

Figure 4. Cytotoxicity of Wilms' tumor gene 1 (WT1)- T cell receptor (TCR)-cytotoxic T lymphocyte (CTL) against various target cells. Two sets of WT1-lymphoblastoid cell line (LCL) and green fluorescent protein (GFP)-LCL, either from donor 1 (A24/) or donor 2 (A26/33), were used as targets in a cytotoxicity assay. Where indicated, cells were loaded with WT1-derived peptide (CMTWNQMNL). Note that WT1-LCL(A24/) was specifically lysed by WT1-TCR-CTL, whereas GFP-LCL(A24/) was barely lysed by the same CTL.

WT1-LCL(A24/) and GFP-LCL(A24/), both derived from the donor 1 lymphocytes, were used as target cells in CTL assays, in the presence or absence of WT1-specific peptide pulsing. When LCLs were pulsed with the peptide, both the WT1-LCL(A24/) and the GFP-LCL(A24/) were efficiently lysed by the WT1-TCR-CTL (Figure 3a). In the absence of the peptide, the GFP-LCL(A24/) was poorly lysed by the WT1-TCR-CTL. By contrast, the WT1-LCL (A24/) was efficiently lysed by WT1-TCR-CTL (>40% specific lysis at effector: target ratio of 5:1) (Figure 4).

We then examined whether the observed killing by WT1-TCR-CTL was HLA-A24 restricted. For this purpose, a set of WT1-LCL(A26/33) and a GFP-LCL(A26/33), both derived from the donor 2 lymphocytes, were used in the CTL assay. Both WT1-LCL(A26/33) and the control LCL(A26/33) were not lysed by WT1-TCR-CTL, regardless of WT1-peptide pulsing. These results indicate that the WT1-derived peptide was presented on the WT1-LCL in an HLA-restricted manner. Therefore, we conclude that the WT1-LCLs serve as target cells of WT1-specific CTLs, and that our experimental strategy provides useful research tools in the field of cancer immunotherapy.

DISCUSSION

EBV-LCLs are regarded as convenient tools for a variety of immunological studies, as they can be easily established from peripheral blood of any individual, and it is relatively easy to obtain large quantities without the aid of exogenous cytokines. We demonstrated here that a novel 'maxi-EBV' with a WT1 transgene can be used to readily convert resting B-lymphocytes to WT1-expressing LCLs *ex vivo*.

A strategy to establish LCLs with transgene expression requires two successive procedures: first, establishing LCLs using the B95-8 strain of EBV and, second, transducing the established LCLs by plasmid transfection or recombinant virus infection (either retrovirus or adenovirus).^{11,12} A previously reported mini-EBV system is an attractive alternative, as one can convert PBMC to transgene-expressing LCLs via just single infection of mini-EBV.¹³ On the other hand, production of mini-EBVs requires EBV packaging cell lines, and, as a result, recombinational events between mini-EBVs

and helper virus genomes can be problematic. The advantage of maxi-EBV system is its simplicity; once HEK293 cells harboring maxi-EBVs are established, maxi-EBVs are stably maintained in the cells and can repeatedly produce viruses. A maxi-EBV lacking viral transactivator BZLF1, a critical switch gene for progeny virus production, was previously reported,³³ and similar modification is expected to solve the safety issue of our maxi-EBV system in the future.

We, for the first time, quantitatively evaluated the expression level of various transgenes in the established LCLs. Fluorescence-activated cell sorting, immunofluorescence and western analyses demonstrated the excellent levels of transgene expression. Latently infected maxi-EBV are maintained as episomes and do not integrate into host chromosomes. Therefore, the WT1 transgene is essentially free from position effects associated with chromosomal integration.³⁴ As a result, high-level expression of WT1 protein can be maintained during proliferation of WT1-LCLs.

This strategy is applicable for individuals with any HLA type. Our data clearly indicate that WT1 is processed and presented as an HLA-binding peptide on WT1-LCLs. Although we examined only one HLA class I-associated epitope, it is highly likely that others, including epitopes complexed with HLA class II, are also presented on WT1-LCLs. In addition, the strategy can be applied for any other tumor antigens, unless they impair the growth of LCLs. LCLs expressing tumor antigens can be ideal target cells of tumor antigen-specific CTLs.

On the other hand, using WT1-LCLs for *ex vivo* CTL induction can be problematic. Our attempts to utilize WT1-LCLs for *ex vivo* expansion of WT1-specific CTL were hampered by several technical limitations. For example, as the blood donors were EBV-seropositive and their PBMC contained pre-existing EBV-specific memory T cells, the expansion of EBV-specific CTLs became predominant after co-cultivation with autologous WT1-LCLs. We also tested a WT1-LCL derived from EBV-seronegative donor (donor 3, see Figure 3b) for *ex vivo* WT1-specific CTL induction, expecting that the lack of EBV-specific memory T cells favor the induction of WT1-specific CTLs. However, induction of WT1-specific CTL again failed (data not shown). Clearly, we need to test alternative experimental strategies. One possibility is to utilize WT1 peptide-pulsed dendritic cells for initial priming before the co-cultivation with WT1-LCLs. Another possibility is to utilize truncated WT1 protein instead of the full-length WT1 for transgene expression. This may augment immunogenicity because previous studies demonstrated that human WT1 truncated at the carboxy-terminal end localizes to the cytoplasm and is more immunogenic than its full-length counterpart.^{35,36}

In summary, we developed a novel maxi-EBV strategy to establish LCLs potentially capable of expressing various tumor antigens as transgenes. These LCLs expressing tumor antigens can be used as target cells in CTL assay, providing useful research tools in the field of cancer immunotherapy. The utility of these LCLs for *ex vivo* CTL expansion is currently unclear, and should be pursued in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antitumor activities of valproic acid on Epstein–Barr virus-associated T and natural killer lymphoma cells

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Epstein–Barr virus (EBV), which infects B cells, T cells, and natural killer (NK) cells, is associated with multiple lymphoid malignancies. Recently, histone deacetylase (HDAC) inhibitors have been reported to have anticancer effects against various tumor cells. In the present study, we evaluated the killing effect of valproic acid (VPA), which acts as an HDAC inhibitor, on EBV-positive and -negative T and NK lymphoma cells. Treatment of multiple T and NK cell lines (SNT13, SNT16, Jurkat, SNK6, KAI3 and KHYG1) with 0.1–5 mM of VPA inhibited HDAC, increased acetylated histone levels and reduced cell viability. No significant differences were seen between EBV-positive and -negative cell lines. Although VPA induced apoptosis in some T and NK cell lines (SNT16, Jurkat and KHYG1) and cell cycle arrest, it did not induce lytic infection in EBV-positive T or NK cell lines. Because the killing effect of VPA was modest (1 mM VPA reduced cell viability by between 22% and 56%), we tested the effects of the combination of 1 mM of VPA and 0.01 μ M of the proteasome inhibitor bortezomib. The combined treated of cells with VPA and bortezomib had an additive killing effect. Finally, we administered VPA to peripheral blood mononuclear cells from three patients with EBV-associated T or NK lymphoproliferative diseases. In these studies, VPA had a greater killing effect against EBV-infected cells than uninfected cells, and the effect was increased when VPA was combined with bortezomib. These results indicate that VPA has antitumor effects on T and NK lymphoma cells and that VPA and bortezomib may have synergistic effects, irrespective of the presence of EBV. (*Cancer Sci* 2012; 103: 375–381)

The ubiquitous Epstein–Barr virus (EBV) infects most individuals by early adulthood and typically remains latent throughout life. Not only does EBV infect B cells, T cells, and natural killer (NK) cells, but it is also associated with multiple lymphoid malignancies, including Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, post-transplant lymphoproliferative disorders, nasal NK/T-cell lymphoma, hydroa vacciniforme-like lymphoma, aggressive NK cell leukemia, and chronic active EBV disease.^(1–4) Epstein–Barr virus plays an important role in the pathogenesis of many of these malignancies via its ability to establish latent infection and induce the proliferation of infected cells.⁽⁵⁾ Some of these EBV-associated lymphoid malignancies are refractory and resistant to conventional chemotherapies. Rituximab, a humanized monoclonal antibody against CD20, targets B cell-specific surface antigens present on EBV-transformed malignant cells. Currently, rituximab is used for the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders.^(6,7) However, the need remains for effective treatments for T and NK cell lymphoid malignancies and novel approaches to molecular targeting are desirable.

