proliferation of EBV-infected B cells.[12] In this review, focused on CAEBV as an EBV-associated T/NK-cell LPD (EBV+ T/NK-LPD), we summarize the current understanding of the disease and describe the authors' own recent work subsidized by grants from the Ministry of Health Labour and Welfare of Japan.

## Clinical characteristics of CAEBV and other EBV-associated T/NK-LPD

As described in the previous section, IM-like symptoms are the main symptoms of CAEBV.[4–7] Other clinical manifestations include thrombocytopenia, anemia, pancytopenia, diarrhea, and uveitis. Peripheral blood EBV-DNA load regularly exceeds $10^{2.5}$ copies/μg DNA.[13] High-level production of various cytokines, including interleukin (IL)-1β, IL-10, and interferon (IFN)-γ has been detected in CAEBV patients and is thought to play an important role in inflammatory symptoms of the disease.[14–16] CAEBV can be classified into the T-cell and NK-cell types, depending on which lymphocyte subset is mainly infected with EBV. A survey of Japanese CAEBV patients found that the T-cell type is associated with less favorable prognosis than the NK-cell type.[17,18] CAEBV was included in the 2008 World Health Organization (WHO) classification of lymphomas as the systemic EBV+ T-cell LPD of childhood.[19]

Although the clinical course of CAEBV is chronic, patients often develop fatal complications such as multi-organ failure, disseminated intravascular coagulopathy (DIC), digestive tract ulcer/perforation, coronary artery aneurysms, and malignant lymphomas, as well as EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH).[7] HLH is a hyper-inflammatory condition caused by overproduction of cytokines by excessively activated T cells and macrophages. Clinical characteristics of HLH include fever, hepatosplenomegaly, pancytopenia, hypertriglyceridemia, DIC, and liver dysfunction.[20] EBV-HLH usually occurs following primary EBV infection and is itself characterized by clonal proliferation of EBV-infected T or NK cells (most often CD8+ T cells).[21,22] EBV-HLH can also occur in association with X-linked lymphoproliferative disease (XLP) and XIAP deficiency.[23]

Patients with CAEBV may have characteristic cutaneous complications, namely hypersensitivity to mosquito bites (HMB) and hydroa vacciniforme (HV), that are themselves distinct EBV+ T/NK-LPD characterized by clonal proliferation of EBV-infected T or NK cells. Both HMB and HV can occur independently or in association with CAEBV. HV is a childhood photosensitivity disorder, characterized by necrotic vesiculopapules on sun-exposed areas.[24] EBV-DNA level is elevated in patients' peripheral blood, and histochemical analysis of skin lesions indicates infiltration of T cells expressing EBV-encoded small RNA (EBER).[25] Although most cases of HV resolve by early adulthood, HV overlapping with CAEBV may eventually develop into EBV-positive malignant lymphoma, which was included in the 2008 WHO classification of lymphoma as the hydroa vacciniforme-like lymphoma.[19,26] HMB is characterized by severe local skin reactions to mosquito bites including erythematous swelling with bullae, necrotic ulcerations, and depressed scars.[27] These local reactions may be accompanied by general symptoms such as high fever, lymphadenopathy, and liver dysfunction. Most HMB patients have EBV infection in NK cells in skin lesions and peripheral blood.[28,29] HMB patients without systemic symptoms may eventually develop CAEBV.[28]

