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タイトル
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Clonal expansion of Epstein-Barr virus (EBV)-infected γδ T cells in patients with chronic active EBV disease and hydroa vacciniforme-like eruptions

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Running head: Clonal expansion of EBV-infected γδ T cells
Abstract

Chronic active Epstein-Barr virus (EBV) disease (CAEBV) is a systemic EBV-positive lymphoproliferative disorder characterized by fever, lymphadenopathy, and splenomegaly. Patients with CAEBV may present with cutaneous symptoms including hypersensitivity to mosquito bites and hydroa vacciniforme (HV)-like eruptions. HV is a rare photodermatosis characterized by vesicles and crust formation after exposure to sunlight, with onset in childhood, and is associated with latent EBV infection. While $\gamma\delta$ T cells have been recently demonstrated to be the major EBV-infected cell population in HV, the immunophenotypic features of EBV-infected $\gamma\delta$ T cells in CAEBV with HV-like eruptions or HV remain largely undetermined. Herein we describe 3 patients with CAEBV whose $\gamma\delta$ T cells were found to be the major cellular target of EBV. HV-like eruptions were observed in two of them. A clonally expanded subpopulation of $\gamma\delta$ T cells that were highly activated and T-cell receptor $\gamma9$- and $\delta2$-positive cells was demonstrated in all patients. We also showed that the clonally expanded $\gamma\delta$ T cells infiltrated into the HV-like eruptions in one patient from whom skin biopsy specimens were available. These results suggest the pathogenetic roles of clonally expanded $\gamma\delta$ T cells infected by EBV in patients with CAEBV and HV-like eruptions.

Keywords: Epstein-Barr virus, clonal proliferation, $\gamma\delta$ T cells, hydroa vacciniforme
Introduction

Epstein Barr virus (EBV) is a herpes virus that is ubiquitous in the human population. Primary EBV infection occasionally causes infectious mononucleosis that is an acute and usually benign self-limited disease [1]. The target of EBV infection is primarily B lymphocytes. However, EBV infection has also been etiologically associated with various malignancies and lymphoproliferative disorders including chronic active EBV infection (CAEBV), in which monoclonal or oligoclonal proliferation of EBV-infected T or natural killer (NK) cells may play important roles in disease development [2]. CAEBV is characterized by persistent or recurrent infectious mononucleosis-like symptoms such as fever, lymphadenopathy and hepatosplenomegaly, and by extremely high viral loads in peripheral blood [3]. Patients with CAEBV may also suffer from cutaneous lesions including hypersensitivity to mosquito bites or hydroa vacciniforme (HV)-like eruptions. Based on the cellular targets of EBV, CAEBV can be divided into T-cell type and NK-cell type infections [4]. T-cell type CAEBV is further subdivided into CD4+, CD8+, and γδ T cell-type infections.

HV is a rare photosensitivity disorder characterized by vesicle, crust and scar formation on sun-exposed areas with onset in childhood. Although the origin of this condition remains unknown, HV resolves spontaneously by adulthood in most cases [5]. However, there also have been severe cases accompanied by systemic manifestations including fever, lymphadenopathy and liver dysfunction. These patients may progress to cutaneous T-cell lymphoma [6]. Accumulating evidence has suggested that both typical and severe HV are associated with latent EBV infection [7-9]. In addition, γδ T cells have
recently been demonstrated to be the major cellular target of EBV infection in HV [10-12]. However, the immunophenotypic features of EBV-infected γδ T cells and their involvement in skin lesions in CAEBV and/or HV are not fully understood. In this report, we describe 3 patients with CAEBV whose γδ T cells were predominantly infected by EBV, and show their selective activation, clonal expansion, and recruitment to HV skin lesions.
Materials and Methods

