Clinical Significance of Cloned Expansion and CD5 Down-Regulation in Epstein-Barr Virus (EBV)-Infected CD8+ T Lymphocytes in EBV-Associated Hemophagocytic Lymphohistiocytosis

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Epstein-Barr virus (EBV) is the pathogen that most commonly triggers infection-associated hemophagocytic lymphohistiocytosis (HLH) and ectopically infects CD8+ T cells in EBV-associated HLH (EBV-HLH). We recently described an EBV-HLH patient who had a clonally expanded population of EBV-infected CD8+ T cells with CD5 down-regulation. To determine whether this finding could serve as a useful marker for EBV-HLH, we investigated 5 additional patients. We found a significant increase in the subpopulation of CD8+ T cells with CD5 down-regulation and bright human leukocyte antigen (HLA)–DR expression in all patients with EBV-HLH but not in patients with infectious mononucleosis or in control subjects. Such T cells were frequently found to be larger cells that stained positive for a specific T cell receptor VB. We also demonstrated that those cells were the major cellular target of EBV, and their numbers progressively declined in parallel with the serum ferritin levels. All together, our findings reveal the immunophenotypic characteristics of EBV-infected CD8+ T cells and may provide a valuable tool for the diagnosis of EBV-HLH.

Hemophagocytic lymphohistiocytosis (HLH) is a rare and potentially fatal disease that is characterized by uncontrolled proliferation of activated T cells and macrophages with overproduction of cytokines [1, 2]. Patients with HLH may present with fever, cytopenia, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, and hemophagocytosis [1, 2]. HLH consists of 2 different conditions. Genetic HLH includes familial HLH that is caused by genetic defects, including mutations of the Perforin, UNC13D, or Syntaxin-11 genes, and immunodeficiency syndromes such as Chédiak-Higashi syndrome, Griscelli syndrome, and X-linked lymphoproliferative syndrome. Most cases of X-linked lymphoproliferative syndrome that should be considered in all males with Epstein-Barr virus (EBV)–associated HLH (EBV-HLH) are caused by mutations of the SAP/SH2D1A or XIAP/BIRC4 genes. On the other hand, acquired HLH is associated with malignancies, autoimmune diseases, and infections. Infection-associated HLH can be caused by a variety of viral, bacterial, fungal, and parasitic infections; however, EBV is the most frequent triggering agent [3]. Most patients with acquired HLH show no obvious signs of underlying immunodeficiency.
EBV is a ubiquitous herpes virus that infects the majority of the world’s adult population and persists in B cells for the lifetime of normal individuals, generally without causing disease. Primary EBV infection is usually inapparent but occasionally causes acute infectious mononucleosis, which is a benign self-limited disease characterized by vigorous proliferation of activated antigen-specific and nonspecific cytotoxic T cells [4]. EBV infection has been also associated with a number of malignancies, as well as lymphoproliferative disorders including chronic active EBV infection (CAEBV) and HLH, where clonal proliferation of EBV-infected T and natural killer cells may play pathogenetic roles in disease development [5–8]. We recently described differences between HLH and CAEBV, which are cellular targets of EBV; EBV predominantly infects CD8+ T cells in EBV-HLH, whereas the majority of EBV-infected cell populations are non-CD8+ lymphocytes in CAEBV [9]. The immunophenotypic features of those EBV-infected cells, however, have not been fully characterized, especially in EBV-HLH. Our recent observation of clonal proliferation of EBV-infected CD8+ T cells with down-regulation of CD5 expression in a patient with EBV-HLH [10] prompted us to conduct a series of studies to characterize EBV-infected cells in EBV-HLH.

Human CD5 is a membrane glycoprotein that is normally expressed on the majority of circulating T cells and a small population of B cells, but not natural killer cells, and is involved in the modulation of antigen-specific receptor-mediated activation and differentiation signals [11, 12]. It has recently been reported that CD5 is recruited and colocalizes with CD3 at the immunological synapse and inhibits TCR signaling in T cells interacting with antigen-presenting cells, and that CD5 protects circulating tumor antigen–specific cytotoxic T cells from tumor-mediated activation-induced cell death [13, 14]. Despite accumulating evidence, the physiological signals that down-regulate CD5 expression in T cells and the nature of CD8+ T cells with down-regulation of CD5 remain undetermined [15]. In this report, we describe the clinical significance of down-regulation of CD5 in clonally expanded EBV-infected CD8+ T cells in EBV-HLH.

