pathophysiology, diagnosis, monitoring, and therapy. However, the fundamental cause of the disease has not been elucidated. The recent development of novel technologies for genetic analysis,including new-generation sequencing, may enable identification of genetic alterations responsible forCAEBV.Since CAEBV is anuncommon disease and its knowledge is not prevailing in both physicians and in general society,it may sometimes take years for a patient to have the right diagnosis. Advanced techniques requiredfor it also make the diagnosis of CAEBV difficult. Although there is a consensus that early HSCT gives a better result, the decision to have HSCT is often difficult especially when the patient is in stable condition without severe symptoms. Establishing a standard clinical guideline for the diagnosis and treatment of CAEBV will alleviate these problems and facilitate quick and accurate diagnosis followed by timely intervention with a right choice of treatment.

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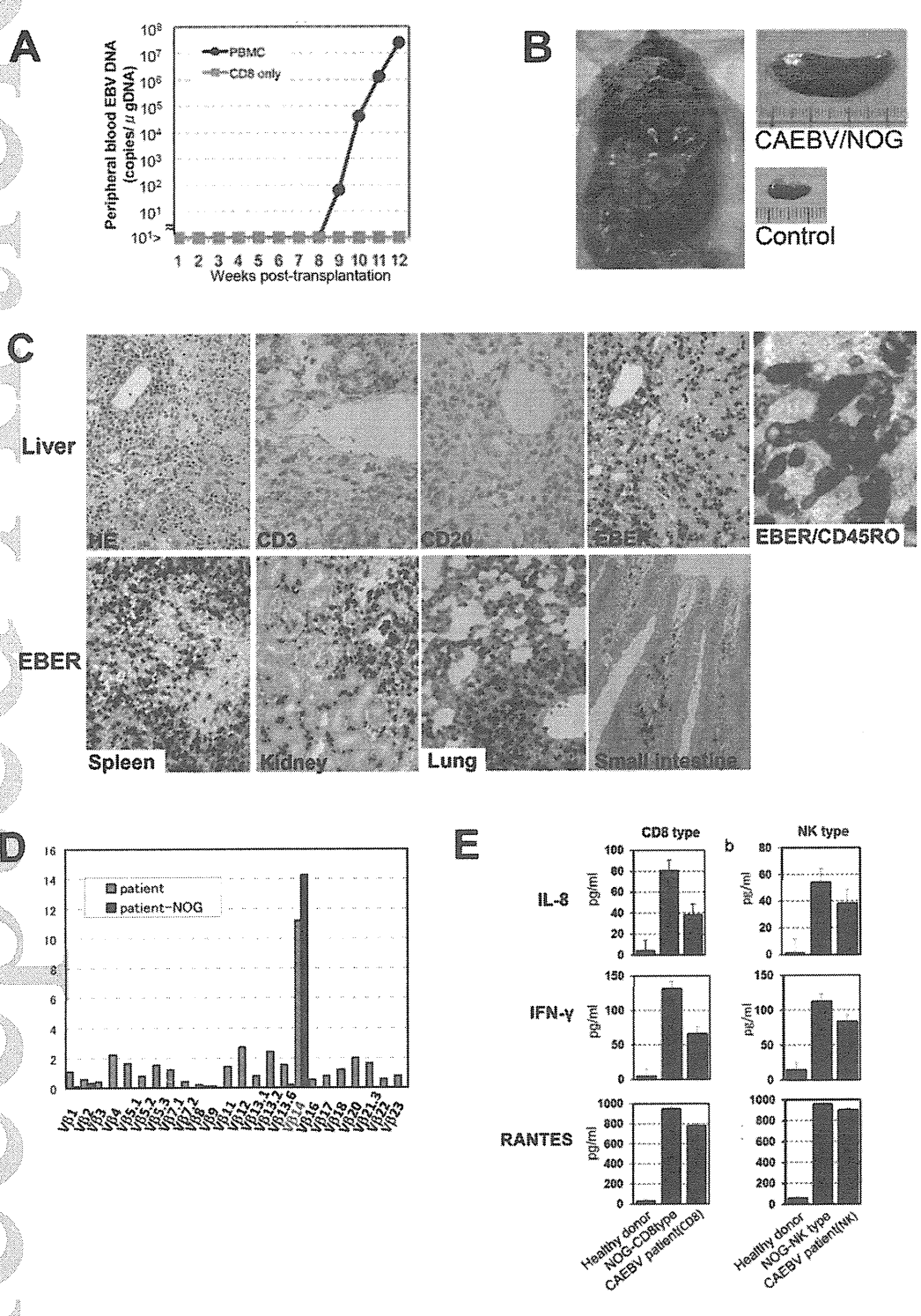
Figure Legends

Figure 1. Mouse xenograft model of CAEBV. PBMCs of a patient with the CD8 type CAEBV were transplanted intravenously to NOG mice. **A.**Measurement of peripheral blood EBV DNA. EBV DNA load increased rapidly from ~9 weeks post-transplantationwhen whole PBMC but not isolated $CD8^+$cells were transplanted. **B.** Splenomegaly of a model mouse. **C.** Pathological analysis. Histochemical analyses revealed massive infiltration of $EBER^+/CD20^-/CD3^+/CD45RO^+$ cells in most major organs including the spleen, kidneys, lungs, and small intestine. **D.** TCR repertoire analysis of peripheral blood T cells isolated fromthe patient and a mouse that received her PBMC. An identical clone of EBV-infected T cells expressing Vβ14 is proliferating in the patient and the corresponding mouse. **E.** Human cytokine levels in CAEBV model mice. Serum levels of IL-8, IFN-γ, and RANTES were measured in mice that were transplanted with PBMCs isolated from either a CD8-type or an NK-type CAEBV patient. The same set of cytokines were also quantitated in the sera of the original patients and healthy donors. Modified from PLoS Pathog 7(10): e1002326.