Sodium valproate (VPA) is a short chain fatty acid that is widely used to treat epilepsy. It is easily accessible and has a

well-established safety profile. Recently, VPA was reported to be a potent histone deacetylase (HDAC) inhibitor and inducer of DNA demethylation.⁽⁸⁾ It has been found that HDAC inhibitors have potent anticancer activities, with remarkable tumor specificity, and some have even demonstrated therapeutic potential.⁽⁹⁾ The HDAC inhibitors can affect tumor cell growth and survival through multiple biological effects. For example, they induce tumor cell death with all of the biochemical and morphological characteristics of apoptosis. Several HDAC inhibitors have been used in the treatment of leukemias and lymphomas, such as cutaneous T cell lymphoma, myelodysplastic syndrome, and diffuse B cell lymphoma.⁽⁹⁾ They have been used alone or in combination with DNA demethylating agents or other anticancer chemotherapies. Valproate has been reported to induce cell death in human leukemia cell lines⁽¹⁰⁾ and endometrial tumor cells,⁽¹¹⁾ and to enhance the efficacy of chemotherapy in EBV-positive tumors.⁽¹²⁾ Furthermore, VPA was shown to activate lytic viral gene expression in cells infected with EBV.^(12,13)

Previously, we reported that the proteasome inhibitor bortezomib induced apoptosis in T and NK lymphoma cells.⁽¹⁴⁾ Bortezomib produced a stronger killing effect in EBV-infected tumor cells compared with uninfected cells from patients with EBV-associated lymphoproliferative diseases, although the killing effect of bortezomib in cell lines was not affected by the presence of EBV. In the present study, we administered VPA to EBV-positive and -negative T cell lines and NK cell lines, and evaluated its antitumor effects by analyzing cell viability, the induction of apoptosis, cell cycle arrest, and expression of EBV-encoded genes. Finally, we evaluated the antitumor effect of the combination of VPA and bortezomib using both *in vitro* cell lines and *ex vivo* primary cultures of EBV-infected T and NK lymphoma cells.

Materials and Methods

Cell lines and reagents. Of the cell lines used in the present study, SNT13 and SNT16 are EBV-positive T cell lines,⁽¹⁵⁾ SNK6⁽¹⁵⁾ and KAI3⁽¹⁶⁾ are EBV-positive NK cell lines, and Jurkat⁽¹⁷⁾ and KHYG1⁽¹⁸⁾ are EBV-negative T and NK cell lines, respectively. The SNT13, SNT16, SNK6, and KAI3 cells were derived from patients with chronic active EBV disease or nasal NK/T-cell lymphoma. The MT2/rEBV/9-7 cell line⁽¹⁹⁾ was established through infection of MT2 cells with the hygromycin-resistant B95-8 strain.⁽²⁰⁾ The MT2/hyg cell line was transfected with a hygromycin resistance gene. Similarly, the NKL cell line⁽²¹⁾ was derived from a patient with NK cell leukemia, and the TL1 cell line⁽²²⁾ was established from NKL cells infected with an Akata-transfected recombinant EBV strain carrying a neomycin resistance gene.

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Valproate (Sigma, St Louis, MO, USA) was dissolved in distilled water. Bortezomib, a gift from Millennium Pharmaceuticals (Cambridge, MA, USA), was dissolved in PBS.

Cell viability. Cell viability was quantified by Trypan blue exclusion. These experiments were performed in duplicate.

Immunoblotting. Cells were lysed directly in SDS sample buffer. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies. Antibodies directed against acetyl-Histone 3, caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Beverly, MA, USA), and β -actin (Sigma) were used.

Flow cytometry apoptosis assays. Apoptosis was measured by flow cytometry using an annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) apoptosis assay kit (BD Pharmingen Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Cell cycle assay. Cells were treated with 1 mM of VPA for 48 h, fixed with 70% ethanol, and then washed with ice-cold PBS. Fixed cells were treated with 10 μ g/mL DNase-free RNase and stained with 5 μ g/mL propidium iodide (Sigma).

Real-time RT-PCR. Viral mRNA expression was quantified by RT-PCR, as described previously.^(23,24) β 2-Microglobulin (β 2m) was used as an endogenous control and reference gene for relative quantification.⁽²⁵⁾ Each experiment was performed in triplicate. The Mann-Whitney *U*-test was used to compare expression levels and *P* < 0.05 were considered significant.

Patients. Mononuclear cells (MNC) were collected from three patients with EBV-associated diseases. Patients T-1 (a 7-year-old boy) and T-2 (a 6-year-old girl) had hydroa vaccini-forme-like lymphoma, a newly classified EBV-associated T cell lymphoma.⁽²⁾ In these patients, approximately 10% of the MNC were infected with EBV and the EBV-infected cells were primarily $\gamma\delta$ T cells.⁽²⁶⁾ The third patient, NK-1 (a 14-year-old boy), had chronic active EBV disease, NK cell type.⁽²⁷⁻²⁹⁾ Chronic active EBV disease is now considered an EBV-associated T/NK lymphoproliferative disease.^(30,31) In this patient, approximately 40% of the MNC were infected with EBV and the EBV-infected cells were NK cells. Mononuclear cells from three healthy donors were used as controls. Informed consent was obtained from all participants or their guardians. The present study was approved by the Institutional Review Board of Nagoya University Hospital.

Flow cytometric *in situ* hybridization (FISH). To quantify EBV-infected cells and to identify the cell type(s) infected by EBV, a FISH assay was performed.⁽²⁶⁾ Briefly, 5×10^5 MNC were stained with monoclonal antibodies for 1 h at 4°C. Cells were fixed, permeabilized, and hybridized with a fluorescein-labeled EBV-encoded small RNA (EBER)-specific peptide nucleic acid probe (Y5200; Dako, Glostrup, Denmark). Stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

Magnetic cell sorting. Primarily infected cell fractions were separated by magnetic sorting using a TCR γ/δ^+ T Cell Isolation kit or CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity and recovery rates were 98.3% and 80.0%, respectively, with the TCR γ/δ^+ T Cell Isolation kit, and 96.4% and 80.9%, respectively, with the CD56 MicroBeads.

Results

Effects of VPA on HDAC in T and NK cell lines. Acetylated histone 3 levels were determined in T cell lines (SNT16 and Jurkat) and NK cell lines (KAI3 and KHYG1) after 24 h exposure to 0.1-5 mM of VPA. Valproate increased acetylated histone 3 levels in a dose-dependent manner (Fig. 1a), indicating that VPA inhibits HDAC in these cell lines.

Effects of VPA on the viability of T and NK cell lines. To evaluate the effects of VPA on cells viability, EBV-positive T cell

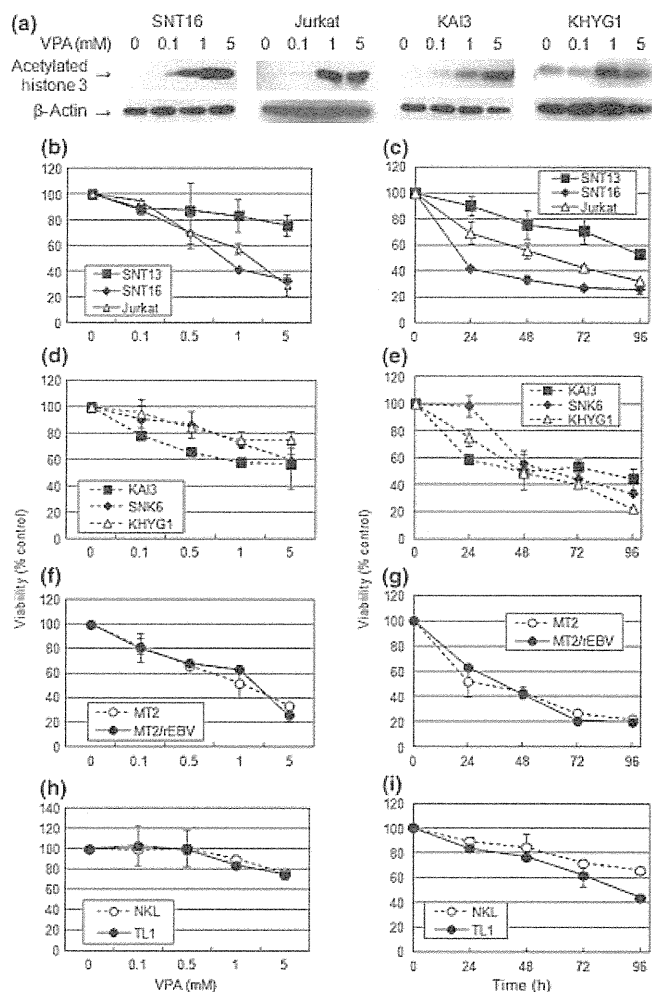


Fig. 1. Valproate (VPA) inhibits histone deacetylase (HDAC) and reduces viability of T and natural killer (NK) cell lines. (a) Acetylated histone 3 was detected by immunoblotting in T and NK cell lines treated with various concentrations of VPA for 24 h. β -Actin was used as a loading control. Viability of (b,c) Epstein-Barr virus (EBV)-positive T cell lines (SNT13 and SNT16) and an EBV-negative T cell line (Jurkat), (d,e) EBV-positive NK cell lines (KAI3 and SNK6) and an EBV-negative NK cell line (KHYG1), (f,g) an EBV-positive T cell line (MT2/rEBV) and its parental cell line (MT2/hyg), and (h,i) an EBV-positive NK cell line (TL1) and its parental line (NKL) that were either treated with VPA at the concentrations indicated for 24 h (b,d,f,h) or with 1 mM VPA for 96 h (c,e,g,i). Viability is shown as the ratio of viable cells in the different treatment groups to distilled water-treated cells, as assessed by Trypan blue exclusion. Data are the mean \pm SEM.

