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

Hideo Asada, MD; Sachiko Miyagawa, MD; Yasuyuki Sumikawa, MD; Yuji Yamaguchi, MD; Satoshi Itami, MD; Setsuo Suguri, MD; Masakazu Harada, MD; Yoshiki Tokura, MD; Shigeishi Ishihara, MD; Shiro Ohshima, MD; Kunihiko Yoshikawa, MD

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. 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. Furthermore, Ishihara et al 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. 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. 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 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...
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increased (as follows: the anti-EB viral capsid antigen (VCA) IgG and against EBV was compatible with chronic active EBV infection suggested to be NK cells. The pattern of serum antibody titers or CD19+ B cells. Southern blot analysis using EBV-terminal action (PCR) analysis after sorting lymphocytes demonstrated that EBV DNA existed in NK cells but not in CD4+ T, CD8+ T, 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).

**Tissue Preparation**

Skin 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.

**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 µL of phosphate-buffered saline (PBS), filtrated with a 45-µm 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 µg/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.

**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. Briefly, deparaffinized tissue sections of the site were treated with 10 µg/mL of proteinase K (Boehringer Mannheim GmbH Biochemical, Mannheim, Germany) for 30 minutes at 37°C, 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 Biochemical) 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-
taminations. 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 μmol/L of each of the forward and reverse primers. To detect expression of B2LF1 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 VCA (Chemicon, Temecula, Calif). Each cell population prepared by magnetic cell sorting was highly purified (>98%) by flow-cytometric analysis using a fluorescence-activated cell sorter FACScan (Becton Dickinson).

PROLIFERATION ASSAY

RPMI-1640 medium supplemented with penicillin-streptomycin mixture, 5×10^−5 M 2-mercaptoethanol, 2 mM glutamine, 10 mM 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 (5×10^6 cells/mL) were mixed with antigen-presenting cells (5×10^5 cells/mL), and cultured with 4 μg/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 5×10^6 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 μg/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). Cultures of unexposed CD4+ T cells with NK cells was performed in parallel. The cultured cells were cytocentrifuged 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.

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 cm^2 (Aedes albopictus), 0 cm^2 (Anopheles sinensis), 0 cm^2 (Anopheles stephensi), 8.3 cm^2 (Culex pipiens pallens), 2.1 cm^2 (Culex tritaeniorhynchus), and 0 cm^2 (phosphate-buffered saline solution [PBS]) (Figure 2A). Erythema with bulla was observed after 48 hours by scratch patch testing with the salivary gland extract from A albopictus.

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-
phocytes. In situ hybridization with EBER1 probe demonstrated that approximately 5% of infiltrating cells were positive for Epstein-Barr virus in the same lesion as panel A (original magnification ×100.)

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 µg/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).

EXPRESSON 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 non-stimulated 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 (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).

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-
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 repeat 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

Figure 5. A, Peripheral blood mononuclear cells (PBMCs) (5 × 10⁶ cells/mL) from the patient and healthy volunteers (negative controls [NCs] 1-3) were stimulated with 4 µg/mL of the mixture of salivary gland extracts from 5 species of mosquito (MIX). CD4⁺ T, CD8⁺ T, and natural killer (NK) cells (5 × 10⁶ 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 × 10⁶ cells/mL) from peripheral blood of the patient were stimulated with 4 µg/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 CD34⁺ T cells stimulated (A-B) or nonstimulated (C-D) by the salivary gland extract of Aedes albopictus (original magnification ×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).

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.
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 vivo 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. 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. Taken together, we propose a possible process of HMB in our patient as summarized in Figure 8, 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. 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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Corresponding author and reprints: Hideo Asada, MD, Department of Dermatology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan (e-mail: asadah@naramed-u.ac.jp).

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