Figure 2. Flow-cytometric in situ hybridization (FISH).**A.** Protocol of FISH. **B.** Results of FISH in a patient with hydroavacciniforme. EBER-positive cells are shown in red and EBER-negative cells in blue. Most EBV-infected cells in the peripheral blood of this patient had the phenotype $CD3^+/CD4^-/CD8^-/TCR\gamma\delta^+$. **C.**Results of FISH in a patient with the NK-cell type CAEBV. EBER-positive cells are shown in red and EBER-negative cells in blue. The majority of EBV-infected cells in the peripheral blood of this patient were $CD56^+$ NK cells. Besides, a fraction of $TCR\alpha\beta^+/CD3^+/CD4^+$ cells also contained EBV.



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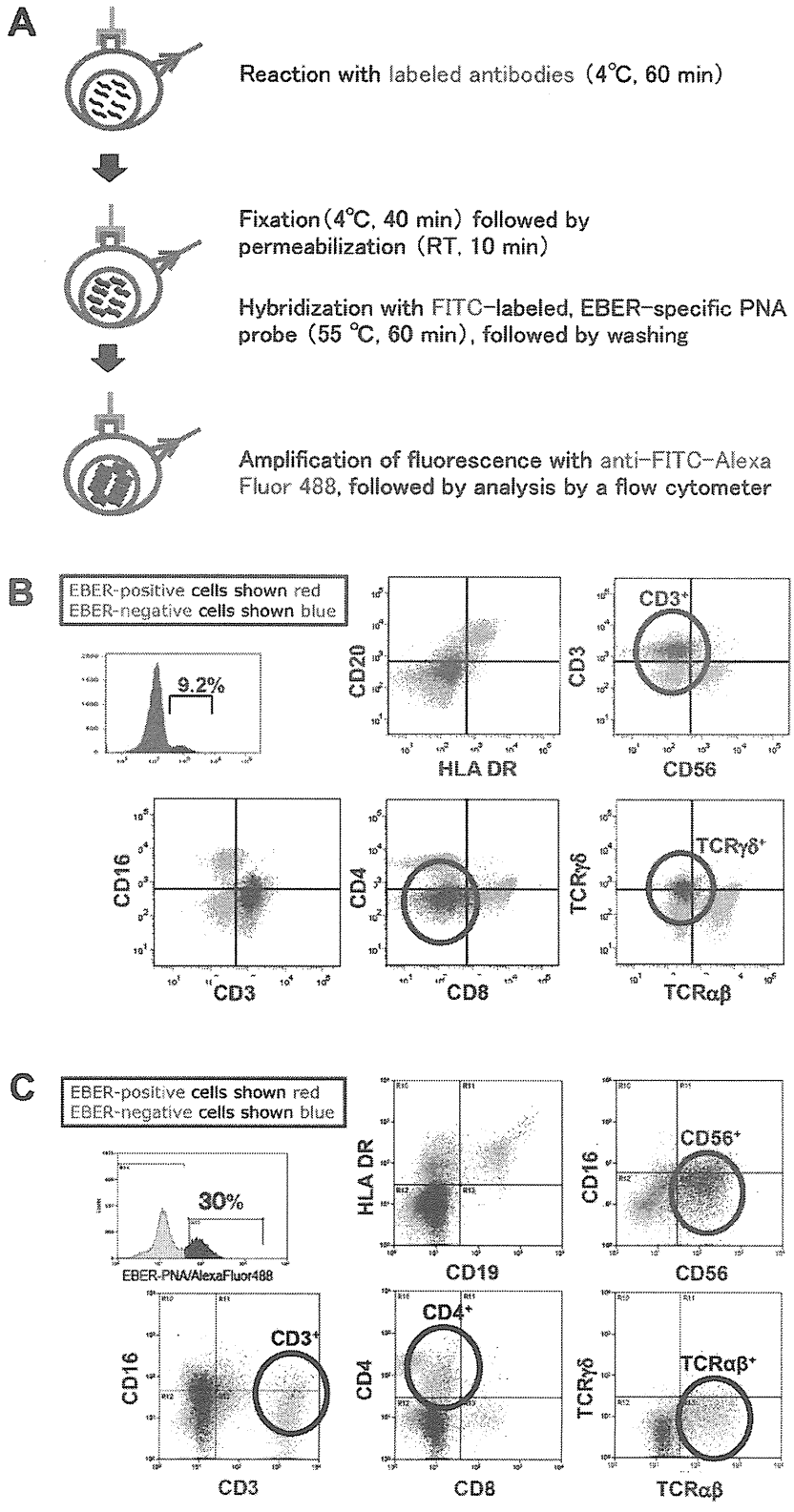

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Application of flow cytometric *in situ* hybridization assay to Epstein–Barr virus-associated T/natural killer cell lymphoproliferative diseases

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Epstein–Barr virus (EBV) infects various types of lymphocytes and is associated with not only B cell-origin lymphoma, but also T or natural killer cell lymphoproliferative diseases (T/NK LPD). Recently, we established a novel assay to identify EBV-infected cells using FISH. Using this assay, dual staining with antibodies to both surface antigens and an EBV-encoded small RNA (EBER) probe can be performed. In the present study, we applied this recently developed FISH assay to EBV-associated T/NK LPD to confirm its diagnostic utility. Using FISH, we prospectively analyzed peripheral blood from patients with suspected EBV-associated T/NK LPD. The results were compared with those obtained using immunobead sorting followed by quantitative PCR. In all, 26 patients were included study. Using FISH, 0.15–67.0% of peripheral blood lymphocytes were found to be positive for EBER. Dual staining was used to determine EBER-positive cell phenotypes in 23 of 26 subjects (88.5%). In five of seven patients with hydroa vacciniforme-like lymphoma (an EBV-positive cutaneous T cell lymphoma), EBER-positive cells were identified as $CD3^+CD4^-CD8^-TCR\gamma\delta^+$ T cells. Furthermore, in a 25-year-old male patient with systemic EBV-positive T cell LPD, two lymphocyte lineages were positive for EBER: $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. Thus, we confirmed that our newly developed assay is useful for quantifying and characterizing EBV-infected lymphocytes in EBV-associated T/NK LPD and that it can be used not only to complement the pathological diagnosis, but also to clarify the pathogenesis and to expand the spectrum of EBV-associated diseases. (*Cancer Sci* 2012; 103: 1481–1488)

Epstein–Barr virus (EBV) is ubiquitous and infects not only B cells, but also T and natural killer (NK) cells. There are a number of EBV-associated T/NK lymphoproliferative diseases (LPD) and lymphoma/leukemia, such as EBV-associated hemophagocytic lymphohistiocytosis (HLH), systemic EBV-positive T cell lymphoproliferative disease of childhood (systemic EBV^+ T-LPD), hydroa vacciniforme (HV)-like lymphoma, extranodal NK/T-cell lymphoma, nasal type (ENKL), and aggressive NK cell leukemia (ANKL).[1–5] Severe chronic active EBV disease (SCAEBV), which is seen mainly in East Asia, is now considered to be an LPD caused by clonal expansion of EBV-infected T or NK cells.[6–9] However, the definition of each EBV-associated T/NK LPD is unclear and there is significant overlap between them.[5,9–13] Therefore, diagnosis of EBV-associated T/NK LPD can be problematic.