lines (SNT13 and SNT16), an EBV-negative T cell line (Jurkat), EBV-positive NK cell lines (KAI3 and SNK6), and an EBV-negative NK cell line (KHYG1) were exposed to 0.1-5 mM of VPA for 24 h. The cell viability of all six cell lines tested was reduced by VPA in a dose-dependent manner (Fig. 1b,d). In another series of experiments, the same six cell lines were exposed to 1 mM VPA for 4 days, with viability evaluated every 24 h. In these experiments, VPA reduced the viability of all six cell lines by between 22% and 52% after 96 h (Fig. 1c,e). There were no obvious differences between the effects of VPA on EBV-positive and -negative cell lines. Furthermore, to directly compare the effects of VPA on EBV-positive and -negative cell lines, we exposed MT2/hyg and MT2/rEBV/9-7 (Fig. 1f,g) and NKL and TL1 (Fig. 1h,i) cells to VPA and found that 0.1-5 mM of VPA had almost identical effects on the EBV-positive and -negative cell lines.

Effects of VPA on the apoptosis of T and NK cell lines. To determine whether VPA induces apoptosis in these cell lines, the cleavage of caspase-3 and PARP was analyzed by immunoblotting. One mM of VPA increased levels of cleaved caspase-3 and PARP in Jurkat and KHYG1, which are EBV-negative T and NK cell lines, respectively (Fig. 2a), suggesting that VPA induces apoptosis in these two cell lines. Analysis of the induction of apoptosis by flow cytometry showed that VPA only increased the number of apoptotic cells in the SNT16 cell line

(Fig. 2b). In the other cell lines tested, increases in the number of apoptotic cells were not confirmed, although the number of dead cells increased. A representative result for KHYG1 cells is shown in Figure 2(c).

Effects of VPA on the cell cycle in T and NK cell lines. To investigate the effects of VPA on the cell cycle, cells were treated with 1 mM VPA for 48 h, stained with propidium iodide, and then analyzed by flow cytometry. The population of cells in the G₁ phase was increased following exposure

Fig. 2. Effects of valproate (VPA) on apoptosis. (a) T and natural killer (NK) cell lines were treated with 1 mM VPA for 24 or 48 h. β -Actin was used as a loading control. Valproate induced the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in Jurkat and KHYG1 cells. (b,c) T and NK cell lines were treated with 1 mM VPA for 48 h. Viable cells were defined as those negative for annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD). (b) The number of early apoptotic SNT16 cells, defined as those positive for annexin V-PE and negative for 7-AAD, was increased, as was (c) the numbers of dead KHYG1 cells, defined as those positive for both annexin V-PE and 7-AAD.

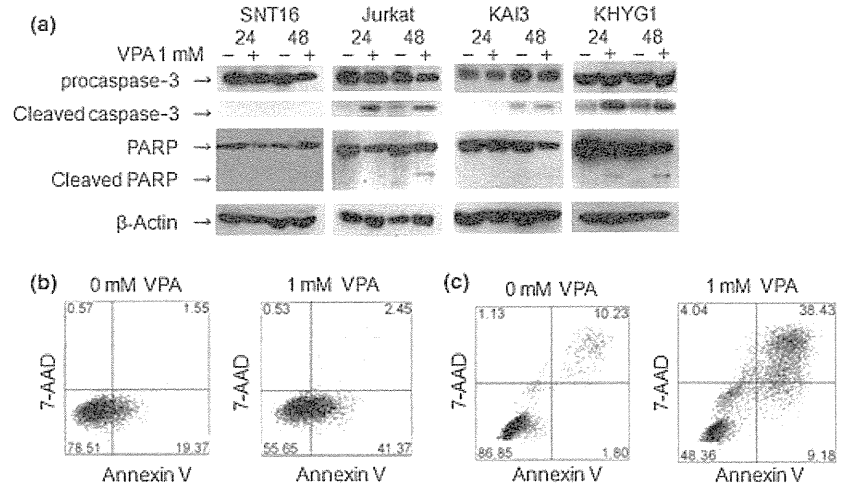
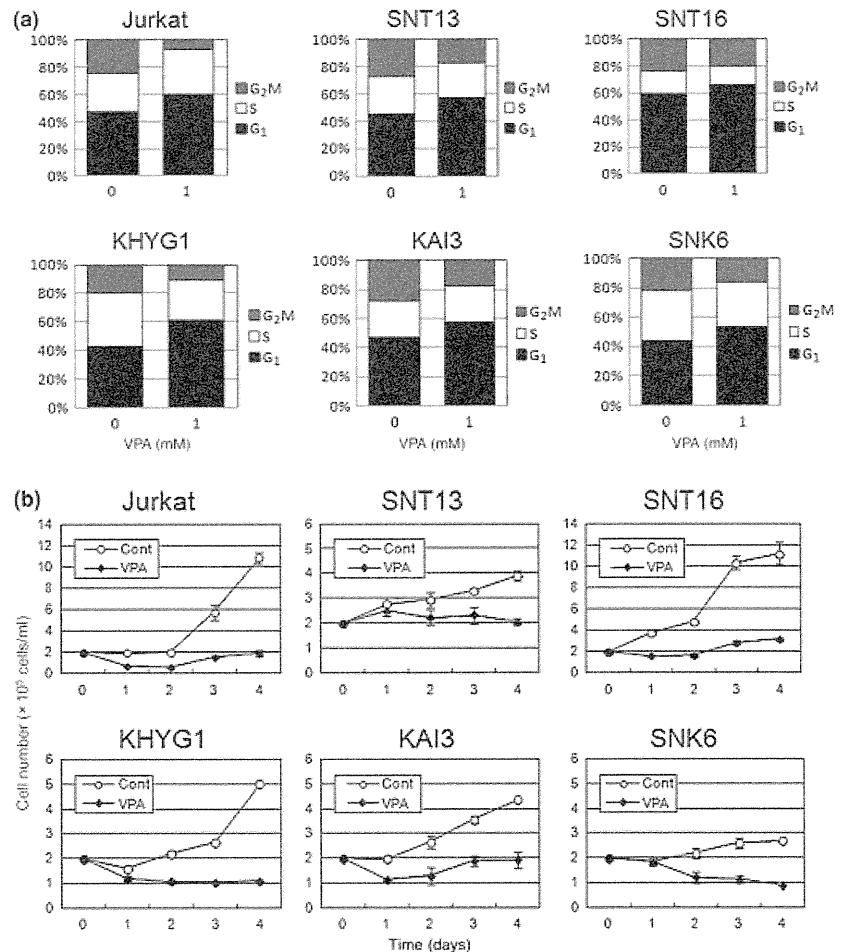


Fig. 3. Effects of valproate (VPA) on the cell cycle. (a) T cell lines (SNT13, SNT16, and Jurkat), and natural killer (NK) cell lines (KAI3, SNK6, and KHYG1) were treated with 1 mM VPA or distilled water for 48 h, fixed, and stained with propidium iodide. Cell cycle profiles were assessed by flow cytometry. (b) Cells were treated with 1 mM VPA or distilled water (control) and viable cells were counted using the Trypan blue exclusion test. Experiments were performed in duplicate. Data are the mean \pm SEM.



to VPA and VPA arrested the cell cycle in all T and NK cell lines tested (Fig. 3a). To confirm that VPA arrested the cell cycle, proliferation was compared in the presence and absence of VPA. Proliferation was inhibited in all VPA-treated cells compared with control cells (Fig. 3b).

