

CD4⁺ T-Lymphocyte–Induced Epstein-Barr Virus Reactivation in a Patient With Severe Hypersensitivity to Mosquito Bites and Epstein-Barr Virus–Infected NK Cell Lymphocytosis

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Background: Natural killer (NK) cell lymphocytosis associated with Epstein-Barr virus (EBV) infection often shows severe hypersensitivity to mosquito bites (HMB) characterized by intense local skin reactions and systemic symptoms such as high fever, lymphadenopathy, and hepatosplenomegaly. However, the induction mechanism of HMB is still unclear.

Observations: We investigated a typical case of HMB with EBV-positive NK cell lymphocytosis. CD4⁺ T cells dominantly infiltrated the site of the mosquito bite, while EBV-positive cells were few in comparison. CD4⁺ T cells, but not CD8⁺ T cells or NK cells, responded to the mosquito salivary gland extracts. Interestingly, coculturing of the NK cells and CD4⁺ T cells activated by mosquito extracts induced expression of EBV lytic-cycle proteins in the NK

cells. Furthermore, the expression of *BZLF1*, a viral lytic-cycle transactivator, was detectable at the skin lesion induced by scratch patch testing with mosquito extract. The EBV DNA copy number levels in the plasma were elevated in systemic HMB symptoms compared with the normal condition.

Conclusions: CD4⁺ T cells are important for the primary skin reaction to mosquito bites and might play a key role in reactivation of latent EBV infection in NK cells. This viral reactivation contributed to the pathogenesis of the infectious mononucleosis-like systemic symptoms of HMB in our present case.

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HYPERSENSITIVITY to mosquito bites (HMB) is characterized by intense local skin symptoms, which consist of not only erythema or bulla but also ulceration or scarring and systemic symptoms such as high fever, lymphadenopathy, and hepatosplenomegaly.¹ It was reported that natural killer (NK) cells infiltrate the skin lesions and that NK cells proliferate in the peripheral blood in a patient with HMB.² Furthermore, Ishihara et al³ demonstrated clonal lymphoproliferation of EBV DNA–positive NK cells in patients with HMB and suggested that HMB is not an allergic disease but an EBV-associated lymphoproliferative disease. However, the mechanism of HMB is still poorly understood. We previously demonstrated that EBV-carrying NK cells in patients with HMB overexpressed surface Fas ligand (FasL) or soluble FasL and suggested that the enhanced FasL might be related to organ (or tissue) damage, such as intense skin lesions at mosquito bite sites and liver dysfunction.^{4,5} However, the relationship between EBV and HMB remains unclear.

Herein, we propose a possible mechanism explaining the relation between HMB and EBV-positive NK cell lymphocytosis. We found that CD4⁺ T cells from a patient with HMB markedly responded to certain mosquito salivary gland extracts and showed that these CD4⁺ T cells could induce reactivation of latent EBV infection in NK cells that may be involved in the pathogenesis of HMB.

METHODS

PATIENT

The following study was performed with both the informed consent of the patient and the approval of the responsible committee in our hospitals. The clinical features and laboratory data of the patient have been previously reported.⁵ Briefly, an 18-year-old woman had recurrent necrotizing papules on the face and oral mucosa for 8 years. Since she was 13 years old, she has also had intense skin reactions at mosquito bite sites. The skin reaction usually began with erythema and swelling at 12 to 24 hours after the mosquito bite and developed to bulla, hemorrhagic necrosis, and ulcer formation (**Figure 1**). In addition to these local



Figure 1. Skin lesions at mosquito bite site. A, Erythema and swelling with blister formation 2 days after mosquito bites. B, Skin necrosis and ulcer formation after 10 days.

cutaneous manifestations, she also had systemic symptoms such as high fever, lymphadenopathy, hepatosplenomegaly, and general malaise. Hematologically, the typical cell morphology of large granular lymphocytes with azurophilic granules in an abundant cytoplasm was observed in approximately half of the mononuclear cells in a smear of the patient's peripheral blood. Flow cytometric analysis of lymphocytes showed a marked increase (51.1%) in the CD56⁺, CD16⁺, CD3⁺ populations, which was suggested to be NK cells. The pattern of serum antibody titers against EBV was compatible with chronic active EBV infection as follows: the anti-EB viral capsid antigen (VCA) IgG and anti-EB early antigen (EA) IgG levels were markedly increased ($\times 2560$ and $\times 320$, respectively). Polymerase chain reaction (PCR) analysis after sorting lymphocytes demonstrated that EBV DNA existed in NK cells but not in CD4⁺ T, CD8⁺ T, or CD19⁺ B cells. Southern blot analysis using EBV-terminal repeat probe demonstrated that EBV-positive NK cells oligoclonally proliferated. In situ hybridization using EBV-encoded small nuclear RNA1 (*EBER1*) probe demonstrated that more than 90% of NK cells were positive for EBV. Reverse transcription (RT)-PCR analysis demonstrated no expression of EBV lytic-cycle genes and type II latency expression pattern of EBV latent gene (ie, *EBER1*⁺, EBV nuclear antigen 1 [*EBNA1*]⁺, *EBNA2*⁻, latent membrane protein 1 [*LMP1*]⁺, *LMP2A*⁻, or *LMP2B*⁻).

