in the T-PLL tumor cells derived from the patient. To further confirm this and to detect any EBV antigens in these cells, we prepared cell lysates from the cultured T-PLL tumor cells as well as EBV-negative BJAB cells, for Western blotting. First, we used the serum prepared from the patient as a primary antibody. This serum was pre-treated by incubation with BJAB cell lysate for 72 hours. Since the patient was EBV positive, there should be a broad spectrum of antibodies against different EBV antigens (Callahan et al., 1999; Robertson and Kieff, 1995). The Western blotting demonstrated that the T-PLL cell contained proteins corresponding to EBNA3C, EBNA2, EBNA1 and probably LMP1, based on the molecular sizes, respectively (Fig. 3A). Although these bands were specific, they were absent in the control BJAB cells, the molecular size alone was not sufficient to identify specific antigens. To confirm the specificity of a number of these EBV antigens and to determine if expression of the EBV oncoprotein LMP1 was present, the membrane was stripped completely of signal and then re-probed with specific monoclonal antibodies against EBNA3C (Fig. 3B) and LMP1 (Fig. 3C), respectively. These two proteins were chosen as targets as these antibodies have greater specificity and were available in our lab (Callahan et al., 1999; Knight et al., 2003). The results demonstrated that at least these two major latent EBV oncoproteins were clearly expressed in the T-PLL cells derived from this patient. We did not further determine expression of EBNA1 and EBNA2 with specific antibodies as they were unavailable in our laboratory.

To corroborate the Western blotting data above, we further employed immunofluorescence assays to determine the expression of EBV antigens in the T-PLL cells. We harvested the T-PLL cells from the growing culture at 6 months when there were only large size tumor cells. EBV-negative BJAB cells and the EBV-positive lymphoblastoid cell line (LCL) were used as controls. There were no detectable levels of EBNA3C or LMP1 expression in BJAB cells (Fig. 4A, B, top panels). In contrast, there were approximately 90% of LCL cells contained EBNA3C or LMP1 (Fig. 4A, B, middle panels). Approximately 80% of T-PLL cells were EBNA3C positive, and 40% of T-PLL cells LMP1 positive (Fig. 4A, B, bottom panels). This suggests that the majority of the T-PLL cells were positive for EBV infection as indicated by the positive EBNA3C signals.

3.4. The cultured T-PLL cells express the CD3 marker

CD3 is a pan-T antigen which is expressed on the surface of T lymphocytes (Kanellopoulos et al., 1983). Since CD3 was positive in bone marrow sample as well as the PBMC sample of the patient, we chose this marker to further confirm the origin of the T-PLL cells. We first stained the cells with CD3 antibody for flow cytometry analysis. The results showed that the cultured T-PLL cells had an increase in CD3 expression level. The intensity of CD3 positive increased about 100 times over that of the control (Fig. 5, Upper Panel). These results suggested that the T-PLL cells derived from the patient's blood cells have a T cell origin. To further determine if these T-PLL cells were also EBV-infected, we performed FACS analysis by double staining using CD3 as well as EBV LMP1 antibody. For analysis of the LMP-1 signals, the cells were fixed in 1% paraformaldehyde at room temperature to ensure that the cells were permeabilized for intracellular staining. Our data showed that the

majority of cells which were LMP1 positive were also CD3 positive (Fig. 5, bottom panel). This demonstrated that in the context of this patient, a predominant fraction of the T-PLL cells were expressing EBV LMP1.

4. Discussion

T-PLL is a rare type of post-thymic T-cell neoplasm, the etiology of which is unknown. So far, it is widely accepted that certain chromosomal translocations are the common molecular feature of this tumor (Ravandi et al., 2005). However the cause of these chromosome changes remains unclear. Unlike Adult T-cell leukemia or lymphoma, the Human T-lymphotrophic viruses I and II (HTLV-I/II) are invariably negative by both serology and polymerase chain reaction (PCR) in T-PLL patients and a positive result for these viruses excludes this diagnosis (Pawson et al., 1998; Pawson et al., 1997).

Epstein–Barr virus (EBV), discovered more than 40 years ago from a Burkitt's lymphoma biopsy, was the first virus to be directly associated with human cancer (Epstein, Achong, and Barr, 1964). EBV has two distinct life cycles in the human host; a lytic form of infection that produces new infectious virions, and a latent form of infection that allows the virus to persist in a dormant state for the lifetime of the host (Klein, Kis, and Klein, 2007). EBV has evolved a life cycle that mimics the natural differentiation pathway of antigen-activated B cells, giving the virus access to its site of latent infection, the resting memory B cells (Klein, Kis, and Klein, 2007). By steering infected cells through the various stages of lymphocyte differentiation, EBV is able to enter a cell type suitable for long-term latent persistence and periodic reactivation (Klein, Kis, and Klein, 2007). However, its presence in various stages of B-cell development, and its ability to infect other cell types such as epithelial cells and T cells could have pathogenic consequences, and may also contribute to the development of a diverse group of leukemias, lymphomas and carcinomas (Klein, Kis, and Klein, 2007).