Effects of VPA on lytic infection of EBV-positive T and NK cell lines. The expression of the following eight viral genes were analyzed using real-time RT-PCR: lytic genes encoding BZLF1 and gp350/220; and latent genes encoding EBV nuclear antigen (EBNA) 1, EBNA2, latent membrane protein (LMP) 1, LMP2, EBER1, and *Bam*HI-A rightward transcripts (BARTs). BZLF1, but not gp350/220, was detected in the T cell lines. Conversely, neither BZLF1 nor gp350/220 were detected in the NK cell lines (Fig. 4). The expression of the two lytic genes and six latent genes did not differ significantly between VPA-treated cells and controls. Representative results for two latent genes (those encoding LMP1 and EBER1) are shown in Figure 4.

Effects of the combination of VPA and bortezomib on cell death. Because the antitumor effect of VPA alone was modest (1 mM VPA treatment for 96 h reduced cell viability by between 22% and 56%) (Fig. 1b–e), we evaluated the effects of the combination of VPA (1 mM) and the proteasome inhibitor bortezomib (0.01 μ M) in several cell lines. In Jurkat and KAI3 cells, the combination of VPA plus bortezomib enhanced cell death (Fig. 5); however, in SNT16 and KHYG1 cells, the effects of this combination were difficult to assess because 0.01 μ M bortezomib alone killed almost all the cells (Fig. 5).

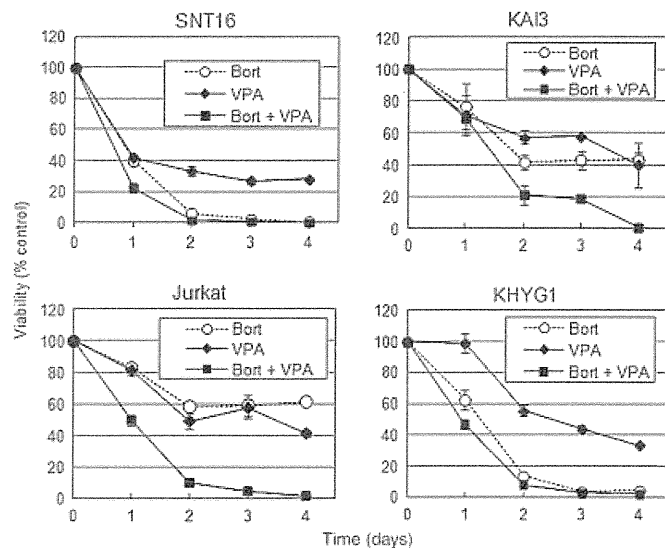


Fig. 5. Combined effects of valproate (VPA) and bortezomib. T cell lines (SNT16 and Jurkat) and natural killer (NK) cell lines (KAI3 and KHYG1) were treated with 1 mM VPA and/or 0.01 μ M bortezomib for 96 h and cell viability was assessed. VPA and bortezomib had additive effects in reducing the viability of T and NK cell lines. Data are the mean \pm SEM.

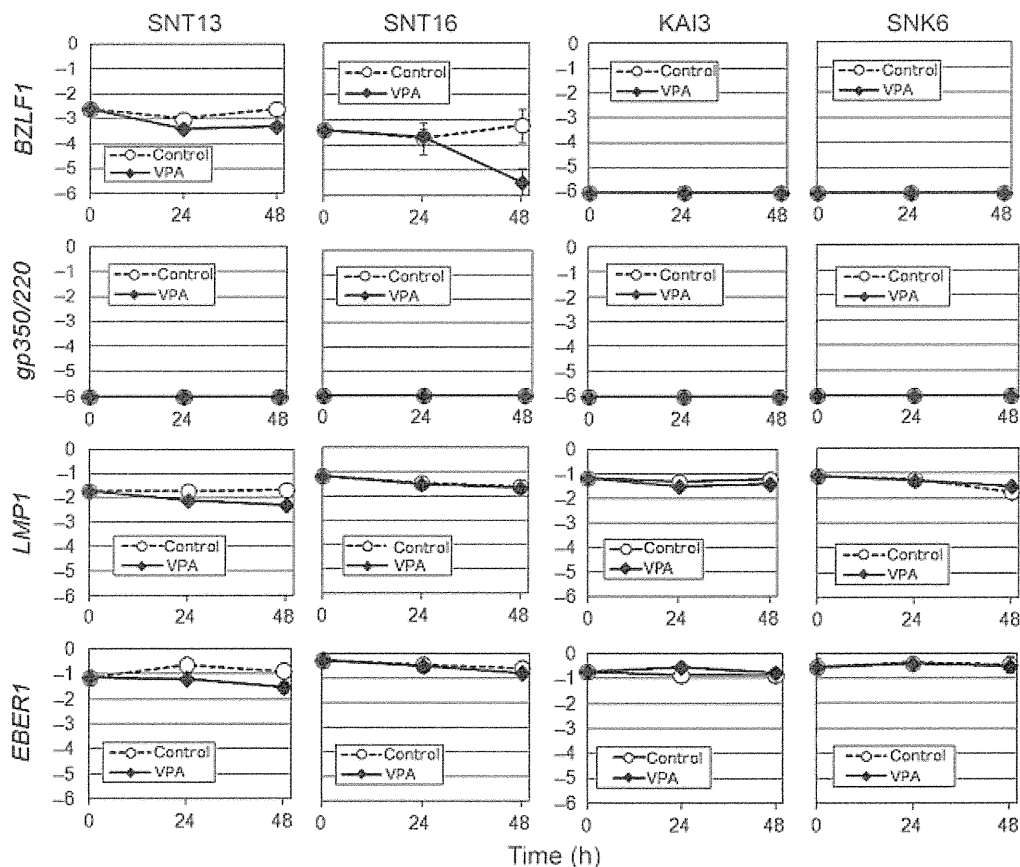


Fig. 4. Effects of valproate (VPA) treatment on the expression of Epstein–Barr virus (EBV)-encoded genes. The EBV-positive T cell lines (SNT13 and SNT16) and EBV-positive natural killer (NK) cell lines (KAI3 and SNK6) were treated with 1 mM VPA and harvested at 0, 24, and 48 h to evaluate gene expression using real-time RT-PCR. *BZLF1* is an immediate early gene and *gp350/220* is a late gene. *LMP1* and *EBER1* are latent genes. β 2-Microglobulin was used as an internal control and reference gene for relative quantification and assigned an arbitrary value of 1 (10^0). Data are the mean \pm SEM.

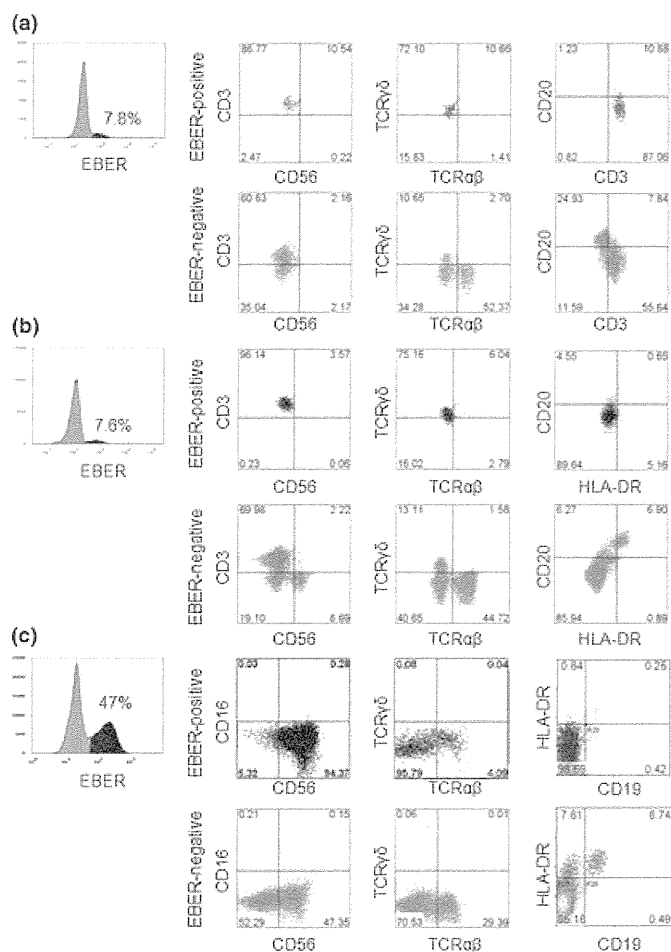


Fig. 6. Identification of Epstein-Barr virus (EBV)-infected cell fractions in patients with EBV-associated T/natural killer (NK) lymphoma. (a, b) Patients T-1 (a) and T-2 (b), who had hydroa vacciniforme-like lymphoma. (c) Patient NK-1, who had chronic active EBV disease, NK cell-type. Mononuclear cells were analyzed in a FISH assay. The EBV-encoded small RNA (EBER)-positive (black) and -negative (gray) lymphocytes were gated and plotted in quadrants.

