EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases

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EBV-associated T/NK–cell lymphoproliferative disease (T/NK-LPD) is defined as a systemic illness characterized by clonal proliferation of EBV-infected T or NK cells. We prospectively enrolled 108 nonimmunocompromised patients with this disease (50 men and 58 women; median onset age, 8 years; age range, 1-50 years) evidenced by expansion of EBV$^+$ T/NK cells in the peripheral blood; these were of the T-cell type in 64 cases and of the NK-cell type in 44, and were clinically categorized into 4 groups: 80 cases of chronic active EBV disease, 15 of EBV-associated hemophagocytic lymphohistiocytosis, 9 of severe mosquito bite allergy, and 4 of hydroa vacciniforme. These clinical profiles were closely linked with the EBV$^+$ cell immunophenotypes. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications. During the follow-up, 13 patients developed overt lymphoma or leukemia characterized by extranodal NK/T-cell lymphoma and aggressive NK-cell leukemia. Fifty-nine received hematopoietic stem cell transplantation, 66% of whom survived. Age at onset of disease (≥ 8 years) and liver dysfunction were risk factors for mortality, whereas patients who received transplantation had a better prognosis. These data depict clinical characteristics of systemic EBV$^+$ T/NK-LPD and provide insight into the diagnostic and therapeutic approaches for distinct disease. (*Blood*. 2012;119(3): 673-686)

Introduction

EBV-associated lymphoproliferative diseases (LPDs) have a vast spectrum from reactive to neoplastic processes in the transformation and proliferation of lymphocytes spanning B, T, and NK cells,[1-3] and are clinically complicated by the interaction between the biologic properties of EBV$^+$ lymphocytes and the host immune status. Our understanding of these diseases is now evolving and has led to the recognition of a variety of EBV$^+$ diseases, including Burkitt lymphoma,[3] age-related EBV$^+$ B-cell LPD,[4] extranodal NK/T-cell lymphoma of nasal type (ENKL),[5] aggressive NK-cell leukemia (ANKL),[6] classic Hodgkin lymphoma,[3] and immunodeficiency-associated lymphoproliferative disorders.[1] EBV-associated T- and NK-cell LPD (T/NK-LPD) was first incorporated into the 4th World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues, in which systemic EBV$^+$ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma are proposed as distinct entities.[7-8] Historically, based on their broad clinical manifestations, these diseases have been described under various nosological terms from indolent (eg, severe mosquito bite allergy[9] and hydroa vacciniforme[10]) to aggressive or fulminant forms (eg, EBV-associated hemophagocytic lymphohistiocytosis [HLH],[11] chronic active EBV disease [CAEBV] of the T/NK–cell type,[12] fulminant EBV$^+$ T-cell LPD of childhood,[13] and fatal infectious mononucleosis[3]).

CAEBV originally referred to chronic or recurrent infectious mononucleosis-like symptoms.[14-16] A severe form of CAEBV was found to be prevalent in east Asian countries and was characterized by clonal expansion of the EBV-infected T or NK cells,[12,17-18] whereas in Western countries CAEBV is mostly associated with EBV-infected B cells.[19-20] The term EBV-associated HLH was coined to describe hemophagocytosis involving BM or other organs and resulting in pancytopenia in the peripheral blood. This disease is also frequently seen in east Asian countries,[11] and involves a clonal expansion of EBV$^+$ T or NK cells, which produce inflammatory cytokines that induce the activation of macrophages and hemophagocytosis.[21-23] Apart from these systemic diseases, accumulating evidence indicates that 2 cutaneous diseases, hydroa vacciniforme and severe mosquito bite allergy, are closely associated with EBV$^+$ T or NK cells. Hydroa vacciniforme is characterized by recurrent vesiculopapules usually occurring on sun-exposed areas and seen in children and adolescents.[10] In some of these patients, systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly have been recorded.[24-26] Severe mosquito bite allergy was determined to be associated with EBV$^+$ NK cells, but rarely with EBV$^+$ T cells, and to progress into overt lymphoma or leukemia in the long-standing clinical course.[9,27] These EBV$^+$ cutaneous diseases had the same geographic distribution as the other EBV$^+$ T/NK–cell lymphomas and LPDs among

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Table 1. Definitions of EBV$^+$ T/NK-LPDs in this study

Disease	Eligibility criteria	Exclusion criteria	Lineages/clonality	References
Clinical category				
CAEBV of T/NK–cell type	(1) Illness ≥ 3 mo in duration (EBV-related illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme-like eruptions, and hypersensitivity to mosquito bites)*	(1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition	T/NK cell	12
	(2) Increased amounts of EBV detected by Southern blot hybridization or EBER$^+$ cells in affected tissues or peripheral blood; ≥ $10^{2.5}$ copies/μg of EBV DNA in PBMCs	(2) Congenital immunodeficiency including X-linked lymphoproliferative disorders	Polyclonal, oligoclonal, monoclonal	16,29
HLH	(1) Clinical criteria (fever and splenomegaly)	(1) Hemophagocytic syndrome in accelerated phase of CAEBV of T/NK cell type	T/NK cell	11
	(2) Laboratory criteria (cytopenia affecting 2 of 3 lineages in the peripheral blood, hypertriglyceridemia, and/or hypofibrinogenemia) (3) Histological criteria (hemophagocytosis in the BM, spleen, or lymph nodes)	(2) Congenital immunodeficiency including familial HLH	Polyclonal, oligoclonal, monoclonal	
Severe mosquito bite allergy*	Hypersensitivity to mosquito bites characterized by high fever after bites, ulcers, necrosis, and scarring*	Any systemic symptoms in addition to the cutaneous lesions were categorized to CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	9,39
Hydroa vacciniforme*	Recurrent vesiculopapules with central umbilication and crust formation mimicking herpetic vesicles usually occurring on sun-exposed areas	Any systemic symptoms in addition to cutaneous lesions categorized as CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	10,39
Pathological classification				
Systemic EBV$^+$ T-cell LPD	(1) Illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, hemophagocytosis, and interstitial pneumonia (2) Can occur shortly after primary EBV infection or in the setting of CAEBV (3) Monoclonal expansion of EBV-infected T cells with an activated cytotoxic phenotype in tissues or peripheral blood	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T-cell, monoclonal	7
Hydroa vacciniforme-like lymphoma	(1) Recurrent vesiculopapules with central umbilication and crust formation usually occurring on sun-exposed areas with or without systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly (2) Monoclonality of EBV-infected cells	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T/NK cell, monoclonal	7

*"Severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme-like eruptions" were used to designate symptoms.

east Asians and Native Americans in Central and South America and Mexico,[8] and were encountered as a part of the initial and accompanying symptoms of the systemic EBV$^+$ T/NK-LPDs.[28-30] However, the mutual relationship and clinicopathologic distinctiveness of these EBV$^+$ T/NK-LPDs are unfounded, posing diagnostic and therapeutic problems for pathologists and hematologists, respectively. These patients appear to exist in the gray zone between systemic EBV$^+$ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma according to the 4th WHO classification. The former encompasses CAEBV of T-cell type, EBV$^+$ HLH, and EBV$^+$ T-cell lymphomas with prodromal phase, whereas the latter may include all cases with EBV$^+$ hydra vacciniforme despite the presence or absence of the systemic disease in the patient's history.

The aim of the present study was to clarify the clinicopathologic characteristics of these EBV$^+$ T/NK-LPDs and the biologic properties of the proliferating cells by analyzing a large number of patients. We previously performed a nationwide survey for CAEBV of T/NK–cell type and determined its prognostic factors.[29] Similarly, a nationwide study for HLH was recently performed in Japan.[31] However, these studies were retrospective and lacked the precise diagnosis of the current level because of their study design. In 1998, we established an EBV-DNA quantification system using real-time PCR,[32-33] which allowed for the determination of the phenotype of EBV-infected cells in the peripheral blood with the combination of fractionation to the lymphocyte subset.[12,34-35] More recently, we developed the simultaneous staining method for surface antigens and nuclear EBV-encoded small RNA (EBER) to more precisely determine EBV-infected cell phenotypes.[36] Using these techniques, we enrolled and prospectively followed patients with definitive cases of EBV$^+$ T/NK-LPDs in 1998. In this study, 108 nonimmunocompromised patients with EBV$^+$ T/NK-LPDs were analyzed for clinical and virological characteristics to obtain an understanding of their pathogenesis and for refining their