PREPARATION OF MOSQUITO EXTRACTS

The mosquito extracts were prepared from salivary glands of 5 species of mosquito, 4 endemic species in Japan (*Aedes albopictus*, *Anopheles sinensis*, *Culex pipiens pallens*, and *Culex tri-*

taeniorhynchus), and 1 exotic species (*Anopheles stephensi*). Forty salivary glands from each of the 5 mosquito species were homogenized, suspended in 500 μ L of phosphate-buffered saline (PBS), filtrated with a 45- μ m filter, and measured for protein concentration by the Lowry method.

SKIN TEST

Scratch patch and prick testing were done by the conventional procedure, using the above-mentioned solutions of mosquito salivary gland extracts (final protein concentration: 40 μ g/mL) and PBS as a control. Scratch patch and prick testing were performed on the upper arm and the back, respectively. The evaluation of scratch patch test reactions was done 48 and 72 hours after challenge. The reactions to prick testing were estimated at 15 minutes, and 24 and 48 hours after challenge.

TISSUE PREPARATION

Tissue samples were obtained from the skin lesions at mosquito bite sites (approximately 72 hours after the bites). Tissue samples for hematoxylin-eosin stain or in situ hybridization were fixed with 4% paraformaldehyde in 0.1M PBS, pH 7.0, and embedded in paraffin; serial sections were prepared. Samples for immunostaining were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and then deep frozen in liquid nitrogen and stored at -80°C prior to sectioning.

IMMUNOSTAINING FOR LYMPHOCYTE SURFACE MARKERS

Monoclonal antibodies (MoAbs) against CD4 (Novocastra Laboratories, Newcastle, England), CD8 (DAKO, Kyoto, Japan), and CD16 (Novocastra Laboratories) were used for immunostaining. Immunohistochemical examination for lymphocyte surface markers was carried out on frozen sections of the skin lesion at the mosquito bite site. Sections were incubated in 0.3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase. The slides were incubated with the primary MoAbs for 18 hours at 4°C . Binding of the primary MoAbs was demonstrated using the LSAB2 Kit (DAKO).

IN SITU HYBRIDIZATION

The presence of EBV in the infiltrating cells at the mosquito bite site was assessed by in situ hybridization for *EBER1*. Hybridization was carried out as previously described.⁶ Briefly, deparaffinized tissue sections of the bite site were treated with 10 μ g/mL of proteinase K (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) for 30 minutes at 37°C , re-fixed with 4% paraformaldehyde in 0.1M PBS for 10 minutes, and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0, for 10 minutes. The sections were hybridized with the digoxigenin-labeled oligonucleotide *EBER1* probe at 42°C for 16 hours. Hybridized digoxigenin-labeled probe was detected by DIG Nucleic Acid Detection Kit (Boehringer Mannheim GmbH Biochemica) according to the manufacturer's instructions.

RT-PCR ANALYSIS

Skin biopsy was performed at the skin lesion induced for 48 hours by scratch patch test with *A. albopictus* salivary gland extract. Total RNA was isolated from the skin biopsy samples by the acid guanidinium thiocyanate-phenol-chloroform method. Samples of RNA were pretreated with 10 U of deoxyribonuclease for 15 minutes at 37°C followed by denaturing of the enzyme for 5 minutes at 99°C to avoid amplification of DNA con-

tamination. One microgram of total RNA of each sample was reverse transcribed using M-MLV RT (Gibco-BRL, Gaithersburg, Md) and random hexamer primers. The resultant complementary DNA was amplified by the PCR using recombinant Taq DNA polymerase (Takara Shuzo Co, Shiga, Japan) and 0.5 $\mu\text{mol/L}$ of each of the forward and reverse primers. To detect expression of *BZLF1* messenger RNA, nested sets of primers were synthesized and used for amplification as described by Prang et al.⁷ B95-8 cells were used as a positive control for detection of EBV lytic-cycle gene expression. As a negative control, we used the tissue samples from nonspecific dermatitis lesion of the patient. For quality control of RNA samples, we used histone 3.3 RT-PCR according to the method of Futscher et al.⁸