Effects of VPA on the viability of EBV-infected cells from patients with EBV-associated lymphoma. The *ex vivo* effect of VPA on lymphoma cells from patients with EBV-associated T/NK lymphoma or lymphoproliferative diseases was evaluated. To identify the fractions that contained EBV-infected cells, MNC were stained with surface marker antibodies and then subjected to *in situ* hybridization with EBER in a FISH assay. In patients T-1 and T-2, who had hydroa vacciniforme-like lymphoma, the FISH assay showed that 7.8% and 7.6% of MNC were EBER positive, respectively. Most of the EBER-positive MNC in these patients were CD3+ and TCR $\gamma\delta$ + T cells (Fig. 6a,b). Conversely, in patient NK-1, who had chronic active EBV disease, 47% of MNC were EBER positive. Most of the EBER-positive MNC in this patient were CD56+ NK cells (Fig. 6c). Magnetic sorting was then used to separate $\gamma\delta$ T cells from other MNC in patients T-1 and T-2, and NK cells from the other MNC in patient NK-1. Bortezomib (0.5 μ M) and/or VPA (1 mM) was administered to each fraction and viable cells were counted over a period of 3–4 days. Individually, bortezomib and VPA had greater killing effects on the fractions containing EBV-infected cells compared with the other MNC, whereas the combination of bortezomib plus VPA produced the strongest killing effect (Fig. 7a–c). In the $\gamma\delta$ T and NK cell fractions,

the absolute number of control viable cells was stable or increased slightly, but was reduced by treatment (data not shown). The viability of cells obtained from blood samples from three healthy donors after combined treatment with bortezomib plus VPA for 4 days ranged between 75% and 100%, indicating that bortezomib and VPA do not affect non-tumor cells (Fig. 7d).

Discussion

Several studies have reported that HDAC inhibitors have anticancer activities and some have even been tested in clinical trials.^(32–34) Valproate is used to treat epilepsy, is easily accessible, and has a well-established safety profile. Therefore, evaluation of an anticancer effect of VPA may be very useful in the treatment of malignant diseases. In the present study, VPA reduced the viability of T and NK lymphoma/leukemia cell lines independently of the presence of EBV. However, the killing effect of VPA was smaller than that of bortezomib, despite the fact that the concentration of VPA tested (1 mM) was higher than that used in the treatment of epilepsy (0.3–0.6 mM).

The HDAC inhibitors affect tumor cell growth and survival via multiple biological effects. For example, they induce tumor cell death with all the biochemical and morphological characteristics of apoptosis. The HDAC inhibitors induce cell cycle arrest at the G₁/S boundary via upregulation of *CDKN1A*, which encodes p21^{WAF1/CIP1}, and/or downregulation of cyclins. They can suppress angiogenesis by reducing the expression of proangiogenic factors and also have immunomodulatory effects, enhancing tumor cell antigenicity and altering the expression of key cytokines, including tumor necrosis factor- α , interleukin-1, and interferon- γ .⁽⁹⁾ In the present study, we analyzed the mechanism by which VPA reduces the viability of T and NK cell lines. In some cell lines, VPA induced apoptosis, whereas in most there was evidence of cell cycle arrest. Thus, VPA probably activates other pathways to kill tumor cells than apoptosis and cell cycle arrest.

The proteasome inhibitor bortezomib has strong killing effects on T and NK lymphoma/leukemia cell lines (independent of the presence of EBV) and EBV-infected tumor cells from patients with EBV-associated T/NK lymphoproliferative diseases.⁽¹⁴⁾ Bortezomib is used in the treatment of myeloma and has also been assessed for efficacy against a variety of other malignancies. Recently, bortezomib and an HDAC inhibitor were reported to have synergistic effects in human and mouse models.^(35,36) Therefore, in the present study we evaluated the effects of the combination of bortezomib and VPA. Bortezomib and VPA were found to have additive killing effects on T and NK cell lines and EBV-infected MNC from patients. In the two cell lines tested, the effect of bortezomib was too strong to evaluate the killing effect of the combination treatment, despite the low bortezomib concentration used. Conversely, in Jurkat and KAI3, in which a low concentration of bortezomib killed approximately half the cells, the combination treatment killed nearly all cells within 4 days. Furthermore, the combination treatment had a stronger killing effect in EBV-infected MNC from patients than in uninfected cells. These results suggest the potential usefulness of the combination of VPA and bortezomib in the treatment of EBV-associated T/NK lymphoproliferative diseases.

Valproate has been reported to induce lytic infection by EBV,^(12,13) human cytomegalovirus,⁽³⁷⁾ and Kaposi sarcoma-associated herpes virus.⁽³⁸⁾ Induction of the lytic cycle is an advantage for the treatment of EBV-associated malignant diseases because of the lysis of EBV-infected tumor cells, the possible availability of antiviral therapy, and the recognition of expressed viral lytic proteins by the host immune system. Furthermore, the combination of VPA and an antiviral drug may

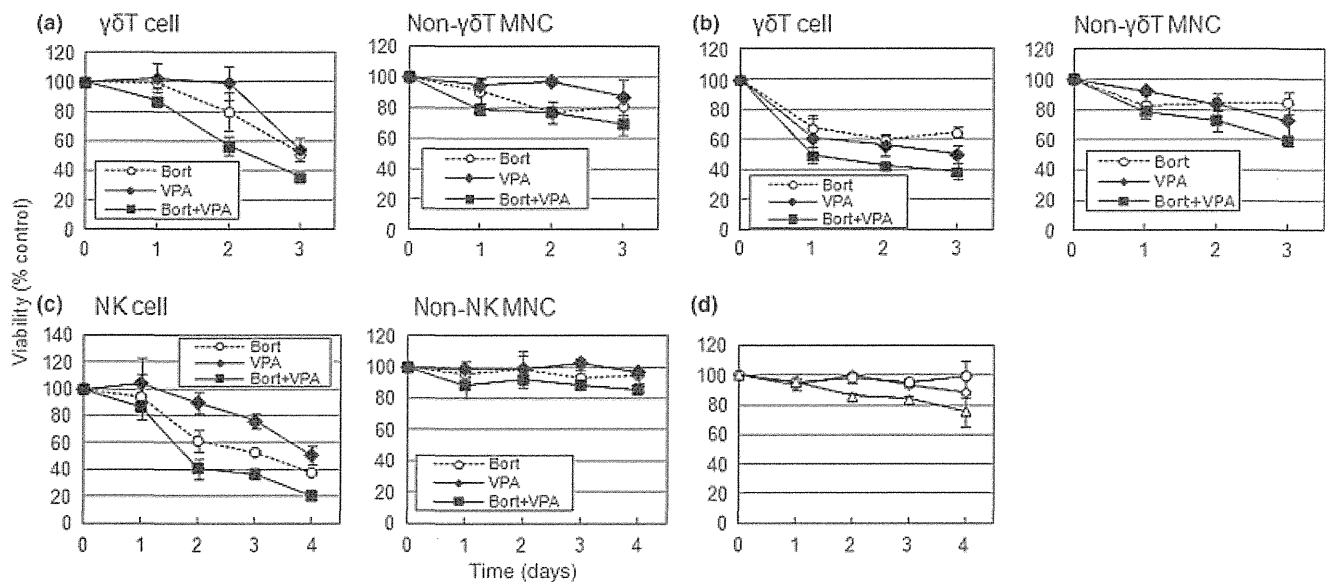


Fig. 7. Effects of the combination of valproate (VPA) and bortezomib on Epstein-Barr virus (EBV)-infected lymphoma cells. Cell populations were separated by magnetic sorting. Each fraction was exposed to VPA (1 mM) and/or bortezomib (0.5 μ M) and viable cells were counted over 3 or 4 days. (a,b) Viability of $\gamma\delta$ T cells and other mononuclear cells (MNC) from patients T-1 (a) and T-2 (b) with hydroa vacciniforme-like lymphoma. (c) Viability of NK cells and other MNC from patient NK-1 with chronic active EBV disease, NK cell-type. (d) Viability of MNC from three healthy donors treated with 1 mM VPA and 0.5 μ M bortezomib for 4 days. Data are the mean \pm SEM.

increase cell killing because some antiviral drugs inhibit virus DNA polymerase and are more effective in the lytic state than in the latent state.⁽³⁹⁾ To our knowledge, this is the first report of the effects of VPA on T and NK cell lines. In previous studies showing that VPA induces the EBV lytic cycle, a gastric carcinoma cell line and B cell lines were used.^(12,13) In the present study, VPA did not induce the EBV lytic cycle in any of the T or NK cell lines tested. In the two EBV-positive T cell lines tested, expression of only the immediate early gene *BZLF1* was detected (expression of the late gene *gp350/220* was not detected). In the NK cell lines, the expression of neither gene was detected. These results are consistent with our previous report.⁽²³⁾ In addition, bortezomib only induced the EBV lytic cycle in EBV-positive T cell lines.⁽²³⁾ Therefore, it seems that lytic infection can be induced in EBV-positive T cell lines. Nevertheless, VPA treatment did not induce lytic infection in EBV-positive T cell lines in the present study.