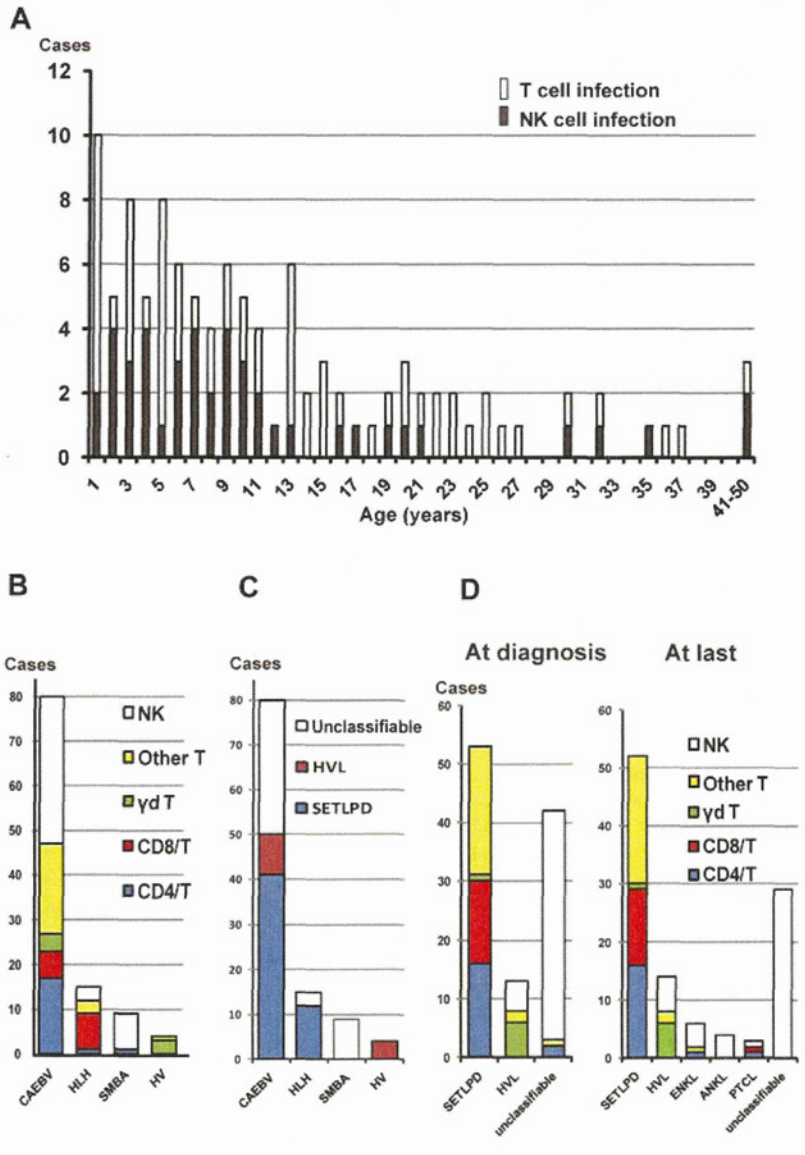

Figure 1. EBV-infected cell phenotypes of EBV⁺ T/NK lymphoproliferative diseases. (A) Age distribution of patients with T-cell and NK-cell types. (B) EBV-infected cells among categories of clinical groups. Infected T cells were further divided into CD4⁺ T cells, CD8⁺ T cells, γδ T cells, and "other T cells." The 25 cases of "other T cells" were defined as either phenotypically different T-cell subsets (2 patients were CD4⁻CD8⁻, 1 patient was CD4⁺CD8⁺, and 1 patient had 2 lineages consisting of CD4⁺CD8⁻ and CD4⁻CD8⁺ cells) or ill-defined T cells (n = 21). In the majority of the ill-defined T-cell patients, Abs against CD4 or CD8 could not be used to define their CD4/CD8 phenotype because the number of recovered PBMCs was not sufficient. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme. (C) The 4th WHO pathologic classification of each clinical group at the time of diagnosis. SETLPD indicates systemic EBV⁺ T-cell lymphoproliferative disease of childhood; and HVL, hydroa vacciniforme–like lymphoma. (D) EBV-infected cells among categories of the pathologic classification at diagnosis and at the last follow-up or death. Patients in CR were classified according to the data and status before remission.

classification. Furthermore, prognostic factors and the efficacy of therapeutic interventions including hematopoietic stem cell transplantation (HSCT) were analyzed.

Methods

Eligibility criteria

Informed consent was obtained from all participants or their guardians in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Nagoya University Graduate School of Medicine. From 1998 to 2010, patients whose samples were sent to Nagoya University Graduate School of Medicine for determination of the EBV-infected cell phenotype and who fulfilled the following criteria were prospectively enrolled in this study: (1) EBV-associated T/NK-LPD suspected or diagnosed based on clinical and/or histopathological findings; (2) high EBV load detected in PBMCs by quantitative PCR ($\geq 10^{2.5}$ copies/μg of EBV-DNA)[12,32]; and (3) EBV infection in T or NK cells in the peripheral blood confirmed by either immunobead sorting followed by quantitative PCR[34-35] or FISH.[36] Exclusion criteria were: (1) pathologically defined ENKL,[5] ANKL,[37] or peripheral T-cell lymphoma (PTCL)[38]; (2) congenital immunodeficiency; (3) HIV positivity; and (4) other immunodeficiencies requiring immunosuppressive therapies or underlying diseases with potential immunosuppression. Patients were recruited through an announcement by the Japanese Association for Research on Epstein-Barr Virus and Related Diseases and on the homepage of our institute's website. Approximately 240 hematology units and 400 departments of pediatrics were included in the association.

On entry into the study, peripheral blood was collected and sent to Nagoya University Graduate School of Medicine to examine EBV-DNA quantification and EBV-infected cell determination along with detailed clinical data. Clonality analyses were also performed at this time if possible. Primary EBV infection was determined based on serological findings, detection of antiviral capsid Ag-IgM, and seroconversion of either antiviral capsid Ag-IgG or anti-EBV nuclear Ag. A total of 108 patients from 40 hospitals were enrolled in the study (25 from Nagoya University Hospital, 13 from Osaka Medical Center and Research Institute for Maternal and Child Health, 9 from Fukushima Medical University, and 61 from other hospitals). Each patient enrolled in the study was treated according to physician decision at each hospital. The physicians completed questionnaires regarding the administered treatment and outcome every 3 years (2001, 2004, and 2007); the final questionnaire was sent and collected in December 2010. Compared with data provided by previous national surveys for CAEBV and HLH,[29,31] we estimated that approximately 15%-20% of systemic EBV⁺ T/NK-LPD cases during the study period were recruited by this registry.

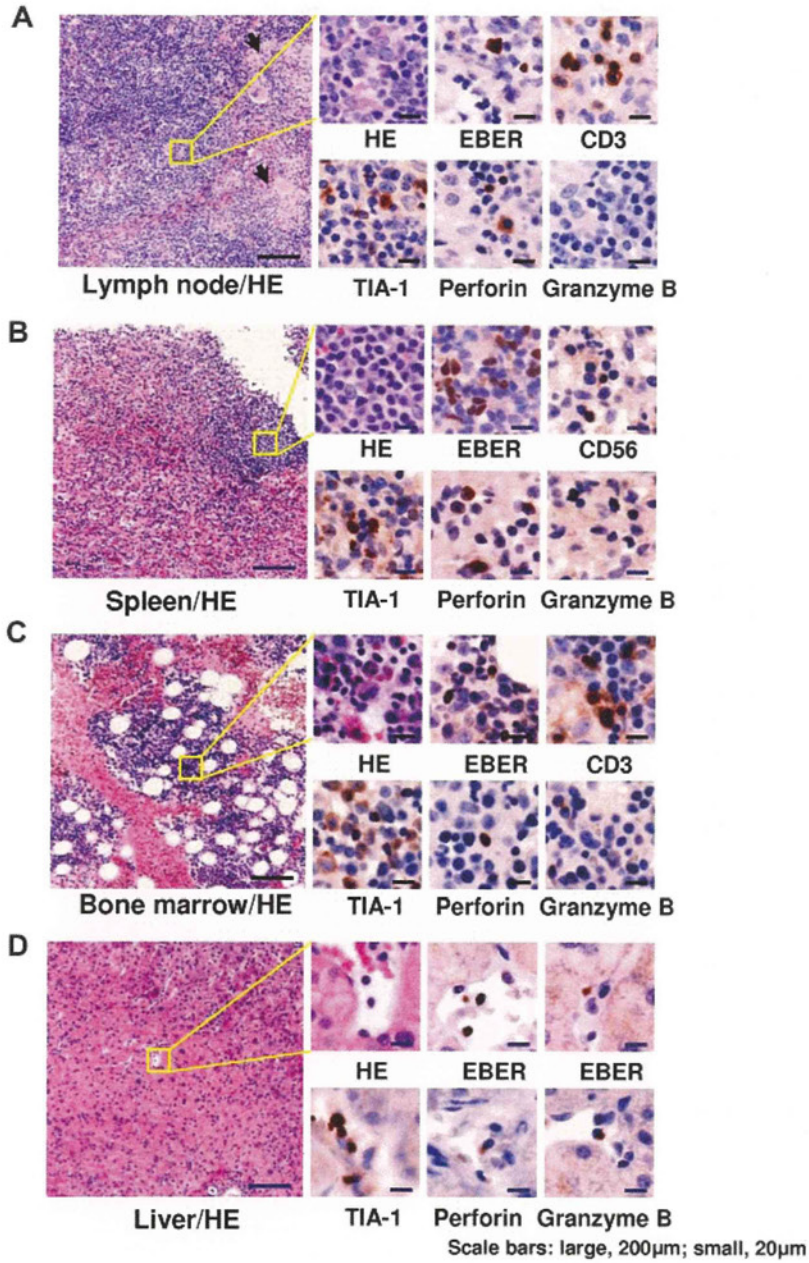

Figure 2. Histopathological findings of representative patients. (A) Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (patient 3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions were detected (arrows). Medium-sized transformed lymphocytes in the paracortex were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (B) Spleen from a 13-year-old boy with chronic active EBV disease with NK-cell infection (patient 6). White pulp was atrophic and red pulp showed congestion. Small lymphocytes infiltrating in the red pulp were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (C) BM from a 25-year-old female with chronic active EBV disease with T-cell infection (patient 17). In the mild hyperplastic BM, small lymphocytes were positive for EBER. TIA-1, perforin, and granzyme B were positive. (D) Liver from a 42-year-old female with chronic active EBV disease with NK-cell infection (patient 60). Small lymphocytes infiltrating in vessels and sinusoid were positive for EBER. TIA-1, perforin, and granzyme B were positive. HE indicates H&E staining. Images of sections were obtained by a microscopy (BX50, Olympus Corp) with CCD camera (D5-5M-L1, Nikon Corp). Each micrograph was represented at either a 100× or 400× magnification using 10× or 40× objective lens (UPlanFL, Olympus Corp), respectively.