Because EBV is ubiquitous and latently infects various lymphocytes, detection of EBV alone is insufficient for diagnosis of EBV-associated diseases[14] To diagnose EBV-associated diseases and to explore their pathogenesis, EBV load must be determined; however, the EBV-infected cells must also be identified. *In situ* hybridization (ISH) using the EBV-encoded small RNA (EBER) is widely used to detect EBV-infected cells in tissue specimens.[15–17] However, biopsies are invasive and cannot always be obtained. To overcome these problems, we recently established a novel assay to simultaneously quantify and identify EBV-infected cells using FISH.[18] Both nuclear EBER and surface lymphocyte antigens can be stained using a fluorescein-conjugated probe that specifically hybridizes to EBER. This assay is a more convenient and less invasive procedure than EBER ISH and can be performed on peripheral blood. Using this assay, we determined the phenotype of EBV-infected B cells in patients with EBV infection after stem cell/liver transplantation.[19]

In the present study, we applied the FISH assay to peripheral blood from 26 patients with EBV-associated T/NK LPD to confirm its utility for the diagnosis of EBV-associated T/NK LPD and to further elucidate the pathogenesis of this disease. The results of the FISH assay were validated by comparison with EBV DNA loads determined by quantitative PCR. Furthermore, lymphocyte phenotypes were compared with those determined by immunobead sorting followed by quantitative PCR.

Materials and Methods

Patients and samples. From January 2009 to July 2010, patients who fulfilled the following criteria were prospectively enrolled in the present study: (i) EBV-associated T/NK LPD was suspected or diagnosed based on clinical and histopathological findings, and determination of EBV-infected cell phenotypes was requested from Nagoya University Graduate School of Medicine; (ii) high EBV DNA levels ($\geq 10^{2.5}$ copies/μg DNA) in PBMCs, as determined by quantitative PCR[7,20,21]; and (iii) both the FISH assay and immunobead sorting followed by quantitative PCR could be performed and results compared. Exclusion criteria were as follows: (i) patients with diseases involving infection of B cells, such as infectious mononucleosis and immunodeficiency-associated LPD; (ii) cases of congenital immunodeficiency; (iii) human immunodeficiency virus-positive cases; and (iv) patients who had received either hematopoietic or organ transplantation prior to enrolment.

In all, 28 patients were initially enrolled in the study. However, two subjects, who were initially suspected of having EBV-associated HLH, were excluded from the study because

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Table 1. Determination of Epstein–Barr virus-infected cell phenotypes using FISH and immunobead sorting/quantitative polymerase chain reaction

Pateint no.	Sex	Age (years)	Disease	EBV clonality	TCR gene rearrangement	FISH			EBV DNA (copies/μg DNA)							
						EBER$^+$ cells (%)	EBER$^+$ cell phenotypes	EBV-infected cells	PBMC	CD3$^+$	CD4$^+$	CD8$^+$	CD19$^+$	CD56$^+$	TCRαβ	TCRγδ
1	M	10	SCAEBV	Monoclonal	β	1.0	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	8300	**18 000**	1900	**9900**	5700	5400	ND	ND
2	F	22	SCAEBV	Negative	None	0.31	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	310 000‡	280 000	ND	ND	110 000	190 000	ND	ND
3	M	15	SCAEBV	Monoclonal	None	0.54	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	7200	1700	ND	ND	3600	2300	ND	ND
4	M	36	SCAEBV	Monoclonal	None	5.7	CD3$^+$CD56$^+$	CD56$^+$ T	44 000	3400	3900	**47 000**	39 000	**480 000**	ND	ND
5	M	8	SCAEBV	Monoclonal	β	29.9	CD3-CD56$^+$ CD3$^+$CD4$^+$TCRαβ$^+$	NK 82% CD4$^+$T 8%	240 000	17 000	27 000	21 000	90 000	**3 900 000**	ND	ND
6	F	11	SCAEBV	ND	γ	5.3	CD16$^+$CD56$^+$	NK	57 000	17 000	ND	ND	**18 000**	**93 000**	ND	ND
7	M	14	SCAEBV	Monoclonal	None	49.0	CD56$^+$	NK	600 000‡	1000	ND	ND	ND	**2 000 000**	1200	17 000
8	M	34	SCAEBV	Negative	None	0.32	CD56$^+$	NK	1500	0	0	0	0	**28 000**	ND	ND
9	F	13	SCAEBV	Negative	None	0.15	Not identified	Untypable	830	**14 000**	**19 300**	**3700**	140	810	ND	ND
10	F	6	HV-like lymphoma	Oligoclonal	β,γ,δ	9.0	CD3$^+$TCRγδ$^+$	γδT	170 000	**170 000**	150 000	49 000	**270 000**	130 000	ND	**330 000**
11†	M	6	HV-like lymphoma	Monoclonal	δ	25.9	CD3$^+$TCRγδ$^+$	γδT	42 000‡	**47 000**	ND	ND	9100	**49 000**	6400	**190 000**
12†	M	11	HV-like lymphoma	Monoclonal	γ,δ	4.8	CD3$^+$TCRγδ$^+$	γδT	10 000	**13 000**	1100	1300	5900	**19 000**	210	**87 000**
13	M	12	HV-like lymphoma	Monoclonal	β	36.8	CD3$^+$TCRγδ$^+$	γδT	920 000	ND	60 000	94 000	52 000	**1 500 000**	ND	ND
14†	M	16	HV-like lymphoma	Monoclonal	γ,δ	1.7	CD3$^+$TCRγδ$^+$	γδT	6100‡	**16 000**	ND	ND	2300	4400	8300	**100 000**
15	F	22	HV-like lymphoma	ND	β	13.0	CD3$^+$CD56$^+$	CD56$^+$ T	240 000	**420 000**	ND	ND	140 000	**2 000 000**	ND	ND
16	M	3	HV-like lymphoma	Monoclonal	None	67.0	CD16$^+$CD56$^+$	NK	1 200 000	240 000	110 000	500 000	310 000	**15 000 000**	ND	ND
17	F	1	HLH	ND	None	0.20	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	650	**1400**	ND	ND	150	0	ND	ND
18	M	1	HLH	Monoclonal	β	17.5	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	220 000	**760 000**	**360 000**	**1 600 000**	**1 200 000**	**1 600 000**	ND	ND
19	M	1	HLH	Negative	β	0.15	Not identified	Untypable	430	0	20	**510**	120	**1500**	ND	ND
20	F	25	HLH	Polyclonal	None	0.19	Not identified	Untypable	310	**700**	150	**3200**	**8900**	120	ND	ND
21	M	56	ENKL	ND	None	0.32	CD56$^+$	NK	2400	140	0	0	**20 000**	**11 000**	ND	ND
22	F	57	ENKL	ND	None	2.0	CD56$^+$	NK	24 000	12 000	8700	7600	**27 000**	**540 000**	ND	ND
23	M	26	Systemic EBV$^+$ T-LPD	Monoclonal	β, γ	4.5	CD3$^+$CD8$^+$ CD3$^+$CD4$^+$	CD8$^+$T 52% CD4$^+$T 39%	57 000	**110 000**	**110 000**	**130 000**	37 000	**88 000**	ND	ND
24	F	46	Systemic EBV$^+$ T-LPD	Monoclonal	γ	31.3	CD3$^+$CD8$^+$TCRαβ$^+$	CD8$^+$T	940 000	700 000	53 000	**1 410 000**	170 000	160 000	ND	ND
25	M	14	ANKL	Monoclonal	None	31.0	CD56$^+$	NK	310 000	ND	6500	24 000	5800	**2 000 000**	ND	ND
26	M	56	PTCL	Monoclonal	β	0.55	CD3$^+$CD4$^+$TCRαβ$^+$	CD4$^+$T	3300	**6300**	**6800**	1000	4100	3500	ND	ND

