



Vascular lesion in a patient of chronic active Epstein-Barr virus infection with hypersensitivity to mosquito bites: vasculitis induced by mosquito bite with the infiltration of nonneoplastic Epstein-Barr virus-positive cells and subsequent development of natural killer/T-cell lymphoma with angiodestruction[☆]

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Summary This report describes a vasculitis and subsequently developing angiodestructive lymphoma in an 11-year-old Japanese-Filipino girl exhibiting mosquito allergy with the background of chronic active Epstein-Barr virus (EBV) infection. She developed necrotic skin ulcer at the site of mosquito bite, and histopathological examination revealed EBV-positive mononuclear cell infiltration throughout the wall of small-sized muscular artery. These EBV-positive lymphoid cells were oligoclonal in Southern blot analysis for EBV terminal repeats. Effectiveness of steroid therapy also supports the nonneoplastic nature. Approximately 1 year later, she developed progressive large skin ulcer without mosquito bites. Microscopically, the angiocentric or angiodestructive pattern of EBV-positive atypical cells supported the diagnosis of extranodal natural killer/T-cell lymphoma. Southern blot analysis revealed the monoclonal neoplastic nature of EBV-positive cells. In contrast to the primary mosquito bite lesion, natural killer/T-cell lymphoma cells exhibited the higher expression of EBV latent membrane protein 1 mRNA and the apparent protein expression detected by immunohistochemistry.

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Abbreviations: CAEBV, chronic active EBV infection; EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; EBV, Epstein-Barr virus; HMB, hypersensitivity to mosquito bites; IHC, immunohistochemistry; ISH, in situ hybridization; LCL, lymphoblastoid cell line; LMP, latent membrane protein; NBF, neutral-buffered formalin; NK, natural killer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; RT, reverse transcription; SAB, streptavidin-biotin; VCA, viral capsid antigen

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1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous virus in human beings. Most individuals are infected with EBV by early adulthood. Primary EBV infection is usually asymptomatic, but sometimes, it results in infectious mononucleosis, which is basically self-limited because of the development of EBV-specific immunity. However, some individuals in Asia, mostly in Japan, are reported to develop chronic infection with EBV. Chronic active EBV (CAEBV) infection is characterized by chronic recurrent infectious mononucleosislike symptoms over a long period and by an unusual pattern of anti-EBV antibodies [1]. Chronic active EBV infection is a disease with a high mortality and life-threatening complications, such as virus-associated hemophagocytic syndrome probably caused by cytokinemia induced by EBV-infected lymphoid cells, EBV-positive lymphoid neoplasia mainly in T-cell and natural killer (NK) cell lineage, and cardiovascular diseases and large-vessel arteritis with infiltration of EBV-positive lymphoid cells [1-3].

Interestingly, some CAEBV patients show skin symptoms, such as hypersensitivity to mosquito bite (HMB) and hydroa vacciniforme-like eruptions. Approximately one third of CAEBV patients exhibit the history of HMB, and the patients often develop HMB as a primary symptom before their cases are diagnosed as CAEBV [4]. Chronic active EBV patients with HMB develop severe local skin reactions at the sites of mosquito bite, which result in necrotic skin ulcerations and subsequent depressed scars. Because HMB in CAEBV is a relatively rare disease, the pathogenesis of skin lesion is little understood.

In this report, we described the vasculitis lesion at the site of mosquito bite in a patient of CAEBV with HMB. After approximately 1-year interval, EBV-positive NK/T-cell lymphoma developed, which showed spontaneous skin ulcer without mosquito bites, and the histopathology exhibited the angiodestructive pattern resembling the vasculitis lesion in the primary mosquito bite. We further characterized the EBV infection of both lesions.

