研究の刊行に関する一覧表

書籍

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Clonal origin of Epstein-Barr virus (EBV)-infected T/NK-cell subpopulations in EBV-positive T/NK-cell lymphoproliferative disorders of childhood

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ABSTRACT

Background: In Japan, chronic active Epstein-Barr virus infection (CAEBV) may manifest with infection of T-cells or NK-cells, clonal lymphoid proliferations, and overt lymphoid malignancy. These EBV-positive lymphoproliferative disorders (EBV$^+$LPD) of childhood are related to, but distinct from the infectious mononucleosis-like CAEBV seen in Western populations. The clonal nature of viral infection within lymphoid subsets of patients with EBV$^+$LPD of childhood is not well described.
Objectives: Viral distribution and clonotype were assessed within T-cell subsets, NK-cells, and CD34$^+$stem cells following high purity cell sorting.
Study design: Six Japanese patients with EBV$^+$LPD of childhood (3 T-cell LPD and 3 NK-cell LPD) were recruited. Prior to immunochemotherapy, viral loads and clonal analyses of T-cell subsets, NK-cells, and CD34$^+$stem cells were studied by high-accuracy cell sorting (>99.5%), Southern blotting and real-time polymerase chain reaction.
Results: Patient 1 had a monoclonal proliferation of EBV-infected γδT-cells and carried a lower copy number of EBV in αβT-cells. Patients 2 and 3 had clonal expansions of EBV-infected CD4$^+$T-cells, and lower EBV load in NK-cells. Patients 4, 5 and 6 had EBV$^+$NK-cell expansions with higher EBV load than T-cells. EBV-terminal repeats were determined as clonal bands in the minor targeted populations of 5 patients. The size of terminal repeats indicated the same clonotype in minor subsets as in the major subsets of four patients. EBV was not, however, detected in the bone marrow-derived CD34$^+$stem cells of patients.
Conclusions: A single EBV clonotype may infect multiple NK-cell and T-cell subsets of patients with EBV$^+$LPD of childhood. CD34$^+$stem cells are spared, suggesting infection of more differentiated elements.

1. Background

More than 90% of adults have been infected with Epstein-Barr virus (EBV). Primary infection often occurs in children and adolescents and may manifest as an acute infectious mononucleosis (IM) in the latter group. The γ herpes virus enters CD21$^+$B cells, and persists throughout life by establishing latent infection in memory B-cell pools and evading immune elimination by EBV-specific cytotoxic T-cells.[1] Chronic active EBV infection (CAEBV) is a rare mononucleosis syndrome affecting otherwise immunocompetent individuals and is characterized by high levels of circulating EBV DNA and recurrent IM-like symptoms, along with cardiac, cerebral, and intestinal involvement.[2,3] This typical CAEBV was

Abbreviations: Abs, antibodies; BM, bone marrow; CAEBV, chronic active Epstein-Barr virus infection; EBV, Epstein-Barr virus; EBER, Epstein-Barr virus-encoded mRNA; EBNA, Epstein-Barr virus-nuclear antigen; FITC, fluorescein isothiocyanate; HSC, hematopoietic stem cells; HLH, hemophagocytic lymphohistiocytosis; HIV, human immunodeficiency virus; HMB, hypersensitivity to mosquito bites; Ig, immunoglobulin; IM, infectious mononucleosis; LMP, latent membrane protein; LPD, lymphoproliferative disorder; MNC, mononuclear cell; Lin, lineage; NK, natural killer; PB, peripheral blood; PCR, polymerase chain reaction; PE, phycoerythrin; TCR, T-cell receptor; TR, terminal repeat.

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Table 1
Clinical profiles of patients studied.

