Epstein-Barr Virus (EBV) Gene Expression in EBV-Positive Peripheral T-Cell Lymphomas

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Received 16 February 1993/Accepted 28 June 1993

Epstein-Barr virus (EBV) DNA has been detected in peripheral T-cell lymphomas. In this study, analysis of the EBV termini indicated that the infection was clonal and nonpermissive. Analysis of viral expression detected the processed, spliced mRNAs representing EBNA1, LMP1, LMP2, and BamHI A transcripts in all EBV-positive peripheral T-cell lymphomas. The LMP1 protein was detected by immunofluorescence in a single specimen. In contrast, neither the EBNA2 protein nor the spliced EBNA2 mRNA were detected. These data indicate that EBV-infected T-cell lymphomas are clonal expansions of a single EBV-infected cell with a pattern of gene expression which is distinct from that detected in Burkitt’s lymphomas or posttransplant lymphomas but similar to viral expression in nasopharyngeal carcinomas.

Epstein-Barr virus (EBV) is the cause of infectious mononucleosis and posttransplant lymphoma and is associated with African Burkitt’s lymphoma and nasopharyngeal carcinoma (NPC) (6, 19, 20). EBV has also been detected in Hodgkin’s disease and in peripheral T-cell lymphoma (PTL) (9, 11, 29, 30). Analysis of the EBV termini suggests that most of these malignancies are clonal expansions of a single EBV-infected cell (9, 11, 12). The evolution of malignancy in a cell infected with a virus capable of inducing cellular proliferation suggests that the virus is an important determinant of the malignant pathway. Therefore, it is important to compare patterns of viral expression in these distinct malignancies.

Studies of viral infection in vitro have identified nine viral proteins which are expressed in latently infected cells, including six nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) (12). Several studies have characterized viral expression in various human malignancies by using human sera or monoclonal antibodies (MAbs). These studies have identified several differences in viral expression. In Burkitt’s lymphomas, only EBNA1 is believed to be expressed, whereas in posttransplant lymphomas, EBNA1, EBNA2, and LMP1 have been detected (23, 33). In NPCs, EBNA1, LMP1, and LMP2 and a family of transcripts from the BamHI A fragment are expressed (3, 4, 7, 8, 10). In Hodgkin’s disease, LMP1 and the EBV replication activator protein, ZEBRA, have been detected in Reed-Sternberg cells (16, 17). Detection of LMP1 and EBNA2 in several cases of midline granuloma histologically characterized as PTL has also been reported (9).

In this study, EBV infection in PTL was further characterized by analysis of viral expression by using immunofluorescence with monoclonal antibodies to LMP1 and EBNA2 and polymerase chain reaction (PCR) amplification of cDNA. Tumor samples from six patients with histopathologically and phenotypically characterized PTL were obtained from National Taiwan University Hospital. The histopathologic and immunophenotypic characteristics have been previously described (29, 30).

To identify and characterize EBV DNA in PTLs, Southern blots were prepared with dilutions of Raji DNA representing approximately 1, 5, and 50 copies of episomal DNA and sample DNA, digested with BamHI. Hybridization with the XhoI fragment, representing unique DNA adjacent to the right terminus of EBV, identified a single EBV DNA restriction enzyme fragment at a low copy number in specimens 2 and 4 (Fig. 1 and Table 1). The low level of EBV DNA in specimen 2 may reflect dilution of tumor tissue by infiltrating cells or normal tissue. However, in other hybridizations, approximately two genome copies were detected in specimens 2 and 1 (data not shown). A single restriction enzyme fragment was detected in each of the EBV-positive PTLs. The same fragment was also detected by hybridization with the EcoRI fragment representing unique DNA adjacent to the terminal repeats at the left end of the genome (data not shown). The detection of a single fused restriction enzyme fragment representing the fused EBV termini of the episome indicates that a PTL is a clonal proliferation of a single EBV-infected cell. Ladder arrays of smaller terminal fragments representing virion DNA were not detected, indicating that the EBV infection in PTLs was predominantly latent. In contrast, in NPCs, abundant EBV DNA was detected with additional heterogeneous smaller fragments possibly representing linear DNA (Fig. 1). This suggests that, in some cells in NPCs from Taiwan, EBV replicates, resulting in an increase in the abundance of EBV DNA.

Two types of EBV have been defined by divergent sequences within the EBNA2 and EBNA3 genes (2, 5, 27). To identify the EBV types in PTLs, a Southern blot was hybridized to probes which distinguish the EBNA2 sequences (2, 35). Hybridization with the EBV1 and EBV2 EBNA probes to DNA from three EBV-positive specimens identified EBV2 in specimen 1 and EBV1 in specimens 2 and 4 (Table 1). The detection of EBV2 in a Taiwanese PTL was unexpected, as a similar analysis of Taiwanese NPCs identified the EBV1 strain in 10 of 10 specimens. The EBV1 strain detected in both PTLs and NPCs contains the restric-

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FIG. 1. Identification of EBV DNA in PTLs. Hybridization to Southern blots prepared with DNA digested with BamHI from PTLs and NPCs with an XhoI α RNA probe identified a single fragment in PTL samples 2 and 4. EBV DNA was not detected in samples 5 and 6. Dilutions of Raji DNA representing 50 copies, 5 copies, or 1 copy of EBV were included as positive controls.