PURIFICATION OF CD4⁺ T, CD8⁺ T, AND NK CELLS

Whole blood was collected from the patient and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. CD4⁺ T, CD8⁺ T, and NK cells were purified by magnetic cell sorting using StemStep-TM Kit (Stem Cell Technologies Inc, Vancouver, British Columbia) as described elsewhere.⁹ StemStep-TM is a negative selection system in which the unwanted cells are immunomagnetically labeled and bound to a magnetic column. Purified cell phenotypes were examined by flow cytometric analysis after staining with MoAbs against CD4, CD8, CD16, and CD56 conjugated with fluorescein isothiocyanate or phycoerythrin (Becton Dickinson, Mountain View, Calif). Each cell population prepared by magnetic cell sorting was highly purified (>98% by flow cytometric analysis using a fluorescence-activated cell sorter FACSscan; Becton Dickinson).

PROLIFERATION ASSAY

RPMI-1640 medium supplemented with penicillin-streptomycin mixture, $5 \times 10^{-5}\text{M}$ 2-mercaptoethanol, 2mM glutamine, 10mM HEPES, and 10% heat-inactivated fetal bovine serum were used as the culture medium. CD4⁺ T, CD8⁺ T, and NK cells were purified from the mononuclear cells of the patient and 5 healthy volunteers, and autologous PBMCs were x-ray irradiated (3000 rad [30 Gy]) and used as antigen-presenting cells. CD4⁺ T, CD8⁺ T, and NK cells (5×10^5 cells/mL) were mixed with antigen-presenting cells (5×10^4 cells/mL), and cultured with 4 $\mu\text{g/mL}$ of extracts from mosquito salivary glands in 96-well U-bottom plates. After a 4-day incubation period with salivary extracts, cultures were pulsed with 18.5 kBq [³H]-thymidine (Amersham, Aylesbury, England) per 5×10^4 cells. Cultures were harvested 4 hours later onto glass fiber filter paper using a semiautomatic cell harvester. Radioactivity was measured with liquid scintillation. These assays were performed 3 times for each sample.

IMMUNOSTAINING FOR EBV LYTIC-CYCLE PROTEINS

CD4⁺ T cells from the patient were exposed to 4 $\mu\text{g/mL}$ of salivary gland extract of *A albopictus* for 3 days and cocultured with NK cells from the same patient for 3 days (CD4⁺ T cells–NK cell ratio = 1:10). Coculture of unexposed CD4⁺ T cells with NK cells was performed in parallel. The cultured cells were cytopun onto glass slides fixed in cold acetone. To detect expression of EA and VCA of EBV in the NK cells, 2-color immunofluorescence analysis was performed. The fixed cells were incubated with MoAbs against VCA (Chemicon, Temecula, Calif) or EA (Chemicon) for 1 hour, washed 3 times, incubated for 30 minutes with fluorescein isothiocyanate-conjugated goat F(ab')₂ antibody fragment to mouse IgG. After washing 3 times, the cells were incubated for 1 hour with biotinylated MoAb against CD56



Figure 2. Skin testing with mosquito salivary gland extracts. A, Prick testing was done with the salivary gland extracts from 5 species of mosquito. The erythematous area at 48 hours of each prick test was 23.6 cm² (*Aedes albopictus*), 0 cm² (*Anopheles sinensis*), 0 cm² (*Anopheles stephensi*), 8.3 cm² (*Culex pipiens pallens*), 2.1 cm² (*Culex tritaeniorhynchus*), and 0 cm² (phosphate-buffered saline solution [PBS]). B, Erythema with bulla was observed after 48 hours by scratch patch testing with the salivary gland extract from *A albopictus*.

(Pharmingen, San Diego, Calif), washed 3 times, and incubated for 30 minutes with Texas Red-conjugated streptavidin (Pharmingen). Finally, the slides were washed 3 times and examined with an immunofluorescence microscope.