Patients

We evaluated 3 Japanese patients with γδ T cell-type CAEBV. The clinical and immunological data of patient 1 have been reported elsewhere [13]. Patient 2 was a 10-year-old girl who had frequent episodes of fever, stomatitis, and liver dysfunction since the age of 4 years. She had mild hepatosplenomegaly. The copy numbers of the EBV genome were found to be extremely high in the peripheral blood at the age of 9 years (1.2 x10^5 copies/μg DNA). There was no history of hypersensitivity to mosquito bites and HV in this patient. Patient 3 was a 14-year-old female who developed vesiculopapules on sun-exposed skin at the age of 4 years. A diagnosis of HV was made at the age of 5 years, when skin biopsy specimens exhibited the presence of EBV genome. The EBV viral loads in the peripheral blood were in the range of 3.5 x 10^3 to 5.4 x10^4 copies/μg DNA. Despite the absence of hepatosplenomegaly or lymphadenopathy, her clinical history was remarkable for occasional febrile episodes and frequent severe gingivitis in addition to HV. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

Cell preparations and in situ hybridization for EBV-encoded small RNA1 (EBER-1)

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation from patients and controls. Peripheral blood lymphocytes (PBLs) were prepared from PBMCs by depletion of monocytes using anti-CD14 monoclonal
antibody (mAb)-coated magnetic beads. CD4⁺ T, CD8⁺ T, CD19⁺ B, and CD56⁺ NK cells were then purified by positive selection from PBLs using mAb-coated magnetic beads [14]. To obtain γδ T cells, PBLs were stained with biotin-conjugated anti-T-cell receptor (TCR) γδ mAb, followed by incubation with streptavidin-coated magnetic beads (all from Becton Dickinson, San Diego, CA). The purity of the isolated γδ, CD4⁺, and CD8⁺ T cells from patient 2 was 95.6, 98.6, and 97.8%, respectively, as determined by flow cytometric analysis. The purity of the isolated γδ T, CD4⁺ T, CD8⁺ T, CD19⁺ B, and CD56⁺ NK cells from patient 3 was 98.2, 98.4, 94.7, 96.2, and 92.8%, respectively. In situ hybridization for EBER-1 was performed as described previously [14].

Flow cytometry

Expression of Vδ chains and human leukocyte antigen (HLA)-DR was evaluated on γδ T cells using a FACSCalibur flow cytometer with CellQuest software (BD Bioscience, Tokyo, Japan) [14]. The following mAbs were used: fluorescein isothiocyanate-conjugated anti-TCR Vδ1 (Thermo Scientific, Rockford, IL), anti-TCR Vδ2 (Immunotech, Marseille, France), anti-TCR Pan γδ (Immunotech), and phycoerythrin-labeled anti-HLA-DR (Becton Dickinson).

Analysis of TCR Vδ and Vγ mRNA

Complementarity determining region 3 (CDR3) spectratyping was performed as described previously, with minor modifications [15, 16]. Briefly, each fragment of TCR Vδ and Vδ was amplified from cDNA obtained from PBMCs with one of 3 Vδ-specific or 3 Vγ-specific primers, respectively. Thirty-five or 30 cycles of amplification (94°C for
45 sec, 60°C for 45 sec, and 72°C for 45 sec) were used, and the polymerase chain reaction (PCR) products were then subjected to run-off reactions using a nested 6-fluorescein phosphoramidite-labelled Cδ or Cγ primer, respectively. The size distribution of each of the fluorescent PCR products was determined as described previously [17]. Quantitative reverse-transcription (RT)-PCR of TCR Vδ2 was performed on an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR GreenER qPCR SuperMix for ABI Prism (Invitrogen, Carlsbad, CA) [14]. We used β-actin as the reference gene. The PCR products of Vδ2 cDNA were subcloned with a TOPO TA cloning kit (Invitrogen) and subcloned PCR products were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit on an ABI 3100 automated sequencer (Applied Biosystems).