**METHODS**

**Patients.** We evaluated 6 Japanese patients who were affected by EBV-HLH (Tables 1 and 2). All patients were previously healthy, and no history of other affected members was noted in each family where no parental consanguinity was present. The clinical and immunological data of patient HLH1 have been reported elsewhere [10]. The remaining patients showed typical clinical features of HLH such as persistent fever, hepatomegaly, splenomegaly, cytopenia, abnormal liver function, hyperferritinemia, elevated levels of soluble interleukin-2 receptor, and hemophagocytosis in the bone marrow (Tables 1 and 2). Thus, all 6 patients met the diagnostic criteria for HLH [16]. The number of EBV DNA copies in the peripheral blood was markedly increased to 3.8 × 10^6 copies/mL (normal range, <1 × 10^3 copies/mL) in patient HLH2, 1.4 × 10^7 copies/mL in patient HLH3, 8.0 × 10^6 copies/mL in patient HLH4, 8.7 × 10^5 copies/10^6 cells (normal range, <2.0 × 10^5 copies/10^6 cells) in patient HLH5, and 1.9 × 10^5 copies/mL in patient HLH6. Serological tests for EBV revealed that anti-EBV nuclear antigen titers were negative in the presence of antiviral capsid antigen immunoglobulin M and/or immunoglobulin G antibody, indicating a primary EBV infection in each patient (Table 2). Of the 5 patients for whom Southern blot analysis of EBV terminal repeats were available, all showed evidence of mono-
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Normal range 4.0–9.0 2.5–5.0 11.5–15.5 150–350 119–229 18–40 30–149 183–381 4.6–204.0 145–519 No <10 <10 <10

**NOTE.** EBV titers were measured by immunofluorescence assay and are expressed as reciprocal serum dilutions. EBNA, Epstein-Barr virus nuclear antigen; IgG, immunoglobulin G; IgM, immunoglobulin M; IM, infectious mononucleosis; LDH, lactate dehydrogenase; NA, not available; NK, natural killer; sIL-2R, soluble interleukin-2 receptor; VCA, viral capsid antigen; WBC, white blood cell.

*a Hemophagocytosis in bone marrow.
clonal expansion of EBV-infected cells in the peripheral blood. No detectable mutations within the SAP/SH2D1A or XIAP/BIRC4 genes were observed in the 4 male patients (patients HLH2, HLH3, HLH4, and HLH6) [17, 18]. All cases but 1 were successfully treated with immune-modulating chemotherapy, including corticosteroids, cyclosporine A, and etoposide (Table 1). None of the patients exhibited CAEBV with persistent hepatosplenomegaly and viral loads.

We also investigated 7 cases of infectious mononucleosis as control cases. Primary EBV infection was serologically confirmed for all patients with infectious mononucleosis. All of the patients with infectious mononucleosis exhibited an acute self-limited episode, and 2 of them received only a short course of corticosteroid (Table 1). Although 3 patients with infectious mononucleosis (patients IM1–IM3) initially exhibited hypercytopenia or hemophagocytosis in the bone marrow, there was no evidence of ectopic EBV infection in the T cell or natural killer cell population in those patients.

Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and written informed consent was obtained according to the Declaration of Helsinki.

**Cell preparations.** Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation from patients and control participants. In patient HLH4, frozen PBMCs were utilized. Peripheral blood lymphocytes (PBLs) were prepared from PBMCs by depletion of monocytes using anti-CD14 monoclonal antibody (mAb)–coated magnetic beads (Becton Dickinson). CD4+ T, CD8+ T, CD19+ B, and CD56+ natural killer cells were then purified by positive selection from PBLs using mAb-coated magnetic beads. To obtain TCR VB3+ cells, PBMCs were stained with phycoerythrin–conjugated anti-TCR VB3 mAb (Immunotech), followed by incubation with anti-phycoerythrin mAb-coated magnetic beads. After enrichment of CD8+ cells from PBLs by depletion of CD4+, CD19+ and CD56+ cells, CD5−CD8+ T cells were purified by negative selection with phycoerythrin–conjugated anti-CD5 mAb (Becton Dickinson), followed by positive selection with anti-CD8 mAb-coated magnetic beads. The purity of each isolated cell population was assessed by flow cytometric analysis.