Bold letters indicate that Epstein–Barr virus (EBV) DNA was concentrated in the fraction. †These cases have been reported previously.[18] ‡Samples were obtained on different days when FISH was performed. ANKL, aggressive NK cell leukemia, nasal type; ENKL, extranodal natural killer (NK)/T cell lymphoma, nasal type; HLH, hemophagocytic lymphohistiocytosis; HV-like lymphoma, hydroa vacciniforme-like lymphoma; ND, not done; PTCL, peripheral T cell lymphoma; SCAEBV, severe chronic active EBV disease; systemic EBV$^+$ T-LPD, systemic EBV-positive T lymphoproliferative disease of childhood; TCR, T cell receptor.

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they were shown to have severe infectious mononucleosis and had only B cell infection, leaving 26 patients in the study: nine cases of SCAEBV, seven of HV-like lymphoma, four of HLH, two of systemic EBV$^+$ T-LPD, two of ENKL, one of ANKL, and one of peripheral T cell lymphoma (PTCL). Diagnoses of HV-like lymphoma, systemic EBV$^+$ T-LPD, ENKL, ANKL, or PTCL were made based on biopsy or bone marrow findings according to World Health Organization (WHO) criteria.[10,22–24] Diagnoses of HLH were made on the basis of criteria proposed by an international treatment study group,[25] whereas SCAEBV was diagnosed using previously proposed criteria.[7,26] Briefly, for a diagnosis of SCAEBV to be made, patients had to fulfill the following diagnostic criteria: (i) an illness of >6 months duration (an EBV-related illness or symptoms including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, or hypersensitivity to mosquito bites); (ii) increased quantities of EBV in either affected tissues or peripheral blood; and (iii) no evidence of any prior immunologic abnormalities or of any other recent infection that may explain the condition. There were several overlapping cases. For example, in one patient, ANKL developed at the end stage of SCAEBV. In some patients, HLH developed during the course of other EBV-associated T/NK LPD. In such cases, pathological diagnoses (HV-like lymphoma, systemic EBV$^+$ T-LPD, ENKL, ANKL, and PTCL) were used in preference to SCAEBV and HLH. Of the 26 patients in the study, 14 underwent biopsy (skin, $n = 6$; liver, $n = 3$; intestine, $n = 2$; others, $n = 3$), 19 underwent bone marrow examination, and one underwent an autopsy. Seventeen healthy volunteers who were seropositive for EBV were included in the study as negative controls.

Blood was usually taken at the time of diagnosis, although some subjects had already received treatment, such as steroids, cyclosporin A, and chemotherapies. In six subjects, repetitive sampling was performed with or without treatment. Heparinized blood samples were obtained and PBMCs were separated on density gradients. The PBMCs were cryopreserved at −80°C until required.

Informed consent was obtained from all subjects or their guardians, as well as from the healthy controls. The Institutional Review Board of Nagoya University Hospital approved the use of all specimens that were examined in the present study.

