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Hypersensitivity to Mosquito Bites Conceals Clonal Lymphoproliferation of Epstein-Barr Viral DNA-positive Natural Killer Cells

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In order to clarify the relationship between Epstein-Barr (EB) virus and hypersensitivity to mosquito bites (HMB), and to search for the mechanism which induces EB virus-associated lymphoproliferative diseases, we investigated patients with HMB, using hematological, immunological and virological techniques. Among 5 cases of HMB, $CD56^+$ cells had proliferated and $CD3^+$ cells were diminished in 4 cases. Although anti-EB virus antibody titers were not consistent with chronic active EB virus infection, EB viral DNA was detected in the peripheral blood mononuclear cells in all 5 cases. Moreover, EB viral DNA-positive cells had proliferated monoclonally in 4 cases, and biclonally in 1 case. It was proved that most of the EB viral DNA existed in natural killer (NK) cells through polymerase chain reaction analysis. These findings suggest that the basis of HMB may be clonal lymphoproliferation of EB viral DNA-positive NK cells and this hematological abnormality may induce the characteristic symptoms of HMB. In some cases, the proliferating NK cells can metamorphose into leukemic cells, and hemophagocytic syndrome, which has been assumed to be a complication of HMB, may then occur.

Key words: Hypersensitivity to mosquito bites — Epstein-Barr virus — Natural killer cell — Clonal lymphoproliferation — Granular lymphocyte proliferative disorder

Hypersensitivity to mosquito bites (HMB) is characterized by intense local skin symptoms, which consist of not only erythema or bulla, but also ulcer or scar, and general symptoms such as high fever following mosquito bites.[1] This disorder is so rare that there have been only about 40 cases reported in the literature. The first case of HMB was reported from Florida, USA, in 1938.[2] However, subsequent cases have been reported from East Asia, Japan[3–5] and Taiwan.[6] Many patients with HMB have died of hemophagocytic syndrome (HPS), such as malignant histiocytosis (MH) and virus-associated hemophagocytic syndrome (VAHS).[7, 8]

Recently Epstein-Barr (EB) virus has been considered to be associated with not only B-cell malignancy, but also T-cell malignancy[9, 10] and natural killer (NK) cell granular lymphocyte proliferative disorder (NK-GLPD).[11] When we investigated cases of chronic active EB virus infection (CAEBV) to clarify the relationship between lymphoproliferative diseases (LPDs) and EB virus, 6 out of 11 cases of CAEBV had advanced to LPDs and 4 of these 6 cases had a clinical history of HMB.[12] This finding suggests that EB virus-associated LPDs may have a relationship with HMB, and the HPS following HMB might have been associated with EB virus.

To clarify the relationship between EB virus and HMB and to search for the mechanism which induces EB virus-associated LPD, we investigated several cases of HMB using hematological, immunological and virological techniques.

MATERIALS AND METHODS

Patients Six Japanese patients with HMB were entered in this study. The profiles of patients are shown in Table I. None of them had any blood relationship with the others and their places of residence were all different.

Clinical symptoms of the patients were typical, namely intense local skin symptoms and high fever lasting for 2 to 3 days following mosquito bites. One patient (case 3) presented with hepatosplenomegaly and another patient (case 4) with hepatomegaly. All but one patient had been diagnosed as having HMB and they were treated with

Table I. Profiles of the Patients

Case	Sex	Age at onset	Age at present	IgE (IU/ml)	WBC (/μl)	Band (%)	Seg (%)	Lym (%)	Mo (%)	Eo (%)	Ba (%)	AtLy (%)	GL (%)
1	F	12	14	11,000	5,400	2	27	60	6	1	0	4	0
2	F	17	21	2,000	6,600	5	80	14	0	0	1	0	0
3	M	4	10	4,990	8,600	1	15	33	4	4	0	0	43
4	F	2	10	1,000	4,700	1	10	25	6	1	0	0	57
5	M	10	20	2,931	4,510	3	35	46	11	4	1	0	0
6	M	1	10	20,854	7,800	2	42	46	5	4	0	1	0

Abbreviations: WBC, white blood cells; Band, band forms; Seg, segmented forms; Ly, lymphocytes; Mo, monocytes; Eo, eosinophilic leukocytes; Ba, basophilic leukocytes; AtLy, atypical lymphocytes; GL, granular lymphocytes.

symptomatic therapy only when symptoms of HMB occurred. One patient (case 6) had been diagnosed as having IgA nephropathy, and he has been treated with oral prednisolone.