### *Prospective clinicopathologic analysis of CAEBV and other EBV+ T/NK-LPD*

Chronic active EBV infection, EBV-HLH, HMB, and HV are thus distinct but overlapping entities categorized as EBV+ T/NK-LPD. The higher incidence of these diseases in East Asian countries and their occasional coincidence in a single patient imply a common pathogenesis.[7,30] Kimura *et al.* performed a large-scale prospective study of Japanese EBV+ T/NK-LPD.[31] A total of 108 cases of EBV+ T/NK-LPD (80 cases of CAEBV, 15 cases of EBV-HLH, nine cases of HMB, and four cases of HV) were analyzed. They found that the clinical profile of EBV+ T/NK-LPD is closely linked with the lineage of EBV-infected cells. More than half (53%) of EBV-HLH patients had EBV in the CD8+ T-cell subset, in contrast to the low incidence of EBV infection in this subset in the other EBV+ T/NK-LPD. Most HMB patients (89%) had EBV-infected NK cells, whereas the majority (75%) of HV patients had EBV-infected γδT cells. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications and 13 (12%) developed overt lymphoma or leukemia. Age of onset ≥8 years and liver dysfunction were risk factors for mortality, and transplant patients had better prognosis. Patients with CD4+ T-cell infection had shorter survival as compared with those with NK-cell infection. Because shorter time from onset to hematopoietic stem cell transplantation (HSCT) and inactive disease at HSCT were associated with longer survival, earlier HSCT in good condition was considered preferable. Among the 108 patients enrolled, four patients developed aggressive NK-cell leukemia (ANKL) and six patients developed extranodal NK/T-cell lymphoma (ENKL). It is thus conceivable that a certain fraction of patients with ANKL and ENKL developed these malignancies as a consequence of CAEBV.[32,33]

### *Characteristics of adult CAEBV*

Chronic active EBV infection has been described mainly as a disease of childhood and young adulthood; the mean age of onset was estimated to be 11.3 years.[18] Recently, however, an increasing number of adult patients fulfilling the criteria of CAEBV has been reported. This may be a true increase in the incidence of adult-onset CAEBV or reflect improved recognition of this disease by physicians. Arai *et al.* reviewed 23 cases of adult-onset CAEBV and described the characteristics.[34] In 87% of adult cases, T cells were infected with EBV, whereas in childhood-onset cases, the T- and NK-cell types were equally frequent. Adult-onset cases appeared rapidly progressive and more aggressive, although the number of patients analyzed was limited. Further investigation with a larger number of patients is required to elucidate the characteristics of adulthood CAEBV and its relation to the childhood counterpart.

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### *Recurrence of CAEBV with EBV-infected, donor-derived T cells following HSCT*

The relative prevalence of CAEBV in East Asia and in natives of Central and South America implies a genetic background for its pathogenesis. Recently HLA-A*26, a major histocompatibility complex class I allele relatively common in East Asia, was found to be associated with an increased risk for EBV$^+$ T/NK-LPD.[35] Although the possible involvement of EBV strains with increased propensity to induce T/NK-cell lymphoproliferation cannot be formally denied, it is highly unlikely because outbreaks and familial transmission of CAEBV have not been reported. Arai *et al.* reported an intriguing case of CAEBV in which the patient experienced relapse after bone marrow transplantation.[36] A 35-year-old female patient with CAEBV of the CD8 type had HSCT from an unrelated male donor following myeloablative preconditioning with total body irradiation. The serologic HLA types of the patient and the donor were identical, whereas the DNA types were different in two HLA-DR alleles. Although the peripheral blood EBV-DNA was undetectable at 1 month after HSCT and remained so for nearly 12 months, the patient's EBV-DNA load increased again and reached $1.0 \times 10^5$ copies/μg DNA. EBV was found primarily in CD8$^+$ T cells again, but the EBV-infected cells now had an XY karyotype, clearly indicating their donor origin. Sequencing analysis of the variable region of the EBV-encoded *LMP1* gene showed that the virus strain infecting the CD8$^+$ T cells was different before and after bone marrow transplantation, suggesting that the repeated episodes of CAEBV were not caused by a rare EBV strain with an unusual biological activity. If we do not suppose that these two consecutive episodes of CAEBV in a single patient occurred only by chance, these findings suggest that the patient may have had a certain genetic background that exerts its direct effects on cellular lineages unrelated to hematopoietic stem cells.