Patient criteria

Patients were clinically divided into 4 groups according to the clinical categorization at the 2008 National Institutes of Health meeting: (1) CAEBV of T/NK–cell type, (2) EBV-associated HLH, (3) hydroa vacciniforme, and (4) severe mosquito bite allergy.[39] The clinical diagnosis was made at entry into the study. Definitions of each clinical category are listed in Table 1. CAEBV was defined according to previously proposed criteria.[16,29] HLH was defined based on the criteria proposed by an international treatment study group.[11] Severe mosquito bite allergy and hydroa vacciniforme were applied for cases with only skin symptoms and lacking systemic symptoms. In this study, "severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme–like eruptions" were used as terms for symptoms; "hydroa vacciniforme–like lymphoma" was used as a term for pathologic classification.

Patients were also classified according to the 4th WHO classification for tumors of hematopoietic and lymphoid tissues.[7] The definitions of pathologic classification are listed in Table 1. The classification was made both at the diagnosis and at the last follow-up or death. Patients diagnosed with ENKL, ANKL, or PTCL were excluded from the study, but some developed these diseases during the follow-up period. Of 108 patients, 54 were biopsied (liver, n = 15; skin, n = 15; lymph nodes, n = 10; intestine, n = 3; spleen, n = 2; muscle, n = 2; others, n = 7), and 6 were autopsied. For differential diagnosis, BM examination was performed in most patients (79%), even though there were no hematologic abnormalities of the peripheral blood. When abnormal findings were detected in BM or peripheral blood, EBER/immunohistochemical staining was performed. Histopathology was reviewed by the Central Pathology Review Board (Shigeo Nakamura, Nagoya University and Koichi Ohshima, Kurume University).

Disease status was defined as follows: stable disease, partial remission (PR), and complete remission (CR). Patients with PR had no symptoms but had significant EBV loads in PBMCs (EBV-DNA $\geq 10^{2.5}$ copies/μg of DNA).[12,32] CR patients had no symptoms and continuously low or no EBV loads in PBMCs (EBV-DNA $< 10^{2.5}$ copies/μg DNA). Disease activity was assessed before HSCT and was classified as either active or inactive as described previously.[40] Active disease was defined by the existence of symptoms and signs such as fever, persistent hepatitis, lymphadenopathy,

Table 2. Comparison of characteristics based on EBV-infected cell type in 108 patients with EBV⁺ T/NK-LPD

	Total cells (n = 108)	T cells (n = 64)	NK cells (n = 44)	P*
Sex (male/female)	50/58	27/37	23/21	NS
Age at disease onset, y	12.1 ± 10.6	12.7 ± 10.3	11.3 ± 11.0	NS
Clinical category at diagnosis, n				
CAEBV	80	47	33	NS
HLH	15	12	3	.066
Severe mosquito bite allergy	9	**1**	**8**	**.003**
Hydroa vacciniforme	4	4	0	NS
Past history of infectious mononucleosis, n (%)	37 (34)	24 (22)	13 (12)	NS
Primary infection at diagnosis, n (%)	19 (18)	**16 (15)**	**3 (3)**	**.012**
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA, mean	4.3 ± 0.9	4.2 ± 0.9	4.5 ± 0.8	NS
Plasma, log copies/mL, mean	3.3 ± 1.7	3.5 ± 1.6	3.1 ± 2.0	NS
EBV clonality, monoclonal/oligoclonal/polyclonal	64/8/4	36/4/3	28/4/1	NS
TCR rearrangement, any rearrangement/none	42/48	**36/20**	**6/28**	**< .001**
Chromosomal aberration (abnormal/normal cases)	6/84	4/50	2/34	NS
Symptoms and signs at diagnosis, n (%)				
Fever	98 (91)	59 (92)	39 (89)	NS
Liver dysfunction	83 (77)	49 (77)	34 (77)	NS
Splenomegaly	64 (59)	39 (61)	25 (57)	NS
Thrombocytopenia	47 (44)	26 (41)	21 (48)	NS
Anemia	46 (43)	29 (45)	17 (39)	NS
Lymphadenopathy	41 (38)	27 (42)	14 (32)	NS
Hemophagocytic syndrome	38 (36)	23 (36)	15 (34)	NS
Hypersensitivity to mosquito bites (HMB)	32 (30)	**3 (5)**	**29 (43)**	**< .001**
Hydroa vacciniforme-like eruption (HV-LE)	15 (14)	8 (13)	7 (16)	NS
HMB⁺HV⁻LE⁺	5 (5)	**0 (0)**	**5 (11)**	**.001**
HMB⁻HV⁻LE⁺	10 (9)	**8 (13)**	**2 (5)**	NS
Chemotherapy, n (%)	70 (65)	45 (70)	25 (57)	NS
HSCT, n (%)	59 (55)	32 (50)	27 (61)	NS
Outcome, n (%)				
Dead	47 (44)	27 (42)	20 (45)	NS
Alive	61 (57)	37 (58)	27 (61)	NS
Stable disease	11 (10)	8 (13)	3 (7)	NS
Complete remission	46 (43)	26 (41)	20 (20)	NS
Partial remission	4 (4)	3 (5)	1 (2)	NS

NS indicates not significant.

*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

hepatosplenomegaly, pancytopenia, or progressive skin lesions along with an elevated EBV load in the peripheral blood. Liver dysfunction was defined as an increase in alanine transaminase levels to 2 times above the upper limit of normal on at least 2 consecutive occasions.

Analyses of EBV and determination of EBV-infected cells

DNA was extracted from 1×10^6 PBMCs or 200 μL of plasma and real-time quantitative PCR was then performed as described previously.[12,32] EBV clonality was assessed by Southern blotting with a terminal repeat probe, as described previously.[12,41] To determine which cell population harbored EBV, either immunobead sorting followed by quantitative PCR or FISH assay was performed. For the former method, PBMCs were fractionated into $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, $CD56^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ cells using an immunobead method (IMag Cell Separation System; BD Biosciences) that resulted in 97%-99% purity.[34-35] Purified cells were analyzed by real-time quantitative PCR. The infected-cell phenotypes were determined in comparison with unfractionated (whole) PBMCs, as described previously.[34-35] For example, patients were defined as $CD3^+$ when $CD3^+$ cells contained higher amounts of EBV DNA than whole PBMCs. The FISH assay was performed as described previously.[36] Briefly, PBMCs were stained with fluorescence labeled mAbs against surface marker, fixed, permeabilized, and hybridized with EBER-specific PNA Probe/FITC (Y5200; Dako). After enhancing fluorescence, stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest Version 5.1.1 software (BD Biosciences). More than 0.1% of $EBER^+$ cells was considered to be significant and such subset was designated EBV^+. This frequency was chosen based on previous data using EBV^+ cell lines.[36]

TCR gene rearrangement

TCR gene rearrangement was determined by multiplex PCR using the T-cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies), which was developed and standardized in a European BIOMED-2 collaborative study.[42]

Histopathology

Immunostaining was performed using an avidin-biotin peroxidase complex method with mAbs against CD3 (Dako), CD56 (Novocastra Laboratories), perforin (Novocastra Laboratories), T cell–restricted intracellular Ag 1 (TIA-1; Immunotech), and granzyme B (Monosan).[43] FISH was performed using the EBER probe (Dako) as described previously.[43] Hybridization was detected using mouse monoclonal anti-FITC Ab (Dako) and a Vectastain ABC kit (Vector).

Statistical analysis

Statistical analysis was performed using SPSS for Windows Version 18.0. For univariate analysis, either the χ^2 or the Fisher exact test (single-sided) was used to compare categorical variables. The Mann-Whitney U test was used to compare quantitative variables. Logistic regression analysis was used for multivariate analysis. Comparison between quantities of EBV-DNA in PBMCs and plasma was performed by regression analysis. The Kaplan-Meier method and the log-rank test were used for survival analysis. $P < .05$ was considered statistically significant for all analyses.