**Flow cytometry.** Expression of CD5 and human leukocyte antigen (HLA)–DR was evaluated on CD8+ T cells among the total population of mononuclear cells with a FACSCalibur flow cytometer using CellQuest software (BD Bioscience). The fluorescence-activated cell sorting profiles done on the patients with HLH and infectious mononucleosis were performed on different days with different fluorescence-activated cell sorting settings. Because the cutoff settings was similar for 95% of the cells to be CD5+ among CD8+ T cells from several healthy donors, PBMCs from healthy donors can be used as simultaneous controls for CD5 expression. CD5 expression on CD8+ T cells was considered dim if it was lower than the cutoff level but was higher than levels of natural killer cells and most of B cells that did not express CD5. The following mAbs were used: FITC–conjugated anti-CD5 and phycoerythrin–conjugated anti-HLA-DR (Becton Dickinson); R-phycocerythrin–cyanine 5–conjugated anti-CD4 or anti-CD8 (Dako, Glostrup, Denmark). Analysis of differences among the data groups was performed using a Student t-test for unpaired samples. P-values <.05 were considered to indicate significant differences.

**Analysis of TCR VB Repertoire and In Situ Hybridization for EBV-Encoded Small RNA1 (EBER-1).** Flow cytometric analysis of the TCR VB repertoire, complementarity-determining region 3 (CDR3) spectratyping, and in situ hybridization for EBER-1 were performed as described elsewhere [19–21]. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) of TCR VB13.3 was performed on an ABI 7700 sequencer (Applied Biosystems) using the SYBR GreenER qPCR SuperMix for ABI Prism (Invitrogen). We used β-actin as a reference gene. The following primers were used: TCR VB13.3, forward 5’-CAAGGAGATTCAATCCCT-3’, reverse 5’-TTCTGATGGCTCAAACAC-3’; β-actin, forward 5’-GACAGATGCGAGAGATTACT-3’, reverse 5’-TGATCCACATCCTGCTGAAGGT-3’. EBER-1 cells were detected at a frequency of 0.0016%–1.26% among PBMCs from patients with infectious mononucleosis, but <0.001% within PBMCs from EBV-seropositive adult control participants, as reported elsewhere [9]. The polymerase chain reaction (PCR) products of VB complementary DNA were subcloned with a TOPO TA cloning kit (Invitrogen), and sequencing was performed on subcloned PCR products using the ABI Prism BigDye Terminator Cycle sequencing kit on an ABI 3100 automated sequencer (Applied Biosystems).

**RESULTS**

**Increased subpopulation of CD5−/dim HLA-DR++ CD8+ T cells.** We have previously demonstrated that EBV infection occurs predominantly in CD8+ T cells in EBV-HLH and that such clonally expanded CD8+ T cells exhibit unique immunophenotypic profiles including down-regulation of CD5 (CD5−/dim) and bright expression of HLA-DR (HLA-DR++) in a single patient (patient HLH1) [9, 10]. Therefore, we first assessed whether this unusual subset of CD5−/dim HLA-DR++ CD8+ T cells was present in the acute phase of other EBV-HLH patients. As shown in Figure 1, all of the EBV-HLH patients showed significantly increased subpopulations of CD5−/dim HLA-DR++ CD8+ T cells, which were negligible among control participants and patients with infectious mononucleosis, even when the patients with infectious mononucleosis exhibited hypercytokinemia or hemophagocytosis in their bone marrow. Because
of the predominance of larger cells among those cells, the total sample of mononuclear cells, but not lymphocytes, was gated for the analysis to avoid underestimation, followed by gating of CD8+ cells to eliminate contaminating monocytes that did not express CD5 but did express HLA-DR antigens. EBV-HLH patients also tended toward a greater absolute number of CD5+/HLA-DR++ CD8+ T cells compared with patients with infectious mononucleosis, but there was no significant difference, likely because of the high variability of lymphocyte counts (Figure 1B). On the other hand, CD5+/HLA-DR++ cell populations were not detected in CD4+ T cells in EBV-HLH patients, or in control participants and patients with infectious mononucleosis (data not shown).