## Pathophysiology of CAEBV

The pathogenesis of CAEBV is not understood. Most T and NK cells do not express the EBV receptor CD21, and the mechanism of their infection with EBV is not clear. Transfer of CD21 from B cells to NK cells through immunological synapse may render the latter cells accessible to EBV.[37] The mechanism by which EBV induces proliferation of T and NK cells is not known either. EBV-induced expression of CD40 and its engagement by CD40L may have a role in the survival of EBV-infected T and NK cells of CAEBV patients.[38] Given that EBV-positive T or NK cells have been occasionally found in the tonsil and peripheral blood of IM patients, ectopic EBV infection in T or NK cells does not necessarily lead to the development of CAEBV.[39–41] Although EBV-infected T and NK cells in CAEBV patients and cell lines derived from them do not express the most immunodominant EBNA3 and EBNA2, they express EBNA1, latent membrane protein 1 (LMP1) and LMP2 (the latency II type EBV gene expression) that are frequently recognized by EBV-specific CTL.[3,42–45] Hosts with normal immune functions are thus expected to have the capacity to recognize EBV-infected T and NK cells. It is thus conceivable that patients with CAEBV have a certain defect in immunologic functions that causes inefficient recognition and/or killing of EBV-infected latency II cells. Indeed, deficiency in cellular immune responses to EBV has been detected in patients with CAEBV.[46–48]The defect in T-cell responses to LMP2A might be particularly relevant to this issue.[47] Interestingly, a patient with clinical manifestations similar to CAEBV, although the virus was found in his B cells, was found to have mutations in the gene encoding perforin, which has a critical role in granule-mediated killing of target cells.[49] None of the other patients with CAEBV, however, were found to have a mutation in the *perforin* gene. Mutations of the genes responsible for XLP, XIAP deficiency, and familial HLH (except for the type 2 that is caused by mutations of *perforin*) have not been reported for patients with CAEBV.[7]

Clonal proliferation of EBV-infected T or NK cells in CAEBV and other EBV$^+$ T/NK-LPD implies that these diseases have a malignant nature. CAEBV, however, is a chronic disease and patients with clonal expansion of EBV-infected T or NK cells may remain in a stable condition for years without treatment.[18] Overt malignant lymphoma occurs usually after a long course of disease. Therefore CAEBV may represent, at least in its early phase, a premalignant or smoldering phase of EBV-positive leukemia/lymphomas. Ohshima *et al.* proposed a pathological categorization of CAEBV into a continuous spectrum ranging from a smoldering phase to overt leukemia/lymphoma.[50] Clonality of EBV-infected T or NK cells in CAEBV may not necessarily indicate a malignant phenotype; acquisition of clonality might be a result of other selective processes such as immune escape.

### *Mouse xenograft models for EBV$^+$ T/NK-LPD*

Animal models for EBV$^+$ T/NK-LPD have not been available, rendering research on their pathogenesis and therapy difficult. Imadome *et al.* transplanted peripheral blood mononuclear cells (PBMC) isolated from patients with CAEBV and EBV-HLH into immunodeficient mice of the NOD/Shi-*scid*/IL-2R$\gamma^{null}$ (NOG) strain, and successfully reproduced major features of these diseases including systemic monoclonal proliferation of EBV-infected T or NK cells and hypercytokinemia (Fig. 1).[51] Although many features were common to CAEBV and EBV-HLH model mice, hemorrhagic lesions in the abdominal and thoracic cavities and extreme hypercytokinemia were unique to the latter model, indicating that these mouse models reflect the differences in the pathophysiology of the original diseases. Importantly, these models revealed an essential role of CD4$^+$ T cells in the engraftment of EBV-infected T and NK cells. *In vivo* depletion of CD4$^+$ T cells following transplantation effectively prevented the engraftment of EBV-infected cells of not only the CD4$^+$ lineage but also the CD8$^+$ and CD56$^+$ lineages. Furthermore, OKT-4 antibody given after engraftment was also effective to reduce EBV-DNA load in the peripheral blood and major organs (Imadome *et al.*, unpubl. data 2012). These results suggest that therapeutic approaches targeting CD4$^+$ T cells may be possible.