Analyses of EBV DNA. After DNA had been extracted from 1×10^6 PBMCs, real-time quantitative PCR was performed as described previously.[7,20] The amount of EBV DNA was calculated as the number of virus copies per μg PBMC DNA. To determine which cell population harbored EBV, the PBMCs were fractionated into CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$, CD56$^+$, T cell receptor (TCR) αβ$^+$, and TCRγδ$^+$ cells using an immunobead method (IMag Cell Separation System; BD Biosciences, Franklin Lakes, NJ, USA) that resulted in 97–99% purity. Purified cells were analyzed by real-time PCR and compared with PBMCs.[27,28] Southern blotting with a terminal repeat probe was used to assess EBV clonality, as described previously.[29]

Determination of TCR gene rearrangement. Multiplex PCR using the T cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies, La Ciotat, France) was used to evaluate TCR gene; this assay was developed and standardized in a European BIOMED-2 collaborative study.[30,31]

FISH assay. The FISH assay was performed as described previously.[18,19] First, for surface marker staining, 5×10^5 PBMCs were stained with phycoerythrin (PE)-labeled anti-CD3 (clone UCHT1; eBioscience, San Diego, CA, USA), anti-CD8 (clone B9.11; Immunotech, Marseille, France), anti-CD19 (clone HD37; Dako, Glostrup, Denmark), and Vδ2 (clone B6; BD Pharmingen, San Jose, CA, USA) mAbs, and phycoerythrin cyanine 5 (PC5)-labeled anti-CD4 (clone 13B8.2; Immunotech), anti-CD16 (clone 3G8; Immunotech), anti-HLA-DR (clone IMMU357; Immunotech), and anti-TCRγδ (clone IMMU510; Immunotech) mAbs for 1 h at 4°C. In cases of weak fluorescence signals or incomplete cell separation likely due to degradation or detachment under the harsh hybridization conditions,[18] biotin-labeled antibodies (anti-CD3 clone UCHT1, anti-CD19 clone HIB19, anti-CD56 clone CB56, and anti-TCRαβ clone IP26 [eBioscience]; anti-CD122 clone Mik-b3 [BD Biosciences]) were used, followed by application of PE- or PC5-conjugated streptavidin (eBioscience). Isotype-matched monoclonal mouse IgG antibodies were used as controls.

Cells were fixed, permeabilized, and hybridized with EBER PNA Probe/FITC (Y5200; Dako) or Negative Control PNA Probe/FITC (Dako).[18,19] An Alexa Fluor 488 Signal Amplification Kit (Molecular Probes, Eugene, OR, USA) was used to enhance fluorescence and photostability.

Stained cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences). Lymphocytes were gated by standard forward and side scatter profiles.[32] Up to 50 000 events were acquired for each analysis. Based on experiments involving mixing of EBV-positive and -negative cell lines, the detection limit of the FISH assay was considered to be 0.1% and 0.01% for T and B cells, respectively.[18]

Statistical analysis. Statistical analyses were performed using SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA). The FISH and real-time PCR assays were compared by regression analysis. The Mann–Whitney *U*-test was used to compare the mean percentages of EBER-positive cells in each group. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Quantification of EBV-infected peripheral blood lymphocytes by FISH. We applied the FISH assay to samples from 26 patients with EBV-associated T/NK LPD. Subject characteristics are given in Table 1. Most subjects were monoclonal, as determined by Southern blot hybridization using an EBV terminal repeat probe. The assay for TCR gene rearrangements detected T cell clonality in 15 patients. The FISH assay detected EBER-positive lymphocytes in each of the 26 patients at levels ranging from

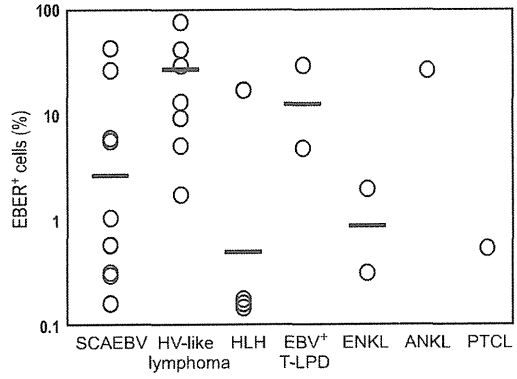

Fig. 1. Quantification of Epstein–Barr virus (EBV)-infected lymphocytes. The FISH assay was used to analyze PBMCs and the percentage of EBV-encoded small RNA (EBER)-positive cells in each disease is shown. Bars indicate the mean for each group. ANKL, aggressive NK cell leukemia; EBV$^+$ T-LPD, systemic EBV-positive T lymphoproliferative disease of childhood; ENKL, extranodal NK/T-cell lymphoma, nasal type; HLH, hemophagocytic lymphohistiocytosis; HV-like lymphoma, hydroa vacciniforme-like lymphoma; PTCL, peripheral T cell lymphoma; SCAEBV, severe chronic active EBV disease.