While all of the patients manifested high levels of IgE, ranging from 1,000 to 20,854 IU/ml, some of them were suffering from other atopic diseases, such as allergic rhinitis (case 1), urticaria (case 2) and atopic dermatitis (case 5). Two patients (cases 3 and 4) exhibited granular lymphocytosis in the peripheral blood, but hematological findings were not unusual in the rest, and none of the patients had other hematological or immunological histories or complications. None had been diagnosed as having infectious mononucleosis or primary EB virus infection, or CAEBV.

Anti-EB virus antibody titers To investigate the relationship between HMB and EB virus infection, we examined the anti-viral capsid antigen (VCA) IgG, IgA and IgM, anti-early antigen (EA) IgG and IgA, and anti-EB virus-associated nuclear antigen (EBNA) antibody in the serum by means of fluorescent antibody methods.

Subsets of lymphocytes To clarify the phenotype of proliferating granular lymphocytes in cases 3 and 4, and to investigate the phenotype of peripheral lymphocytes in the rest, we performed cell surface analysis by flow cytometry using direct immunofluorescence methods with monoclonal antibodies (mAbs). The patients' peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Percoll (v/v, density 1.078 g/ml). After incubation with mAbs labeled with fluorescein isothiocyanate or phycoerythrin, the cells were subjected to flow cytometric analysis (Facscan 420; Becton Dickinson). The mAbs were Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), NKH-1 (anti-CD56) and anti-HLA-DR (Becton Dickinson, Mountain View, CA).

Polymerase chain reaction (PCR) analysis Similarly, to investigate the relation between HMB and EB virus, we performed PCR analysis.

Genomic DNA was extracted from the PBMCs and the serum of the patients according to standard methods. PCR amplification of EB viral DNA was performed as follows. The reaction mixture contained 5 μl of genomic DNA, 5 μl of lysis buffer (100 m*M* Tris-HCl, 200 m*M* NaCl, 10 m*M* EDTA, 4 *M* urea and 0.5% *N*-lauroylsarcosine), 1 μl of 10 m*M* deoxyribonucleotide triphosphate mixture, 1 U of Taq DNA polymerase, 5 μl of internal control DNA and 1 μl of each 15 m*M* primer in 50 μl total volume. The mixtures were overlaid with 50 μl of mineral oil to prevent evaporation. PCR was performed using a Perkin Elmer Cetus Thermal Cycler (Perkin Elmer Cetus, Überlingen, Germany) at the following setting; 94°C, 1 min for denaturation, 60°C, 1 min for annealing and 72°C, 2 min for extension, for 40 cycles. The final product (10 μl) was analyzed by agarose gel electrophoresis (3% agarose in Tris-borate-edetic acid) and visualized with UV light after ethidium bromide staining.

The primers were designed to amplify an EB virus-specific 161 bp fragment which exists in the *Bam*HI-W region. The primer sequences were as follows; F (sense) 5′TCCTCGTCCAGCAAGAAGAG3′, R(anti-sense) 5′CAACTTGAGGCAGCCTAATCC3′.

The positive control was DNA extracted from Raji cells and the negative control was DNA extracted from the PBMCs of a healthy volunteer who had had infectious mononucleosis. DNA from human placenta was used as an internal control. In addition, to confirm the specificity of HMB in PCR analysis for EB virus, we used DNA extracted from the PBMCs of a patient with mosquito allergy who did not present with general symptoms, but showed remarkable local skin symptoms.

Southern blot hybridization To confirm the presence of EB viral DNA in the peripheral lymphocytes and to investigate the clonality of proliferating lymphocytes, we carried out Southern blot hybridization. DNA was extracted from PBMCs stored at −80°C, and 5 μg of DNA was digested with 50 units of restriction endonuclease,

*Bam*HI or *Eco*RI, at 37°C for 3 h. The digested DNA was subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon filter by the Southern blotting technique.[13] Filter-bound DNA fragments were hybridized with ^{32}P-labeled probes and visualized on an autoradiogram.