2. Materials and methods

2.1. Case report

An 11-year-old Japanese-Filipino girl had exhibited HMB since the age of 5. She had prolonged coldlike symptoms at the age of 4, and her illness was diagnosed as CAEBV. She presented in July 2002 with a high fever of 39°C after a mosquito bite. She exhibited a swollen erythematous lesion at right heel, with a walnut-sized necrotic plaque. The peripheral blood picture showed red blood cells $525 \times 10^4/\mu\text{L}$, platelets $28.8 \times 10^4/\mu\text{L}$, and white blood cells $11.7 \times 10^3/\mu\text{L}$ with 55% lymphocytes. Atypical lymphocytes were not detected. Cell surface

marker analyses of peripheral blood mononuclear cells (normal ranges in parentheses) showed CD4⁺ cells, 23.4% (30.5%-53.7%); CD8⁺ cells, 34.6% (17.4%-43.0%); CD16⁺ cells, 15.8% (1.5%-25.5%); and CD56⁺ cells, 16.2% (3.8%-28.6%). Serum immunoglobulin (Ig) E was 524 IU/mL (measured in August 2001), and mosquito-specific IgE antibodies were not detected. Epstein-Barr virus-related antibody titers, measured in June 2002, were anti-viral capsid antigens (VCA) 1280 $\times$ (IgG), <10 $\times$ (IgM), and 40 $\times$ (IgA); anti-early antigens 640 $\times$ (IgG) and 80 $\times$ (IgA); and anti-EBV nuclear antigens (EBNA) 40 $\times$. Skin biopsy from the periphery of the necrotic plaque was performed for evaluation of the inflammatory cell infiltration and the relationship to EBV infection. The diagnosis of vasculitis with the infiltration of EBV-positive cells was made, and the steroid therapy (prednisolone 30 mg/d) was started. Her skin lesion had improved, and she was discharged and was followed at outpatient clinic. In August 2003, skin ulcers developed again at left elbow and left leg; furthermore, hen-egg-sized necrotic skin ulcer appeared in abdominal region without mosquito bites in October. Prednisolone (30 mg/d for a month) and cyclosporine (150 mg/d for a week) were not effective, and the resection of ulcer lesion was performed. The diagnosis of EBV-positive NK/T-cell lymphoma was made for the resected tissue, and antineoplastic chemotherapy (etoposide and prednisolone) was started. However, the bacterial infection of the skin ulcer hampered the chemotherapy, and her skin ulcer enlarged. Her general condition deteriorated, and she died in March 2004. An autopsy was not performed.

2.2. Immunohistochemistry

Biopsy specimens were fixed in 10% neutral-buffered formalin (NBF) or in 4% paraformaldehyde (PFA)-phosphate-buffered saline (PBS) and embedded in paraffin. Three-micrometer-thick histological sections were subjected to the immunohistochemical studies with streptavidin-biotin (SAB) method. The mouse monoclonal antibodies used in this study were PS1 (anti-CD3 ϵ ; Nichirei, Tokyo, Japan), 1F6 (anti-CD4; Nichirei), C8/144B (anti-CD8; Nichirei), L26 (anti-CD20; Nichirei), UCHL1 (anti-CD45RO; Dako, Glostrup, Denmark), 1B6 (anti-CD56; Nichirei), KP1 (anti-CD68; Dako), PE2 (anti-EBNA2; Dako), CS1-4 (anti-latent membrane protein [LMP] 1; Dako), and 2E3 (anti-VCA; Biogenesis, Poole, England). Rabbit antisera to human IgG (Dako), IgM (Dako), and C3c (Dako) were also used.

2.3. In situ hybridization

Three-micrometer-thick sections of paraffin-embedded tissue were processed using MicroProbe Staining System (Fisher Scientific, Pittsburgh, Pa) according to the manufacturer's protocol. Epstein-Barr virus-encoded RNA 1 (EBER1) oligonucleotide probe (5'-AGA-CACCGTCCTCACCACCCGGGACTTGTA-3') was la-

beled with digoxigenin, and signals were developed with alkaline phosphatase-labeled antidigoxigenin Fab fragment (Roche, Mannheim, Germany). Sections treated with RNase before hybridization were used as negative controls for hybridization.