QUANTITATION OF EBV DNA BY REAL-TIME PCR

Plasma samples were taken at different time points from the same patient, at times either with or without systemic HMB symptoms. We extracted DNA from the plasma samples and measured EBV DNA concentrations in each sample using a real-time quantitative PCR system that amplified a DNA segment in the *Bam* HI-W fragment region of the EBV genome.¹⁰ Real-time quantitative PCR and reaction setup were performed as previously described.¹⁰

RESULTS

SKIN TEST

Prick testing was done with the salivary gland extracts from 5 species of mosquito (ie, *A albopictus*, *A sinensis*, *A stephensi*, *C p pallens*, and *C tritaeniorhynchus*). As a result of the prick testing, the extracts from 3 species of mosquito induced sharply demarcated erythema with edema after 24 hours, but no immediate reaction was observed. The erythematous area at 48 hours of each prick test was 23.6 cm² (*A albopictus*), 0 cm² (*A sinensis*), 0 cm² (*A stephensi*), 8.3 cm² (*C p pallens*), 2.1 cm² (*C tritaeniorhynchus*), and 0 cm² (PBS) (**Figure 2A**). Scratch patch testing was done with the salivary gland extract from *A albopictus*, and as a result, erythema with bulla was observed after 48 hours (**Figure 2B**).

DETECTION OF EBV AND ANALYSIS OF SURFACE PHENOTYPE

The biopsy specimens were taken from the skin lesions at the mosquito bite sites. We performed in situ hybridization to detect EBV infection and immunohistochemistry to study the surface phenotype of infiltrating lym-

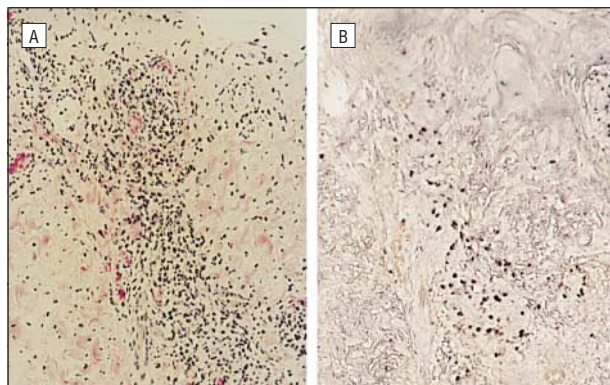


Figure 3. A, Hematoxylin-eosin staining of a skin lesion at mosquito bite site demonstrated dense infiltration of lymphoid cells in the dermis. B, In situ hybridization with *EBER1* probe demonstrated that about 5% of infiltrating cells were positive for Epstein-Barr virus in the same lesion as panel A (original magnification $\times 100$.)

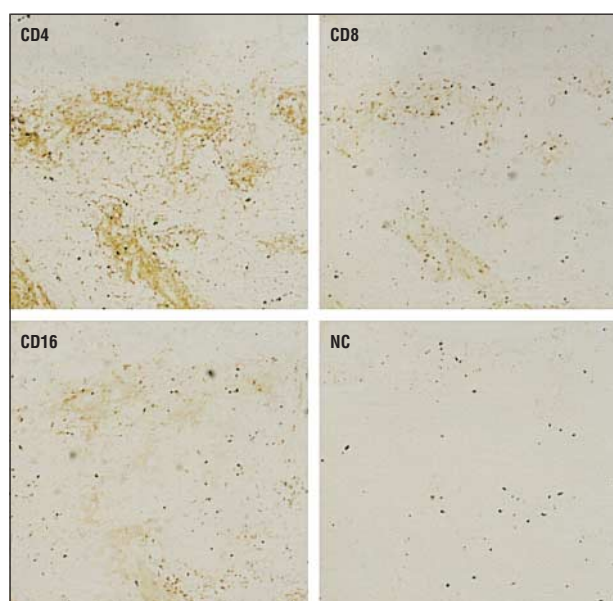


Figure 4. Immunohistochemical analysis for lymphocyte surface markers was carried out on frozen sections of the skin lesion at the mosquito bite site using monoclonal antibodies against CD4, CD8, CD16 and negative control monoclonal antibodies (NC). CD4⁺ cells dominantly infiltrated the skin lesions, while CD8⁺ and CD16⁺ cells were observed less frequently (original magnification $\times 40$).

phocytes. In situ hybridization with *EBER1* probe demonstrated that approximately 5% of infiltrating cells were positive for EBV (**Figure 3**). Immunohistochemical analysis showed that CD4⁺ cells dominantly infiltrated the skin lesions, while infiltration by CD8⁺ and CD16⁺ cells were less frequently observed (**Figure 4**).

MARKED RESPONSE OF PBMCs TO SALIVARY GLAND EXTRACTS

Peripheral blood mononuclear cells derived from the patient and healthy volunteers were examined in terms of their proliferative response to the mixture of the salivary gland extracts from *A albopictus*, *A sinensis*, *C p pallens*, and *C tritaeniorhynchus*. Marked proliferative response of PBMCs was observed in the patient, while

PBMCs from the healthy volunteers showed no or low proliferative responses. Then, CD4⁺ T, CD8⁺ T, and NK cells purified from peripheral blood of the patient were stimulated with the salivary gland extracts. CD4⁺ T cells, but neither NK cells nor CD8⁺ T cells, markedly reacted to the extracts (**Figure 5A**).