Consistent with our previous observation [9], all of the EBV-HLH patients exhibited increased percentages of CD8+ T cells, concomitant with a decrease in the percentage of CD4+ T cells in the peripheral blood (data not shown). The majority of CD8+ T cells expressed CD45RO and HLA-DR antigens, indicating an activating phenotype. However, these findings were also observed in patients with infectious mononucleosis [22], thus not allowing us to differentiate EBV-HLH from infectious mononucleosis.

**TCR VB repertoire of CD8+ T cells.** We next investigated the diversity of the TCR VB repertoire in CD8+ T cells from EBV-HLH using mAbs and CDR3 spectratyping. Similar to the findings for CD8+ T cells from patient HLH1 [10], a massive expansion of a specific TCR VB was demonstrated for CD8+ T cells from other EBV-HLH patients, except for patient HLH4. Flow cytometric analysis of the TCR VB repertoire showed an expansion in the percentages of CD8+ T cells that stained positive for TCR VB3, with a few cells expressing most of the TCR VB families in patients HLH2, HLH5, and HLH6 (Figure 2A). Those clonally expanded CD8+ T cell populations exhibited down-regulation of CD5 and bright expression of HLA-DR. By contrast, none of the patients with infectious mononucleosis exhibited a high percentage (>30%) of CD8+ T cells expressing
Figure 2. Analysis of the T cell receptor (TCR) VB repertoire on CD8⁺ T cells. A, Expression of the TCR VB. Peripheral blood samples were stained with monoclonal antibodies (mAbs) for the TCR VB subfamilies together with anti-CD8 and anti-CD5 or anti-HLA-DR mAbs. *Cocktail of mAbs for TCR VB1, 2, 3, 5.1, 5.2, 5.3, 7, 8, 9, 11, 12, 13.1, 14, 17, 18, 20, 22, and 23. The percentage of cell gated in each quadrant is shown. B, Reverse-transcription polymerase chain reaction (RT-PCR) of the TCR VB subfamilies. Each TCR VB fragment was amplified from complementary DNA with 1 of the 24 VB-specific primers. Lane M, 100-bp molecular size marker. C, TCR VB13.3 (13.1) messenger RNA copies, determined by real-time quantitative RT-PCR. Data were normalized to β-actin expression and represent the mean (± standard deviation) of 3 independent experiments. D, CDR3 spectratyping, showing the size distribution of the PCR products of TCR VB13.3. E, Functional amino acid sequence of TCR VB13.3. The same sequence was detected in 100% of 12 clones. HLH, hemophagocytic lymphohistiocytosis. NA, not available.
rearrangement was also detected in patients HLH2, HLH3, HLH4, and HLH6 (data not shown).

**Characterization of EBV-infected cells.** To determine which populations of lymphocytes were infected with EBV, in situ hybridization for EBER-1 was performed on various samples isolated from EBV-HLH patients (Figure 3). In patient HLH2, 90.4% of TCR VB3+ cells, which were 99.7% pure and had 89.5% of CD8+ T cells, were EBER-1 positive, whereas 1.6% of TCR VB3+ cells, which comprised 2.8% of the contaminating TCR VB3+ CD8+ T cells, were found to be EBER-1 positive. In patient HLH3, EBER-1+ cells constituted 75.5% of CD8+ T cells but were not detected among CD4+ T cells; both of these T cell populations were more than 98% pure. In patient HLH4, EBER-1+ cells were detected in 68.8% of CD5+CD8+ T cells (purity, 85.8%) and in 0.1% of CD5+ cells (purity, 88.3%). In patient HLH6, EBER-1+ cells were detected in 78.5% of CD8+ T cells (purity, 99.1%), but in only 0.16% of CD8+ cells (purity, 93.5%). We were not able to analyze cell-specific infection of EBV in patient HLH5 because no appropriate sample was available.

**Percentages of infected cells and clinical course.** To evaluate whether the presence of CD5*dim* HLA-DR++ CD8+ T cells varied over the initial course of the treatments, serial flow cytometric analysis was performed. As shown in Figure 4, the percentages of CD5*dim* HLA-DR++ CD8+ T cells declined progressively in all patients with available samples, in parallel with the serum ferritin level and the viral load, which were specific and sensitive indicators that reflected the disease activity [2, 7].