Patient	1	2	3	4	5	6
Sex	f	f	m	f	m	m
Age (yrs) at onset, at the study	6, 7	8, 25	2, 5	4, 4	1, 10	3, 8
Involvement						
CAL	no	no	no	yes	no	no
Enteral	no	no	no	yes	no	no
HMB	no	yes	no	yes	yes	yes
HV	yes	yes	no	no	yes	yes
Anti EBV-Abs						
VCA-IgG	160	640	1280	640	160	160
-IgM	<10	<10	<10	<10	<10	<10
-IgA	<10	40	20	<10	<10	<10
EADR-IgG	<10	160	640	40	10	10
-IgA	na	na	<10	160	<10	<10
EBNA	40	10	40	40	80	80
EBV DNA[a] PB/MNC	$100/10^6$	$300/10^4$	600/na	3000/na	$300/10^5$	2000/na
Major targeted subsets	γδT	CD4	CD4	CD56	CD56	CD56
CD3/CD19/CD56 (%)	70/28/2	75/18/7	69/24/7	82/5/13	47/17/36	58/15/27
$CD3^+HLA\text{-}DR^+$ (%)	14.4	21.5	15.8	14.2	0.6	5.9
CD4/CD8	1.6	0.8	5.3	2.0	3.3	2.9
Clonality[b] EBV-TR	M	B	M	B	M	M
TCR	R	R	na	G	G	G
Outcome	AOD	Death	ADF	ADF	AOD	AOD
		Post-SCT	Post-SCT	Post-SCT		

[a] Each value means the copy number of EBV DNA (PB: /ml, MNC: /μg DNA).
[b] Clonality was screened by Southern blotting for PB-MNC derived DNA probed with EBV-TR, TCR and IgH genes. There was no evidence of clonally proliferating B cells. EBV, Epstein-Barr virus; CAL, coronary artery lesion; CNS, central nervous system; HMB, hypersensitivity to mosquito bites; HV, hydroa vacciniforme; VCA, viral capsid antigen; EBNA, EBV nuclear antigen; PB, peripheral blood; MNC, mononuclear cells; TR, terminal repeat; TCR, T-cell receptor; M, monoclonal; B, biclonal; R, rearrangement; G, germ line; AOD, alive on disease; ADF, alive on disease free state; SCT, stem cell transplantation.

first described in Western populations and rarely progresses to lymphoproliferative disease (LPD). In contrast, CAEBV in Asian populations, including Japan, is characterized by ectopic infection of natural killer (NK)-cells and T-cell subsets.[4,5] Emergence of clonal T-cell or NK-cell proliferations and often frank lymphoid malignancy is common in this setting.[6] Affected patients need allogeneic stem cell transplantation (SCT) for the cure of disease.[7] The World Health Organization, to avoid confusion, recommends that these clonal EBV-positive T/NK-cell lymphoid proliferations be referred to as EBV^+LPD of childhood rather than CAEBV.[8,9] The Asian Hematopathology association recommends a grading system wherein polyclonal and oligoclonal EBV^+T/NK-cell expansions are classified as EBV-associated T/NK-cell LPD.[10] The EBV^+LPD are often complicated by hemophagocytic lymphohistiocytosis (HLH). EBV-associated HLH is similarly driven by ectopic infection of $CD8^+$T-cells. Despite the increasing number of reports,[11] the cause of T/NK-cell infection remains unclear.

The episome of EBV DNA has a variable reiteration of terminal repeat (TR) sequences that are joined by random recombination upon circularization of the linear genome at entry into cells. The TR number is maintained in the progeny of the infected cells and is an indicator of clonal infection. The study of TR sequences may further elucidate the nature of EBV infection in NK-cells and T-cells in EBV^+LPD of childhood.

2. Objectives

This study aimed to identify the clonal origin of EBV-infected T/NK-cell subsets in EBV^+LPD patients using high-accuracy cell sorting and molecular analysis.

3. Study design

3.1. Patients

Six Japanese patients with CAEBV[12] treated in Kyushu University between 2002 and 2008, were recruited (Table 1). The presence of clonal T-cells in Patient-1 and development of frank lymphoma in Patient-2 (see Section 4.1) met WHO criteria for EBV^+T-cell LPD.[8] Monoclonal/biclonal TR band(s) in other patients (see Section 4.2) were consistent with lower grade EBV^+LPD according to Asian Hematopathology guidelines.[10] The median age at onset was 3.5 years. The unique symptoms included hypersensitivity to mosquito bite (HMB) ($n=4$), hydroa vacciniforme ($n=4$), and coronary artery lesion ($n=1$). Three patients had abnormal patterns of anti EBV-antibodies (Abs). The primary targets were T-cells in 3 or NK-cells in 3 patients. Peripheral blood (PB) or bone marrow (BM) samples were collected before chemotherapy, after obtaining informed consent. Previously tested EBV-seropositive patients were used as controls.