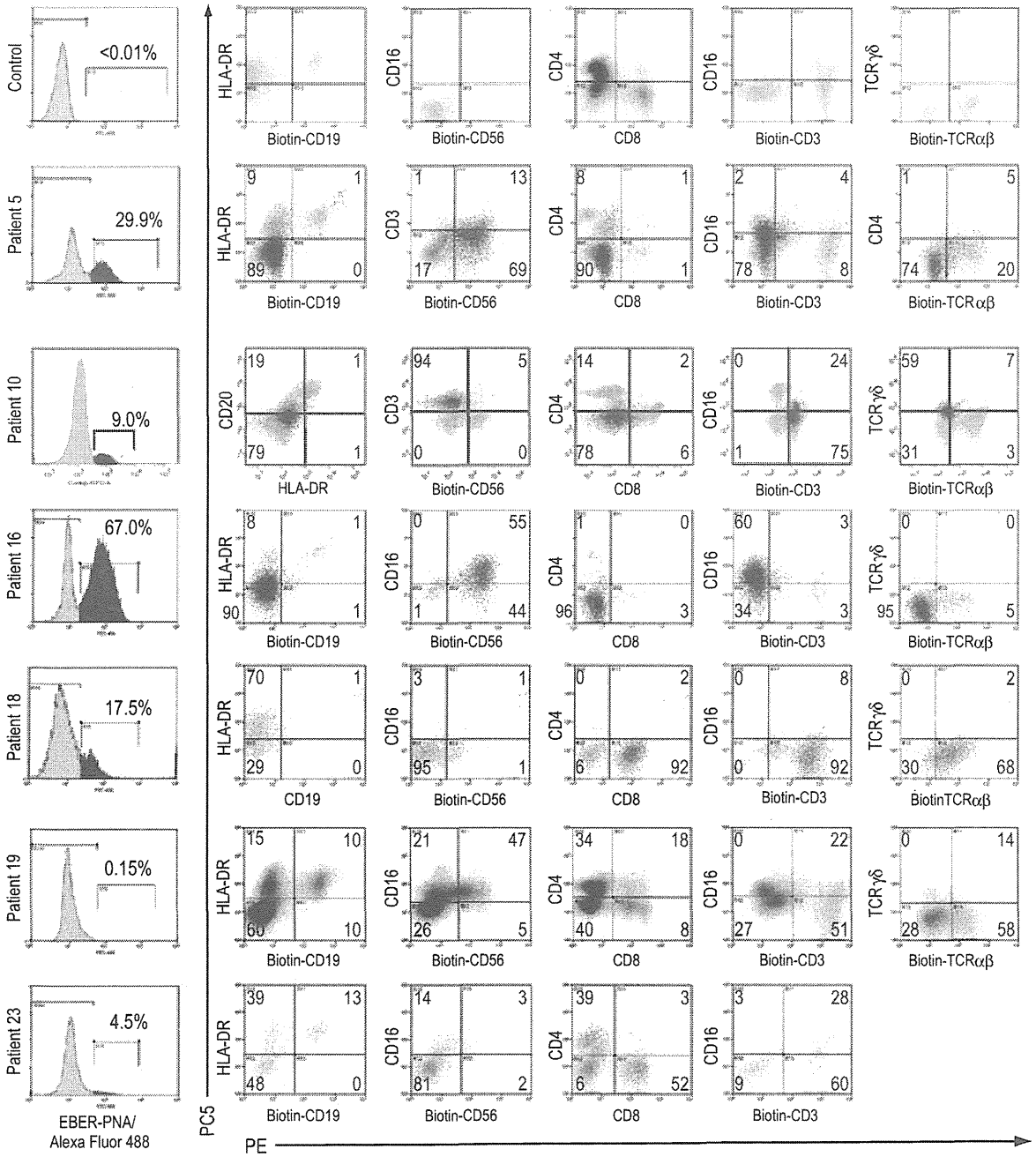

Fig. 2. Characterization of Epstein–Barr virus (EBV)-infected lymphocytes in representative patients. The numbers in each histogram represent the percentage of EBV-encoded small RNA (EBER)-positive lymphocytes. The EBER-positive (red) and EBER-negative (blue) lymphocytes were gated and plotted in quadrants. The numbers in the quadrants indicate the percentage of EBER-positive cells for each surface immunophenotype. Control, a healthy EBV-seropositive volunteer. Patient numbers are the same as given in Table 1. PC5, phycoerythrin cyanine 5; PE, phycoerythrin; PNA, peptide nucleic acid; TCR, T cell receptor.

0.15% to 67.0% (Table 1). The percentage of EBER-positive cells according to disease were as follows: SCAEBV, 2.6 ± 1.8%; HV-like lymphoma, 12.9 ± 1.6%; HLH, 0.6 ± 3.1%; systemic EBV$^+$ T-LPD, 11.9 ± 2.6%; and ENKL, 0.8 ± 2.6% (Fig. 1). The levels of EBER-positive cells were slightly higher in HV-like lymphoma patients than in patients with SCAEBV or HLH, but the differences did not reach statistical significance (P = 0.08 and P = 0.06, respectively).

To confirm the specificity of the assay, PBMCs were obtained from 17 healthy volunteers who were seropositive for EBV. However, EBV DNA was detected in the PBMCs of only one volunteer using real-time PCR. The same PBMCs were subjected to the FISH assay and no EBER-positive cells were detected (detection limit >0.1%).

Determination of EBV-infected cell phenotypes by FISH assay. The EBER-positive cell phenotypes were determined by dual staining with antibodies to surface antigens and the EBER probe in 23 of 26 patients (88.5%; Table 1). Representative results of the dual staining are shown in Figure 2. In Patient 5, the EBV-infected cells were predominantly

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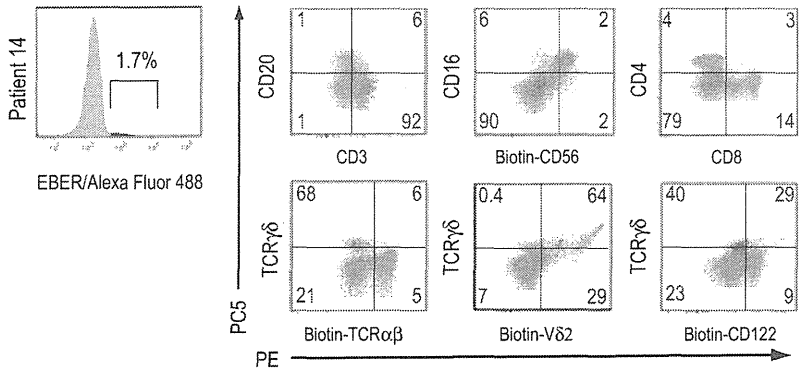

Fig. 3. Characterization of Epstein–Barr virus (EBV)-infected cell phenotypes in a 16-year-old boy with hydroa vacciniforme (HV)-like lymphoma. The EBV-encoded small RNA (EBER)-positive (red) and EBER-negative (gray) lymphocytes were gated and plotted in quadrants. The numbers in the quadrants indicate the percentages of EBER-positive cells for each surface immunophenotype. PC5, phycoerythrin cyanine 5; PE, phycoerythrin; TCR, T cell receptor.

$CD3^-$ $CD56^+$ $TCR\alpha\beta^-$ NK cells; in Patient 10 they were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells; in Patient 16 they were $CD3^-$ $CD16^+$ $CD56^+$ NK cells; and in Patient 18 they were $CD3^+$ $CD4^-$ $CD8^+$ $TCR\alpha\beta^+$ T cells (Fig. 2). We were unable to determine the phenotypes of EBV-infected cells in Patient 19, in whom only 0.15% of cells were EBER positive. Interestingly, in Patient 23, a 26-year-old man with systemic EBV^+ T cell LPD, almost half of the EBER-positive cells were CD4 positive, with the remainder CD8 positive. Thus, two lymphocyte lineages were present in the peripheral blood of this patient. Immunobead sorting followed by quantitative PCR revealed that the quantity of EBV DNA was high in the $CD3^+$, $CD4^+$, and $CD8^+$ fractions (Table 1), supporting the FISH data. Furthermore, TCR gene rearrangement analysis showed two peaks of the rearranged TCR Vγ chain in this patient (data not shown). Similarly, in Patient 5, whose main EBV-infected cells were $CD3^-$ $CD56^+$ $TCR\alpha\beta^-$ NK cells, the $CD3^+$ $CD4^+$ $TCR\alpha\beta^+$ population also included EBER-positive cells (Fig. 2). This observation suggests that the majority of EBV-infected cells in this patient were NK cells, but that there was also a minor population of EBV-infected T cells. In this patient, TCR rearrangement was recognized in the Vβ chain, which would theoretically not be detected in NK cell LPD (Table 1).

