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Case report

## **Flow cytometric analysis of skin blister fluid induced by mosquito bites in a patient with chronic active Epstein-Barr virus infection**

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**Running title:** Skin blister fluid of CAEBV

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## Abstract

In chronic active Epstein-Barr virus (EBV) infection (CAEBV), ectopic EBV infection has been described in T or natural killer (NK) cells. NK cell-type infection (NK-CAEBV) is characterized by large granular lymphocytosis, high IgE levels, and unusual reactions to mosquito bites, including severe local skin reactions, fever and liver dysfunction. However, the mechanisms underlying these reactions remain undetermined. Herein, we describe a patient with NK-CAEBV whose blister fluid after mosquito bites was analyzed. The patient exhibited significant increases in the percentage of CD56<sup>+</sup> NK cells in the fluid compared with a simple mosquito allergy, in which the majority of infiltrated cells were CD203c<sup>+</sup> cells, indicating basophils and/or mast cells. His fluid also contained CD203c<sup>+</sup> cells, and his circulating basophils were activated by mosquito extracts *in vitro*. These results suggest that CD203c<sup>+</sup> cells as well as NK cells may play pathogenic roles in the severe skin reactions to mosquito bites in NK-CAEBV.

**Key words:** Chronic active Epstein-Barr virus infection, NK cells, Mosquito bites, Skin blister, CD203c

## Introduction

Epstein-Barr virus (EBV) is a ubiquitous herpes virus that infects the majority of the world's population by adulthood [1]. Primary EBV infection is usually inapparent but occasionally presents as acute infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [1]. After acute infection, EBV persists in B cells for the lifetime of the seropositive normal host. EBV infection has been also linked with a variety of malignancies, as well as lymphoproliferative disorders of T and natural killer (NK) cells, including hemophagocytic lymphohistiocytosis, chronic active EBV infection (CAEBV), and nasal-type lymphomas [2]. In these cases, T or NK cells are the cellular targets of EBV infection, and the pathogenic roles of ectopic EBV infection have been described [3].

CAEBV is characterized by chronic and recurrent infectious mononucleosis-like symptoms and extremely high viral loads in peripheral blood [4]. Based on the cellular targets of EBV, CAEBV is largely divided into 2 clinically distinct subtypes; T cell-type and NK cell-type infections [5]. T cell-type infection is associated with fever, high titers of anti-EBV antibodies, and higher mortality, whereas NK cell-type infection is characterized by large granular lymphocytosis, elevated serum IgE levels, and unusual reactions to mosquito bites. In the latter subtype, affected subjects may present with severe local skin reactions including large erythematous swellings, blister formation, and necrotic ulcerations in addition to systemic symptoms, such as fever, lymphadenopathy, and liver dysfunction after exposures to mosquito bites. This condition is so called "hypersensitivity to mosquito bites", however, the immunological mechanism leading to the severe reactions that are quite different from

those of simple mosquito allergy [6] remains largely unknown. In this report, we describe a patient with NK cell-type CAEBV who exhibited unusual reactions to mosquito bites, and discuss the immunological reactions associated with mosquito bites.