RESPONSES OF CD4⁺ T CELLS TO SALIVARY GLAND EXTRACTS

CD4⁺ T cells of the patient and a healthy volunteer were stimulated with each of salivary gland extracts from *A albopictus*, *A sinensis*, *A stephensi*, *C p pallens*, and *C tritaeniorhynchus* (4 μ g/mL). Marked proliferation was found in response to salivary gland extract of *A albopictus*, while moderate responses to *C p pallens* and *C tritaeniorhynchus* and low responses to *A sinensis* and *A stephensi* were observed (**Figure 5B**).

EXPRESSION OF EBV LYTIC-CYCLE PROTEINS IN NK CELLS

Natural killer cells from the patient were cocultured for 72 hours with autologous CD4⁺ T cells stimulated or nonstimulated by the salivary gland extract of *A albopictus*. The cells were double-stained with anti-CD56 MoAb and MoAbs to lytic-phase EBV proteins (VCA and EA). We obtained cytoplasmic staining for VCA (**Figure 6**) and EA (data not shown) proteins in 1% to 3% of CD56⁺ cells cocultured with mosquito antigen-stimulated CD4⁺ T cells. In contrast, no VCA or EA signal could be detected in either CD56⁺ cell culture with nonstimulated CD4⁺ T cells or CD56⁺ cell culture with mosquito salivary gland extract without CD4⁺ T cells.

EBV LYTIC-CYCLE GENE EXPRESSION IN THE SKIN LESION

Skin biopsy was performed from the skin lesion induced by scratch patch testing with salivary gland extract of *A albopictus*. Reverse transcriptase-PCR analysis demonstrated the expression of a viral lytic-cycle transactivator *BZLF1* gene at the skin lesion induced by mosquito extract but not at the nonspecific dermatitis lesion (**Figure 7**).

PLASMA LEVELS OF EBV DNA AT DIFFERENT TIME POINTS

With use of real-time quantitative PCR, we evaluated EBV genome concentrations in the plasma samples of the patient at different time points, at times either with or without systemic HMB symptoms. Plasma EBV DNA levels increased in systemic HMB symptoms compared with healthy conditions without systemic HMB symptoms (**Table**).

COMMENT

There were at least 3 characteristic features in our present case. The first was marked increase of NK cells, which were morphologically large granular lymphocytes that ex-