Analysis of TCR Vδ genomic sequences in skin

TCR-δ gene rearrangements were detected by family-specific multiplex PCR, as described previously [18]. Genomic DNA extracted from paraffin-embedded skin biopsy specimens with DEXPAT (Takara Bio Inc., Shiga, Japan) was subjected to PCR amplification using 6 TCR Vδ-specific forward primers and 4 TCR Jδ-specific reverse primers that could detect all major Dδ(D)Jδ rearrangements. PCR products were then subcloned and sequenced.
Results

Ectopic EBV infection of γδ T cells

To determine which populations of lymphocytes were infected with EBV, in situ hybridization for EBER-1 was performed on samples isolated from the patients. As shown in Fig. 1, γδ T cells were found to account for the majority of EBER-1+ cells, with negligible EBER-1 identified in CD4+ T, CD8+ T, CD19+ B, and CD56+ NK cells in all cases. Immunophenotypic analysis of lymphocyte subsets exhibited markedly increased percentages of γδ T cells in their peripheral blood (Fig. 2A). More than 80% of those γδ T cells expressed the activation marker HLA-DR, which is generally negative in normal γδ T cells (Fig. 2B). The ratio of CD4+ to CD8+ T cells and the percentage expression of CD45RO+ memory marker in CD4+ and CD8+ T cells were normal in all patients (data not shown).

Clonal expansion of Vγ9+ Vδ2+ γδ T cells

We next analyzed the diversity of the TCR Vδ repertoire in γδ T cells from the patients using Vδ-specific mAbs and CDR3 spectratyping. Flow cytometric analysis clearly demonstrated a massive expansion of TCR Vδ2+ cells among the γδ T cells from these patients (Fig. 2A). In age-matched controls, CDR3 spectratyping of the TCR Vδ1, δ2, and δ3 segments exhibited a Gaussian curve reflecting the polyclonal Vδ repertoire (Fig. 3A). Although all 3 Vδ segments were amplified from the patients’ cDNA, preferential usage of TCR Vδ2 was seen on their gel images in the patients compared with controls (data not shown). Quantitative RT-PCR analysis showed that the levels of
Vδ2 mRNA in PBMCs from the patients were much higher than those in PBMCs from controls (Fig. 3B). CDR3 spectratyping of their TCR Vδ2 segments exhibited a single peak, whereas their Vδ1 and Vδ3 segments exhibited polyclonal peaks (Fig. 3A). The junctional amino acid sequence of their TCR Vδ2 segments also exhibited monoclonal profiles (Table 1). The clonally expanded γδ T cells of patients 1, 2 and 3 were Vδ2-Jδ2+, Vδ2-Jδ1+, Vδ2-Jδ3+ cells, respectively. We also analyzed mRNA of TCR Vγ1, Vγ4 and Vγ9. Similar to those found in TCR Vδ2, the patients’ TCR Vγ9 segments were preferentially amplified (data not shown), and showed monoclonal profiles (Fig. 3C). Consistent with these monoclonal TCR Vδ2 and Vγ9 patterns, Southern blot analysis of EBV terminal repeats showed monoclonal EBV in the peripheral blood from all patients (data not shown).

**Skin infiltration of Vδ2+ γδ T cells**

The availability of skin biopsy specimens from patient 1 offered us the unique opportunity to investigate whether the clonally expanded γδ T cells existed in the HV-like eruptions. Although the Vδ clones obtained from her skin lesion included only Vδ2, the Vδ2 segments exhibited a couple of different Jδ. Of note, we were able to show that one of those Vδ2 clones were identical to that of the clonally expanded Vδ2-Jδ2+ γδ T cells in the peripheral blood (Fig. 4, Table 1). These results suggested that EBV-infected Vδ2+ γδ T cells infiltrated into the skin lesion.
Discussion

HV has been recently reported to be pathogenically associated with chronic latent EBV infection [7-9]. It is also known that patients with CAEBV may present with various cutaneous manifestations including HV-like eruptions [3]. CAEBV patients with HV-like eruptions sometimes develop cutaneous T-cell lymphoma that is defined as HV-like lymphoma in the 2008 World Health Organization classification of lymphoid neoplasms [19]. This condition is increasingly being described in children and adolescents from Asia or Latin America [19]. In these diseases, the EBV-infection is usually thought to occur in T or NK cells, however, precise immunophenotyping and clonality of those cells remains to be elucidated [7-9].