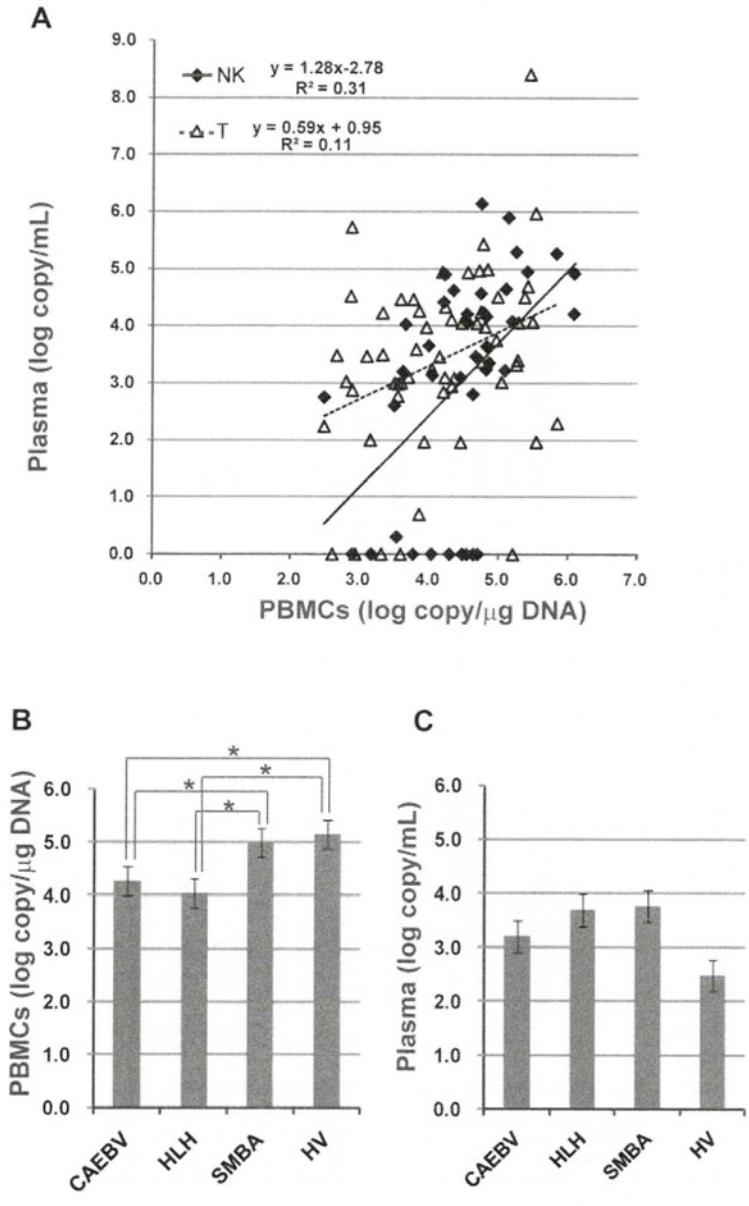

Figure 3. Viral load in the peripheral blood at the time of diagnosis. EBV-DNA was quantified by real-time PCR. (A) Correlation of viral load between PBMCs and plasma. The correlation was separately estimated in patients with T-cell infection and those with NK-cell infection. (B) Quantity of EBV-DNA in PBMCs among categories of clinical groups. $^*P < .05$. (C) Quantity of EBV-DNA in plasma among categories of clinical groups. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme.

Results

Characteristics of patients with EBV$^+$T/NK-LPD

A total of 108 patients (50 men and 58 women) were enrolled in this study. Detailed characteristics of each patient are shown in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Age at diagnosis ranged from 1 to 51 years (median, 14 years). At the time of diagnosis, the main phenotypes of EBV-infected cells in the peripheral blood were T cells and NK cells in 64 and 44 patients, respectively. Onset age ranged from 1 to 50 years (median, 9 years). Most patients (91%) were children and young adults less than 30 years of age, but there were some middle-aged patients (age range, 30-50 years) also existed (Figure 1A). There was no difference in onset age between patients with the T-cell type and those with the NK-cell type. The former were further subdivided into the CD4$^+$ T-cell type (n = 18), the CD8$^+$ T-cell type (n = 14), the γδ T-cell type (n = 7), and other or ill-defined T-cell type (n = 25). In 2 patients (patients 92 and 100, supplemental Table 1), 2 lineages of cells were infected with EBV.

After entry into the study, patients were clinically categorized into 4 groups based on clinical symptoms and diagnostic criteria: CAEBV (n = 80), EBV-associated HLH (n = 15), severe mosquito bite allergy (n = 9), and hydroa vacciniforme (n = 4; Figure 1B). The CAEBV group consisted of 47 patients with the T-cell type (59%) and 33 with the NK-cell type (41%); the former were further subdivided into the CD4$^+$ T-cell type (21%), the CD8$^+$ T-cell type (8%), and the γδ T-cell type (5%). Eight of 15 (53%) EBV-associated HLH patients had EBV-harboring CD8$^+$ T cells, in contrast to their low occurrence in the other clinical groups. In addition, most patients (89%) with severe mosquito bite allergy had EBV-infected NK cells, whereas many (75%) with hydroa vacciniforme had EBV-infected γδ T cells (Figure 1B). Therefore, clinical profiles were closely linked with the EBV$^+$ cell immunophenotype.

Between 1 and 349 months from the onset of disease (median, 46 months), 47 patients had died, whereas 61 patients were alive for follow-up periods of 13-263 months (median, 82 months). The

main causes of death were multiple organ failure (n = 10), hepatic failure (n = 6), heart failure (n = 5), pulmonary failure (n = 5), sepsis (n = 5), intracranial hemorrhage (n = 5), intestinal hemorrhage or perforation (n = 3), hemophagocytic syndrome (n = 2), and other (n = 6). Of the 47 patients who died, 20 (42%) died after transplantation. Of the 61 surviving patients, 41 were in CR and 4 were in PR without any symptoms, whereas 16 remained in stable disease at the last follow-up.

Clonality analysis

At the time of diagnosis, viral clonality was analyzed by Southern blot analysis using EBV terminal repeat. Of 76 patients with available DNA, EBV-infected cells were monoclonal in 64 (84%) and oligoclonal in 8 (11%). Polyclonal EBV-infected cells were detected in only 4 patients (5%). TCR rearrangement was analyzed in 90 patients at the time of diagnosis, 42 of whom had monoclonal rearrangements. Six patients with NK-cell infection demonstrated TCR rearrangement. Because this analysis uses a PCR-based method, erroneous detection of a seemingly clonal cell population (pseudoclonality) or reduced TCR diversity caused by the prevalence of a few Ag-selected subclones, which are often seen in EBV infection, may occur.[42] Chromosomal aberrations were detected in the peripheral blood or lymph nodes at diagnosis in 6 patients, whereas an additional 6 patients later developed chromosomal aberrations in their clinical course of 1-9 years (median, 5 years). Patterns of chromosomal aberrations in each patient are shown in supplemental Table 2. These results provided additional support to the assertion that patients with EBV$^+$ T/NK-LPDs had clonality at early stages and subsequently developed overt lymphoma or leukemia with an increase of chromosomal aberrations in their clinical course.

Pathologic categories based on the 4th WHO classification

At the time of diagnosis, based on the 4th WHO classification, 53 and 13 patients were classified into systemic EBV$^+$ T-LPD of childhood and hydroa vacciniforme–like lymphoma, respectively. The proportion of these pathologic categories in each clinical group is shown in Figure 1C. Four patients clinically categorized to hydroa vacciniforme without any cellular atypia or systemic symptoms were classified into hydroa vacciniforme–like lymphoma based on the monoclonality of cells with TCR rearrangements. In systemic EBV$^+$ T-cell LPD, T-cell subsets of EBV-infected cells were variable (Figure 1D). In hydroa vacciniforme–like lymphoma, 6 of 13 patients had γδ T-cell infection. Conversely, 42 patients were not classified into either of these pathologic categories because they failed to correspond to criteria in the current WHO classification. Classification of each patient is shown in supplemental Table 1.

At the last follow-up or death, there were 29 patients who were unclassifiable, most of whom had CAEBV of the NK-cell type and severe mosquito bite allergy with NK-cell infection (Figure 1D). In the clinical course, ENKL developed in 6 patients (patients 2, 5, 20, 34, 60, and 81 in supplemental Table 1) after 9 months to 12 years of follow-up after onset (median, 1.5 years), whereas ANKL developed in 4 patients (patients 8, 43, 66, and 80) after 2-17 years of follow-up (median, 12 years); most of these patients had NK-cell infection. EBV$^+$ PTCL developed in 3 patients after 1 year (patient 83), 5 years (patient 93), and 20 years (patient 53) of follow-up. The EBV$^+$ PTCL patients in this study were characterized by their expression of cytotoxic molecules, nodal manifestation, lack of CD56 expression, and TCR gene rearrangement. These features

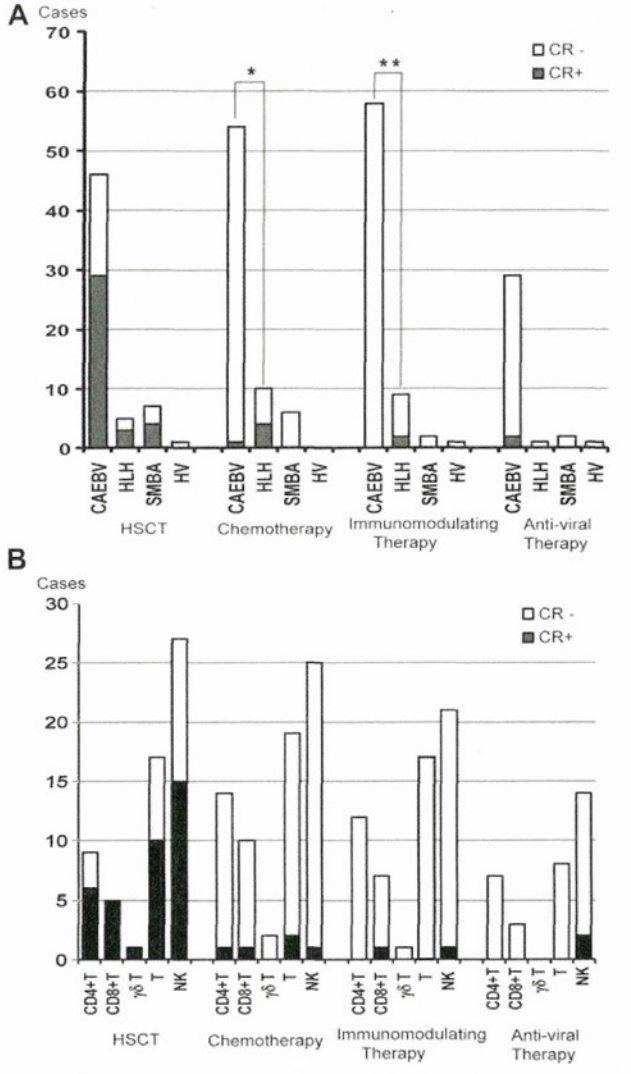

Figure 4. Efficacy of therapeutic interventions. (A) Number of patients treated with each therapy and patients who maintained CR are shown among categories of clinical groups. SMBA indictes severe mosquito bite allergy; and HV, hydroa vacciniforme. *P = .002; **P = .02. (B) Numbers of patients who received each therapy and those who maintained sustained CR are shown among categories of EBV-infected cells.

suggest a pathologic distinction between these EBV$^+$ PTCL and extranasal ENKL.