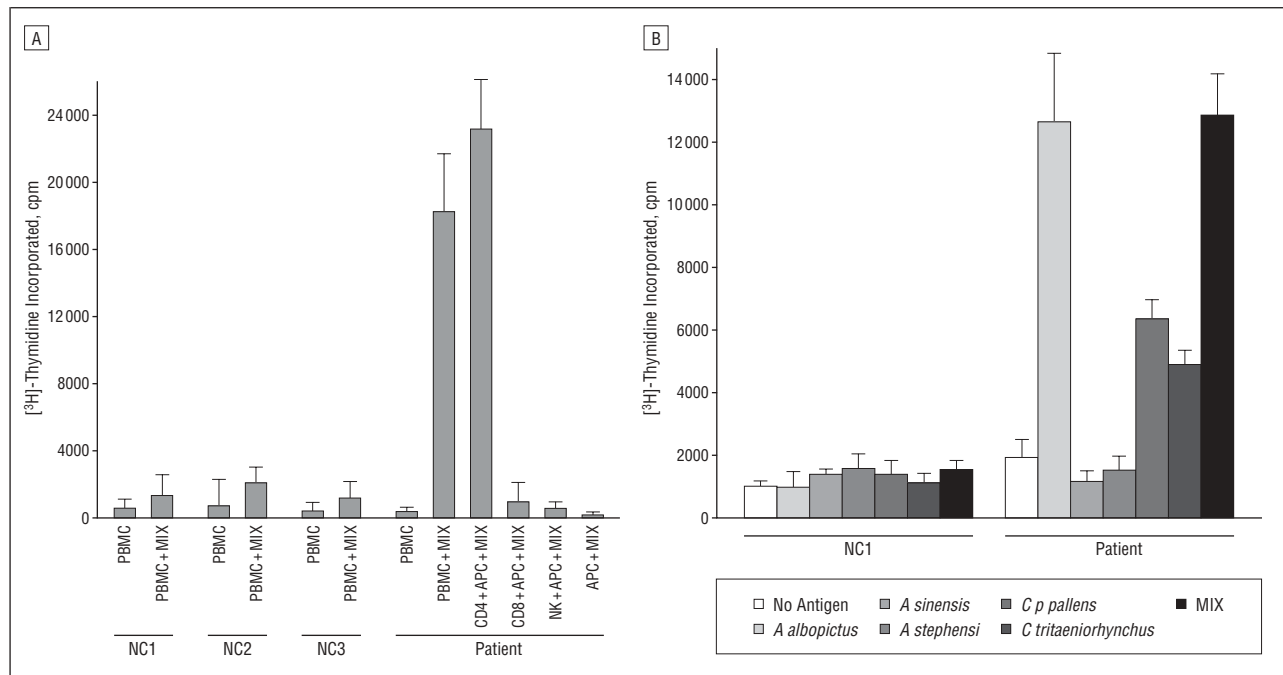


Figure 5. A, Peripheral blood mononuclear cells (PBMCs) (5×10^5 cells/mL) from the patient and healthy volunteers (negative controls [NCs] 1-3) were stimulated with 4 μ g/mL of the mixture of salivary gland extracts from 5 species of mosquito (MIX). CD4⁺ T, CD8⁺ T, and natural killer (NK) cells (5×10^5 cells/mL) from peripheral blood of the patient were also stimulated with MIX. Marked proliferative response of PBMCs was observed in the patient, while PBMCs from the healthy volunteers showed no or low proliferative responses. CD4⁺ T cells, but neither NK cells nor CD8⁺ T cells, reacted to MIX. B, CD4⁺ T cells (5×10^5 cells/mL) of the patient and healthy volunteer (NC1) were stimulated with 4 μ g/mL of each salivary gland extract from *Aedes albopictus*, *Anopheles sinensis*, *Anopheles stephensi*, *Culex pipiens pallens*, *Culex tritaeniorhynchus*, or MIX. Marked proliferation was found in response to salivary gland extract of *A. albopictus* in the patient, while moderate responses to *C. p. pallens* and *C. tritaeniorhynchus* and no responses to *A. sinensis* and *A. stephensi* were observed. APC indicates antigen-presenting cell. Error bars represent SE.

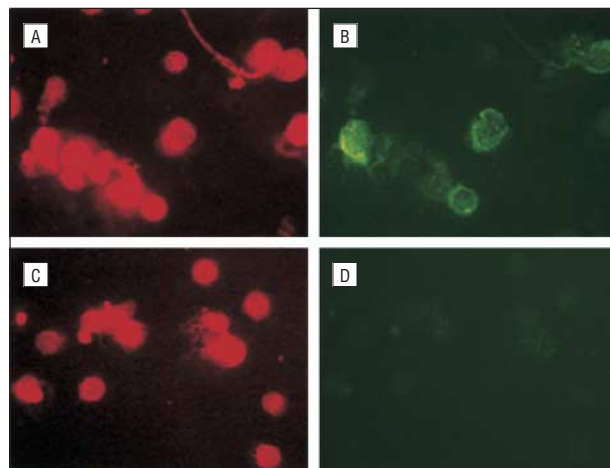


Figure 6. Natural killer cells from the patient were cocultured for 72 hours with autologous CD4⁺ T cells stimulated (A-B) or nonstimulated (C-D) by the salivary gland extract of *Aedes albopictus* (original magnification $\times 400$). The cells were double stained with anti-CD56 monoclonal antibody (red) and anti-Epstein-Barr virus viral capsid antigen monoclonal antibody (green). We observed cytoplasmic staining for viral capsid antigen in CD56⁺ cells cocultured with mosquito antigen-stimulated CD4⁺ T cells (A-B). In contrast, no specific viral capsid antigen signal could be detected in CD56⁺ cells cocultured with nonstimulated CD4⁺ T cells (C-D).

pressed CD16 and CD56, but not CD3, CD4, or CD8. Second, the expanding NK cells were infected with EBV. In situ hybridization with *EBER1*, PCR analysis of EBV DNA, and RT-PCR analysis of EBV messenger RNA revealed that the NK cells were latently infected with EBV. Moreover, Southern blot analysis using EBV-terminal re-