In the present study, we demonstrated ectopic EBV infection of $\gamma\delta$ T cells and increased subpopulations of $\gamma\delta$ T cells in the peripheral blood in 3 patients with CAEBV. Interestingly, two of them showed HV-like eruptions. These results were in agreement with recent studies that demonstrated associations between HV and increased numbers of EBV-infected $\gamma\delta$ T cells [10-12]. In addition, we found that circulating $\gamma\delta$ T cells were in a highly-activated state in our patients. In normal individuals, $\gamma\delta$ T cells usually comprise a small proportion of the circulating lymphocyte population. Expansion of the $\gamma\delta$ T-cell subset has been also documented in the context of various infections including Mycobacterium and cytomegalovirus [20]. However, selective activation and expansion of $\gamma\delta$ T cells may provide us with clinical clues to suspect ectopic EBV infection of $\gamma\delta$ T cells in children suffering from HV or lymphoproliferative disease. Further investigations are necessary to clarify the major cellular targets of EBV infection and to understand the
pathophysiological link between ectopic EBV infection and the clinical manifestations of
the disease.

There was clear evidence of clonal expansion of $V_\gamma 9^+ V_\delta 2^+ \gamma\delta$ T cells in our
patients. We assume that the EBV infection predominantly targeted $V_\gamma 9^+ V_\delta 2^+ \gamma\delta$ T cells
and expanded clonally in the patients. Human $\gamma\delta$ T cells exhibit a restricted repertoire
using a limited set of $V_\gamma$ and $V_\delta$ gene elements compared with $\alpha\beta$ T cells. There are only
6 functional $V_\gamma$ and 8 functional $V_\delta$ genes in human [21]. At birth, the repertoire of $\gamma\delta$ T
cells in cord blood is broad with no apparent restriction [22]. However, the initially small
subset of $V_\gamma 9^+ V_\delta 2^+$ cells has been shown to gradually increase with age, resulting in a
high frequency of polyclonal $V_\gamma 9^+ V_\delta 2^+$ cells in adult peripheral blood [23]. Although
careful interpretation of the results of monotypic $V_\gamma 9/V_\delta 2$ domain expression is needed,
in our cases the predominance of $V_\gamma 9^+ V_\delta 2^+ \gamma\delta$ T cell was not likely to be age-dependent.
The absolute counts of $V_\delta 2^+ \gamma\delta$ T cells, as well as the $V_\delta 2$ mRNA levels, in PBMCs were
strikingly elevated in our young patients. CDR3 spectratyping of their $V_\delta 2$ segments
showed monoclonal peaks that were confirmed by sequence analysis. In addition,
Southern blot analysis of EBV terminal repeats indicated monoclonal EBV infection. On
the other hand, studies of T cell lines established from 3 patients with nasal T-cell
lymphoma or CAEBV have also indicated preferential expansion of EBV-infected
$V_\gamma 9/V_\delta 2 \gamma\delta$ T cells [24]. However, these and our results are insufficient to allow us to
draw any conclusion regarding higher susceptibility to EBV infection of $V_\gamma 9^+ V_\delta 2^+ \gamma\delta$ T
cells compared with other $\gamma\delta$ T cells. Frequent identification of EBV-infected $V_\gamma 9^+ V_\delta 2^+$
$\gamma\delta$ T cells could simply be due to a higher percentage of $V_\gamma 9/V_\delta 2 \gamma\delta$ T cells at the time of
infection. The mechanism underlying the ectopic infection also remains elusive.
In patient 1, we have previously demonstrated that approximately 25% of the cells infiltrating into the HV-like eruptions were EBV positive by *in situ* hybridization for EBER-1, and that about 30% of the infiltrating cells appeared to be CD3⁺CD4⁻CD8⁻ T cells by immunohistochemical analysis [13]. However, the presence of EBV-infected γδ T cells in the skin could not be confirmed because of the lack of a suitable anti-TCR γδ antibody for paraffin-embedded tissues. In the present study, our molecular analysis clearly demonstrated the skin infiltration of Vδ2⁺γδ T cells, which were identical to the clonally expanded Vδ2⁺γδ T cells in the peripheral blood and were thus infected by EBV in this patient. In normal individuals, γδ T cells take residence mainly in epithelial tissues, such as skin, and represent the major T-cell population there, and modulate epithelial homeostasis through their cytokine secretion and cytolytic properties [25]. It is well known that skin lesions of HV can be induced by repeated ultraviolet exposure [26]. Studies in a murine model for drug-induced cutaneous lupus erythematosus demonstrated that ultraviolet might enhance the function of skin γδ T cells that were involved in skin lesions [27]. Therefore, EBV-infected γδ T cells may play an important role in the skin lesions of HV. Additional investigation will be required to assess whether the presence of EBV-infected γδ T cells in the skin lesions results from ectopic EBV infection into skin-resident γδ T cells or migration of EBV-infected γδ T cells from peripheral blood after sun exposures.