In summary, the results of the present study suggest that VPA has potential antitumor activity, regardless of whether EBV is present, although its efficacy may not be sufficient. The combination of VPA plus bortezomib may be a useful treatment because of the potential synergistic effects. Our results indicate that VPA has killing effects on T and NK lymphoma cells. Other HDAC inhibitors, such as suberoylanilide hydroxamic acid and

depsipeptide, have potent activity against T cell lymphoma⁽⁴⁰⁾ and may produce beneficial effects in EBV-associated malignancies by inducing the lytic cycle or suppressing the expression of EBV-related genes.⁽¹³⁾

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Disclosure Statement

The authors have no conflict of interest.

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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 he-

matopoietic 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 allergy⁹ and hydroa vacciniforme¹⁰) 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 mononucleosis³).

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 \geq 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)* (2) Increased amounts of EBV detected by Southern blot hybridization or EBER ⁺ cells in affected tissues or peripheral blood; $\geq 10^{2.5}$ copies/ μ g of EBV DNA in PBMCs	(1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition (2) Congenital immunodeficiency including X-linked lymphoproliferative disorders	T/NK cell Polyclonal, oligoclonal, monoclonal	12 16,29
HLH	(1) Clinical criteria (fever and splenomegaly) (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)	(1) Hemophagocytic syndrome in accelerated phase of CAEBV of T/NK cell type (2) Congenital immunodeficiency including familial HLH	T/NK cell Polyclonal, oligoclonal, monoclonal	11
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

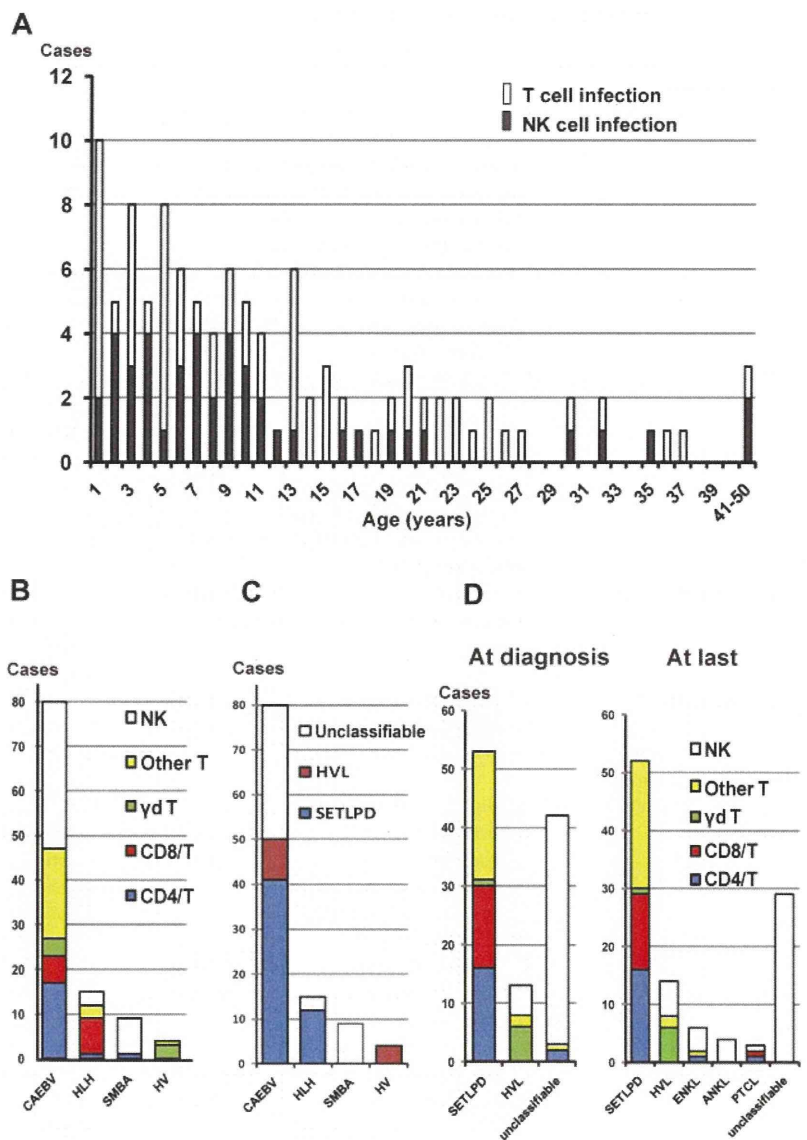
*"Severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme-like eruptions" were used to designate symptoms.

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⁺ hydroa 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, $\gamma\delta$ 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 PCR³⁴⁻³⁵ 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 dis-

eases 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.

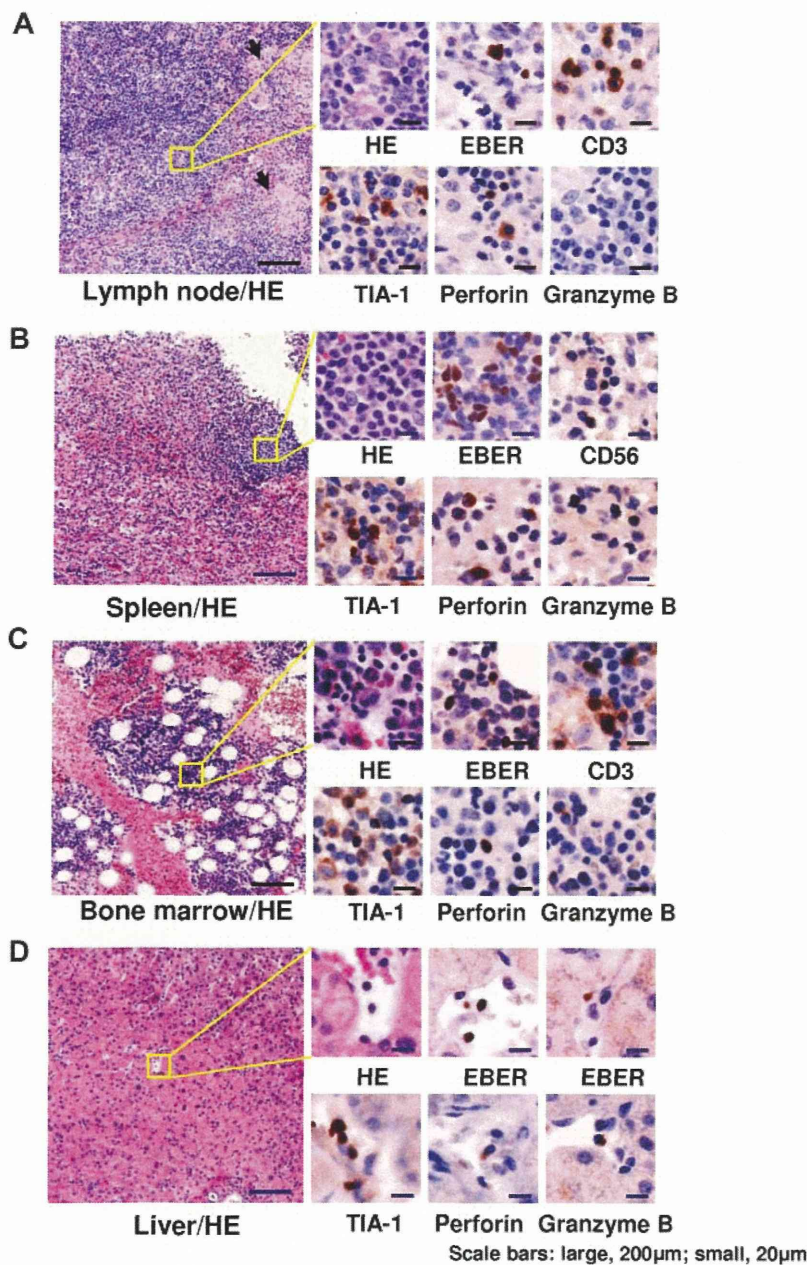


Figure 2. Histopathological findings of representative patients. (A) Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (patient 3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions 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/ μ g of DNA).^{12,32} CR patients had no symptoms and continuously low or no EBV loads in PBMCs (EBV-DNA $< 10^{2.5}$ copies/ μ g 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⁺, TCRαβ⁺, and TCRγδ⁺ 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 EBV-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 EBV⁺ 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 EBV 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.