Representative results of histological examinations are shown in Figure 2. Histological findings and the number of EBER$^+$ cells varied among patients. EBER$^+$ lymphocytes were detected at various frequencies. Infiltrating cells (presumably EBV-infected) expressed cytotoxic molecules such as TIA-1, perforin, and granzyme B. BM aspirations showed various findings, but most patients had normocellular BM without any abnormal findings. Patients with EBV-associated HLH showed normoplastic or hyperplastic BM with mild or moderate hemophagocytosis. In all patients, however, BM findings showed an absence of hematologic malignant disorders at the time of diagnosis.

Differences between patients with T-cell and NK-cell infection

We compared clinical and virological differences between T- and NK-cell infections (Table 2). T-cell infection was characterized by higher rates of primary EBV infection and TCR rearrangement,

Table 3. Univariate and multivariate analyses of factors associated with mortality in 108 patients with EBV⁺ T/NK-LD

	Univariate analysis		Multivariate analysis†	
	OR (95% CI)	P*	OR (95% CI)	P‡
Female sex	**1.40 (0.98-1.97)**	**.048**	1.26 (0.48-3.31)	.64
Age at disease onset (≥ 8 years)§	**1.63 (1.17-2.28)**	**.003**	**4.43 (1.61-12.2)**	**.004**
Past history of infectious mononucleosis	0.62 (0.35-1.11)	.093	0.36 (0.12-1.02)	.054
Primary infection at diagnosis	0.47 (0.18-1.20)	.079	0.32 (0.08-1.25)	.10
Clinical entity at diagnosis				
CAEBV	1.12 (0.90-1.39)	NS		
HLH	0.65 (0.24-1.77)	NS		
Severe mosquito bite allergy	1.04 (0.30-3.65)	NS		
Hydroa vacciniforme	0.43 (0.05-4.03)	NS		
T-cell infection	1.13 (0.69-1.71)	NS		
NK-cell infection	0.95 (0.69-1.30)	NS		
EBV DNA in mononuclear cells (≥ $10^{4.5}$ copies/μg DNA)	1.16 (0.79-1.71)	NS		
EBV DNA in plasma (≥ $10^{3.5}$ copies/mL)	1.23 (0.84-1.72)	NS		
EBV monoclonality	1.08 (0.89-1.31)	NS		
TCR rearrangement	1.13 (0.73-1.76)	NS		
Chromosomal aberration	1.92 (0.34-10.9)	NS		
Symptoms and signs at diagnosis				
Fever	1.10 (0.98-1.24)	NS		
Liver dysfunction	**1.33 (1.09-1.63)**	**.006**	**4.25 (1.23-14.7)**	**.022**
Splenomegaly	**1.38 (1.01-1.88)**	**.033**	¶	
Anemia	**1.84 (1.18-2.88)**	**.005**	1.36 (0.31-6.01)	.68
Thrombocytopenia	**1.75 (1.13-2.71)**	**.009**	1.80 (0.44-7.33)	.41
Lymphadenopathy	1.24 (0.77-2.00)	NS		
Hemophagocytic syndrome	1.30 (0.72-2.32)	NS		
Hypersensitivity to mosquito bites	0.89 (0.69-1.15)	NS		
Hydroa vacciniforme-like eruption	0.86 (0.34-1.97)	NS		
Chemotherapy	0.84 (0.53-1.34)	NS		
HSCT	**0.67 (0.045-0.98)**	**.022**	**0.34 (0.12-0.96)**	**.041**
T-cell infection group	**0.54 (0.30-0.97)**	**.021**		
NK-cell infection group	0.83 (0.51-1.34)	NS		

NS indicates not significant.

*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

†For multivariate analysis, factors with $P < .10$ were included.

‡$P < .05$ (shown in bold) are statistically significant.

§Stratified onset ages were analyzed in advance, and ≥ 8 years was chosen as the age factor.

¶Splenomegaly was excluded from multivariate analysis, because this factor was closely associated with anemia, thrombocytopenia, and liver dysfunction.

whereas a significant number (43%) of patients with NK-cell infection had hypersensitivity to mosquito bites (Table 2). Interestingly, 5 patients had both hypersensitivity to mosquito bites and hydroa vacciniforme–like eruptions; these patients all had NK-cell infection (Table 2). Conversely, 8 of 10 patients with hydroa vacciniforme–like eruptions but without hypersensitivity to mosquito bites had T-cell infections (Table 2).

A comparison of viral load in the peripheral blood between patients with T- and NK-cell infections detected similar levels of EBV-DNA in both PBMCs and plasma (Table 2). Correlation of viral loads between PBMCs and plasma was estimated (Figure 3A). The quantity of EBV-DNA in PBMCs was significantly correlated with that in plasma in both T-cell and NK-cell infections, although EBV-DNA was not detected in the plasma from 15 patients. We also compared viral load among clinical groups (Figure 3B-C). Interestingly, the quantity of EBV-DNA in PBMCs was significantly higher in patients with severe mosquito bite allergy and hydroa vacciniforme, but these patients did not have any systemic symptoms.

Efficacy of therapeutic interventions

Each patient received a variety of therapies. HSCT was administered to 59 patients, which induced sustained CR in 63% of patients with CAEBV, 60% of HLH patients, and 57% of severe mosquito bite allergy patients (Figure 4A). Seventy patients received chemotherapy such as etoposide/cyclosporine A/dexamethasone, cyclophosphamide/doxorubicin/vincristine/prednisolone (CHOP), CHOP plus etoposide, and high-dose cytosine arabinoside therapy. Chemotherapy was effective in some patients, but the effect was usually transient and failed to induce sustained CR in most cases. Chemotherapy induced sustained CR in only 5 patients, 4 of whom had HLH (Figure 4A). Immunomodulating therapies such as prednisolone, cyclosporine A, high-dose IV immunoglobulin, and methyl prednisolone pulse therapy were administered to 58 patients. The immunomodulating therapies induced sustained CR in 2 patients with HLH (Figure 4A). In patients with HLH, both chemotherapy and immunomodulating therapy induced sustained CR more frequently compared with those with CAEBV ($P = .002$ and $P = .02$, respectively). Antiviral therapies such as acyclovir, adenine arabinoside, and ganciclovir were administered to 32 patients. In 2 patients (patients 11 and 45 in supplemental Tale 1), sustained CR was achieved during oral acyclovir therapy and weekly IV administration of adenine arabinoside (Figure 4A). However, because antiviral therapies had been administered for a long time, it was not clear whether CR was induced by them or if it was spontaneously achieved.