## Diagnosis and monitoring of CAEBV

Prolonged or relapsing symptoms of IM are the major clue to the diagnosis of CAEBV. Although elevated serum antibody titers

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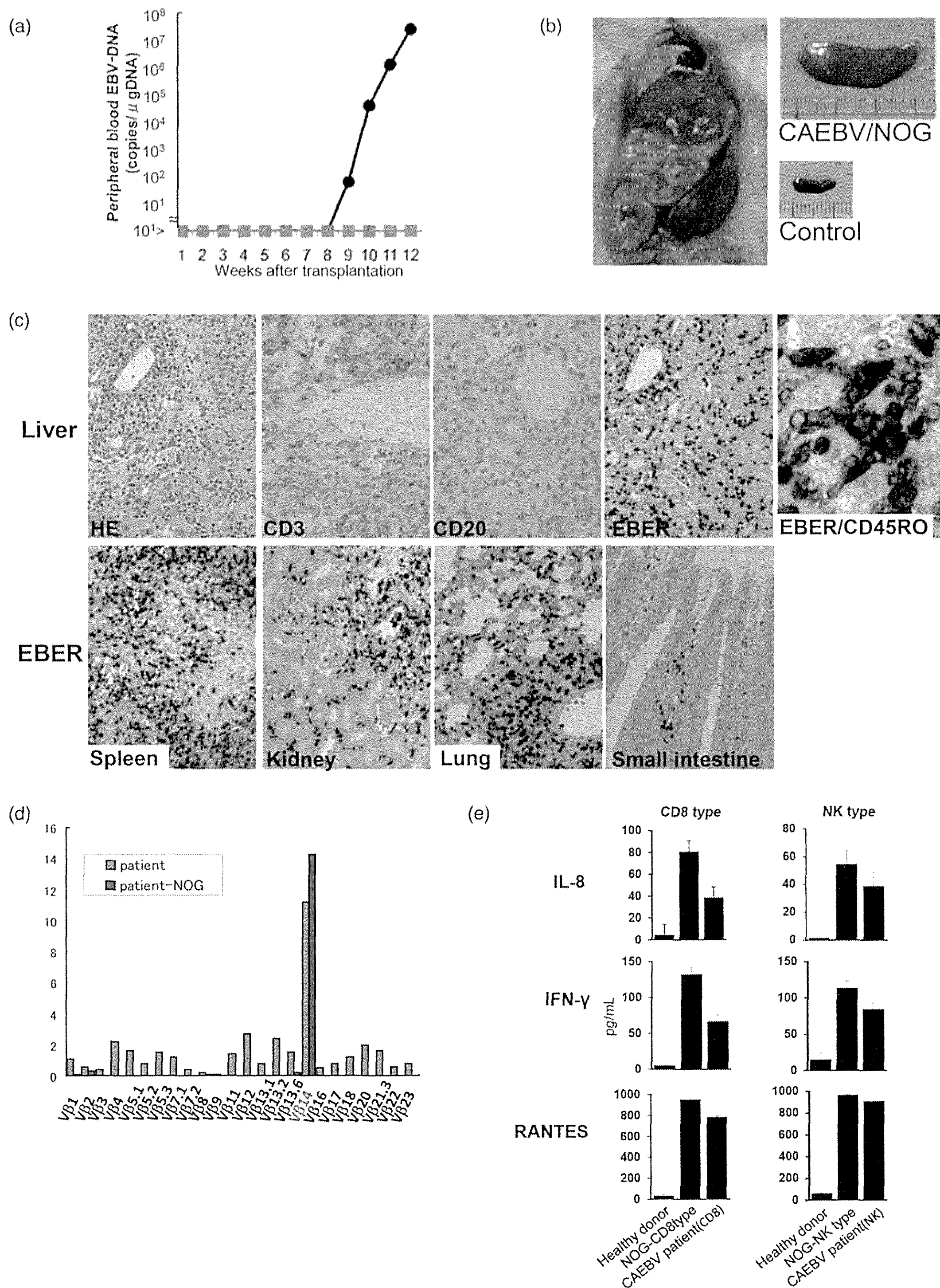


