A Syndrome of Peripheral Blood T-Cell Infection With Epstein-Barr Virus (EBV) Followed by EBV–Positive T-Cell Lymphoma

By Hirokazu Kanegane, Kishor Bhatia, Marina Gutierrez, Hisashi Kaneda, Taizo Wada, Akihiro Yachie, Hidetoshi Seki, Takashi Arai, Sei-ichi Kagimoto, Minoru Okazaki, Tsutomu Oh-ishi, Amir Moghaddam, Fred Wang, and Giovanna Tosato

The role of Epstein-Barr virus (EBV) in the pathogenesis of severe, chronic active EBV infection and its complications is unclear. We investigated two Japanese patients diagnosed with severe, chronic active EBV infection who subsequently developed EBV–positive T-cell lymphoma. The patients displayed abnormally high antibody titers to EBV antigens, and had evidence of peripheral blood CD4+ T-cell infection with EBV, 19 months and 3 months, respectively, before the diagnosis of EBV–positive T-cell lymphoma. The lymphomas were infected with monoclonal EBV and expressed the EBV latency genes EBNA-1, LMP-1, and LMP-2. Genetic studies showed that the virus detected in the T-cell lymphoma was indistinguishable, with respect to type and previously defined LMP-1 and EBNA-1 gene variations, from virus detected in the peripheral blood T cells 19 months earlier. These studies support an important pathogenetic role of T-cell infection with EBV in chronic active EBV infection and in the EBV–positive T-cell lymphoma that followed.

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From the Center for Biologics Evaluation and Research, Bethesda MD; National Cancer Institute, National Institutes of Health, Bethesda, Maryland; Kanazawa University School of Medicine, Kanazawa, Ishikawa, Japan; Saitama Children’s Medical Center, Iwatsuki, Saitama, Japan; and the Infectious Disease Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA.

Address correspondence to Dr G. Tosato, Center for Biologics Evaluation and Research, Building 29A, Room 2D16, HFM-535, 1401 Rockville Pike, Rockville, Maryland 20852.

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antibodies to hepatitis A, B, and C were detected by passive hemagglutination tests. Antibodies to cytomegalovirus were determined by complement fixation or immunofluorescence.

In situ hybridization for EBV RNA. The presence of EBV in cell populations was assessed by in situ hybridization for EBV–encoded small nuclear RNAs (EBER).22,23 CD8+ T, CD8+ B, and NK cells were separated from the patient’s peripheral blood mononuclear cells by incubating with monoclonal antibodies to CD8, CD4, CD8, and CD16, followed by isolation of each subpopulation by electronic cell sorting, using an Epics Elite flow cytometer (Coulter Immunology, Hialeah, PA) or a FACSStar Plus (Becton Dickinson, San Jose, CA). Each sorted population was more than 97% pure. Subpopulations of CD4+ T, CD8+ T, B, NK, and control cells were centrifuged on 3-aminopropyltriethoxy-silane–coated glass slides and fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer. After rinsing and rehydration, hybridization was performed with an ALP-conjugated sense and antisense oligonucleotide probe to EBER–1, as described.22,24

Southern blot analysis for EBV clonality. DNA was prepared from fresh frozen tissue samples and from B95-8, Raji, and Louches cell lines with the QIAamp Tissue Kit (Qiagen, Inc, Chatsworth, CA) according to the manufacturer’s protocol. Ten µg of genomic DNA was digested with BamHI separated on a 0.7% agarose gel and transferred to a nitrocellulose membrane. The membrane was hybridized with a 32P-labeled B95-8 DNA fragment (167,129-169,566) that contains the LMP-1 open reading frame and detects the right terminal repeats.25 The membrane was washed with 0.2% SSC and 1% SDS at 68 °C and visualized by autoradiography.

