Chronological changes in Epstein-Barr virus genome and subsets of peripheral mononuclear cells in a case of HLH

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ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a rare disorder characterized by high grade fever, heaptosplenomegaly, lymphadenopathy and cytope- nia in association with hypercytokinemia and mac- rophage activation. Epstein-Barr virus (EBV) is a common cause of HLH particularly in Asian countries. Although EBV usually infects B cells and causes infectious mononucleosis, it is detected predominantly in CD8$^+$ T cells in EBV-associated HLH. In the present study we found the EBV genome in various lineages of the lymphoid cells including T, B, and natural killer cells in a 14-months-old boy with mild EBV-associated HLH. Furthermore, chronological changes in the copy number of EBV-DNA in each lineage are reported. Profound decrease in the number of peripheral natural killer cells at the early stage could be involved in the development of HLH.

Keywords: Epstein-Barr Virus; Hemophagocytic Lymphohistio Cytosis; Natural Killer Cell

1. INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a rare disorder characterized by high grade fever, hepatosplenomegaly, lymphadenopathy and cytopenia in association with hypercytokinemia and macrophage activation [1]. HLH is classified into two clinical entities; primary HLH which develops in inherited diseases such as familial hemophagocytic lymphohistiocytosis (FHL) or primary immunodeficiency, and secondary HLH which develops associated with other acquired disorders such as infection, collagen vascular diseases and malignancy [1]. Sixty percent of the secondary HLH in children is associated with Epstein-Barr virus (EBV) in Japan [2]. Although most cases of EBV-HLH have been reported from Asian countries, reports from other regions have been increasing [3,4]. EBV is a ubiquitous human herpes virus which usually infects B cells and causes self-limiting infectious mononucleosis (IM). On the other hand, persistent or recurrent IM-like disease associated with abnormally high levels of both anti-EBV antibodies and EBV genome is known as chronic active EBV infection (CAEBV) [5]. EBV-associated HLH (EBV-HLH) occurs in association with either CAEBV or a primary infection of the virus [1-5]. Recent reports have demonstrated that EBV infects NK or CD4$^+$ T cells in CAEBV, whereas it dominantly infects CD8$^+$ T cells in EBV-HLH [5,6]. We report here a case of HLH associated with a primary infection of EBV and the chronological changes in EBV-DNA of each lymphoid cell subset.

2. CASE REPORT

A previously healthy 14-month-old boy was admitted to our hospital because of fever up to 41°C persisting for four days. He was born to non-consanguineous healthy parents and had no history suggesting immunodeficiency or hypersensitivity to mosquito bite. On the third hospital day, cervical lymphadenopathy and hepatosplenomegaly were noted. Laboratory examination showed: white blood cell count 7.89×10^9/l (neutrophils 17%, lymphocytes 55%), hemoglobin 98 g/l, platelet count 45×10^9/l, aspartate transaminase 543 IU/l, alanine transaminase 204 IU/l, total cholesterol 0.81 g/l, triglyceride 2.24 g/l, C-reactive protein 115.3 mg/l, ferritin 2090 g/l. Coagulation study showed: prothrombin time 17.8 seconds, activated partial thromboplastin time 51.8 seconds, fibrinogen 2.66 g/l (reference range 2.00 g/l - 4.00 g/l) and fibrin degradation products 93.9 g/ml (< 10 g/ml), sug-

gesting disseminated intravascular coagulation (DIC). NK cell activity and a serum soluble CD25 was 14% (normal; 18% - 45%) and 10,300 U/ml (normal < 520 U/ml), respectively. Although hemophagocytosis was not evident in his bone marrow, seven of the eight items of HLH criteria by Histiocyte Society were fulfilled [1]. Nafamostat mesilate was administered for DIC. Because mild dilatation of coronary artery with a high echogenicity of the arterial wall similar to that observed in Kawasaki disease was detected by echocardiography on the 7th day of hospitalization despite slight improvement of both clinical and laboratory findings, high-dose intravenous immunoglobulin (HD-IVIG) therapy (2 g/kg) was started. Serological test for EBV before HD-IVIG therapy showed IgG antibody to viral capsid antigen (VCA) 1:160, IgG antibody to early antigen 1:20, but negative for IgG antibody to EBV nuclear antigen (EBNA) suggesting a primary infection of EBV. Because his clinical and laboratory findings rapidly improved, no further therapy was performed.