2.4. Cells

An EBV-positive Burkitt's lymphoma cell line, Akata, was kindly provided by Prof Kenzo Takada (Hokkaido University, Sapporo, Japan). Epstein-Barr virus-transformed lymphoblastoid cell line (LCL) and a human T-cell line, Jurkat, were also used. For the induction of lytic cycle of EBV infection of Akata cells, 5×10^6 cells were incubated in 5 mL of complete medium containing 1% (vol/vol) rabbit F(ab')₂ fragments specific for human IgG (Chemicon, Temecula, Calif) for 12 hours.

2.5. DNA extraction and Epstein-Barr virus genome analyses

DNAs were extracted from cell lines and biopsy tissues, quantified by measuring OD₂₆₀ and stored at -20°C until use. For polymerase chain reaction (PCR) amplification, a DNA sample was diluted into 25 μL of a solution containing 0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 500 nmol/L of each primer, 0.6 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif), and PCR reaction buffer provided by the manufacturer. Primer sequences for EBV *Bam*HI-W region were 5' -CGGTCGCCAGTCCTAC-CAG-3' and 5' -CCTGGAGAGGTCAGGTTACT-3', and 5' -CTTCTGACACAACCTGTGTTCCTAGC-3' and 5' -TCACCACCAACTTCATCCACGTTTAC-3' for human β -globin. Southern blot analysis for the clonality of EBV terminal repeats was performed as previously described [5], with the exception of the use of nonradioactive probe. Probe was labeled using Gene Images random prime labeling system (Amersham Pharmacia Biotech, Buckinghamshire, England). Southern-blotted filters were hybridized with the labeled probe, and signals were generated with CDP Star detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

2.6. RNA extraction and reverse transcription-polymerase chain reaction analysis for the expression of Epstein-Barr virus genes

Total RNA was extracted from frozen tissues and cell lines using Trizol reagent (Invitrogen, Carlsbad, Calif), quantified by measuring OD₂₆₀, and ethanol-precipitated at -80°C until use. For the reverse transcription (RT) of RNA samples, 1 μg of total RNA was converted to cDNA with TaqMan Reverse Transcription Reagents (Applied Biosystems) in a 50- μL reaction volume in the presence of random hexamers according to the manufacturer's protocol. For PCR amplification, a 2- μL aliquot of the cDNA sample was diluted to 25 μL of the same PCR solutions as described in the previous section. Primer and probe sequences were previously

described [6]. Amplified products were verified with Southern blot analyses as previously described [7]. The recovery of amplifiable RNA was verified by RT-PCR of β -actin transcripts.

3. Results

3.1. Histopathology and immunohistochemical analyses of the lesions

Microscopically, the skin lesion of mosquito bite showed moderate hyperkeratosis and intraepidermal bulla. We observed mononuclear cell infiltration mainly around the small vessels in dermis and subcutaneous adipose tissue. Furthermore, we found mononuclear cell infiltration throughout the wall of small-sized muscular artery located at the border between dermis and subcutaneous fat tissue [Fig. 1(A)]. The cellular thickening of intima and the interruption of internal elastic lamina were also observed [Fig. 1(B)]. Absence of cellular infiltration in the surrounding adipose tissue suggests that the arterial structure was the primary target of inflammatory cells, not merely involved in the inflammation of subcutis. Thus, this vascular lesion was an apparent arteritis. This lesion must be responsible for the vast necrosis of surrounding subcutaneous fat tissue. Most of the infiltrating mononuclear cells were CD56⁺ [Fig. 1(C)] or CD68⁺ (results not shown). There were a few CD3⁺, CD4⁺, or CD45RO⁺ cells and few CD8⁺ or CD20⁺ cells around the vascular lesion (results not shown). These results clearly indicate that the infiltrating mononuclear cells were NK- and macrophage-lineage cells.

In the recurrent skin ulcer lesion, we also observed the vasculitislike lesion with the vast infiltration of mononuclear cells into vessel wall [Fig. 1(F)]. The infiltrating cells, however, showed the nuclear atypia [Fig. 1(E)] and were positive for CD56 [Fig. 1(G)]. Many CD68⁺ cells were also recognized within and around the vascular wall (results not shown). We recognized this histopathology as angiodestructive pattern and the diagnosis of extranodal NK/T-cell lymphoma, nasal type (WHO classification), was strongly suggested. No deposition of IgG, IgM, and C3 was observed in the both lesions (results not shown).