In summary, our studies demonstrate clonal expansion of EBV-infected γδ T cells in γδ T cell-type CAEBV, further support the association between EBV-infected γδ T cells and HV, and provide the basis for characterization of EBV-infected cells to better understand pathogenesis of EBV-associated lymphoproliferative disorders.
Acknowledgments

The authors declare that they have no conflict of interest. We thank Ms Harumi Matsukawa and Ms. Shizu Kouraba for their excellent technical assistance. This work was supported by a grant from Takeda Science Foundation, Osaka; a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.
References


Table 1. Junctional amino acid sequences of γδ T cells

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Figure Legends

Figure 1. Characterization of EBV-infected cells

(A) Detection of EBER-1⁺ cells in γδ T cells from patient 3. (B-D) Frequency of EBV-infected cells. The percentage of EBER-1⁺ cells within the patients’ lymphocyte subsets are shown in patient 1 (b), patient 2 (c), and patient 3 (d).

Figure 2. Immunophenotype of γδ T cells

(A) Expression profiles of T-cell receptor (TCR) Vδ subfamilies. Peripheral blood samples were stained with mAbs for anti-TCR Vδ1 or anti-TCR Vδ2 together anti-TCR Pan γδ mAb. The percentage of cells was analyzed by flow cytometry and expressed with respect to the total lymphocyte population. (B) Activation status of γδ T cells. Expression of HLA-DR was evaluated in γδ T cells.

Figure 3. CDR3 spectratyping and TCR Vδ2 mRNA copies

(A) CDR3 size distribution of TCR Vδ. Each TCR Vδ fragment was amplified from the cDNA with specific primers. The size distribution of the polymerase chain reaction (PCR) products was determined by an automated sequencer and GeneScan software. (B) TCR Vδ2 mRNA was determined by real-time quantitative RT-PCR. Data was normalized to β-actin expression and represent the mean (± standard deviation) of 3 independent experiments. (C) CDR3 size distribution of TCR Vγ. Each TCR Vγ fragment was amplified from the cDNA with specific primers.
Figure 4. TCR Vδ genomic sequences in the skin lesion from patient 1

(A) Each TCR Vδ fragment was amplified from skin DNA with one of 6 TCR Vδ-specific forward primers and a combination of 4 TCR Jδ-specific reverse primers. (B) Each TCR Jδ fragment was amplified with the TCR Vδ2-specific forward primer and one of 4 TCR Jδ-specific reverse primers. Lane M, 100-bp molecular marker.
Figure 1

(a) Image of cells

(b) Bar graph showing EBER-1+ cells (%) across γδ, CD4, CD8, CD19, and CD56 categories.

(c) Bar graph showing EBER-1+ cells (%) across γδ, CD4, and CD8 categories.

(d) Bar graph showing EBER-1+ cells (%) across γδ, CD4, CD8, CD19, and CD56 categories.
Figure 2

(a) % of γδ cells

(b) % of HLA-DR$^+$ in γδ$^+$ cells
Figure 3

(a) 

(b) 

mRNA copies (relative units)
Figure 4