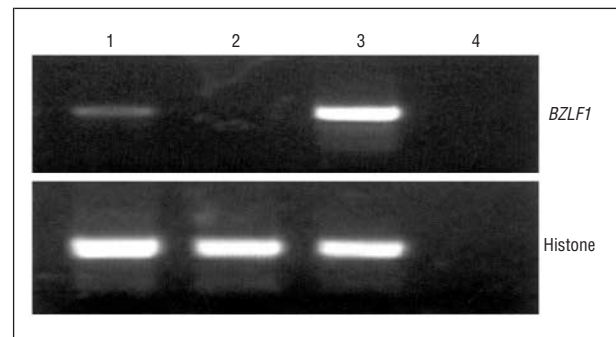


Figure 7. Skin biopsy was performed from the skin lesion induced by scratch patch testing with salivary gland extract of *Aedes albopictus* or nonspecific dermatitis lesion. Reverse transcriptase-polymerase chain reaction analysis using RNA isolated from the skin biopsy samples demonstrated the expression of a viral lytic-cycle transactivator *BZLF1* gene in the skin lesion (lane 1) but not in the nonspecific dermatitis lesion (lane 2). Reverse transcriptase-polymerase chain reaction analysis for histone was used to indicate that the RNA samples were intact and the input was similar for each reaction. Lane 1, skin lesion induced by scratch patch testing with the salivary gland extract of *A. albopictus*; lane 2, nonspecific dermatitis lesion; lane 3, B95-8 cells; lane 4, no RNA.

peat probe demonstrated that EBV DNA-positive cells oligoclonally proliferated. Third, the patient had repeated episodes of HMB with erythematous swelling and skin ulcer at the mosquito bite site associated with high fever, lymphadenopathy, and hepatosplenomegaly. Scratch patch testing with mosquito extracts also induced similar skin reactions. Approximately 50 cases of HMB have been reported in Japan,^{1-5,11} and several reports of such cases are observed in Korea, Taiwan,^{12,13} and

Epstein-Barr Virus (EBV) DNA Concentrations in Plasma Samples

Sample No.	EBV DNA Levels, Copies/mL	Days After Mosquito Bite	Conditions of Patient
1	1700	2	Skin lesions, high fever, and lymphadenopathy
2	540	3	Skin lesions, high fever, and lymphadenopathy
3	340	2	Skin lesions and high fever
4	<100	*	No systemic symptoms
5	<100		
6	<100		

*These blood samples were taken from the patient without systemic symptoms. Around these time points, there was no clear evidence of mosquito bites.

Mexico.¹⁴ As the cases accumulate, there is growing evidence indicating a close association among severe HMB and EBV-carrying NK cell lymphocytosis.^{11,15,16} The clinical and laboratory findings of our present case were in accordance with these previous reports. We previously demonstrated that the EBV-carrying NK cells in the HMB patients overexpressed surface FasL or soluble FasL and suggested that the enhanced FasL might be related to organ (or tissue) damage, such as intense skin lesions at mosquito bite sites and liver dysfunction.^{4,5} However, the relationship between EBV and HMB was still unclear because leukemic NK cells either with or without EBV usually express FasL constitutively.¹⁷

In the present study, we first examined which subset of lymphocytes is responsible for the severe skin reactions to mosquito bites. We immunostained the surface markers of the infiltrated cells in the skin lesion at the mosquito bite site and showed that CD4⁺ T cells were dominant, while CD8⁺ T and CD16⁺ NK cells were less frequently observed. In situ hybridization demonstrated that there were few EBV-positive cells, which accounted for approximately 5% of the total infiltrated lymphocytes. These results suggest that CD4⁺ T cells are the main contribution to skin reaction at mosquito bite sites. To examine which subset of lymphocytes reacts to mosquito antigens, CD4⁺ T, CD8⁺ T, and NK cells were purified from the patient's peripheral blood and were stimulated with mosquito salivary gland extracts. The proliferation assay showed that CD4⁺ T cells, but not CD8⁺ T cells or NK cells, responded to the mosquito extracts. To examine whether the CD4⁺ T cell response shows antigen specificity, we assessed the proliferative responses against each extract prepared from salivary glands of 5 species of mosquito. The extract from salivary gland of *A albopictus* induced the highest proliferative response of CD4⁺ T cells. In contrast, very low or no proliferations were observed in response to the extracts of *A sinensis* and *A stephensi*. Such proliferative responses to each kind of mosquito extracts correlated with the extent of skin reactions induced by prick testing. These results suggest that the patient has CD4⁺ T cells that may recognize certain antigens in mosquito salivary gland extracts, and these antigen-specific CD4⁺ T cells have an important role in the intensive skin reaction to mosquito bite.