Flow cytometric analysis of his peripheral blood at the onset of HLH demonstrated a high level of CD8$^+$HLA-DR$^+$ cells, whereas the number of CD19$^+$, CD20$^+$, CD56$^+$, CD16$^+$, and CD57$^+$ cells was low (**Table 1**). The number of CD8$^+$HLA-DR$^+$ cells declined in 20 days which was associated with recovery of CD16$^+$ or CD57$^+$ cells. The ratio of each subset has returned to normal ranges 3 months after the onset.

The copy number of EBV-DNA was analyzed by a real-time polymerase chain reaction after separation of his peripheral blood mononuclear cells (PBMC) into CD19$^+$, CD3, CD4$^+$, CD8$^+$, CD16$^+$ and 56$^+$ cells by an immunobeads method (DynaBeads; Dynal A/S, Oslo, Norway) as previously reported [7]. DNA was extracted from 2×10^6 PBMC using a QIAmp Blood Kit (Qiagen, Hilden, Germany). A real-time quantitative PCR assay with a fluorogenic probe was performed using TaqMan PCR kit (PE Applied Biosystems, Foster City, CA). The amount of EBV-DNA was calculated as the number of virus copies per microgram PBMC DNA. The copy number of EBV-DNA was extremely high at the onset in all of CD19$^+$, CD3, CD4$^+$, CD8$^+$, CD16$^+$ and 56$^+$ cells. A high level infection with CD16$^+$ or 56$^+$ cells persisted at least for five months, although the copy number of the viral DNA declined with the clinical improvement (**Table 2**). One year after the onset EBV-DNA was detectable only in CD19$^+$ cells at a latent infection level comparable to normal individuals with a past infection of the virus.

After the recovery from HLH, he has not shown any IM-like symptoms or abnormal laboratory findings during 2 years of follow up. IgG antibody to EBV early antigen disappeared soon, whereas serum levels of IgG antibodies to VCA and EBNA gradually elevated to 1:1280 and 1:40 in 5 months, respectively. NK activity restored to 45%. No mutation was detected in either *SH2D1A* or *XIAP* gene.

3. DISCUSSION

In the present study, we analyzed chronological changes of EBV-DNA in each subset of PBMC in a case of EBV-HLH. From his clinical course and the profile of anti-EBV antibodies, HLH was likely associated with a primary infection of EBV rather than CAEBV. Although coronary lesion was transiently observed, the diagnostic criteria for Kawasaki disease was not fulfilled [8]. In addition to vasculitis such as Kawasaki disease, coronary lesions develop in association with infection of the virus to the endothelial cells in CAEBV or with systemic inflammation as observed in systemic-onset juvenile idiopathic arthritis [9,10].

Our case showed increased number of peripheral CD8$^+$HLA-DR$^+$ cells and a decrease in both the number and the activity of NK cells at the diagnosis, which is consistent with the previous reports of EBV-HLH [11].

Table 1. Flow cytometric analyses of the peripheral mononuclear cells.

Date	Apr 5	Apr 25	Jul 11	Nov 6
Absolute number of PBMC (/mm^3)	3666	3846	3872	5728
CD4	19.2%	41.6%	44.1%	36.4%
CD8	70.7%	40.6%	38.7%	34.0%
CD8×HLA-DR	56.7%	17.7%	11.2%	8.9%
CD16	2.3%	17.2%	12.2%	20.0%
CD56	1.4%	ND	6.5%	14.9%
CD57	4.4%	7.6%	6.6%	12.2%
CD19	0.9%	0.7%	12.7%	18.2%
CD20	1.2%	0.6%	9.2%	12.9%

Abbreviation: PBMC, peripheral mononuclear cells; ND, not done.

Table 2. Chronological changes of EBV-DNA (copies/g DNA) in each subset.