We used *Bam*HI-W fragments to investigate the presence of EB viral DNA.[14] With the samples in which EB viral DNA had been detected, we used the terminal repeat sequences (kindly provided by Dr. K. Hirai, Department of Virology and Immunology, Tokyo Medical and Dental University, Tokyo) to clarify the clonality of EB viral DNA-positive cells.[15] In addition, we used the immunoglobulin heavy chain J_H gene probe[16] and the T-cell receptor $C_\beta I$ gene probe[17] to confirm the genotype of proliferating cells.

PCR analysis after sorting lymphocytes To establish the phenotype of EB viral DNA-positive cells, we sorted PBMCs by flow cytometry, utilizing the direct immunofluorescence method with mAb, NKH-1. The obtained CD56$^+$ cells and CD56$^-$ cells were used for PCR analysis.

RESULTS

Anti-EB virus antibody titers The anti-EB virus antibody titers of the six patients are shown in Table II.

Table II. Anti-EB Virus Antibody Titers

Case	VCA			EA		EBNA
	IgG	IgA	IgM	IgG	IgA	
1	1,280	160	<10	5,120	160	160
2	2,560	160	<10	160	<10	<10
3	160	ND	<10	10	ND	<10
4	320	40	<10	40	<10	20
5	160	<10	<10	<10	<10	20
6	80	<10	<10	20	<10	80

Abbreviations: VCA, viral capsid antigen; EA, early antigen; EBNA, EB virus-associated nuclear antigen; ND, not done.

Anti-VCA IgA was positive in three of five cases examined, anti-EA IgG was positive in all but one case, and anti-EA IgA was positive in one case. Interestingly, anti-EBNA was negative in two cases, although anti-VCA IgG was positive in all cases.

Subset of lymphocytes The phenotype of peripheral lymphocytes of five patients is shown in Table III. CD56$^+$ cells had proliferated and CD3$^+$ cells were diminished in not only cases 3 and 4, in which granular lymphocytosis was observed, but also cases 1 and 5. Since prednisolone had been given to patient 6, this patient was not investigated here.

PCR analysis The results of PCR analysis are shown in Table III. EB viral DNA was detected in the PBMCs in all six cases and in the serum in three of four examined

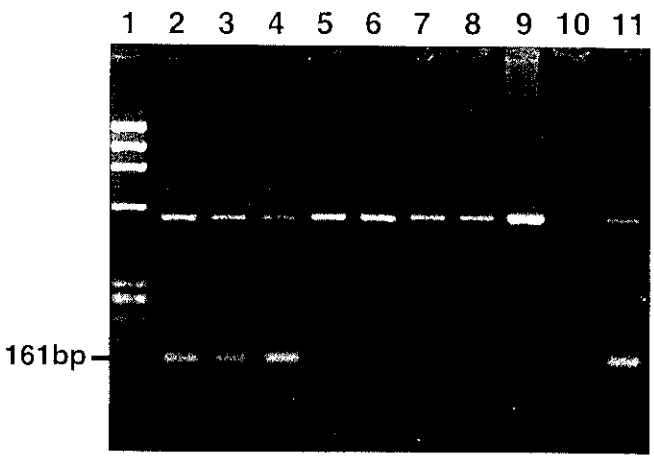

Fig. 1. The results of PCR analysis. The PCR products were detected in the cases of HMB (cases 1, 2, 3, 4, 5 and 6), but not in a case of mosquito allergy which involved only remarkable local skin symptoms (case 7) or in negative controls. Lane 1, markers; lanes 2–8, cases 1–7; lane 9, negative control (human DNA); lane 10, negative control (buffer); lane 11, positive control (Raji cells).

Table III. Phenotype, PCR and Southern Blot Hybridization

Case	Phenotype (%)					PCR		Southern blot	
	CD3	CD4	CD8	CD56	HLA-DR	PBMC	Serum	*Bam*HI-W	TR
1	29.0	16.0	23.0	64.0	3.0	+	ND	+	D
2	53.0	36.0	19.0	14.0	56.0	+	ND	+	S
3	27.4	17.1	12.4	56.5	62.6	+	+	+	S
4	37.0	24.0	13.3	45.3	56.7	+	+	+	S
5	39.9	24.5	17.8	33.5	44.4	+	+	+	S
6	ND	ND	ND	ND	ND	+	−	ND	ND

Abbreviations: PBMC, peripheral blood mononuclear cell; TR, terminal repeat; ND, not done; D, double band; S, single band.

cases. These data suggest that EB virus may have had some effect on these six cases (Fig. 1).