Spliced, untranslated leader sequences (12, 25). The individual mRNAs can therefore be identified by amplification across the specific splice into the coding sequences. EBV transcription was analyzed by reverse transcriptase-based PCR with five of the six PTLs. PTL 3 was not analyzed because of the limited biopsy sample size.

The EBNA1 protein is required for replication of the EBV episome and has been detected in all EBV-positive cell lines and associated malignancies. At least three promoters which are used in various states of infection for EBNA1 transcription have been identified (3, 24, 25). Regardless of promoter usage, all EBNA1 cDNAs which have been described contain an exon within the BamHI U fragment (24, 25). The ability to detect the EBNA1 mRNA was therefore used as a marker for RNA integrity and the ability to detect low-copy-number mRNAs. For all PCR analyses, equal aliquots of RNA were used to prepare cDNA with or without reverse transcriptase. The spliced mRNA encoding EBNA1 was amplified with primers representing exons in BamHI U and BamHI K, generating a 216-bp product representing the mRNA (Fig. 3A). The 40-kb intron between these exons inhibits amplification of genomic DNA or unprocessed transcript, thus increasing the sensitivity of detection by PCR (25). After gel electrophoresis and transfer to nitrocellulose, the blot was hybridized to an EBNA1-specific oligonucleotide probe. The EBNA1 mRNA was detected in the three EBV-positive specimens which were tested (specimens 1, 2, and 4) but was not detected in the two examples of EBV-negative T-cell lymphoma (specimens 5 and 6).

The EBNA2 mRNA was amplified with primers within the Y2 exon to primers within the YH exon, which contains the coding open reading frame (2). The spliced cDNA and genomic DNA PCR products are 209 and 595 bp long, respectively. Although the 209-bp product representing EBNA2 mRNA was readily detected in the cDNA preparation from the B95-8 lymphoid cell line, the spliced product representing the EBNA2 mRNA was not detected in any of the T-cell lymphoma specimen (Fig. 3B).

Strains with the EBNA2 gene deleted have been found in cell lines maintained in vitro, in samples from normal individuals in vivo, and in hairy leukoplakia specimens (5, 21, 28, 31). To confirm that the EBV strains in PTLs contained the EBNA2 coding sequences, DNA was amplified with a set of primers which span the EBNA2 open reading frame (Table 2). The 1.4-kb fragment representing the intact EBNA2 open reading frame was detected in the EBV-positive samples (specimens 1, 2, and 3), indicating that

<table>
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<th>Case</th>
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<th>Detection of protein by IF</th>
<th>Detection of mRNA by RT-PCR</th>
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<tr>
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<td>+</td>
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<td>6</td>
<td>−</td>
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<td>EBNA2+</td>
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* EBV DNA detection is indicated by + for positive hybridizations with the XhoI α probe, \( \sim + \) for detection by in situ hybridization (29), and \(+/−\) for detection by PCR. The EBV type is given in parentheses.
* IF, immunofluorescence.
* RT-PCR, reverse transcriptase-based PCR amplification of cDNA.
* BA, BamHI A fragment.
* ND, not determined.
FIG. 2. Identification of the LMP1 protein in PTLs. Frozen sections were fixed in a chilled 1:1 mixture of methanol and acetone and stained with the S12 and CS1-4 MAb for LMP1 and the PE2 MAb for EBNA2 and then with biotin-conjugated goat anti-mouse immunoglobulin G antibody (Vector Laboratories, Burlingame, Calif.) and fluorescein avidin D (Vector) (15, 33, 34). EBV-positive (Raji, B95-8, and BHM) and EBV-negative (BIAB and Louckes) lymphoblastoid cell lines were used as positive and negative controls. (A) Detection of LMP1 with MAb CS1-4 in the lymphoblastoid cell line BHM identified the characteristic capped-membrane staining. (B) LMP1 in sample 1 was detected as cytoplasmic punctate staining in all tumor cells. Immunofluorescence stains with fluorescein isothiocyanate are shown. Magnification, ×1,000.
FIG. 3. Detection of EBV mRNA in PTLs by reverse transcriptase-based PCR. cDNA was synthesized and PCR amplification was performed as described previously with equal aliquots for reactions with (RT+) or without (RT−) reverse transcriptase (5). Controls containing all PCR reagents and containing H2O instead of the template were included for all reactions (H2O). The oligonucleotides (arrow) used for PCR which spanned the specific introns for the EBNA1, EBNA2, LMP1, LMP2A, LMP2B, and BamHI A transcripts are presented in Table 2. The BamHI fragments and open reading frames are indicated. TR, terminal repeat. After transfer to nitrocellulose, the products were hybridized with internal 32P-labeled oligonucleotide probes (Table 2). B95-8 cDNA was used as a positive control for PCR products representing the processed mRNAs. HeLa cell cDNA was used as a negative control in LMP2 reactions. (A) Detection of EBNA1 mRNA with primers from the BamHI U and BamHI K fragments in the B95-8 EBV genome. The PCR product representing the spliced mRNA of EBNA1 is 216 bp long. (B) Analysis of EBNA2 mRNA transcription in PTLs. The PCR products of cDNA representing the spliced EBNA2 mRNA and of genomic DNA are 209 and 595 bp long, respectively. Transcription was not detected in the PTL samples, although the spliced EBNA2 PCR product was readily detected with RNA from the B95-8 cell line. (C) Detection of LMP1 mRNA in PTLs. The sizes of the PCR products representing spliced mRNA and genomic DNA of LMP1 are 296 and 450 bp long, respectively. The LMP1 mRNA was detected in all EBV genome-positive samples. A PCR product representing EBV genomic sequences was detected in sample 5 with and without reverse transcriptase. This result may reflect amplification of DNA from EBV-positive B lymphocytes. (D) Detection of LMP2 mRNA in PTLs. The LMP2 mRNAs have distinct first exons. The PCR products for each mRNA utilized primers from the two distinct first exons and primers from exon 2, generating products of 309 bp for LMP2A and 301 bp for LMP2B. The LMP2A mRNA was detected in the EBV-positive samples and in sample 5. The LMP2B product was detected in sample 4 only.
EBNA2 was not deleted in the EBV strains in PTLs (data not shown).