Thus, the main EBV-infected cells were identified as NK cells in eight patients, γδ T cells in five patients, $CD8^+$ T cells in five patients, $CD4^+$ T cells in three patients, and $CD56^+$ T cells in two patients (Table 1). These data are mostly in agreement with those generated by immunobead sorting and EBV DNA quantification. For example, in Patient 1 (EBV-infected $CD3^+$ $CD8^+$ $TCR\alpha\beta^+$ T cells), EBV DNA was detected mainly in the $CD3^+$ and $CD8^+$ populations. Conversely, in Patient 6 (EBV-infected NK cells as determined by the FISH assay), EBV DNA was most abundant in the $CD56^+$ population.

In the nine patients with SCAEBV, the main EBV-infected cells were $CD8^+$ T cells in two patients, $CD4^+$ T cells in one patient, and NK cells in five patients; typing was unsuccessful in one patient (Table 1). Thus, the main EBV-infected cells were variable in SCAEBV. Conversely, in five of seven patients with HV-like lymphoma, an EBV-positive cutaneous lymphoma, the EBER-positive cells were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells (Table 1). We further investigated the phenotypes of these $\gamma\delta^+$ T cells, which were positive for Vδ2 but negative for CD122. A representative result (Patient 14) is shown in Figure 3.

We could not identify the EBV-infected cell phenotypes in three patients (Patients 9, 19, and 20), although immunobead sorting and quantitative PCR could identify the predominant population of infected cells. In all three patients, EBER-positive cells accounted for <0.2% of the total population.

Comparison between EBER-positive cells and EBV DNA in peripheral blood. Finally, we compared the FISH assay with real-time quantitative PCR. The number of $EBER^+$ cells determined by the FISH assay was significantly correlated with the EBV DNA load determined by real-time PCR ($P < 0.0001$; Fig. 4a). Patients were divided into NK and T cell infection groups, and the same comparison was performed. A significant correlation was observed and the slope of the correlation was similar in both groups, suggesting that the number of EBV episomes per cell was similar in both groups (Fig. 4b).

We repeated both FISH and real-time PCR on samples from six patients and the resultant longitudinal analyses are shown in Figure 5. In the four patients who had not received any chemotherapy owing to localization of symptoms to the skin or the stability of their condition (Patients 7, 11, 12, and 14), the percentage of EBER-positive cells determined by the FISH assay was stable. However, in the two patients who received hematopoietic stem cell transplantation, the proportion of EBER-positive cells decreased thereafter (Patients 5 and 24).

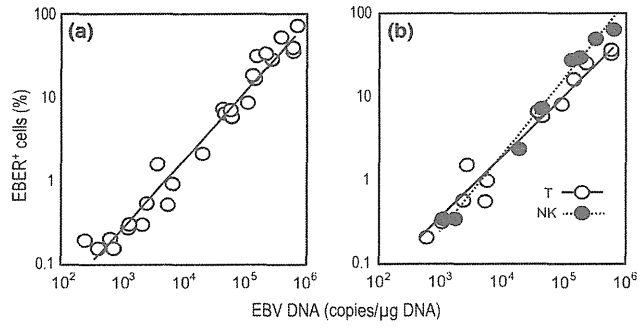

Fig. 4. Correlation between the percentage of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes as determined by FISH and the EBV DNA load determined by real-time PCR. (a) All 26 patients with EBV-associated T or natural killer cell lymphoproliferative diseases (T/NK LPD). (b) Patients were divided into T cell ($n = 13$) and NK cell ($n = 8$) infection groups, and the correlations were evaluated.

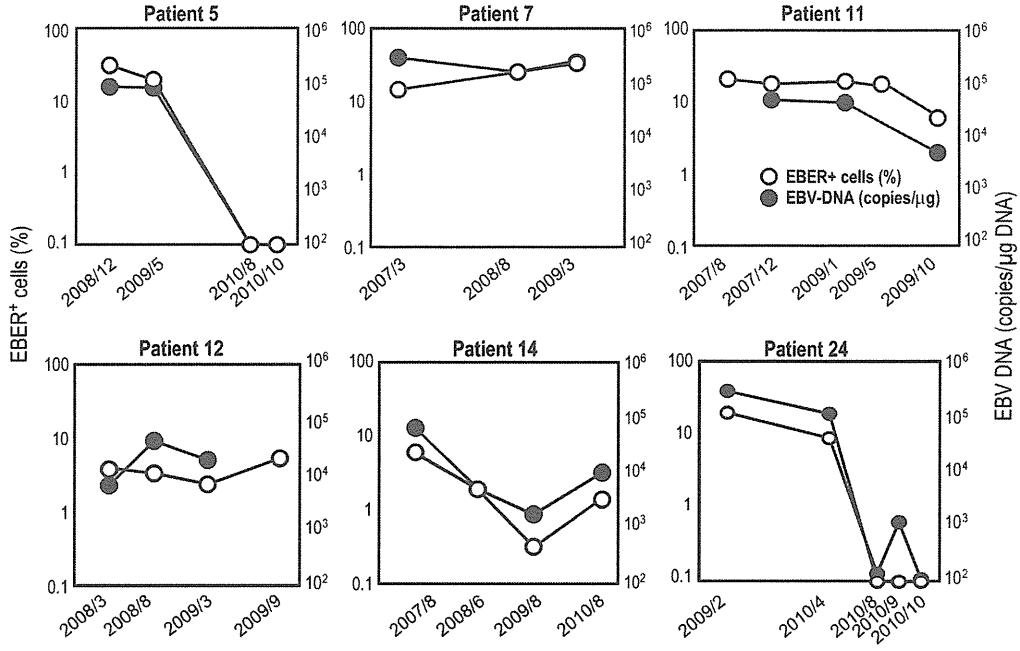

Fig. 5. Longitudinal quantification of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes. Samples of PBMCs were obtained repeatedly on the dates indicated and were analyzed by the FISH assay. The results of EBV DNA quantification are also shown for comparison. Patients 7, 11, 12, and 14 did not receive any chemotherapy owing to the stability of their condition. Patients 5 and 24 received hematopoietic stem cell transplantation.