Date	Apr 5	Apr 25	Jun 7	Sep5	Jan 9	Apr 16, 2008
PBMC	110×10^4	4.77×10^4	269	858	54	86
CD3	17.9×10^4	3.46×10^4	20	823	ND	ND
CD4	1.22×10^4	0.74×10^4	17	249	ND	ND
CD8	40.2×10^4	3.55×10^4	0	527	ND	ND
CD16	ND	5.14×10^4	1483	311	99	ND
CD56	97.8×10^4	5.13×10^4	1295	1769	ND	ND
CD19	13.9×10^4	2.16×10^4	119	932	173	508

Abbreviation: PBMC, peripheral mononuclear cells; ND, not done.

The dominant population of EBV-infected cells is reported to be CD8^{+} T cells in EBV-HLH, whereas the major targets of EBV in CAEBV are CD4^{+} T cells or NK cells [5-7]. EBV-infected CD8^{+}Tcells are activated and produce cytokines such as IFN-γ, TNF-α and interleukin-6, which activate macrophages and endothelial cells resulting in hemophagocytosis, DIC, and multiorgan failure [1,5,12]. NK cells, as well as EBV-specific cytotoxic T cells (CTL), play a critical role in the elimination of EBV-infected cells [1]. This is supported by the fact that the functional defects in cytotoxic activity of CTL and NK cells such as X-linked lymphoproliferative syndrome and FHL predispose for EBV-HLH [1]. In acute IM the number of NK cells, although it usually increases, is inversely correlated with the severity of the disease [13]. Thus, the numerical defect of NK cells as observed in our case could cause the development of EBV-HLH or severe IM. Our case showed subsequent recovery of both NK activity and the number of CD16^{+}, 56^{+} or 57^{+}cells as well as decline in CD8^{+}HLA-DR^{+} cells, which was associated with clinical improvement. Furthermore, no mutation was detected in causative genes of XLP such as *SH2DA* or *XIAP* gene. Thus, although mutations of other FHL-related genes have not been tested, the numerical and functional abnormality was unlikely to be intrinsic to his NK cells. Of note was the finding that high levels of EBV-DNA was detected in CD16^{+} and 56^{+} cells, in addition to T and B cells, despite the decreased number of the subsets in our case. Although other methods such as *in situ* hybridization and immunohistochemistry were not performed, a high level of infection in CD 16[+] and 56^{+} cells was confirmed by serial examination for several months. The copy number of EBV-DNA in PBMC of our case was comparable to that in previously reported cases of EBV-HLH, CAEBV and post-transplantational lymphoproliferative disorder [14]. Although EBV-HLH has a tendency to have larger viral burdens than acute phase of IM, it is difficult to differentiate between these two diseases simply by viral load in whole PBMC [15]. The copy number of EBV-DNA gradually declined in all of the subsets and was finally detectable in B cells only at a latency level one year after the onset of the disease. These findings are in contrast to NK cell-type CAEBV or NK cell lymphoma which shows a clonal expansion of EBV-infected NK cells [5]. It is known that EBV is able to infect NK cells at an early stage of IM, although the precise mechanisms of infection remain unclear [16]. As well, EBV genome is also detected in both NK and B cells under some conditions such as EBV-HLH but usually at a lower level than CD8^{+} T cells [6]. Isobe et al have reported that *in vitro* infection of EBV induces apoptosis of NK cells [17]. Thus, it is possible that the infection by EBV in-

duced apoptosis of NK cells *in vivo* and allowed unregulated activation of EBV-infected CD8^{+}T cells. Decreased number of peripheral B cells in association with HLH as observed in our case has also been reported [18]. Imashuku et al have demonstrated depletion of B cells in the spleen of the patients with HLH and suggested involvement of cytokines produced by activated T or NK cells and/or Fas-FasL-mediated apoptosis [18].

Although our case showed both clinical and laboratory improvement before the commencement of HD-IVIG therapy suggesting self-limiting nature, it is possible that the therapy accelerated the improvement by its anti-inflammatory mechanism [19]. Further studies are required to clarify the mechanisms of the decrease in the number of peripheral NK cells in EBV-HLH without underlying primary immunodeficiencies or FHL.

In conclusion, EBV infected various subsets of PBMC including NK cells in a case of HLH associated with a primary infection of EBV. EBV genome gradually declined in association with his clinical improvement and was finally detectable in only B cells at a latency level. Transient decrease in NK cells could be involved in the development of EBV-HLH in our case.

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