## Patient and Methods

### Case report

A 7-year-old Japanese boy was admitted to our hospital because of high fever, malaise, and severe local skin reactions after mosquito bites (Fig. 1A, B). His familial and past histories were unremarkable. Physical examination showed aphthous stomatitis and lymphadenopathy, but the patient had no hepatosplenomegaly. Laboratory studies revealed a WBC count of 7,400/ $\mu$ L, a hemoglobin level of 12.2 g/dL, and a platelet count of  $21.8 \times 10^4$ / $\mu$ L. The differential leukocyte count indicated 63.9% neutrophils, 31.2% lymphocytes, 3.8% monocytes, 0.8% eosinophils, and 0.3% basophils. The biochemical tests showed moderate liver dysfunction (aspartate aminotransferase 151 IU/L, alanine aminotransferase 109 IU/L). The serum soluble interleukin-2 receptor level was elevated to 2,053 IU/mL (normal, 230-550 IU/mL). Immunoglobulin (Ig) levels were normal (IgG 1,340 mg/dL, IgA 386 mg/dL, IgM 97 mg/dL) except for IgE (1,055 IU/mL). Immunophenotypic analysis of the lymphocytes showed a markedly increased percentage of CD56<sup>+</sup> NK cells (51.8%; normal,  $8.0 \pm 4.4\%$ ) with a decrease in the percentage of CD3<sup>+</sup> T (36.3%; normal,  $69.5 \pm 4.6\%$ ), CD4<sup>+</sup> T (23.1%; normal,  $43.1 \pm 6.0\%$ ), CD8<sup>+</sup> T (9.5%; normal,  $22.0 \pm 5.4\%$ ), and CD20<sup>+</sup> B (3.5%; normal,  $11.2 \pm 3.5\%$ ) cells in the peripheral blood. More than 90% of CD56<sup>+</sup> NK cells expressed the activation marker HLA-DR that is generally negative in normal NK cells [7]. Serological test for EBV revealed that anti-viral capsid antigen IgM was negative in the presence of anti-viral capsid antigen IgG (40 times) and anti-EBV nuclear antigen antibody (40 times). The number of EBV DNA copies in the peripheral blood was increased markedly to  $1.3 \times 10^6$  copies/ $10^6$  cells (normal,  $< 2.0 \times 10^1$  copies/ $10^6$  cells). A skin biopsy from the bite sites exhibited

epidermal necrosis and massive infiltration of mononuclear cells around small vessels in the dermis and subcutaneous adipose tissues (Fig. 1C). Most of the infiltrating cells were EBV positive by *in situ* hybridization for EBER-1 (Fig. 1D). Southern blot analysis of EBV terminal repeats showed monoclonal expansion of EBV-infected cells in the peripheral blood (Fig. 1E). Informed consent was obtained according to the Declaration of Helsinki, and approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science.

### **Cell preparation and *in situ* hybridization for EBER-1**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation. CD4<sup>+</sup> T, CD8<sup>+</sup> T, CD19<sup>+</sup> B, and CD56<sup>+</sup> NK cells were purified from PBMCs using mAb-coated magnetic beads (BD PharMingen, San Diego, CA) [8]. The purity of the separated cells was 91.4%, 90.6%, 97.7%, and 84.0%, respectively, as determined by flow cytometric analysis. *In situ* hybridization for EBER-1 was performed as described previously [9].

### **Flow cytometric analysis of skin blister fluid**

Blister fluid was collected into syringes from the patient and 3 children with mosquito allergy who exhibited erythema and blister formations within 24 hours after mosquito bites. Despite the presence of the blister formation, there were no systemic symptoms and no evidence of lymphoproliferative diseases in these 3 subjects, two of which suffered from mild bronchial asthma. Flow cytometric analysis of the blister lymphocytes was performed immediately after sample collection using the following monoclonal antibodies (mAbs): PE-conjugated anti-CD3 and anti-CD56 (BD PharMingen); PE-conjugated anti-CD203c and FITC-conjugated anti-CD16

(Immunotech, Marseille, France); FITC-conjugated anti-CD45RO (Dako, Glostrup, Denmark). All incubation and washing steps were carried out in ice-cold phosphate-buffered saline (PBS). After lysis of the erythrocytes and washing, stained cells were analyzed with a FACSCalibur flow cytometer using the CellQuest software (BD Bioscience, Tokyo, Japan) [8].

### **Mosquito extracts and basophil activation test**

Mosquito extracts were prepared from whole bodies of 2 species that are endemic in Japan (*Aedes albopictus* and *Culex pipiens pallens*). One hundred mosquitoes were homogenized in 2 mL of PBS, and the samples were centrifuged at 14,000g for 10 minutes. The supernatants served as the mosquito extracts and were stored at -80°C until use. The total protein concentrations were 7.0 mg/mL and 6.6 mg/mL, respectively, as assessed by BCA protein assay (Pierce, Rockford, IL) [10]. Mite allergen was obtained from Torii Pharmaceutical Co., Ltd (Osaka, Japan). For the basophil activation test [11], peripheral whole blood was obtained, washed twice in PBS, and suspended in RPMI medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum and antibiotics. Aliquots (200 µL) were incubated with serial dilutions of the mosquito extracts at 37°C for 40 minutes. Cells were then washed, and incubated with FITC-conjugated anti-CD63 (Immunotech) and PE-conjugated anti-CD203c mAbs. Basophils were detected on the basis of forward light-scatter characteristics and CD203c expression.