3.2. Cell sorting

Magnetic activated cell sorting (MACS) was performed on PB mononuclear cells (MNCs) using Vario-MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) after staining with anti-CD3, CD4, CD8 and CD56 immunobeads (Miltenyi Biotec). Anti-fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) (Miltenyi Biotec) immunobeads were used to collect αβT-cells and γδT-cells after staining with FITC-conjugated anti-T-cell receptor (TCR)Vδ2 and PE-conjugated TCRpanα/β monoclonal Abs (Immunotech; Beckman Coulter, Marseille, France), respectively. $CD56^+$NK-cells were collected using Lymphocyte Separation column, after depleting $CD3^+$cells by Lymphocyte Depleting column (Miltenyi Biotec). The procedures yielded >97% purity. $CD3^+$T-cells, γδT-cells, and αβT-cells were further purified (>99.9%) using the FACS Aria Flow Sorter (BD Biosciences, San Jose, CA, USA). $CD34^+$cells were enriched from BM cells by Indirect CD34 MicroBead Kit (Miltenyi Biotec).[13] To further purify $CD34^+$cells (>99.9%), lin^-CD34^+cells were sorted from BM by FACS Aria after staining with conjugated lineage mixtures of (a) PE-Cy5-conjugated anti-CD3, CD4, CD8, CD10, CD20, CD11b, CD14, and CD235a (Immunotech), (b) FITC-conjugated anti-CD3, CD4 and Vδ2 (Immunotech), (c) PE-conjugated anti-TCRpanα/β, CD16 and CD56 (Immunotech), and

(d) allophycocyanin-conjugated anti-CD34 (BD Biosciences). Non-viable cells were excluded by propidium iodide staining (MBL, Nagoya, Japan). Isotype-matched control Abs determined backgrounds. Second sorting avoided contaminations. Flow-cytometric data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). More than 99.9% of purity was confirmed by re-analysis.

3.3. Real-time polymerase chain reaction (PCR) for EBV DNA

TaqMan real-time PCR for EBV DNA was performed as described previously.[5] Gene dosages were analyzed by ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). DNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems), primers, and TaqMan probe. PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. EBV-seropositive healthy persons show <200 copies EBV/ml and <40 copies EBV/μgDNA in PB and MNCs, respectively.

3.4. Southern blotting for EBV-TR or TCR/immunoglobulin (Ig) genes

Blotting analyses were performed by the established protocols.[14] Briefly, 5 μg of high molecular weight DNA were digested with *Eco*RI and/or *Bam*HI, and were electrophoresed on 0.9% agarose gels. DNA was transferred to Byodine-B membranes (Pall Life Sciences, Ann Arbor, MI, USA) and hybridized with ^{32}P-labeled probe of a 5.2-kb *Bam*HI–*Eco*RI fragment containing the EBV-TR sequence, TCR genes (Cβ1, Jβ1, Jβ2, and Jγ) and IgH gene (JH). Normal control DNA was extracted from MNC of healthy EBV-seropositive adults.

3.5. Sequencing of TCRδ and TCRγ gene rearrangements

Genomic DNA was extracted from γδT-cells by conventional methods. Direct sequencing of PCR product of N-regions was completed for TCRδ-gene and TCRγ-gene. The exon and exon-intron boundary regions of each gene were amplified by PCR, and the products were then subjected to direct sequencing using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and 3130*xl* Genetic Analyzer (Life Technologies Corp., CA, USA).