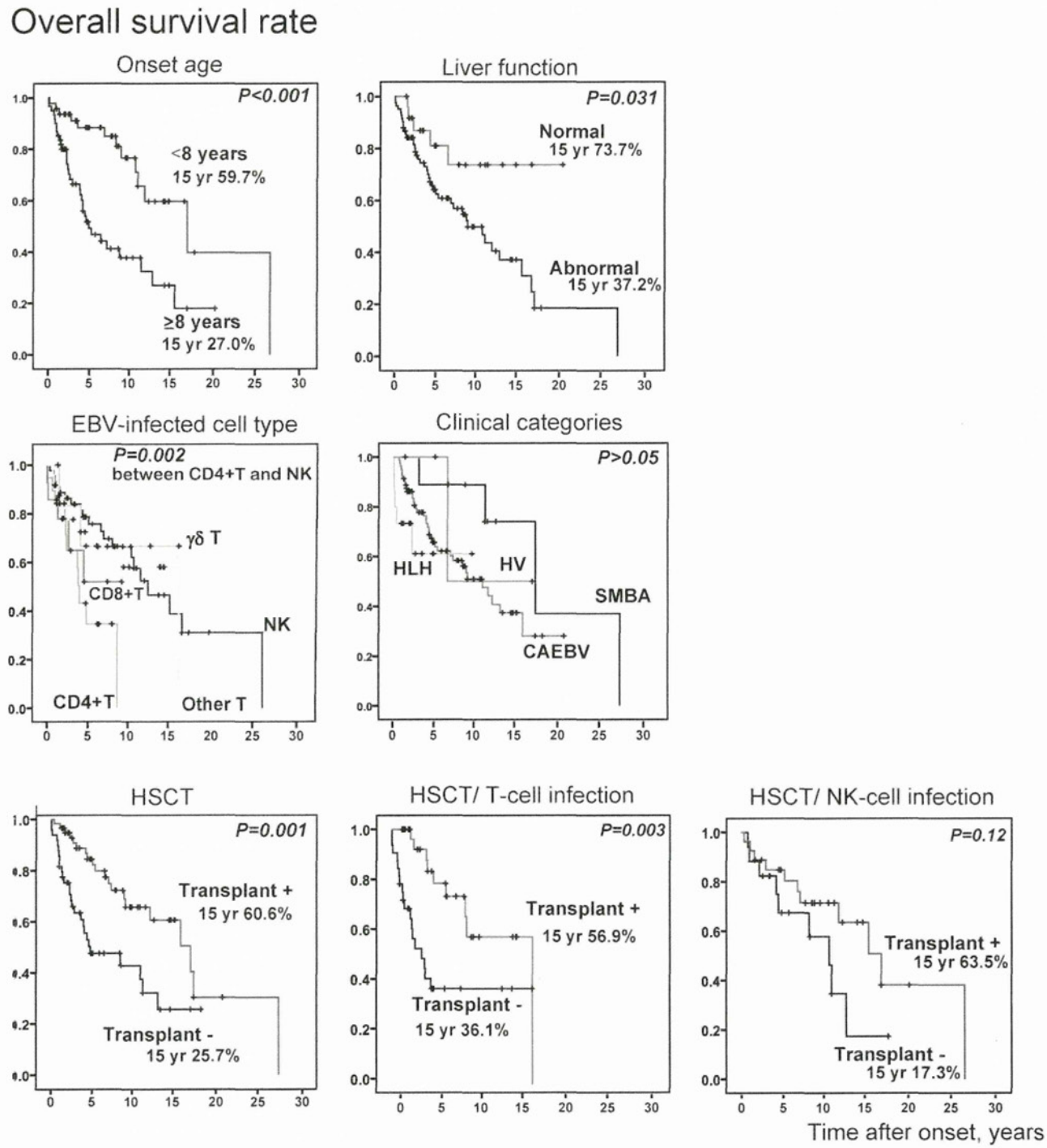

Figure 5. Probability of survival rates from time of disease onset. Overall survival rates from onset (n = 108) were calculated from Kaplan-Meier estimates between each subgroup (onset age ≥ 8 years or < 8 years, with or without liver dysfunction, EBV-infected cell types, clinical categories, and with or without HSCT). HSCT patients were divided into groups based on T-cell infection (n = 64) and NK-cell infection (n = 44) and independently analyzed. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme.

The effects of each therapy among cell types are shown in Figure 4B. There was no statistical difference in the CR rate of each therapy among cell types.

Factors associated with mortality

The factors associated with mortality were analyzed (Table 3), and univariate analysis showed that sex (female), onset age (≥ 8 years), liver dysfunction, splenomegaly, anemia, and thrombocytopenia were significantly associated with mortality. Conversely, HSCT was inversely correlated with mortality rate (odds ratio, 0.67), and this was statistically significant only in patients with T-cell infection. Multivariate analysis using factors for which $P < .10$ revealed that onset age and liver dysfunction were independently significant factors that increased mortality (Table 3); again, HSCT was an independent factor that decreased mortality rate.

Table 4. Comparison of characteristics based on outcome in 59 patients after transplantation

	Total (n = 59)	Alive (n = 39)	Dead (n = 20)	P*
Sex, male/female	29/30	22/17	7/13	NS
Age at disease onset, y	11.8 ± 9.2	11.0 ± 9.0	13.6 ± 9.5	NS
Clinical category at diagnosis, n				
CAEBV	46	32	14	NS
HLH	5	3	2	NS
Severe mosquito bite allergy	7	4	3	NS
Hydroa vacciniforme	1	0	1	NS
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA	4.5 ± 0.8	4.4 ± 0.9	4.5 ± 0.89	NS
Plasma, log copies/mL	3.3 ± 1.6	3.3 ± 1.3	3.3 ± 2.0	NS
T-cell infection, n	32	23	9	NS
NK-cell infection, n	27	16	11	NS
Age at HSCT, y	17.5 ± 9.23	**15.6 ± 9.1**	**21.2 ± 8.3**	**.034**
Time from onset to HSCT, mo	65.0 ± 68.2	52.2 ± 54.7	90.0 ± 84.8	.059
Disease status at transplantation, active/inactive	25/34	**13/26**	**12/8**	**.046**
Preceded chemotherapy, n (%)	42 (71)	27 (69)	15 (75)	NS
Stem cell source, BM/peripheral blood/cord blood	35/11/13	22/8/9	13/3/4	NS
Donor, MRD/MUD/MMRD/MMUD	18/11/4/26	10/9/3/17	8/2/1/9	NS
No of mismatched HLA	0.76 ± 0.9	0.76 ± 0.9	0.75 ± 0.9	NS
Preconditioning regimen, myeloablative/reduced	21/38	11/28	10/10	.086

NS indicates not significant; MRD, matched related donor; MUD, matched unrelated donor; MMRD, mismatched related donor; and MMUD, mismatched unrelated donor.
*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

We compared overall survival rates between each subgroup to confirm association of the above factors with mortality (Figure 5). Overall survival rate in patients whose onset was more than 8 years was significantly low ($P < .001$). Patients with liver dysfunction at the time of diagnosis had lower survival rate ($P = .031$). When patients were divided into 5 groups based on EBV-infected cells, patients with $CD4^+$ T-cell infection had a significantly lower survival rate compared with those with NK-cell infection ($P = .002$). However, there was no statistical difference in survival rate among clinical groups, although the numbers in some groups were small. Patients who received HSCT survived longer ($P = .001$) and, again, this was statistically significant only in patients with T-cell infection ($P = .003$).

Characteristics of patients after HSCT

Of 59 patients who underwent HSCT, 39 patients (66%) survived 1-144 months after transplantation (median, 35.5 months). Conversely, 20 patients (34%) died 1 day to 48 months after transplantation (median, 1.8 months). Detailed characteristics of each patient are shown in supplemental Table 3. Main causes of death were multiple organ failure (n = 5), intracranial hemorrhage (n = 5), sepsis (n = 2), and other (n = 8). Of the 20 deaths, 15 were considered to be treatment related. We compared various factors between patients who lived and those who died after HSCT (Table 4). Univariate analysis showed that age at HSCT was higher and patients with active disease status at the time of HSCT died more frequently after HSCT (Table 4). Time from disease onset to HSCT and intensity of the conditioning regimen (either myeloablative or reduced) were marginally associated with death ($P = .059$ and $P = .086$, respectively). To determine independent risk factors, we performed multivariate analysis using factors for which $P < .10$, and found that none was an independent risk factor for death (data not shown).

We compared overall survival rates (Figure 6A) and event-free survival rates (Figure 6B) of transplanted patients between each subgroup. Although disease status at HSCT was not an independent risk factor by multivariate analysis, overall survival rate was significantly higher in patients with inactive disease at the time of HSCT ($P = .014$); however, its significance diminished for the event-free survival rate. Patients who received HSCT at an age less than 15 years had significantly higher overall ($P = .013$) and event-free survival rates ($P = .015$). Patients whose time from onset to HSCT was less than 30 months also had significantly higher overall ($P = .036$) and event-free survival rates ($P = .033$). Interestingly, these were statistically significant only in patients with T-cell infection.

Discussion

Determining the phenotype of EBV-infected cells is mandatory for our further understanding of the pathogenesis of EBV^+ T/NK-LPDs and related biologic behaviors. In the present study, we used unfixed peripheral blood to determine the phenotypes of EBV-infected cells. One caveat of this study is that we may have missed EBV-associated T/NK-LPDs if EBV-infected cells failed to migrate into the peripheral blood.[33] Furthermore, EBV-infected cells in the peripheral blood might be different from those existing in tissues, although there was no discordant result between tissue biopsy and peripheral blood.

In the present study, EBV-infected cells in EBV^+ T/NK-LPDs were immunophenotypically divided into $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, and NK cells, the variable proportions of which were observed in each of the clinical categories. Kasahara et al reported that CAEBV and EBV-associated HLH were largely caused by $CD4^+$ T or NK cells and $CD8^+$ T cells, respectively.[22] We demonstrated that CAEBV was caused by not only $CD4^+$ T and NK cells but also by $CD8^+$ T and γδ T cells. We also demonstrated that EBV-infected cells in nearly half of hydroa vacciniforme or hydroa vacciniforme–like lymphoma patients were γδ T cells, which is in agreements with our previous observations.[36] Interestingly, all of these cells express molecules characteristic of cytotoxic cells. In fact, $EBER^+$ lymphocytes in EBV^+ T/NK-LPDs usually express cytotoxic molecules including perforin, granzyme B, and TIA-1, as shown in this study and in previous studies.[7,44]