Discussion

Epstein–Barr virus is associated with various types of T/NK LPD. Some are well defined and listed in the revised WHO *Classification of Tumours of Haematopoietic and Lymphoid Tissues*, whereas others are not.[10,13] One of the reasons why these entities are not well defined is that they are relatively rare, especially in the West. Most EBV-associated T/NK LPD are more prevalent in East Asia and Latin America.[8,10] In addition, the diagnosis of such conditions is often problematic. When possible, staining of virus-associated antigens using specific antibodies is the most direct and easiest method of detecting and characterizing EBV-infected cells. Epstein–Barr virus infection of T/NK cells is "latency type II", in which only a few viral antigens (Epstein–Barr virus nuclear antigen-1, latent membrane protein (LMP-1, and LMP-2) are expressed[1,3,33]; however, there are no antibodies available that can stain their extracellular domains. This, together with their low expression levels and weak antigenicity, makes it difficult to staining EBV-infected cells with antibodies against these antigens.

Using the FISH assay, 0.15–67.0% of peripheral blood lymphocytes were positive for EBER in patients with EBV-associated T/NK LPD. The number of EBER-positive cells was correlated with the EBV DNA load determined by quantitative PCR. These results indicate that the FISH assay is useful for the detection and quantification of EBV-infected lymphocytes in patients with EBV-associated T/NK LPD. Furthermore, this assay is applicable for follow-up and evaluation of responses to therapy, as demonstrated in the present study. Because B-LPD, which is also associated with EBV, sometimes develops after stem cell transplantation, differential diagnosis between relapse of T/NK LPD and B-LPD is needed. Our assay is useful for diagnosing not only EBV-associated T/NK LPD, but also B-LPD,[19] and can help to select mAb-based therapy, such as anti-CD20 (rituximab), anti-CD52 (campath-1), or other humanized mAbs targeting lymphocyte surface antigens.

In the present study, using the FISH assay, two different cell lineages were demonstrated in two patients with EBV-associated T/NK LPD. This is particularly interesting in terms of the pathogenesis of EBV-associated T/NK LPD. Biphasic expansion of EBV-infected lymphocytes has been demonstrated in some patients with SCAEBV.[34–37] A recent study using an immuno-FISH assay, which is similar to the FISH assay used in the present study and can characterize EBV-infected cell phenotypes, revealed that not only T/NK cells, but also monocytes were infected with EBV in patients with EBV-associated LPD.[38] There are several possible explanations as to why multiple cell lineages were infected with EBV. First, these patients may have unknown genetic abnormalities, which are associated with the functions of virus-specific or non-specific lymphocytes and allow for infection of T or NK cells or expansion of EBV-infected cells. Second, EBV may infect hematopoietic stem cells that differentiate to multiple cell lineages. Third, EBV-infected lymphocytes may be capable of differentiation, as suggested recently by Ohga *et al.*[37] Further studies are necessary to clarify the mechanism by which EBV infects multiple lineages.

One possible disadvantage of our assay is its relatively low sensitivity. Preliminary studies using cell lines indicated that the assay could detect the phenotype of EBV-infected cells when they comprised at least 0.1% of the total population.[18] However, when human samples were used, cell phenotypes could not be determined when they accounted for <0.2% of the total. Therefore, this assay would not be suitable for patients with low peripheral blood viral loads.

Hydroa vacciniforme-like lymphoma is a recently defined EBV-positive cutaneous malignancy associated with photosensitivity.[10] It is characterized by a papulovesicular eruption that generally proceeds to ulceration and scarring. In some cases, systemic symptoms, including fever, wasting, lymphadenopathy, and hepatosplenomegaly, may be present.[39–42] In HV-like eruptions, both T and NK cells infiltrate the superficial dermis and

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the subcutaneous tissue.[10] Recently, we reported three cases of HV-like lymphoma with EBV-infected γδ T cells using the FISH assay.[18] In five of seven patients in the present study (the three cases in the previous report were included), the EBER-positive cells were $CD3^+$ $CD4^-$ $CD8^-$ $TCR\gamma\delta^+$ T cells. The other two cases were of NK and possible NK T cell infection, respectively. These results indicate that γδ T cells play a central role in the formation of HV-like eruptions, although other types of cells can also be involved. This observation accords with other recent reports.[43,44] The γδ T cells are the major T cell population in the skin and mucosal epithelium. The γδ T cells secrete various cytokines and have cytolytic properties.[45] In the present study, EBER-positive γδ T cells were positive for Vδ2, suggesting that they were Vγ9Vδ2 T cells. The Vγ9Vδ2 T cells are the predominant γδ T cell subtype in human peripheral blood.[46] The γδ T cells sense not only infection, but also cellular stress. In patients with HV-like lymphoma, circulating EBV-positive Vγ9Vδ2 T cells may sense and react to cells damaged by ultraviolet radiation. Furthermore, EBER-positive γδ T cells were negative for CD122. A recent study showed that $CD122^-$ $\gamma\delta^+$ T cells produce interleukin (IL)-17.[47] Thus, EBER-positive γδ T cells may produce IL-17 and then induce and activate neutrophils and the epithelium, resulting in the formation of papulovesicular eruptions.

In conclusion, we applied the FISH assay to peripheral blood from 26 patients with EBV-associated T/NK LPD and confirmed that this assay was useful for the diagnosis of this condition. Furthermore, we found that two lymphocyte lineages were present in some patients with EBV-associated T/NK LPD. We showed that γδ T cells were present in peripheral blood from most cases of HV-like lymphoma. Thus, this assay is a direct and reliable method for quantifying and characterizing EBV-infected lymphocytes and can be used not only to complement pathological diagnosis, but also to clarify the pathogenesis of EBV-associated diseases and expand the spectrum of conditions known to be associated with this virus.

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Disclosure Statement

The authors have no conflict of interest to declare.

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