3.2. Expression of Epstein-Barr virus genes

By using in situ hybridization (ISH), we observed EBER1 signals in approximately one third of infiltrating cells in the mosquito bite lesion [Fig. 1(D)], and RNase treatment before hybridization completely abolished the signals (results not shown). These results clearly indicate that these signals were derived from EBER1 RNA. Therefore, a considerable fraction of infiltrating mononuclear cells was infected with EBV. No expression of EBER1 was detected in the vascular components, such as endothelial cells and smooth muscle cells [Fig. 1(D)]. We also

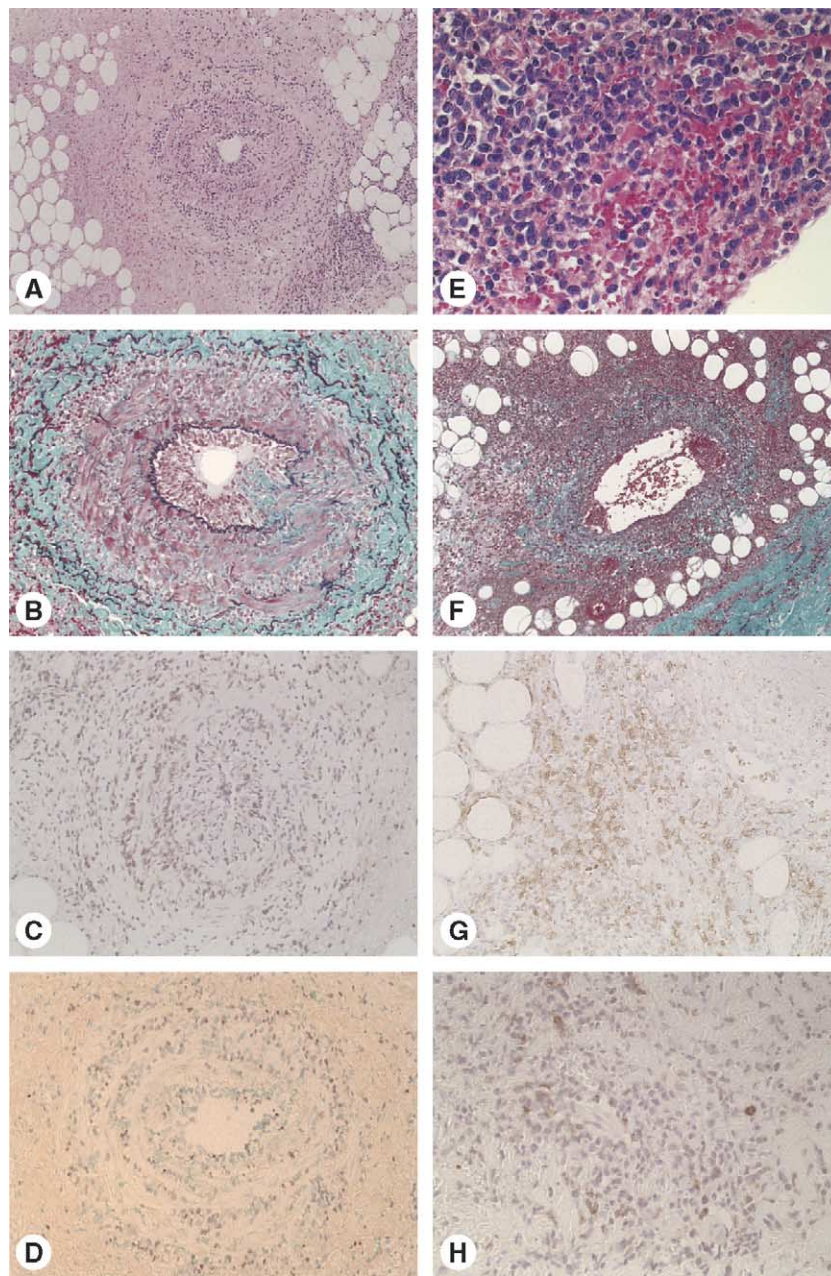


Fig. 1 Histopathology, immunostaining for CD56 and LMP1, and ISH for EBER1 RNA of the biopsy specimens. (A-D) Primary mosquito bite lesion; (E-H) recurrent skin necrosis lesion. (A, B, E, F) Hematoxylin and eosin (A and E) and Elastica-Goldner staining (B, F) of the vascular lesions fixed with 10% NBF. Intimal thickening of the arterial wall and the vast infiltration of mononuclear cells throughout the vessel wall (A) and destruction of internal elastic lamina (B) are observed in the primary mosquito bite lesion. In the recurrent skin necrosis lesion, the mononuclear cells infiltrating into the vessel wall show nuclear atypia (E), and the angiodestructive pattern is observed. (C, G) CD56 staining of the biopsy specimens fixed with 4% PFA-PBS. Positive staining is visible in a considerable fraction of infiltrating cells. Signals were generated with the SAB method. (D) ISH of the biopsy specimen of the primary mosquito bite lesion fixed with 10% NBF using EBER1 probe. Positive staining is visible in many infiltrating cells. (H) LMP1 