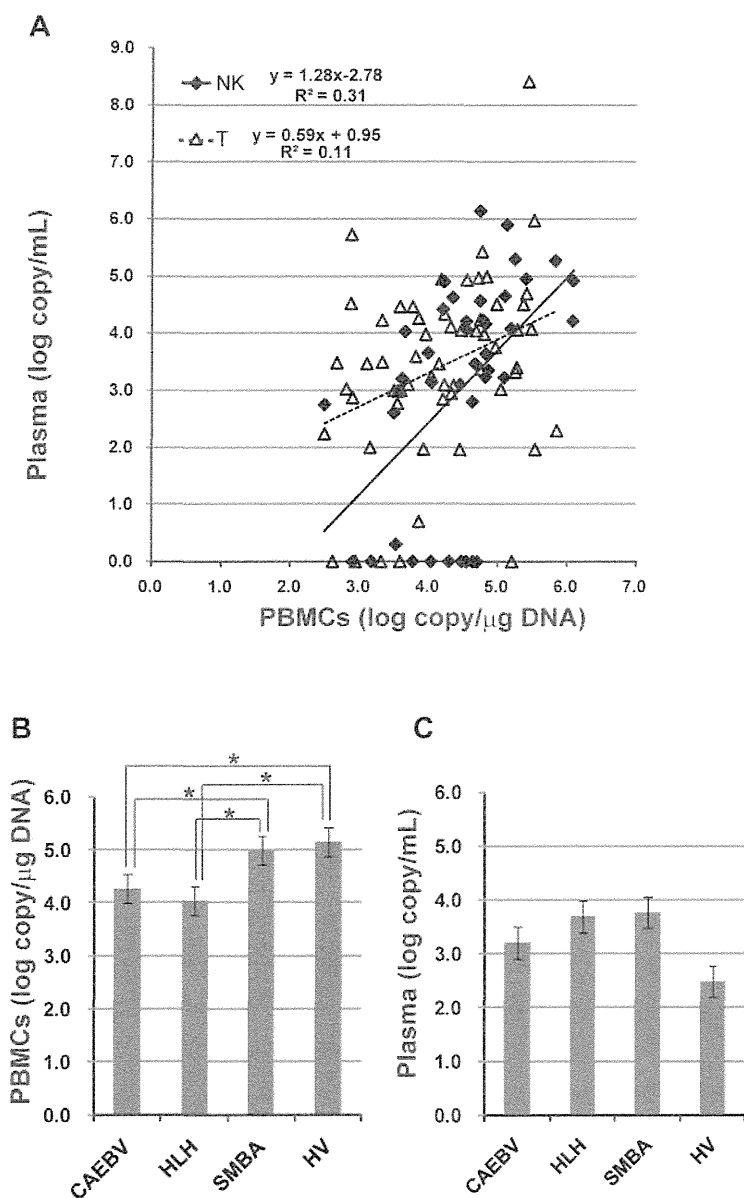


Figure 3. Viral load in the peripheral blood at the time of diagnosis. EBV-DNA was quantified by real-time PCR. (A) Correlation of viral load between PBMCs and plasma. The correlation was separately estimated in patients with T-cell infection and those with NK-cell infection. (B) Quantity of EBV-DNA 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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

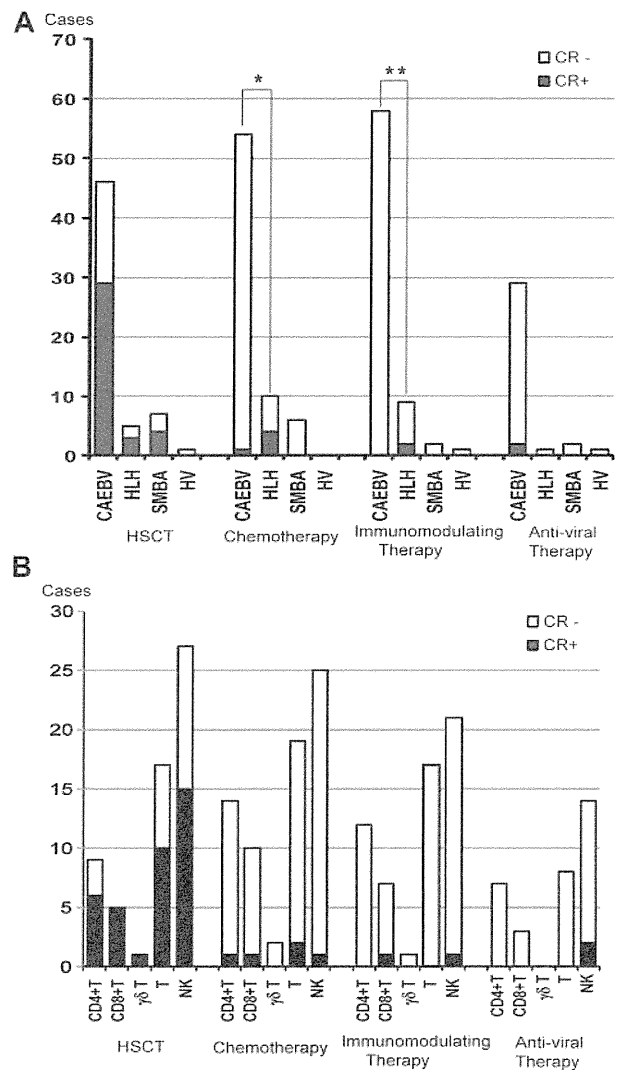


Figure 4. Efficacy of therapeutic interventions. (A) Number of patients treated with each therapy and patients who maintained CR are shown among categories of clinical groups. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme. **P* = .002; ***P* = .02. (B) Numbers of patients who 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 ($\geq 10^{4.5}$ copies/ μ g DNA)	1.16 (0.79-1.71)	NS		
EBV DNA in plasma ($\geq 10^{3.5}$ 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.