Next, to investigate the interaction of CD4⁺ T cells stimulated by mosquito extract with EBV-carrying NK cells, we cocultured these cells in vitro and monitored the expression of EBV antigens. After coculture for 3 days, we detected EBV lytic-cycle antigen expression in the NK cells. In contrast, no lytic-cycle proteins were detected in the coculture of NK cells and nonstimulated CD4⁺ T cells. These phenomena suggest that CD4⁺ T cells, activated by exposure to mosquito extract, might play an important role in reactivation of latent EBV infection in NK cells. Furthermore, to investigate whether such phenomena in vitro also occurred in vivo, we assessed the expression of a viral lytic-cycle transactivator *BZLF1* messenger RNA in the skin lesion induced by scratch patch testing with mosquito salivary gland extract. As a result of the scratch patch testing, the expression of *BZLF1* gene was observed in the skin lesion. Moreover, EBV genome copy number in the plasma also increased in HMB conditions compared with normal conditions. Indeed, the patients with HMB generally have high titers of serum antibody to EBV lytic-cycle proteins such as anti-VCA and EA, which suggests that viral reactivation occasionally occurred in these patients. A recent article demonstrated that EBV-specific CD4⁺ T cells play an important role in reactivation of latent EBV infection in resting B cells through a CD40-dependent pathway.¹⁸ In the present case of EBV-carrying NK cell lymphocytosis, mosquito antigen-specific CD4⁺ T cells, rather than EBV-specific CD4⁺ T cells, seem to be involved in EBV reactivation, although the mechanism by which mosquito antigen-specific CD4⁺ T cells induce EBV reactivation in NK cells is not clear. Our preliminary experiment demonstrated that the supernatant of CD4⁺ T cells stimulated by mosquito extract also induced EBV reactivation in NK cells, which suggests that mosquito antigen-exposed CD4⁺ T cells could secrete soluble factors inducing EBV reactivation in NK cells. Understanding the exact mechanisms involved in EBV reactivation in NK cells will require additional study.

As a result of EBV reactivation in vivo, NK cells expressing EBV lytic-cycle antigens, cell-free EBV, or EBV-infected B cells may induce strong immune reactions and lead the patient to infectious mononucleosis-like systemic symptoms of HMB such as high fever, lymphadenopathy, and hepatosplenomegaly. Recent studies demonstrated that EBV infection to B cells induced the expression of host-encoded superantigens, which elicit potent, antigen-independent T-cell responses, and suggest that the T-cell activation by the superantigens could play a central role in EBV infection.^{19,20} Taken together, we propose a possible process of HMB in our patient as summarized in **Figure 3**, that is, mosquito antigen-specific CD4⁺ T cells are an important part in the primary skin reaction to mosquito bite and may play a key role in reactivation of latent EBV infection in NK cells, and this viral reactivation contributes to the pathogenesis of the infectious mononucleosis-like systemic symptoms of HMB in our patient.

In addition to HMB, other cutaneous manifestations have been reported in the patients with EBV-infected NK or T-cell proliferative disorders.¹⁶ Especially, there have been several reports of patients with

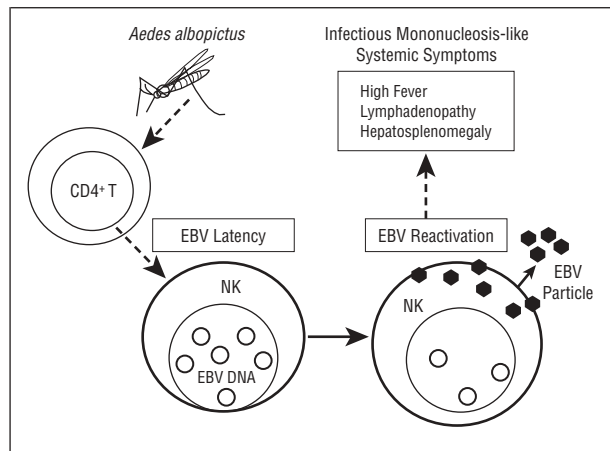


Figure 8. Induction mechanism of systemic hypersensitivity to mosquito bites (HMB) symptoms. CD4⁺ T cells stimulated by mosquito salivary gland extracts may play a key role in reactivation of latent Epstein-Barr virus (EBV) infection in natural killer (NK) cells, and this viral reactivation contributes to the pathogenesis of the infectious mononucleosis-like systemic symptoms of HMB.

severe hydroa vacciniforme-like eruption,^{16,21,22} characterized by recurrent necrotic papulovesicles in light-exposed and nonexposed areas. The patient in the present article also has shown hydroa vacciniforme-like eruption since she was 10 years old. This type of skin lesion was unrelated to mosquito bites. The difference of pathologic mechanism between HMB and hydroa vacciniforme-like eruption should be clarified by further studies.

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