staining of the biopsy specimen of the recurrent skin necrosis lesion fixed with 4% PFA-PBS. Positive staining is visible in some fraction of infiltrating cells. Original magnification $\times 10$ in A and F; $\times 20$ in B, C, D, and G; $\times 40$ in E and H.

observed many EBER1-expressing cells in the recurrent skin necrosis lesion (results not shown). By RT-PCR analyses, the expression of EBNA1 mRNA initiated from FQ promoter and that of LMP1 mRNA were detected in the

RNA samples from the both lesions (Fig. 2). However, the level of expression of LMP1 was different. The recurrent skin necrosis lesion contained much more LMP1 mRNA than mosquito bite lesion (Fig. 2). At protein level, no

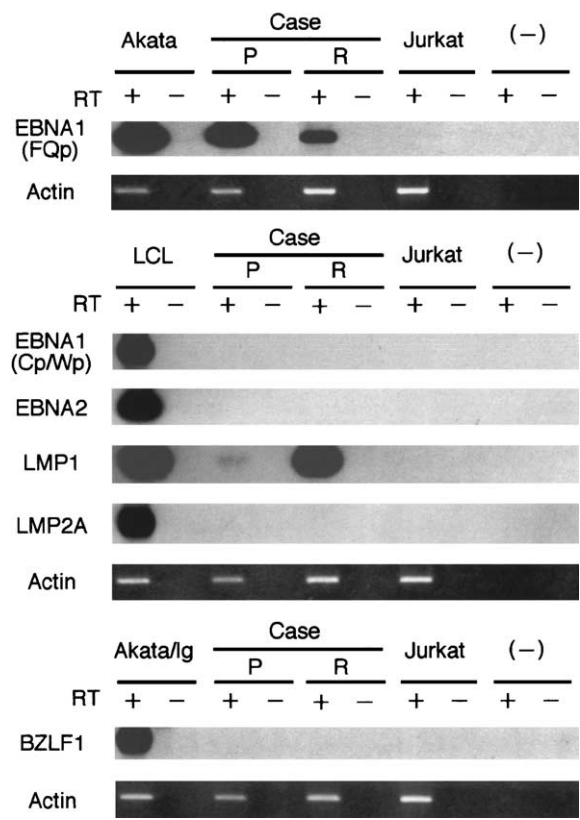


Fig. 2 RT-PCR analyses for EBV genes and for human β -actin. Forty-nanogram samples of total cellular RNA prepared from the indicated cell lines and from the biopsy tissues were reverse-transcribed and amplified by PCR for EBV genes or for human β -actin. Amplified products were electrophoresed in 3% (LMP1) or 2% (others) agarose gels, Southern-blotted, and hybridized with the oligonucleotide probe (EBV genes) or visualized with an ultraviolet transilluminator (β -actin). Total RNA samples without RT were also subjected to the same PCR amplification in the presence of RT reaction mixture. RNA samples from Akata, LCL, and anti-IgG-treated Akata cells (Akata/Ig) worked as positive control samples for each RT-PCR amplification. The biopsy tissues (P indicates primary mosquito bite lesion; R, recurrent skin necrosis lesion) show the band in the amplification for EBNA1 mRNA initiated from FQ promoter (EBNA1 [FQp]) and for LMP1.