**Fig. 1** Mouse xenograft model of chronic active Epstein–Barr virus infection (CAEBV). Peripheral blood mononuclear cells (PBMC) of a patient with the CD8 type CAEBV were transplanted i.v. into NOD/Shi-*scid Il2rg* $^{null}$ (NOG) mice. (a) Measurement of peripheral blood EBV-DNA. EBV-DNA load increased rapidly from approximately 9 weeks after transplantation, when (●) whole PBMC but not (□) isolated $CD8^+$ cells were transplanted. (b) Splenomegaly of a model mouse. (c) Pathological analysis. Histochemical analysis showed massive infiltration of EBV-encoded small RNA $(EBER)^+/CD20^-/CD3^+/CD45RO^+$ cells in most major organs including the spleen, kidneys, lungs, and small intestine. (d) T-cell receptor (TCR) repertoire analysis of peripheral blood T cells isolated from the patient and a mouse that received the patient's PBMC. An identical clone of EBV-infected T cells expressing Vβ14 is proliferating in the patient and the corresponding mouse. (e) Human cytokine levels in CAEBV model mice. Serum levels of interleukin (IL)-8, interferon (IFN)-γ, and regulated on activation, normal T-cell expressed and secreted (RANTES) were measured in mice that were transplanted with PBMC isolated from either a CD8-type or an NK-type CAEBV patient. The same set of cytokines was also quantified in the sera of the original patients and healthy donors. Modified from *PLoS Pathog.* 2011; **7**(10): e1002326.[51]

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against EBV-encoded antigens are often found, this does not always occur, and normal titers of anti-EBV antibodies should not preclude the diagnosis of CAEBV.[7] Diagnostic criteria for CAEBV have been published.[13] Quantification of peripheral blood EBV-DNA is most important for diagnosis and a finding of elevation should be followed by identification of EBV-infected T or NK cells. Quantification of EBV-DNA is, however, influenced by many factors and the results can vary in different laboratories.[52] Recently, therefore, an international standard EBV-DNA sample for normalization became available from the National Institute for Biological Standards and Controls, USA. Given that CAEBV is a chronic disease that may progress to overt malignancy and early HSCT in a better clinical condition is recommended, precise monitoring of patient clinical parameters is particularly important.

### *Flow-cytometric* in situ *hybridization for identification of EBV-infected cells*

Diagnosis of CAEBV requires exact phenotyping of EBV-infected cells. This has usually been done with immunobead sorting of PBMC into lymphocyte subsets, followed by measurement of EBV-DNA in each subset using quantitative polymerase chain reaction. These processes are, however, time-consuming and require specific skills. Kimura *et al.* developed a new method termed "flow-cytometric *in situ* hybridization" (FISH) to phenotype EBV-infected cells (Fig. 2).[53,54] They utilized a fluorescence-labeled peptide nucleic acid (PNA) probe complementary to EBER and succeeded in detecting EBER on flow cytometry. Following reaction with antibodies specific to surface markers, PBMC were permeabilized and subjected to *in situ* hybridization with the PNA probe. EBER probes and surface-bound antibodies were then detected simultaneously on flow cytometry. EBV-infected cells with a certain phenotype can be directly counted using FISH, which is less laborious than the current method. They showed that FISH can be applied for the diagnosis of EBV$^+$ T/NK-LPD, and that EBV infects mainly γδT cells in HV.[53–55]