This T-PLL patient with a high EBV titer allowed the possibility that the virus may be involved in the development of T-PLL. To further confirm this observation, a link between EBV infection and the T-PLL tumor cells must be established from multiple patients. In this report, we continuously cultured patient derived T-PLL cells *in vitro* for approximately a year with loss of continual growth after this period. During the culture of these T-PLL cells *in vitro*, we found that their doubling time was approximately 48–72 hours in the first 2 months after the enhanced population of T-PLL cells survived. However, after this initial 60 days in culture the growth rate of the cells slowed with the supplement of 10% FBS and glutamine in the medium. The growth of these cells was eventually discontinued after 13 months post culture. Improved conditions for culturing T-PLL cells will be necessary for establishment of a T-PLL cell line in the future. Additionally, it would be important to generate multiple cell lines from a number of patients for comparative analyses.

We detected EBV infection of these cells at different stages of culture. Expression of two major latent EBV antigens, EBNA3C and LMP1 was detected in these cells. Therefore, it is likely that EBV had established a Type III latency in these cells as expression of EBNA3C is likely to also be seen with expression on EBNA1P and EBNA2 (Klein, Kis, and Klein, 2007). We also noticed that the percentage of EBNA3C-expressing cells and LMP1-

expressing cells was also varied. This suggests that the T-PLL cells may have expanded from multiple infected T-cells during development of the leukemia and that these cells were likely to be polyclonal in nature as observed by the different EBV latent antigen expression. It would be interesting to determine if there may be a unique type of latency on these cells distinct from type II or type III based on latent antigen expression.

This report provides new evidence that EBV infection may play a role in development of T-PLL. Additional effort will be needed to further focus on the systemic characterization of EBV gene expression profiles in T-PLL cells from multiple patients, and the role of EBV in contributing to the oncogenic process of T-PLL cells.

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Figure 1.

(A) The morphology of the T-PLL cells derived from the patient's periphery blood cells. The lymphocytes were isolated from the periphery blood of the T-PLL patient and then cultured in RPMI1640 supplemented with 10% FBS. The cells were continuously cultured for approximately 13 months. The left picture shows the morphology of the cells in the culture at first day, the size of the cells is almost identical and it is hard to find big size tumor cells. The middle picture shows that the large size tumor cells are visible and the right one shows a typical image obtained for these T-PLL cells. (B) The morphology of the bone marrow sample from the patient which was stained with Haematoxylin and Eosin. There are many mature lymphocytes, and large atypical lymphocytes with small cytoplasm surrounding irregular nuclei, clumped chromatin and distinct nucleoli shown.

Figure 2.

PCR to show the existence of EBV in T-PLL cells. Genomic DNA of T-PLL cells were prepared from the culture which was continuously passaged for 6 months and was used as template for PCR. The DNAs from EBV negative BJAB and positive BC1 cells were used as controls. EBV BamH1 W, C, E regions were amplified and the primer co-ordinates are present in Table 2.

Figure 3.

Western blotting to show the expression of EBV antigens in T-PLL cells derived from patient's peripheral blood cells. The lysate from BJAB cells was used as a negative control. Patient serum (A), A10 mouse monoclonal antibody against EBNA3C (B) and S12 mouse monoclonal antibody against LMP1 (C) were used as primary antibodies, respectively. The membranes stripped completed and re-probed using antibody against actin to be shown as internal loading controls.

Figure 4.

Immunofluorescence analysis to show the expression of EBNA3C (A) and LMP1 (B) in T-PLL cells derived from patient's periphery blood cells. EBV negative BJAB and EBV positive LCL cells were used as controls. The quantification of the positive cells is presented in the bottom.

Figure 5.

FACS analysis shows the T-PLL cells derived from the patient's peripheral blood cells are CD3 positive (A) and dual positive for CD3 and LMP1 (B). The cells were fixed and stained with CD3 antibody or CD3 antibody plus LMP1 antibody and then were subjected to FACS analysis. As shown, the percentages of the cell populations are as following: LMP+ (2%), CD3+ (40%), LMP+/CD3+ (30%), LMP1-/CD3- (25%). BJAB was used as a negative control in this assay.

Table 1

Primers used for PCR analysis

Amplified region	Primer sequences	Primer co-ordinates (NC_007605)
BamHI C fragment	5'GCAGGGCTCGCAAAAGTATAG3'	11095–11855
	5'TGCGGGAAGTGACACCAATA3'	
BamHI E fragment	5'TACTGCCCAACAGTACCAACA3'	99939–100091
	5'GGCCGACATTCTCCAAGATAA3'	
BamHI W fragment	5'CCAGACAGCAGCCCAATTGTC3'	17374–17502
	5'GGTAGAAGACCCCTCTTAC3'	

Table 2

Immunophenotyping Results

Antibody	Major Cell Specificity	Reactivity(Bm)	Reactivity (PB)
CD1a	Thymocyte	-	-
CD2	Pan T, NK	+	+
CD3	Pan T	+	+
CD4	T-helper, Monocyte	-	-
CD5	T, subset of B	+	+
CD7	T, NK		
CD8	T-cytotoxic/suppressor		
CD16	NK, neutrophils	-	-
CD25	pre-B, pre-T	+	+
CD30	Activated T, B	-	-
CD56	NK	-	-
CD57	NK, T-cytotoxic	-	-
TdT	B	-	-
$\gamma\delta$	B	-	-
Cd19	B	-	-