expression of EBV-related antigens (EBNA2, LMP1, and VCA) was observed in the infiltrating cells and in the vascular components by using immunohistochemistry (IHC) in the mosquito bite lesion (results not shown). In contrast, some LMP1-positive cells were detected in the recurrent skin necrosis lesion [Fig. 1(H)]. The expression of *BZLF1* gene of EBV, which is the indicator of lytic infection cycle of EBV, was not observed (Fig. 2). These results indicate that EBV infection in the both lesions was latent and showed latency II type of infection.

3.3. Epstein-Barr virus genome analyses

To estimate the amount of EBV genome loaded in the vascular lesion, we performed the semiquantification of

EBV genome by PCR. Serially diluted (5-fold dilution) DNA samples from the biopsy specimens and a Burkitt lymphoma cell line, Akata, were subjected to PCR amplification of EBV *Bam*HI-W region. The same diluted DNA samples were also subjected to PCR of human β -globin gene to standardize the amount of amplifiable DNA. As the result, the amount of EBV genome in the mosquito bite lesion and that in the recurrent lesion were approximately one fifth and the same of that in Akata cells, respectively (results not shown). These results indicate that the loads of EBV in both lesions were extremely high and that the recurrent lesion contained much more EBV than the mosquito bite lesion. We also performed the Southern blot analysis to verify the clonality of terminal repeats of EBV genome in the biopsy specimens. We observed biconal bands in the DNA samples from the mosquito bite biopsy and monoclonal band in the DNA from the recurrent lesion (Fig. 3). This indicates that the EBV-positive cells in the mosquito bite lesion were nonneoplastic or preneoplastic and that those in the recurrent lesion were neoplastic. Thus, a diagnosis of EBV-positive nasal-type NK/T-cell lymphoma was made for the recurrent skin ulcer lesion.

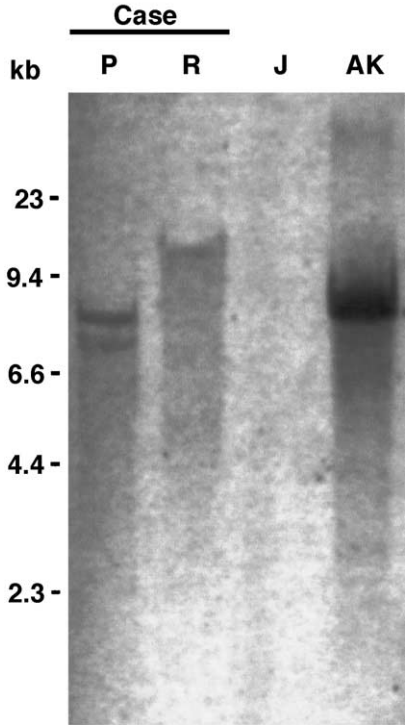


Fig. 3 Southern blot analyses for the clonality of the EBV genome. Ten micrograms of DNA was digested with *Bam*HI, electrophoresed in 0.6% agarose gels, Southern-blotted, and hybridized with EBV-DNA probe containing the adjacent sequence to terminal repeat of EBV genome. DNA size markers are indicated on the left. Biopsy specimens and Akata cells contained a predominant terminal repeat of EBV.