Southern blot hybridization The results of Southern blot hybridization are also shown in Table III. EB viral DNA was detected in all examined cases with *Bam*HI-W fragment as a probe (Fig. 2). These data indicate that HMB may be associated with EB virus infection.

The clonality of EB viral DNA-positive cells was assessed in five cases. This examination showed that the EB viral DNA-positive cells in case 1 were biclonal, and those in cases 2, 3, 4 and 5 were monoclonal (Fig. 3). These data mean that EB viral DNA-positive cells clonally proliferated.

Rearrangement of J_H gene and $C_\beta 1$ gene was not observed in any case (data not shown). This result indicates that the proliferating EB viral DNA-positive cells may not have developed from T-cells or B-cells.

PCR analysis after sorting lymphocytes The results of sorting analysis are shown in Fig. 4. These data indicate that most of the EB viral DNA existed in $CD56^+$ cells (NK cells), and a little in $CD56^-$ cells (possibly B-cells). In addition, the data suggest that clonal proliferation of EB viral DNA-positive $CD56^+$ cells may occur even in cases (e. g., case 2) in which there is no marked proliferation of $CD56^+$ cells.

DISCUSSION

Although HMB is a rare disorder,[1,2] several dermatologists and immunologists have tried to clarify the mechanism which induces these characteristic symptoms.[3–5] However, it has become apparent that about half of the patients with HMB died of MH.[7,8]

In this study, two of five patients manifested NK-GLPD and the other patients had been diagnosed as having clonal lymphoproliferation of NK cells. Moreover, these proliferating cells contained EB viral DNA. As these patients are distributed throughout the course of HMB, it appears that one of the common pathological findings in HMB may be clonal lymphoproliferation of EB viral DNA-positive NK cells.

Although about half of the patients with HMB died of MH,[7,8] most of the recent patients have manifested NK-GLPD[18,19] and this was the cause of death in some cases. The clinical entity of MH was proposed by Rappaport in 1966,[20] but the criteria for MH have varied.[21] Cases which had been diagnosed as MH have since been diagnosed as VAHS or HPS associated with leukemia or lymphoma.[22] These facts suggest that some of the cases of MH following HMB may have been VAHS or HPS associated with leukemia or lymphoma. Our data and the above facts suggest that the pathogen-

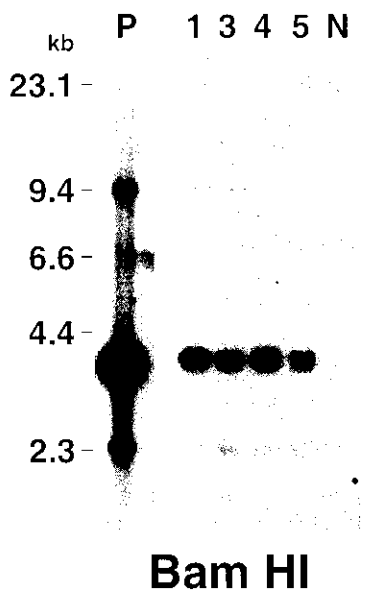

Fig. 2. The results of Southern blot hybridization with *Bam*HI-W as a probe. The *Bam*HI-W fragments in EB viral DNA were detected in the PBMCs derived from cases 1, 3, 4 and 5. In case 2, the *Bam*HI-W fragments were detected in another hybridization. P, positive control; lanes 1–5, cases 1–5; N, negative control.

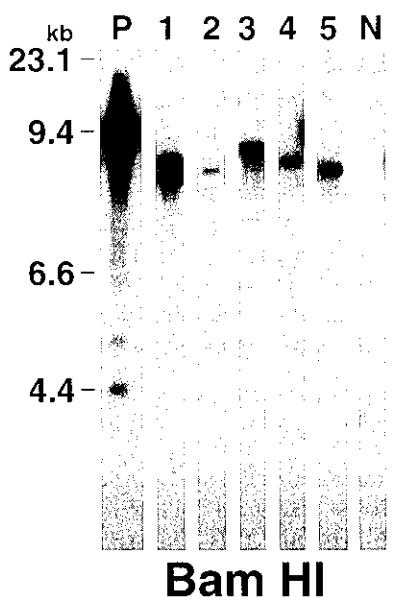

Fig. 3. The results of Southern blot hybridization with terminal repeat sequences as a probe. While a double band was detected in case 1, a single band was detected in cases 2, 3, 4 and 5. P, positive control; lanes 1–5, cases 1–5; N, negative control.