3.6. Double staining for EBV and lymphocyte markers

Double staining of BM clot samples was performed by *in situ* hybridization for EBER and immunostaining for lymphocyte marker to detect EBV-infected cells.[15] Briefly, sections were de-waxed, dehydrated and then treated with proteinase-K. The sections were hybridized with FITC-conjugated EBER probe (Dako, Copenhagen, Denmark). EBER positivity was detected by the combination with anti-FITC Abs, ChemMateENVISION (horseradish peroxidase-labeled polymer reagent, Dako), and diaminobenzidine substrate kit (Dako). The same slide was stained with either Abs against CD34 or each lymphocyte marker to search for EBER$^+$CD34$^+$BM cells.

4. Results

4.1. Target cells and clinical phenotype of patients

4.1.1. T-cell type

Patient-1 had fever and skin lesions. CD4$^-$CD8$^-$γδT-cells increased to 30% of PB-MNC, exclusively expressing Vδ2/Vγ9 (Fig. 1). PCR products amplified by Vδ2/Vγ9 primers indicated clonal proliferation of Vδ2/Vγ9 T-cells. Sequencing of Vγ9-JγP1 transcripts determined an N-region sequence (Table 2). γδT-cells and αβT-cells had 4×10^5 and 4×10^3 copies EBV/μgDNA, respectively. Southern blotting using MNC showed rearranged TCRγ

Table 2
Nucleotide sequence of Vγ9-JγP1 junctional transcripts expressed by γδT-cells.

		Vγ9		N region		JγP1	
Germline	ATT CCG TCA GCC ***TAC	TGT GCC TTG TGG GAG GTG			ACC ACT GGT TGG ATC		
	Vγ9 primer						*JγT3 primer*
Pt		TGT GCC TTG TGG GAG GTG	CAG		ACC ACT GGT TGG TTC AAG ATA TTT GCT GAA GGG ACT AAG		
		C A L W E V	Q		T T G W		

N region, and the 5′ end of JγP1 gene segments are at the top.

The germline sequences of the 3′ end of Vγ9, N region, and the 5′ end of JγP1 gene segments are at the top.