Polymerase chain reaction (PCR) for EBV DNA. The oligonucleotide sequences for amplification of the EBV U2 region encoding EBER-1 were 5'-TTTCCACCAATCATGAAACC-3' and 5'-TG-GCAAAGTGCTGAAAGCAA-3'. Amplification was performed as described previously.22,26 Expected lengths of the amplified products derived from type 1 and type 2 EBV were 378 bp and 483 bp, respectively. Sequences within the C-terminal region of LMP-1 gene were amplified, as described,27 by using the following primers: 5'-GGCAGCTCTGGAGAAATGT-3' and 5'-GACATTGATGCCTAGAAG-3'. Two EBNA-1 fragments were amplified including a 284 bp fragment (nucleotides 50-334) and a 330 bp fragment (nucleotides 1341-1671). The primer pairs used for amplifications were: ACAG-GACCTCGGGAATGCTCTATTGAGGAGA and GGTC-CAGGCGGCAATTCCAA (330 bp fragment). The amplified fragments were directly sequenced from PCR amplified products by using the Sequenase (US Biochem, Cleveland, OH) protocol, as described.28

RNA preparation and reverse transcriptase-mediated PCR (RT-PCR). Total RNA was extracted from cell pellets or tissue, by using guanidine isothiocyanate-phenol (Trizol, Life Technologies). The first-round polymerase chain reaction (RT-PCR) was performed with an ALP-conjugated sense and antisense oligonucleotide probe to EBER–1, as described.22,24

RESULTS

EBV serologies from the two patients were consistent with chronic active EBV infection 19 months (case 1) and 3 months (case 2) before the diagnosis of T-cell lymphoma. Positive IgG, but not IgM, anti-VCA antibodies and positive anti-EBNA antibodies indicated past infection with EBV (Table 1). Abnormally elevated IgG antibodies to VCA and EA and detection of IgA antibodies to VCA and EA suggested ongoing viral replication. In both patients antibody titers to EBV antigens did not change substantially during the following 2 years. Antibodies to hepatitis virus and cytomegalovirus were all negative (data not shown).

To determine which cell populations were infected with EBV in these patients, highly purified (>97% pure) populations of CD4+ T cells, CD8+ T cells, CD16+ NK cells, and CD20+ B cells, obtained by cell sorting of peripheral blood mononuclear cells, were evaluated for the presence of EBV by in situ hybridization for EBER1. In patient 1 (Fig 1), 3.4% of CD4+ T cells were EBER1 positive 19 months before the diagnosis of T-cell lymphoma. As expected, more than 95% of Daudi cells used as a positive control were EBER1 positive, whereas all control purified peripheral blood cell populations from two EBV–seropositive normal individuals were EBER1 negative (data not shown).

Lymphomas were diagnosed in the lymph node from patient 1 and in the spleen from patient 2 at 19 months and 3 months, respectively, after peripheral blood CD4+ T-cell infection with EBV was documented. Both lymphomas were EBV positive by EBER1 in situ hybridization (Fig 2), and were of T-cell lineage as determined by immunohistochemistry with an anti-CD45RO and anti-CD4 MoAb, and Southern analysis that detected rearranged T-cell receptor γ- and β-chain genes with germline immunoglobulin JH genes (not shown).

Both tumors contained monoclonal EBV determined by Southern analysis of EBV terminal repeats (Fig 3). Two tumor samples from patient 1, derived from distinct lymph nodes, displayed indistinguishable clonality. The monoclonal EBV in the tumor sample from patient 2 was distinct from that found in patient 1. As expected, EBV in the B95-8 cell line was present at a high copy number in both episomal and linear forms, whereas

<table>
<thead>
<tr>
<th>Antibodies to EBV Antigens from Patients with Chronic Active EBV Infection</th>
<th>VCA</th>
<th>EA</th>
</tr>
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<tbody>
<tr>
<td>IgG</td>
<td>IgM</td>
<td>IgA</td>
</tr>
<tr>
<td>Case 1</td>
<td>20480</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Case 2</td>
<td>5120</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*Antibody titers to EBV viral capsid antigen (VCA), early antigen (EA), and nuclear antigen (EBNA) were determined by immunofluorescence in patient serum at presentation.*
EBV was monoclonal in the Raji cell line; no EBV was detected in the EBV-negative Louckes cell line (Fig 3).

RT-PCR analysis for EBV–gene expression in the lymphoma tissue specimens showed a type II form of EBV latency. EBER-1, EBNA-1, LMP-1, and LMP-2A transcripts could be amplified readily from both lymphoma specimens, whereas no EBNA-2 or LMP-2B transcripts were derived from these tissues (Fig 4). The mRNAs for the EBV replication genes BZLF1 and BCRF1 (vIL-10) were amplified from both lymphomas, consistent with the occurrence of viral replication in these tissues. The mRNA for the cellular genes hIL-10 and GAPDH were amplified from all samples tested. As expected, the EBV–producing marmoset cell line B95-8 expressed all EBV genes, and the EBV–negative lymphoma cell line BJAB expressed the cellular hIL-10 and GAPDH genes, but no EBV genes.