## Results and Discussion

The patient's clinical and laboratory findings were consistent with CAEBV [4], and NK cell-type infection was likely the case because of the presence of NK cell lymphocytosis, high serum IgE levels, and unusual reactions to mosquito bites [5]. To confirm the ectopic EBV infection of NK cells, we performed *in situ* hybridization for EBER-1 on PBMCs that were isolated from the patient and sorted. As shown in Figs. 1F and 1G, CD56<sup>+</sup> NK cells were found to account for the majority of EBER-1<sup>+</sup> cells, with negligible EBER-1 identified in CD4<sup>+</sup> T, CD8<sup>+</sup> T, and CD19<sup>+</sup> B cells. Although the precise mechanism underlying the severe skin reactions after mosquito bites in patients with NK cell-type CAEBV remains mostly unclear, it has been recognized recently that skin biopsy specimens from such patients are notable for inflammatory reactions composed primarily of EBV-infected NK cells [3, 12, 13]. In addition, studies of CD4<sup>+</sup> T cells from patients with NK cell-type CAEBV showed that co-culturing of NK cells and CD4<sup>+</sup> T cells activated by mosquito extracts induced the expression of EBV lytic-cycle transcripts in NK cells, and that the proliferative response to mosquito extracts was strongly enhanced by the co-existence of NK cells, thus suggesting the pathogenic roles of mosquito antigen-specific CD4<sup>+</sup> T cells in disease development [14, 15]. Indeed, skin biopsy specimens from our patient exhibited migration of many EBV-infected cells to the bite sites along with the infiltration of CD3<sup>+</sup> T cells (data not shown). On the other hand, in subjects with mosquito allergy, reactions to mosquito bites are caused by allergic reactions to mosquito salivary allergens [6, 16]. Immediate wheals and flares induced by mosquito bites correlate with both mosquito salivary gland-specific IgE and IgG levels, whereas bite-induced indurations correlate with T lymphocyte proliferation responses to

mosquito allergens [6, 16]. However, it still remains unknown whether these allergic reactions to mosquito salivary allergens observed in allergic individuals are also involved in the NK cell-type CAEBV.

To address this issue, we performed immunophenotypic analysis of lymphocytes infiltrated in skin blister fluid after mosquito bites using flow cytometry. As shown in Fig. 2, in individuals with simple mosquito allergy, the majority of blister-infiltrated cells were found to be CD45RO<sup>+</sup> cells that did not express T-cell markers but did express CD203c, indicating basophils and/or mast cells [17]. Small populations of CD3<sup>+</sup> T and CD56<sup>+</sup> NK cells were also identified in the fluid. Thus, this approach may allow us to obtain a reliable assessment of the local immunological conditions at the bite site. We next analyzed the patient's blister fluid on two different occasions and found significant increases in the percentage of CD56<sup>+</sup> NK cells in contrast to mosquito allergy (Fig. 2). Most of these NK cells expressed activation marker HLA-DR (data not shown) suggesting their involvement of the local skin reactions. Activation status did not differ between blister-infiltrated NK cells and circulating NK cells. It is also important to note that CD203c<sup>+</sup> cells were present in the patient's blister fluid despite the variable percentage of positive cells.

To evaluate the contribution of CD203c<sup>+</sup> cells to the severe skin reactions in the patient, we assessed the responses of circulating basophils to the mosquito extracts in terms of upregulation of surface CD63 and CD203c. As shown in Fig. 3, we did not observe any significant increase of the percentage of CD63<sup>+</sup> cells or the expression of CD203c on basophils after stimulation with the mite antigen, as well as the extracts from *Culex pipiens pallens* (data not shown). In contrast, the patient's basophils demonstrated a clear increase of CD63 and CD203c expression in a dose-dependent manner after stimulation of the extracts from *Aedes albopictus* that were not able to