The LMP1 introns are relatively small and total 154 bp. Amplification across these introns is less sensitive than that across large introns; therefore, two nested pairs of primers which generated a 450-bp genomic signal and a 296-bp product representing the spliced mRNA were used. The 296-bp product representing the spliced form was detected in specimens 1, 2, and 4 (Fig. 3C). The 450-bp PCR product representing genomic DNA was detected in both the reverse transcriptase-positive and -negative reaction mixtures for specimen 5, indicating the presence of contaminating DNA. As EBV DNA was not detected on Southern blots for specimen 5, the detection of EBV by PCR for specimen 5 may represent amplification of DNA from infiltrating EBV-positive B cells.

The detection of LMP1 mRNA in specimens 1, 2, and 4 with detection of protein only in specimen 1 may indicate that the level of protein is undetectable in the other samples or suggest that the LMP1 mRNA is not always translated. Similar results have been reported for NPC (3, 34).

The spliced mRNAs encoding the LMP2A and LMP2B proteins are transcribed across the terminal repeats (13, 26). The two forms of the protein differ, as the first exon of the LMP2A mRNA encodes a hydrophilic cytoplasmic domain at the amino terminus while the first exon of LMP2B mRNA is noncoding. Amplification with primers from the two distinct first exons for LMP2A and LMP2B to primers in exon 2 generates 309- and 301-bp products, respectively (4). Like the EBNA1 mRNA, the large intron between the first and second exons inhibits the amplification of genomic DNA and unprocessed transcription. The LMP2A spliced product was detected in the EBV-positive specimens 1, 2, and 4. The LMP2B product was detected only in specimen 4. Interestingly, the LMP2A mRNA was also detected in specimen 5, in which EBV DNA was only detectable by PCR (Fig. 3). In a previous study, the LMP2A mRNA was the only transcript detected by PCR in peripheral blood lymphocytes (18). Thus the data presented here suggest that specimen 5 contained EBV-positive lymphocytes which also expressed LMP2A.

Abundant transcription from the BamHI A fragment has been consistently detected in NPCs. The cDNAs representing these transcripts are intrinsically spliced (7, 10). Amplimers which span a small 171-bp splice generate 150- and 321-bp products that are spliced and genomic products, respectively (7). In all three of the EBV-positive specimens (1, 2, and 4), the signals representing both the spliced and unspliced products were detected only in the reaction mixtures which contained reverse transcriptase (data not shown). This result reveals the presence of both spliced and unspliced transcription from these sequences in PTLs.

In summary, the data presented here indicate that a PTL is a clonal proliferation of a single EBV-infected cell, like other malignancies associated with EBV. In addition, the absence of small linear fragments detected by the assay used in this study indicates that PTL is predominantly a latent infection. The pattern of viral expression in PTL is distinct from that which has been shown to occur in the B-cell malignancies associated with EBV, Burkitt’s lymphoma and posttransplant lymphoma, and is similar to that detected in NPC with consistent transcription of EBNA1, LMP1, LMP2A, and BamHI A (4, 5, 7, 10). The absence of EBNA2 expression in PTL, which is an additional example of EBV-infected lymphoid tissue, suggests that the expression of EBNA2 and the coordinately regulated EBNA3 proteins is apparently B-cell specific. Moreover, these similar patterns of expression in two malignancies associated with EBV suggest that these gene products either are essential for maintaining viral infection or contribute to tumor growth and clonal predominance.

This study was supported by grants from the National Institutes of Health (CA32979), the American Cancer Society (MV354A), and the National Science Council of Taiwan, Republic of China.

REFERENCES