Overall survival rate

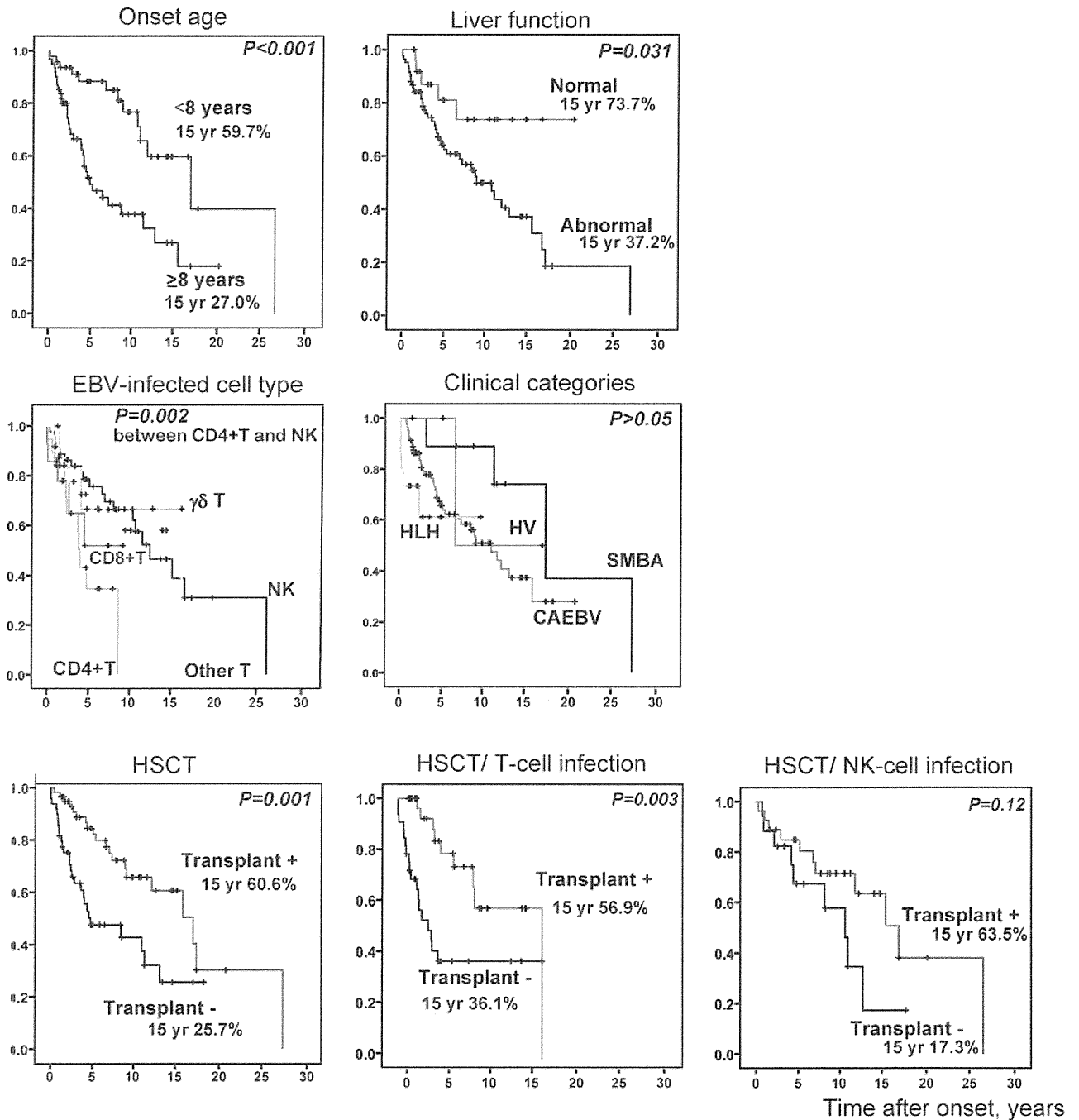


Figure 5. Probability of survival rates from time of disease onset. Overall survival rates from onset (n = 108) were calculated from Kaplan-Meier estimates between each subgroup (onset age ≥ 8 years or < 8 years, with or without liver dysfunction, EBV-infected cell types, clinical categories, and with 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

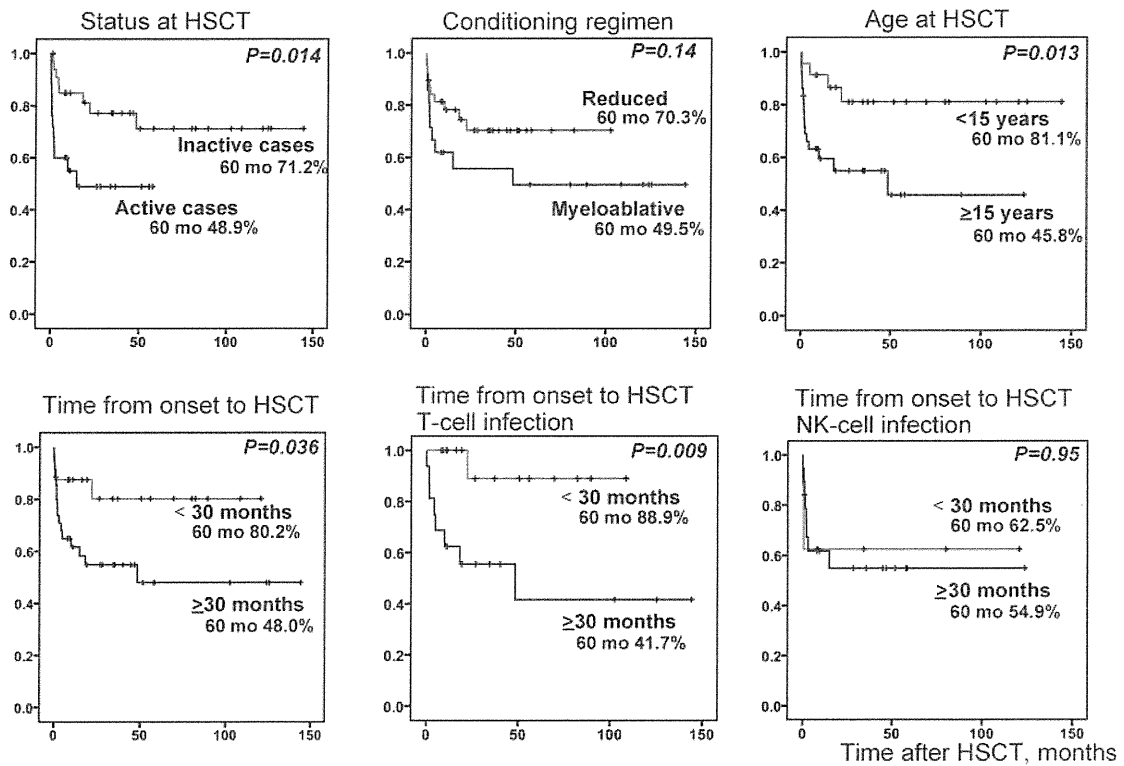
significantly higher in patients with inactive disease at the time of HSCT ($P = .014$); however, its significance diminished for the event-free survival rate. Patients who received HSCT at an age less than 15 years had significantly higher overall ($P = .013$) and event-free survival rates ($P = .015$). 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, $\gamma\delta$ 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 $\gamma\delta$ T cells. We also demonstrated that EBV-infected cells in nearly half of hydroa vacciniforme or hydroa vacciniforme-like lymphoma patients were $\gamma\delta$ 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}

A Overall survival rate after HSCT



B Event free survival rate after HSCT

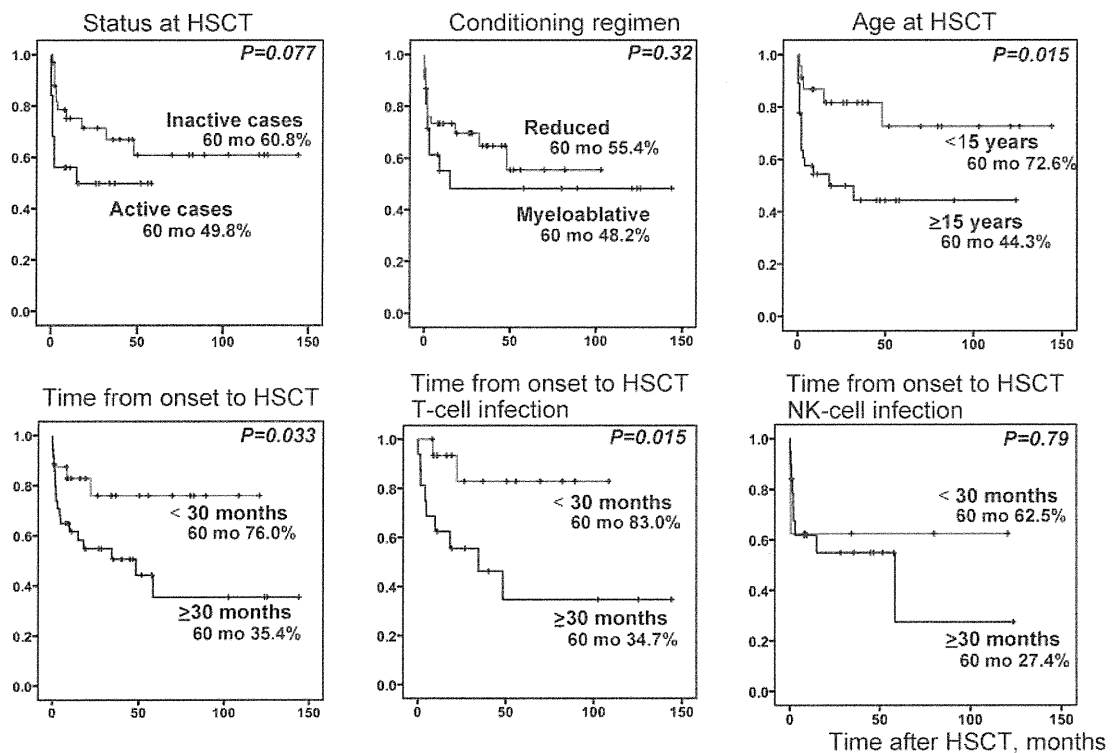


Figure 6. Probability of survival rates after HSCT. Survival rates after HSCT were calculated from Kaplan-Meier estimates from each subgroup (inactive or active cases at HSCT, reduced or myeloablative conditioning, age ≥ 15 years or < 15 years at HSCT, and time from onset to HSCT ≥ 30 months or < 30 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