stimulate basophils from normal adult volunteers (data not shown). Mosquito-specific IgE attached to the Fcε receptors of the basophils might be cross-linked by the mosquito extracts in this system in the patient. These results indicated that CD203c<sup>+</sup> cells might be important to the evolution of the intense skin reactions to mosquito bites in the patient. Recent evidence indicates that basophils play a crucial role in the development of IgE-mediated chronic allergic inflammation in the skin as initiators rather than effectors of the inflammatory response [18]. It is therefore tempting to speculate that mosquito bites activate basophils and/or mast cells to secrete soluble factors that recruit inflammatory cells including EBV-infected NK cells to the bite site, resulting in the severe local skin reactions in NK cell-type CAEBV. The elevated serum IgE levels that are seen in most cases of NK cell-type CAEBV [5] might support this hypothesis. It has been demonstrated recently that basophils produce macrophage-derived chemokine (MDC) in response to IgE [19]. Because MDC is a potent chemoattractant for CC chemokine receptor 4-expressing cells including NK cells [20], MDC might contribute to attract EBV-infected NK cells to the bite site. Further studies will be necessary to understand hypersensitivity reactions to mosquito bites in association with EBV infection.

In summary, our studies demonstrate the usefulness of the analysis of blister-infiltrated cells and provide implications for CD203c<sup>+</sup> cells as well as NK cells in the pathogenesis of severe skin reactions to mosquito bites in NK cell-type CAEBV.

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## Figure Legends

### Figure 1. Skin lesions, skin biopsy and characterization of EBV-infected cells.

Intense inflammatory skin lesions after mosquito bites (A, B). Hematoxylin and eosin staining (C) and *in situ* hybridization for EBER-1 (D) of a skin lesion. Original magnification x100. (E) Southern blot analysis of EBV terminal repeats. Genomic DNA from positive (B95-8; lane 1) and negative (lane 2) controls, and the patient (lane 3) were digested with BamHI and hybridized with a specific probe. M indicates a size marker and the arrow highlights a monoclonal band. (F) Detection of EBER-1<sup>+</sup> cells in the patient's CD56<sup>+</sup> NK cells. Original magnification x400. (G) Frequency of EBV-infected cells. Shown are the percentage of EBER-1<sup>+</sup> cells within the patient's lymphocyte subsets.

### Figure 2. Flow cytometric analysis of skin blister fluid.

(A) Immunophenotype of blister-infiltrated cells. A region of mononuclear cells was gated for fluorescence analysis. The percentage of cells gated in each quadrant is shown. (B) Frequency of T, NK, and CD203c<sup>+</sup> cells. Data are expressed as the percentage of each of the cell subsets. Error bars represent standard deviation.

### Figure 3. Activation of basophils by mosquito extracts.

A region of mononuclear cells was gated (upper left), and a second gate was set for CD203c<sup>+</sup> cells (upper middle). The patient's basophils were cultured with or without stimulation of allergens. The mean fluorescence intensity (MFI) of CD203c<sup>+</sup> cells and the percentage of CD63<sup>+</sup> cells within CD203c<sup>+</sup> cells are shown.