### *MicroRNA as a potential biomarker of CAEBV*

MicroRNA (miRNA) is a small non-coding RNA of 18–25 nucleotides that plays a critical role in the regulation of cellular proliferation, differentiation, and apoptosis through negatively regulating mRNA translation.[56] miRNAs are encoded not only by cells but also by viruses; EBV is actually the first virus shown to encode miRNAs.[57] Two clusters of EBV-encoded miRNAs have been identified: miR–*Bam*HI fragment H rightward open reading frame 1 (miR-BHRF1) and miR–*Bam* HI A region rightward transcripts (miR-BART).[58] Kawano *et al.* reported that plasma levels of miR-BART 1-5p, 2-5p, 5, and 22 are significantly higher in patients with CAEBV than in those with IM and healthy controls.[59] Plasma miR-BART 2-5p, 4, 7, 13, 15, and 22 levels were significantly elevated in CAEBV patients with active disease compared to those with inactive disease. miR-BART 13 level could differentiate patients with active disease from those with inactive disease, with a clear cut-off. Similarly, plasma miR-BART 2-5p and 15 levels could clearly differentiate patients with complete remission from others. Importantly, plasma EBV-DNA level did not show any significant correlation with these clinical parameters. These results suggest that EBV-encoded miRNA in plasma may be a useful biomarker for the diagnosis and monitoring of CAEBV.

## Therapy of CAEBV

Various therapies have been tried for the treatment of CAEBV, including antiviral, chemotherapeutic, and immunomodulatory drugs, with only limited success. These regimens induced sustained complete remission in only exceptional cases and HSCT is at present the only curative therapy for CAEBV.[60] The current event-free survival rate for CAEBV patients following HSCT is estimated to be $0.561 \pm 0.086$.[61] Very recently, Kawa *et al.* reported excellent results of HSCT following non-destructive pretreatment (reduced intensity hematopoietic stem cell transplantation; RIST).[62] For 18 pediatric patients with CAEBV who were treated with RIST, 3 year event-free survival was $85.0 \pm 8.0\%$ and the 3 year overall survival rate was $95.0 \pm 4.9\%$. HSCT is thus the therapy of choice for CAEBV, but HSCT is still accompanied by substantial risk and CAEBV patients have high risk for transplantation-related complications.[18] It is therefore desirable to develop novel therapies that do not depend on HSCT. Preclinical studies of two candidate drugs for CAEBV have been carried out recently and gave hopeful results.

Bortezomib, known as an inhibitor of 26S proteasome,[63] also has an inhibitory effect on the cellular transcription factor NF-κB. Because the survival and proliferation of EBV-transformed B cells are critically dependent on NF-κB activity, bortezomib has been shown to induce apoptosis in these cells.[64] Iwata *et al.* investigated the effect of bortezomib on EBV-infected T-cell lines including those derived from CAEBV.[65] Bortezomib induced apoptosis in all human T-cell lymphoma cell lines examined, whether or not they were infected with EBV. In addition, bortezomib induced the expression of EBV lytic-cycle genes *BZLF1* and *gp350/220*, as has been reported for EBV-infected B-cell lines.[66] Bortezomib also induced apoptosis specifically in EBV-infected T or NK cells cultured *ex vivo* from patients with EBV$^+$T/NK-LPD.

Valproic acid is a widely used anti-epileptic drug and is also known as a potent histone deacetylase (HDAC) inhibitor. HDAC inhibitors have potent anticancer activities with proven efficacy in various human malignancies. Valproic acid induces lytic infection in EBV-infected B-lymphoblastoid and gastric carcinoma cell lines and thereby potentiates the effects of chemotherapeutic agents both *in vitro* and *in vivo*.[67] Iwata *et al.* examined the effect of valproic acid on EBV-infected T and NK cell lines.[68] They found that this agent induces apoptosis in human EBV-infected T and NK cells. Use of the drug with the NF-κB inhibitor bortezomib had an additive effect. In contrast to the previous results with EBV-infected B-cell lines, valproic acid did not induce lytic infection in the virus-infected T- and NK-cell lines, indicating that the apoptosis-inducing effect of valproic acid is not dependent on induction of EBV lytic cycle.