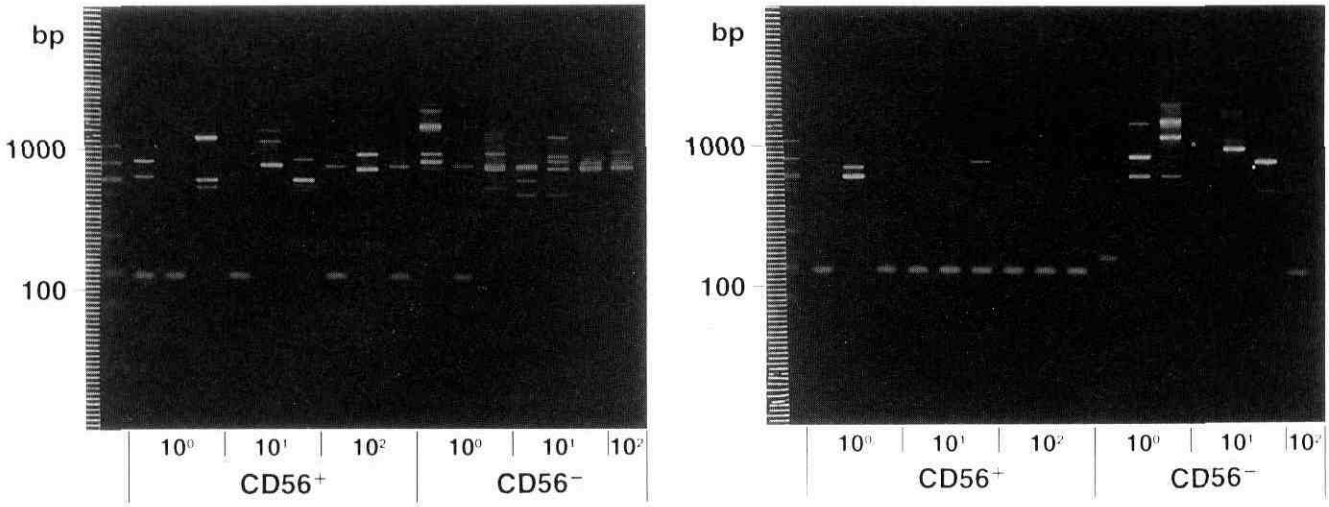

Fig. 4. The results of PCR analysis after sorting lymphocytes with anti-CD56 antibodies. EB viral DNA was amplified at the size of 161 bp in 2 of 3 wells containing 10^0 $CD56^+$ cells, in 3 of 3 wells containing 10^1 cells and in 3 of 3 wells containing 10^2 cells, while it was amplified in 1 of 3 wells containing 10^0 $CD56^-$ cells, in none of 3 wells containing 10^1 and in 1 of 1 well containing 10^2 cells, respectively, in case 5. Even in case 2, EB viral DNA was amplified in 2 of 3 wells containing 10^0 and 10^2 $CD56^+$ cells, and in 1 of 3 wells containing 10^1 cells, although it was amplified in only 1 of 7 wells containing $CD56^-$ cells.

esis of HMB may be clonal lymphoproliferation of EB viral DNA-positive NK cells. This immunohematological abnormality may induce the characteristic symptoms of HMB. In some cases, the proliferating NK cells may metamorphose into leukemic cells, leading to HPS. Indeed, a case of HPS associated with NK cell leukemia has been reported.[23]

On the other hand, the criteria for CAEBV require that there should be no evidence of any prior immunological abnormality which might explain the condition.[24] The mechanism by which the clonal lymphoproliferation of EB viral DNA-positive NK cells is induced is unknown. If this lymphoproliferation is induced by an immunological abnormality, CAEBV with HMB can be regarded as an independent clinical entity from CAEBV without HMB, so HMB alone can be assumed to be a clinical entity which is included in EB virus-associated LPDs.

The mechanism which induces the clonal lymphoproliferation of EB viral DNA-positive NK cells is unknown. We also do not know who may be at risk of HMB. To clarify the mechanism and to elucidate the factors which influence this mechanism, we will need to investigate more cases of HMB from epidemiological, hematological, immunological, virological and etiological viewpoints.

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