Figure 1

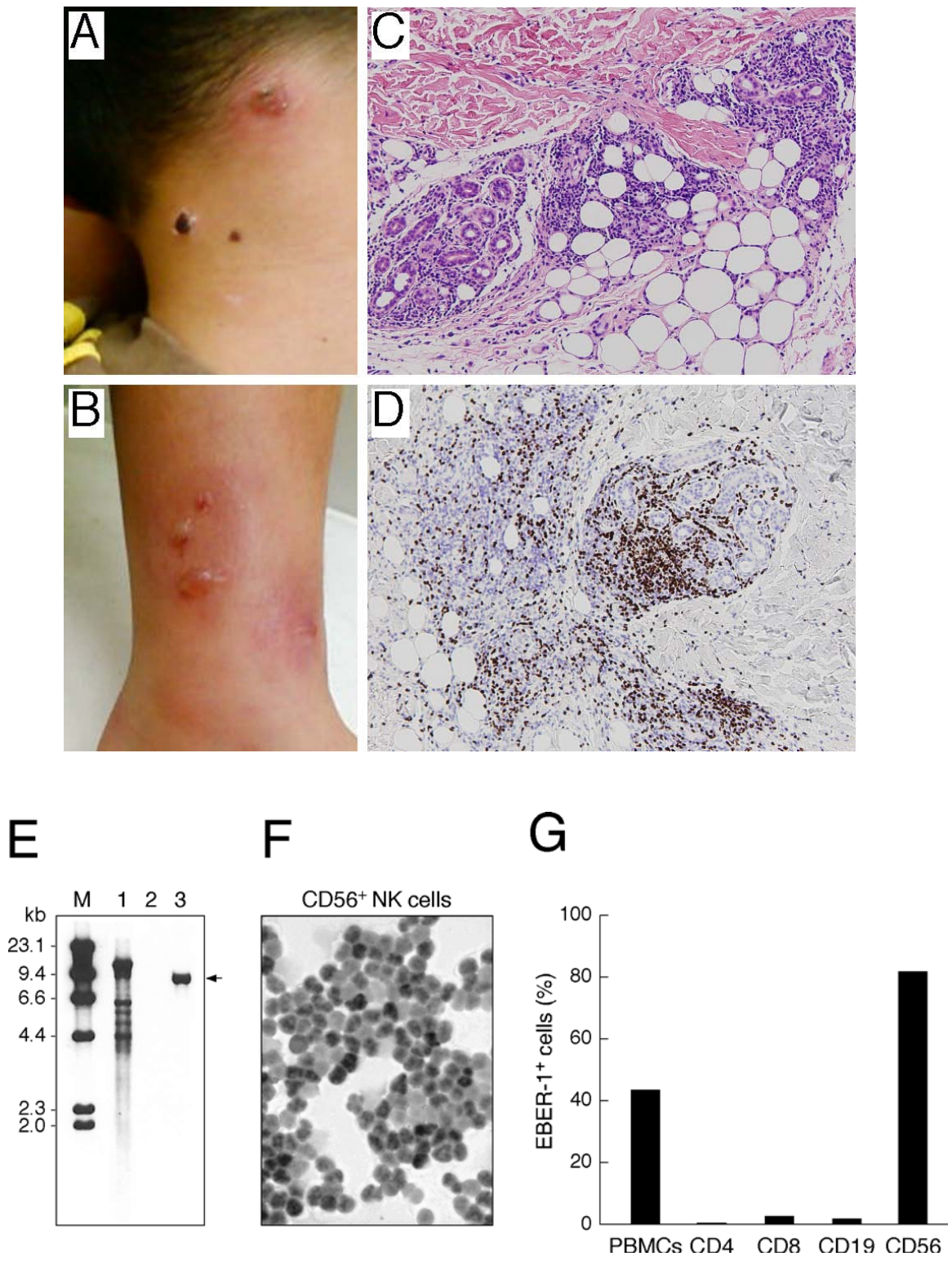
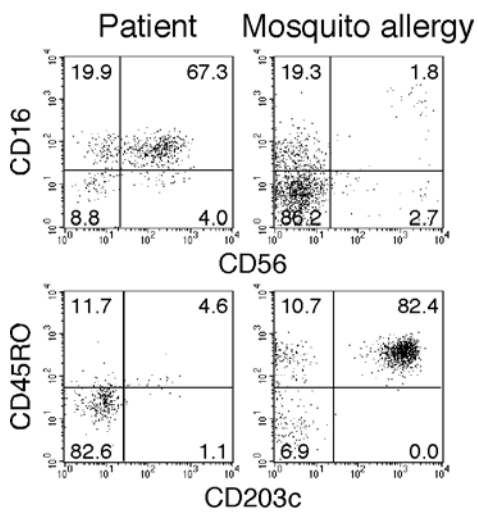


Figure 2

A



B

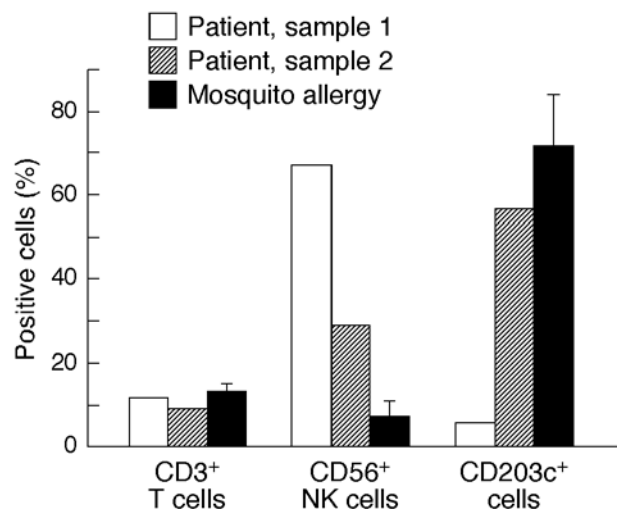


Figure 3