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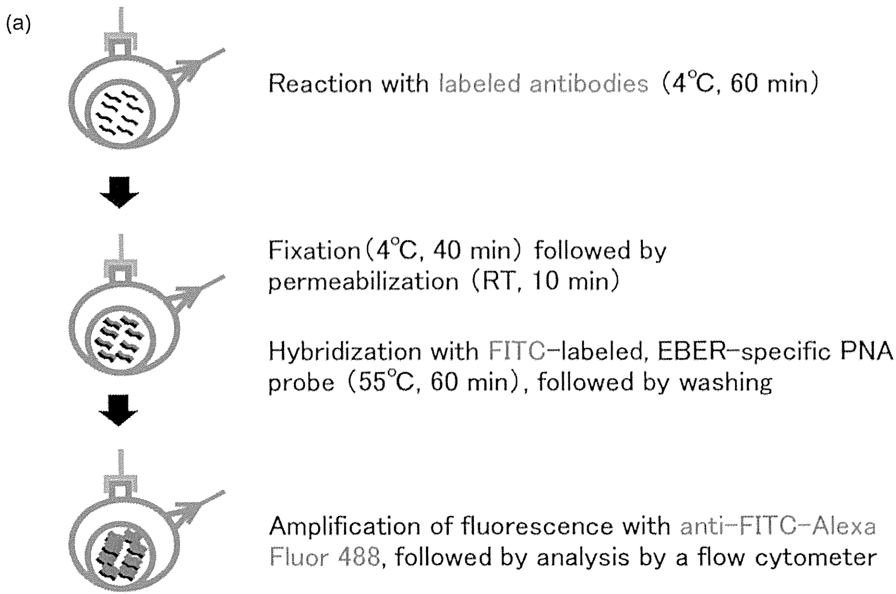
(a)

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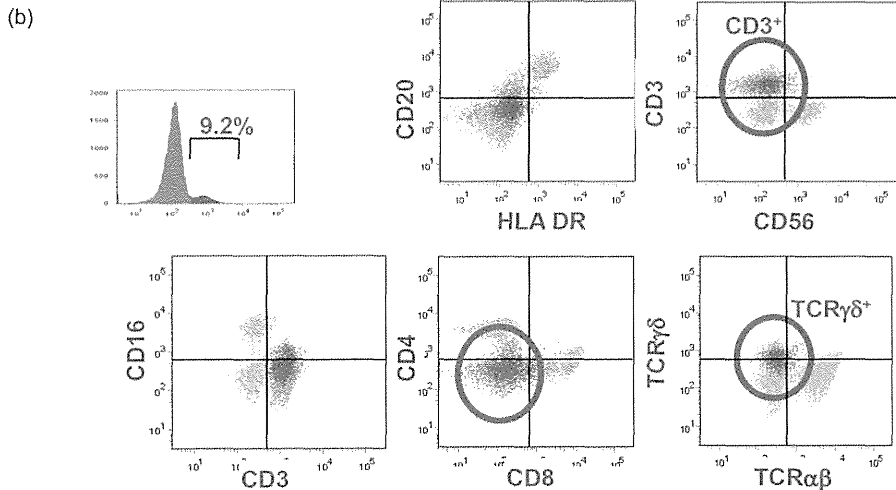