To examine possible links between peripheral blood T-cell infection with EBV and the subsequent development of EBV–positive T-cell lymphoma, we compared EBNA-2, LMP, and EBNA-1 virus variants in the purified CD4+ peripheral blood T cells from patient 1 (obtained 19 months before the diagnosis of T-cell lymphoma was made) with the virus detected in two T-cell lymphoma tissue specimens from the same patient.

![Fig 1. Detection of EBER1 expression by in situ hybridization. Purified populations of CD4+ T cells, CD8+ T cells, CD16+ NK cells, and CD20+ B cells obtained from patient 1, 19 months before the development of lymphoma, were cytocentrifuged and hybridized with an alkaline phosphatase-conjugated EBER1 antisense oligonucleotide probe.](image1)

![Fig 2. Detection of EBER1 expression by in situ hybridization of lymphoma tissues. (A) Lymph node from case 1 (100X magnification); (B) spleen from case 2 (200X magnification).](image2)
Two types of EBV, type 1 and 2, have been defined by differences in the U2 region encoding EBNA-2 resulting in distinct serologic reactivities. PCR amplification with EBNA-2 specific primers indicated that all samples from patient 1, including the peripheral blood CD4 T cells and the two lymphoma tissue specimens, were infected with type-1 EBV (Fig 5). Also infected with type-1 EBV was the lymphoma tissue from patient 2 (Fig 5). As expected, type-1 EBV was detected in the control B95-8 cells (378 bp amplification product), whereas type-2 EBV was detected in Ag876 cells (483 bp amplification product).

The presence of a 30 bp deletion in the LMP1 gene has defined a variant EBV isolate, detectable in the Ag876 cell line (230 bp amplification product), that can be distinguished from the full-length product (260 bp amplification product) of the prototype B95-8 cell line (Fig 5). All samples from patient 1, including peripheral blood CD4-positive cells and lymphoma tissue from the two lymph nodes, yielded the 230 bp PCR product (Fig 5) indicative of the presence of a deleted LMP1 gene. In contrast, the lymphoma tissue from patient 2 yielded a 260 bp PCR product indicative of the presence of a full-length LMP1 gene (Fig 5).

Sequence variations of the carboxy terminal region of EBNA-1 have defined five EBV subtypes distinguished on the basis of several amino acid substitutions, including substitutions at position 487 (alanine, detected in the prototype B95-8 virus, and substitutions with threonine, valine, proline, or leucine). Using PCR to amplify EBNA-1 specific fragments, followed by DNA sequencing, all five previously identified EBNA-1 subtypes were recovered from the peripheral blood CD4+ T cells from patient 1 (Fig 6). In contrast, only the EBNA-1 subtype identified by valine at position 487 was detected in the lymphoma specimens from patient 1 (Fig 6). The same EBNA-1 subtype was also detected in the lymphoma specimen from patient 2 (Fig 6). Thus, patient 1 harbored EBV in the peripheral blood CD4+ T cells that was indistinguishable from the virus detected 19 months later in the T-cell lymphoma, with respect to previously defined EBNA-1, EBNA-2, and LMP-1 variants.

DISCUSSION

In the present studies, we show that approximately 4% of the peripheral blood CD4+ T cells from two Japanese patients with
T-CELL INFECTION WITH EBV

serologic evidence of severe, chronic, active EBV infection were infected with EBV at 19 months and 3 months, respectively, before the development of EBV–positive T-cell lymphoma. Both T-cell lymphomas contained monoclonal EBV and expressed the transforming gene LMP-1, suggesting that the virus might play a role in the pathogenesis of these tumors. Genetic studies showed that EBV infecting both the peripheral blood CD4+ T cells and the T-cell lymphoma diagnosed 19 months later was of type 1 and displayed a 30 bp deletion of LMP-1. In addition, the virus recovered from the T-cell lymphoma tissue was characterized by a substitution at position 487 in the EBNA-1 gene. The same virus variant was also detected in the peripheral blood T cells, albeit in conjunction with four other EBNA-1 subtypes. Thus, we describe the previously unrecognized occurrence of peripheral blood T-cell infection with EBV preceding the development of malignant EBV–positive T-cell lymphomas. Genetic evidence in one of the patients linked the virus from the peripheral blood CD4+ T cells with the monoclonal virus later detected in the T-cell lymphoma in which the pattern of viral gene expression suggested a continued role for EBV as a transforming agent. Thus, peripheral blood T-cell infection with EBV may be important to the pathogenesis of certain EBV–positive T-cell lymphomas.