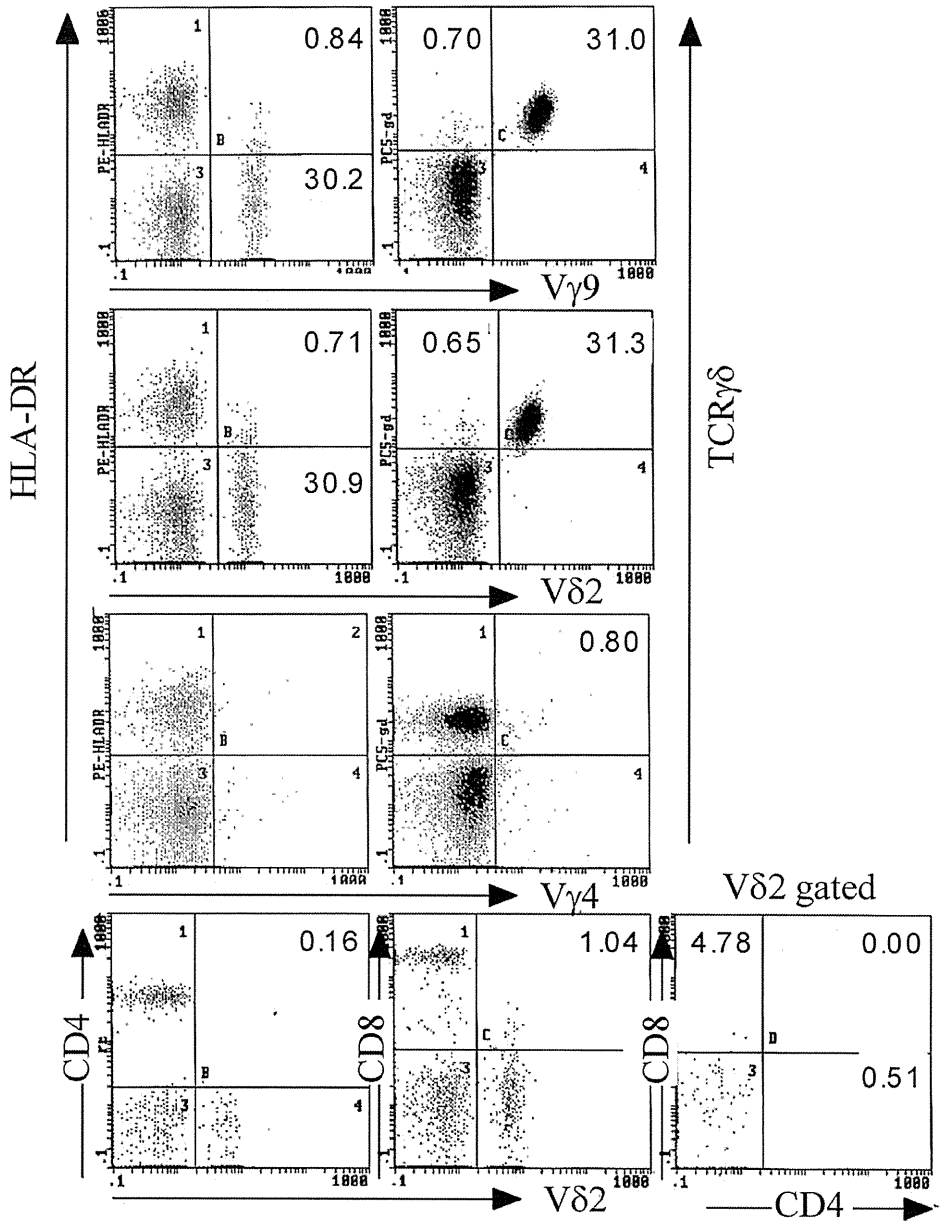

Fig. 1. A representative flow-cytometric analysis of lymphocytes in Patient-1. TCRγδ cells increased to ~30% of peripheral blood lymphocytes, exclusively expressing Vγ9/Vδ2, but with only a negligible expression of HLA-DR. Vγ4$^+$ or Vδ3$^+$ cells were <1% of lymphocytes. More than 99% of Vδ2$^+$ lymphocytes were CD4$^-$CD8$^-$ T-cells.

genes. EBV$^+$Vδ2/Vγ9 T-cells have been sustained in this patient for 6 years after diagnosis. Patient-2 suffered hydroa vacciniforme characterized by cutaneous disease often associated with photosensitivity and HMB. CD3$^+$T-cells, CD8$^+$T-cells and NK-cells carried 2×10^5, 5×10^3 and 9×10^3 copies EBV/μgDNA, respectively. It indirectly indicated a higher percentage of EBV$^+$CD4$^+$T-cells. She received a sibling donor SCT at 25 years of age for cutaneous lymphoma (EBER$^+$CD4$^+$T-cells and NK-cells). Patient-3 had fever and hepatitis. CD4$^+$T-cells, CD8$^+$T-cells and NK-cells had 3×10^4, 90 and <40 copies EBV/μgDNA, respectively. He received a sibling donor BMT.

4.1.2. NK-cell type

Patient-4 showed skin and enteral infiltrations with EBV$^+$NK-cells. CD4$^+$T-cells, CD8$^+$T-cells and NK-cells carried 2×10^3, 1×10^3, and 4×10^5 copies EBV/μgDNA, respectively. She attained EBV-free remission after cord blood transplantation. Patient-5 had fever and HMB. T-cells and NK-cells carried 3×10^4 and 8×10^5 copies EBV/μgDNA, respectively. Low-dose prednisolone has controlled skin infiltrations with EBER$^+$CD4$^+$T-cells. Patient-6 showed fever and HMB. CD4$^+$T-cells, CD8$^+$T-cells and NK-cells had 8×10^3, 4×10^4, and 4×10^5 copies EBV/μgDNA, respectively.

4.2. Clonality of EBV-infected subsets

The clonotype of EBV in purified cells was assessed by the TR size. The blotting sensitivity was determined to be at least 5% EBV-infected cells as determined by add-back experiments. Blot hybridization analysis of EBV-TR sequences using MNC showed a single band in Patient-1. αβT-cells (>99.9%) showed an equal-sized TR to that of γδT-cells in Patient-1 in *Eco*RI or *Bam*HI digestion (Fig. 2). Patient-2 showed a single band in NK-cells, although