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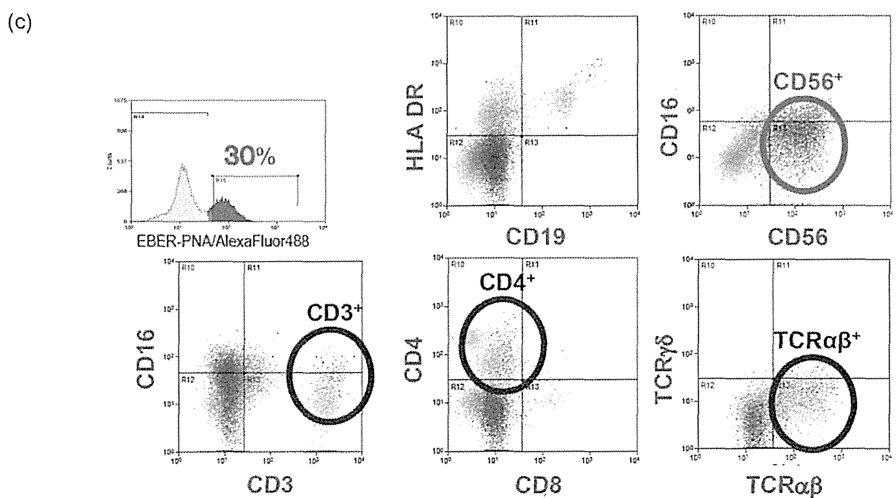


**Fig. 2** Flow-cytometric *in situ* hybridization (FISH). (a) Protocol of FISH. (b) Results of FISH in a patient with hydroa vacciniforme. Red, EBER-positive cells; blue, EBER-negative cells. Most EBV-infected cells in the peripheral blood of this patient had the phenotype $CD3^+/CD4^-/CD8^-/TCR\gamma\delta^+$. (c) Results of FISH in a patient with the NK-cell type chronic active Epstein–Barr virus infection. Red, EBER-positive cells; blue, EBER-negative cells. The majority of EBV-infected cells in the peripheral blood of this patient were $CD56^+$ NK cells. Also, a small proportion of $TCR\alpha\beta^+/CD3^+/CD4^+$ cells also contained EBV. EBER, Epstein–Barr virus-encoded small RNA; FITC, fluorescein isothiocyanate; PNA, peptide nucleic acid; RT, reverse transcription.

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## Perspective

Significant progress has been made in the research of many aspects of CAEBV, including pathophysiology, diagnosis, monitoring, and therapy, but the fundamental cause of the disease has not been elucidated. The recent development of novel technologies for genetic analysis, including new-generation sequencing, may enable identification of genetic alterations responsible for CAEBV. Given that CAEBV is an uncommon disease, it may sometimes take years for the correct diagnosis to be reached. The advanced techniques required for this also make the diagnosis of CAEBV difficult. Although there is a consensus that early HSCT produces a better result, the decision to have HSCT is often difficult, especially when the patient is in a stable condition without severe symptoms. Establishing a standard clinical guideline for the diagnosis and treatment of CAEBV will alleviate these problems and facilitate quick and accurate diagnosis, followed by timely intervention with the right choice of treatment.

## Acknowledgments

The authors' works described in this article have been funded by grants from the Ministry of Health Labour and Welfare of Japan for the Research on Measures for Intractable Diseases (H21-Nanchi-094, H22-Nanchi-080, H24-Nanchi-046).

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厚生労働科学研究委託費 エイズ対策実用化研究事業
「適正な抗HIV療法開発のための研究」班
平成26年度　委託業務成果報告書

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発行日　2015年3月31日

発行者　業務主任者　潟永　博之

発行所　研究班事務局
独立行政法人 国立国際医療研究センター エイズ治療・研究開発センター
〒162-8655　東京都新宿区戸山1-21-1

本報告書は、厚生労働省の厚生労働科学研究委託事業
（エイズ対策実用化研究事業）による委託業務として、
独立行政法人国立国際医療研究センター 総長春日雅人
が実施した平成26年度「適正な抗HIV療法開発のため
の研究」の成果を取りまとめたものです。