4. Discussion

In the current case, the primary mosquito bite lesion contained the oligoclonal EBV genome. This indicates the nonneoplastic or preneoplastic characteristics of EBV-infected cells, and the vasculitis lesion developed at the site of mosquito bite. Healing of the lesion without antineoplastic chemotherapy also supports the nonneoplastic characteristics of EBV-infected cells. The recurrent skin necrosis lesion, on the other hand, exhibited the monoclonal EBV genome, indicating the neoplastic nature of EBV-infected NK-lineage cells. In this condition, the vascular damage, which should be responsible for the necrosis of the skin, developed without mosquito bites. The close association between HMB and NK cell proliferative disorders/lymphomas has been known [8,9]. The affinity of EBV-infected lymphoid cells to blood vessels has been previously reported in EBV-positive NK/T-cell lymphomas [8,10,11]. The term angiocentric or angiodestructive pattern is often used to describe these lesions of the lymphoma tissue. In the mosquito bite lesion, on the other hand, the expression of EBV genes and deposition of immunoglobulins were not detected in the affected arterial wall. Furthermore, mosquito-specific IgE antibodies were not detected in the patient. These suggest that the development of vasculitis at the sites of mosquito bites is not mediated by immunologic mechanisms. Inflammatory mediators would be necessary for nonneoplastic EBV-infected NK cells to develop vasculitis lesions.

When comparing the characteristics of EBV infection, the recurrent lesion contained the monoclonal EBV genome and exhibited the higher expression of LMP1 at both mRNA and protein level than the primary mosquito bite lesion. Latent membrane protein 1 expression in B-lymphoblastoid cell lines induces the increased expression of adhesion molecules, such as lymphocyte function-associated antigen (LFA)-1, LFA-3, and intercellular adhesion molecule-1 [12]. The same alterations might occur in NK-lineage cells, and Takeshita et al [11] reported that NK/T-cell lymphoma cells in cases with angiodestructive lesion frequently expressed adhesion molecules and that those without angiodestruction did not.

There are a few reports describing the histopathology of skin reaction in the patients of HMB with the background of CAEBV [13,14], and diffuse inflammatory cell infiltration without vascular damage, mainly composed of cells positive for NK- or T-cell markers [13,14], was just observed at the lesions. Even in a case of mosquito allergy without analysis for the involvement of EBV infection, only leukoclastic vasculitis of small vessels accompanying diffuse cellular infiltration is reported [15]. Thus, this is the first report describing the apparent vasculitis lesion in these patients. The vasculitis lesion observed in the current case was not accompanied with fibrinoid necrosis nor with the deposition of immune complexes, which would be detectable with IHC to immunoglobulins and complements, and thus is not necrotizing arteritis. Furthermore, the histological picture

of this lesion does not resemble that of granulomatous arteritis, such as temporal arteritis, although many infiltrating CD68⁺ cells were observed in the affected arterial wall. The primary mosquito bite lesion is an apparent arteritis, which would be clinically supported with the responsiveness to the steroid therapy. The nature of the lesion, however, would be the vascular infiltration of preneoplastic cells.

Approximately one fourth of CAEBV patients develop, even without mosquito allergy, inflammatory vascular lesions, which would be fatal [1]. Nakagawa et al [2] reported the histopathology of coronary disease developing in a CAEBV patient. They observed marked lymphoid infiltration in the subepicardial adipose tissue, mild perivascular lymphoid infiltration, fibrinoid necrosis of some coronary arteries, and coronary aneurysms [2]. In their case, the infiltrating lymphoid cells exhibited T-cell markers, such as CD3 and CD45RO. On the other hand, Murakami et al [3] reported the large-vessel arteritis involving coronary arteries, aorta, and its major branches in a CAEBV patient. In their case, destruction of medial elastic laminae and aneurysmal dilation of the lumens were the major pathological findings, and the inflammatory cell infiltration at the vascular lesions was limited. Thus, their case seems to exhibit the late restoring phase of vascular injury. Unfortunately, the detailed analysis of EBV infection was not performed in these cases.

In the current case, we observed the vasculitis lesion induced by mosquito bite with the infiltration of nonneoplastic EBV-positive cells and the subsequent development of NK/T-cell lymphoma with angiodestruction. Monoclonal expansion of EBV-positive cells and increased expression of *LMP1* gene might be responsible for the spontaneous angiodestruction without mosquito bite.

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