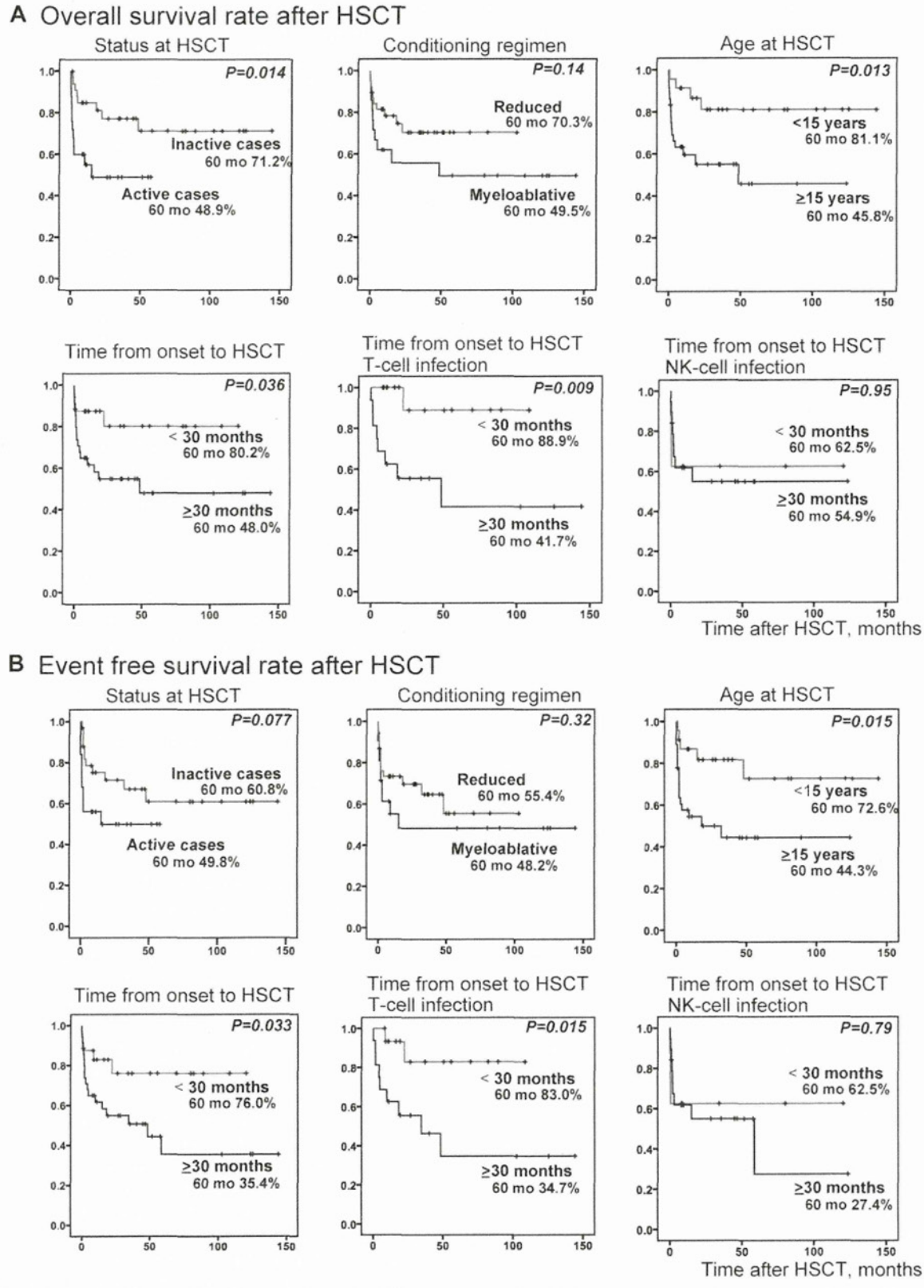

Figure 6. Probability of survival rates after HSCT. Survival rates after HSCT were calculated from Kaplan-Meier estimates between each subgroup (inactive or active cases at HSCT, reduced or myeloablative conditioning, age ≥ 15 years or < 15 years at HSCT, and time from onset to HSCT ≥ 30 months or < 30 months). Stratified ages were analyzed in advance, and ≥ 15 years was chosen as the age factor. Similarly stratified times from onset to HSCT were analyzed in advance, and ≥ 30 months was chosen as the time factor. (A) Overall survival rate after HSCT (n = 59). (B) Event-free survival rate after HSCT (n = 59). For time from onset to HSCT, patients were divided into T-cell infection (n = 32) and NK-cell infection (n = 27) groups and independently analyzed.

The mechanism underlying EBV infection of T and NK cells, which do not express CD21, remains unresolved. It has been shown that NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV binding to

NK-cell hosts.[45] It is plausible that killer cells in close contact with EBV-infected B cells may acquire EBV infection directly and then proliferate with clonality.

In the present study, we evaluated prognostic factors among patients with EBV$^+$ T/NK-LPDs. Multivariate analysis showed that age at onset of disease ($\geq$ 8 years) and liver dysfunction were independent risk factors for mortality, and that patients receiving transplantations had a better prognosis. We found previously that older onset age ($\geq$ 8 years) was associated with mortality in patients with CAEBV.[29] Furthermore, a recent report demonstrated that adult patients with CAEBV had progressive and more aggressive courses than those of childhood onset cases.[46] Interestingly, patients with CD4$^+$ T-cell infection had shorter survival rates than those with NK infection, whereas clinical categories were not correlated with survival rates. Onset age of patients with CD4$^+$ T-cell infection was high (median, 14.5 years). These results suggest that adult patients with CD4$^+$ T-cell infection may have more aggressive features and are likely to develop multiple organ failure. Although the reason is unclear, we should be cautious about rapid progression in patients with CD4$^+$ T-cell infection.

We surveyed administered therapies based on physician questionnaire responses. A potential limitation of this study design was the use of retrospective questionnaires; therefore, we should be cautious about the evaluation of treatment efficacy. Nevertheless, it seems that only HSCT induced CR in patients with EBV-associated T/NK-LPDs except for HLH. Some EBV-associated HLH patients responded well to chemotherapy and immunomodulating therapies,[47] but patients with CAEBV were generally refractory to chemotherapy. Similar findings were reported in patients with CAEBV in the United States.[20] Furthermore, Kaplan-Meier estimates indicated that shorter time from onset to HSCT ($<$ 30 months) and inactive disease at HSCT resulted in long survival times, suggesting that earlier HSCT in patients in good condition is preferred. Patients with CAEBV have a higher risk of transplantation-related complications.[41,48] Recently, Kawa et al reported excellent outcome of HSCT with reduced-intensity conditioning.[40] Although the superiority of reduced-intensity conditioning over myeloablative conditioning did not reach statistical significance in that study, it appears that a reduced-intensity regimen is sufficient to prevent transplantation-related deaths.[40,49]

The concept of EBV$^+$ T/NK-LPD was initially proposed by Kawa et al, and then examined by other researchers.[27,44] This umbrella term encompasses specific clinical diseases of the CAEBV T/NK–cell type, EBV-associated HLH, severe mosquito bite allergy, and hydroa vacciniforme, the distinction of which are differentiated based on clinical manifestations. However, if the clinical data are absent regarding the prodromal phase of expansion of EBV$^+$ T/NK–cells with variable clonality, we cannot discriminate systemic diseases such as ANKL and extranasal ENKL from EBV$^+$ NK-LPDs, because EBV$^+$ proliferating cells are indistinguishable in morphology and phenotype. Recently, this issue was highlighted by Takahashi et al.[50] Interestingly, 4 patients of the present series developed ANKL in their clinical course, 2 of whom had only skin symptoms categorized as severe mosquito bite allergy at the time of the diagnosis. In addition, 6 patients who were clinically categorized as CAEBV NK-cell type (4 cases) and T-cell type (2 cases) developed ENKL; the major clinical difference from de novo ENKL was its early onset (median age, 8.5 years). Three patients had hypersensitivity to mosquito bites. There were no differences in pathologic features between these patients and de novo ENKL patients.[50] Furthermore, new development of chromosomal aberrations was seen in 6 patients during follow-up. In this study, most of the patients with EBV$^+$ T/NK-LPDs had clonality of EBV-infected cells. These results indicate that patients with clonally expanding EBV-infected T or NK cells in EBV$^+$ T/NK-LPD eventually develop overt leukemia and lymphoma, the clinicopathologic findings of which are in keeping with those well documented in extranasal ENKL, ANKL, and PTCL, with additional mutations in cancer genes or tumor-suppressor genes.

In 2008, an international meeting was organized at the National Institute of Health to better define the pathogenesis, classification, and treatment of EBV-associated LPDs in nonimmunocompromised hosts.[39] At that meeting, acute and chronic EBV syndromes of T cells and NK cells were clarified to have a broad spectrum, in which hydroa vacciniforme, hydroa vacciniforme–like lymphoma, severe mosquito bite allergy, and systemic EBV$^+$ T-LPD of childhood were listed as EBV$^+$ T/NK-LPDs under an umbrella term of CAEBV of T/NK–cell type.[39] In the present study, EBV$^+$ T/NK-LPD is characterized by the systemic distribution of EBV$^+$ clones beyond the clinical categorization currently proposed as CAEBV, HLH, severe mosquito bite allergy, and hydroa vacciniforme. Furthermore, we also shed light on the clinicopathologic distinctiveness of patients with NK-cell infection, which has not been well addressed in the past even though these patients comprise approximately 40% of EBV$^+$ T/NK-LPD cases. This phenotype was more closely associated with hypersensitivity to mosquito bite and a relatively indolent clinical course, the biologic significance of which should be clarified in the future.

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Authorship

Contribution: H.K. designed the study, followed the patients, analyzed the data, and wrote the manuscript; Y.I. contributed to the study design, followed the patients, and helped to edit the manuscript; S. Kawabe, K.G., and S.E. performed the experiments; Y.T., S. Kojima, and T.N. followed the patients, collected the clinical data, and helped to edit the manuscript; A.K., A.S., and K.K. followed the patients and collected the clinical data; K.O. performed the experiments and helped to edit the manuscript; and S.N. contributed to the study design, performed the experiments, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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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

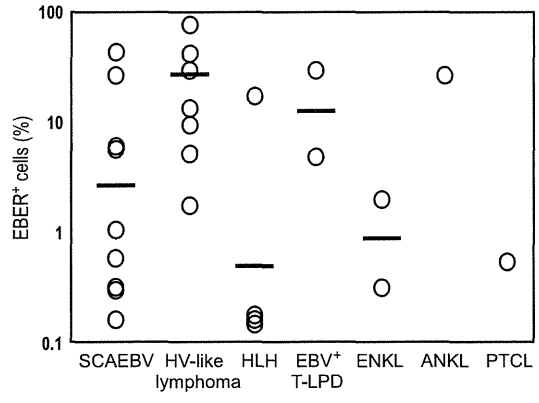

Fig. 1. *Quantification of Epstein–Barr virus (EBV)-infected lymphocytes.* The FISH assay was used to analyze PBMCs and the percentage of EBV-encoded small RNA (EBER)-positive cells in each disease is shown. Bars indicate the mean for each group. ANKL, aggressive NK cell leukemia; EBV$^+$ T-LPD, systemic 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.