Recently, T-cell infection with EBV was reported with increasing frequency in the context of certain leukemias and lymphomas, particularly in the Orient.9-17 Some of these cases have presented as fatal lymphoproliferative disorders associated with primary EBV infection that rapidly progressed toward multiple organ failure, sepsis, and death.9-11 Other cases have presented as extranodal lymphomas localized to the upper respiratory tract exhibiting characteristic histological features of tissue necrosis, vascular damage, and infiltration with inflammatory cells, and have been variously identified as nasal or nasal-type T/NK cell lymphomas, lymphomatoid granulomatosis, lethal midline granuloma, or angiocentric lymphomas.12,13 Other cases included nodal or extranodal T-cell lymphomas with various histologies and phenotypes.14-17 Recently, four EBV–infected T-cell lines were derived from culture of peripheral blood from three patients with severe, chronic active EBV infection.23 In addition, circulating T cells from two patients with severe, chronic active EBV infection were reported to be infected with EBV, raising the possibility that T-cell infection with this virus might be important to the pathogenesis of this illness.23 However, the occurrence of peripheral blood T-cell infection with EBV preceding the development of EBV–positive T-cell lymphoma was not previously described.

In the patients described here, the relationship between the EBV–infected circulating CD4+ T cells and the malignant CD4+ T cells in the lymphomas that subsequently developed could not be established. It is possible that the circulating EBV–infected CD4+ cells were premalignant and contributed to lymphomagenesis. Alternatively, they could have been normal lymphocytes serving as a reservoir for virus later detected in the lymphoma. T-cell receptor clonality analysis could not be performed on the rare circulating CD4+ T cells that were infected with EBV, and thus, cell clonality relationships could not be established.

Normal T-cells are not easily infected with EBV in vitro or in vivo, and generally do not express the EBV receptor, CD21. Some studies have documented expression of CD21 in a proportion of circulating CD4 and CD8+ T cells, and on immature thymic T cells.19,33,34 Some reports mention transient
infection of T cell with EBV, but no EBV–immortalized T-cell
lines have been generated in vitro by exposure to the virus.\textsuperscript{35,36}
Recently, an HTLV-1–infected T-cell line, MT-2, was developed that
expresses functional EBV receptors, and can be persistently
infected with EBV.\textsuperscript{37} Benign T-cell infections with EBV in vivo
have rarely been documented. One report describes a case of a
transient polyclonal benign proliferation of EBV-infected T
cells in a Japanese individual presenting with an infectious
mononucleosis-like syndrome.\textsuperscript{38} Another documented the pres-
ence of EBV–infected T cells in the lymph nodes of individuals
with acute infectious mononucleosis.\textsuperscript{39} Thus, T-cell infection
with EBV was reported rarely, and mostly in the context of
severe illness.

The rare occurrence of T-cell infection with EBV suggests
that the necessary conditions are infrequently met. Susceptible
T-cell targets could be rare or represent an abnormal T-cell
population, and conditions permitting T-cell infection could be
stringent and peculiar. Rare viral mutants may be required for
T-cell infection. Previously, genetic studies of EBV have
documented polymorphism in the EBNA2, EBNA3, LMP-1
genes, and BamHI F region of the genome, but EBV isolates
from normal individuals and EBV–positive tumors in the same
geographic area generally exhibited similar gene polymor-
phisms.\textsuperscript{27} We found that the EBNA-1 variant detected here in
both T cell lymphomas and in the peripheral blood T cells of one
of the patients was not previously identified in peripheral blood
of normal individuals,\textsuperscript{28} including ten normal Japanese (data not
shown), suggesting that further studies are necessary to study
the potential role of EBNA-1 mutations to EBV–associated
diseases. The severity of disease associated with T-cell infection
with EBV may be due, in part, to infection and secondary
functional impairment of the same immune T cells that are
primarily involved in host immunity against the virus.\textsuperscript{40}
In addition, although EBV has evolved an effective strategy for
latent survival in B cells, such strategy may not apply to the
establishment of latency in T cells.

Severe, chronic active EBV infection is a life-threatening
illness leading to the development of lymphoma, myelodyspla-
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