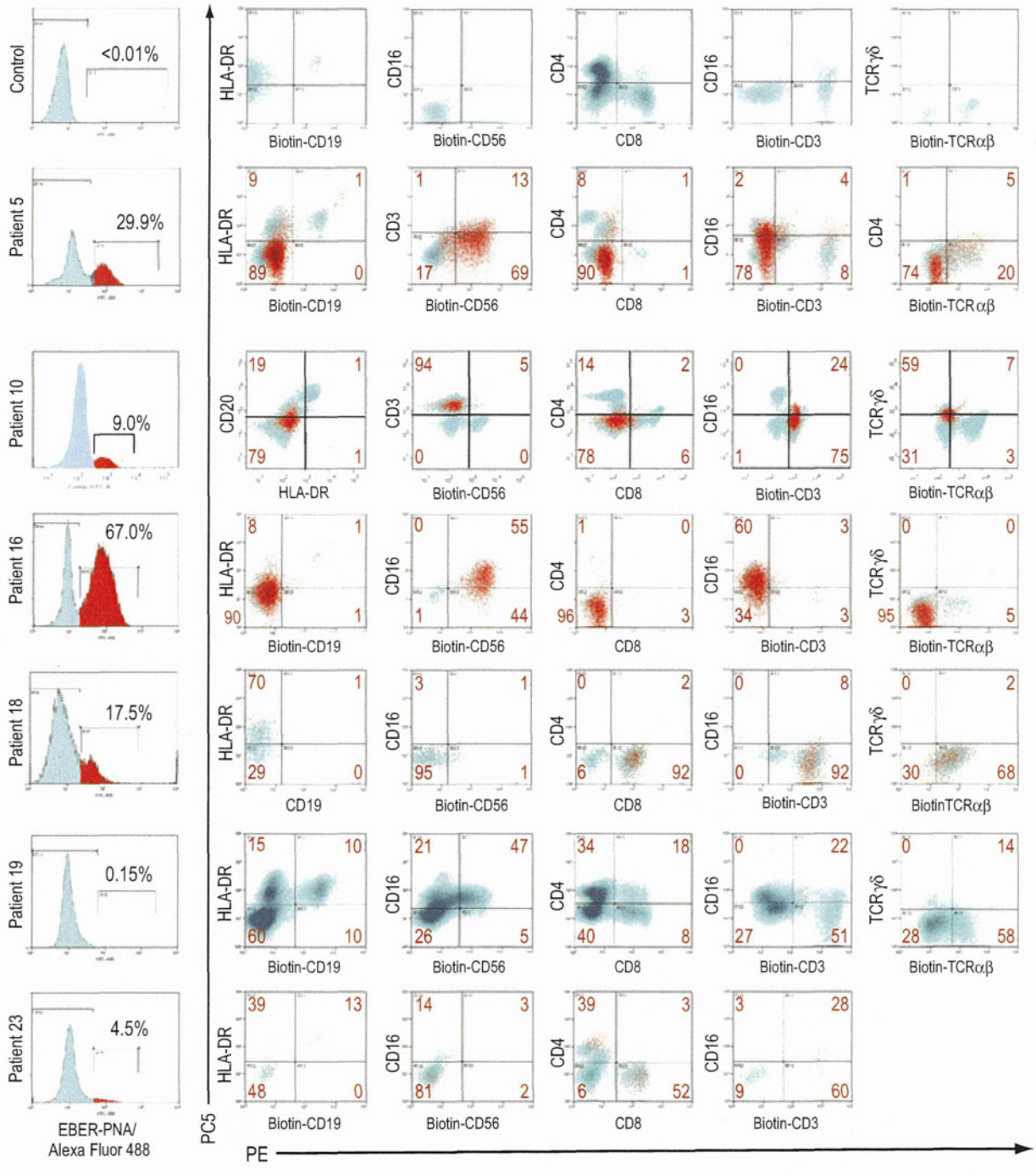

Fig. 2. Characterization of Epstein–Barr virus (EBV)-infected lymphocytes in representative patients. The numbers in each histogram represent the percentage of EBV-encoded small RNA (EBER)-positive lymphocytes. The EBER-positive (red) and EBER-negative (blue) lymphocytes were gated and plotted in quadrants. 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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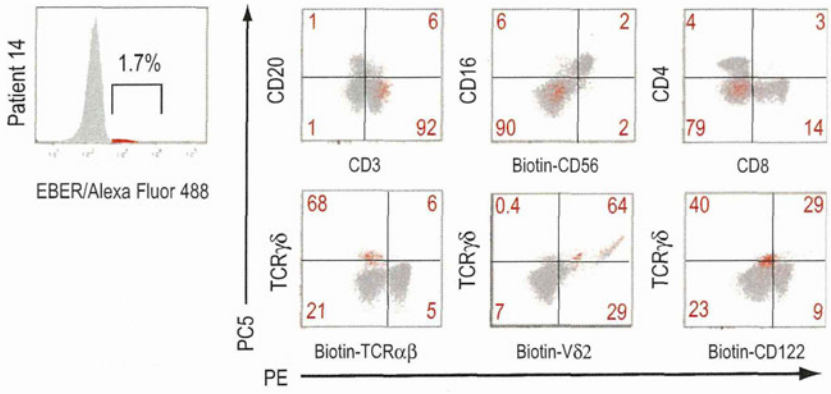

Fig. 3. Characterization of Epstein–Barr virus (EBV)-infected cell phenotypes in a 16-year-old boy with hydroa vacciniforme (HV)-like lymphoma. The EBV-encoded small RNA (EBER)-positive (red) and EBER-negative (gray) lymphocytes were gated and plotted in quadrants. The numbers in the quadrants indicate 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).

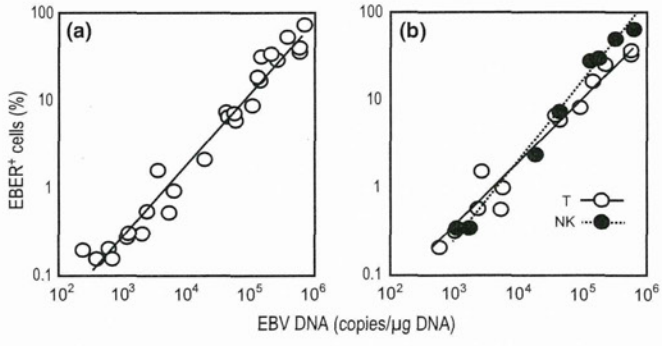

Fig. 4. Correlation between the percentage of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes as determined by FISH and the EBV DNA load determined by real-time PCR. (a) All 26 patients with EBV-associated T or natural killer cell lymphoproliferative diseases (T/NK LPD). (b) Patients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

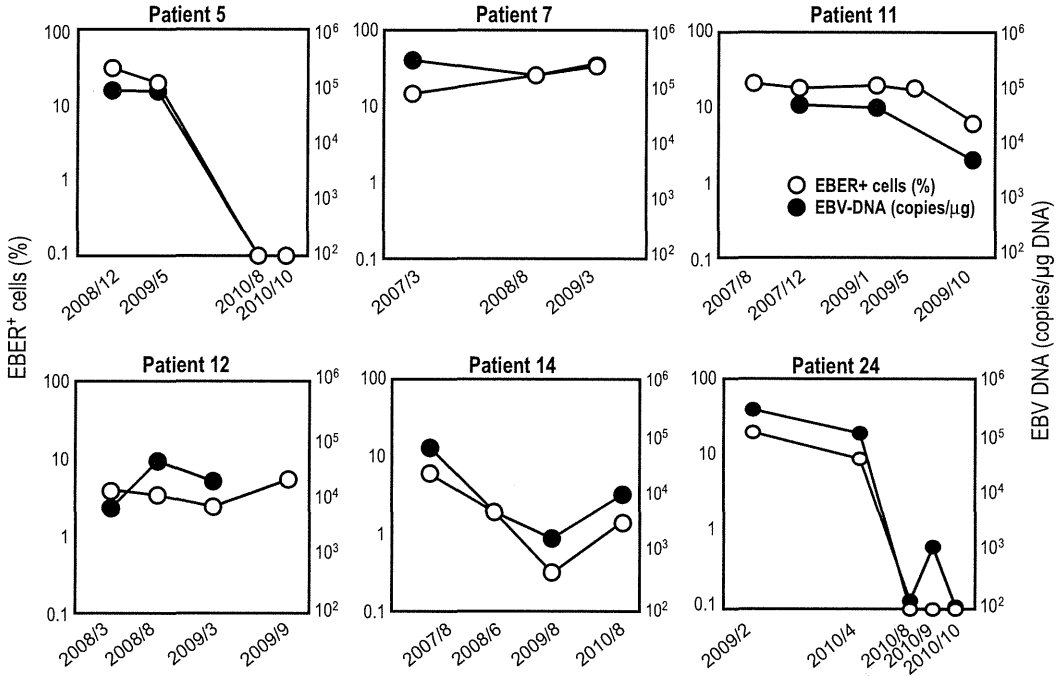

Fig. 5. Longitudinal quantification of Epstein–Barr virus (EBV)-encoded small RNA (EBER)-positive lymphocytes. Samples of PBMCs were obtained repeatedly on the dates indicated and were analyzed by the FISH assay. The results of EBV DNA quantification are also shown for comparison. Patients 7, 11, 12, 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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