**DISCUSSION**

EBV is the most common triggering agent of HLH, a disease that is often fatal when left untreated. Because immunochemotherapy with etoposide and corticosteroids can be life-saving for patients with EBV-HLH [23, 24], early diagnosis is of great importance. Although the HLH-2004 protocol has been shown to be helpful to establish the diagnosis of HLH [16], some of these criteria occur late in the course of the disease [2]. Data on valuable diagnostic parameters, including EBV viral load, levels of proinflammatory cytokines, natural killer cell activity, and mutation analysis of the genes related to genetic HLH, are only available from specialized laboratories. In addition, serological tests for EBV and routine immunophenotypic analysis of lymphocyte subsets are unable to distinguish EBV-HLH from infectious mononucleosis. Patients with infectious mononucleosis sometimes exhibit marked immune responses to regulate EBV-infected B cells and may share some typical findings of EBV-HLH, such as cytopenia, hypercytokinemia, and hemophagocytosis, even though infectious mononucleosis has a benign self-limited episode. On the other hand, it is well known that EBV-HLH can develop as a progressive course after typical infectious mononucleosis-like symptoms, but is not rapidly fatal [7]. Thus, it still remains difficult to diagnose EBV-HLH in the early stage. Because EBV ectopically infects CD8+ T cells without producing a sufficient number of EBV-specific cytotoxic T cells in EBV-HLH [9], a novel diagnostic approach to detect a clonal population of EBV-infected CD8+ T cells is highly desirable. We therefore extended our previous observation [10] and investigated whether combined analysis of the TCR VB repertoire and expression of CD5 and HLA-DR could identify such clonal composition in CD8+ T cells from EBV-HLH patients.

In the present study, we demonstrated a significant increase in the subpopulation of CD5*dim* HLA-DR++ CD8+ T cells in all EBV-HLH patients, compared with patients with infectious mononucleosis and control participants. These cells also expressed CD45RO and were generally larger, suggesting a highly
activated state. In most EBV-HLH patients, the CD5^{dim}\text{--}HLA-DR^{+} CD8 T cells exhibited specific TCR VB, indicating their clonal proliferation. Because the sets of mAbs and primers that we used for analysis of TCR VB repertoire did not assess the entire functional TCR VB repertoire, a clonal population was likely not detected in patient HLH4, for technical reasons. This possibility was supported by the finding that Southern blot analysis of EBV terminal repeats showed the presence of monoclonal EBV in the EBV-HLH patients, including patient HLH4, and by the fact that the total percentages of CD8 T cells that stained positive for each TCR VB mAb was much lower for patients HLH3 and HLH4 than for normal control participants (data not shown). Consistent with our previous results and other studies [6, 9], CD8 T cells were found to account for the majority of EBER-1 cells in all EBV-HLH patients. Moreover, it is important to note that in situ hybridization for EBER-1 clearly demonstrated CD8 T cells that showed clonal proliferation with specific TCR VB or down-regulation of CD5 are the major cellular target of EBV infection in patients with EBV-HLH. Because EBV infects and replicates only in B cells in patients with infectious mononucleosis, the CD5^{dim}\text{--}HLA-DR^{+} cell population might not exist among CD8 T cells from patients with infectious mononucleosis. Taken together, these results suggest that, in EBV-HLH, EBV-infected cells can be detected as clonally expanded CD5^{dim}\text{--}HLA-DR^{+} CD8 T cells that often react with a specific TCR VB mAb among the total population of mononuclear cells by flow cytometry. This may allow us to discriminate EBV-HLH from infectious mononucleosis and diagnose EBV-HLH in the early stage with a small amount of peripheral blood, which would then lead to significant clinical benefit particularly for young children with a suspected diagnosis of HLH.

Recent studies have shown that EBV latent membrane protein-1 suppresses SAP gene expression and leads to T cell activation [25] and that the loss of proapoptotic function of SAP results in uncontrolled T cell proliferation in X-linked lymphoproliferative syndrome [26]. In addition, the EBV genome contains BHRF1, a gene with structural homology to Bcl-2 [4], and this viral Bcl-2 may function to protect infected cells from apoptosis. Therefore, EBV-infected CD8 T cells in patients with EBV-HLH might survive and proliferate with the help of these viral gene products and subsequently exhibit dysregulated...
and harmful cytotoxic functions. Because CD5− T cells have been shown to become hyperresponsive to TCR stimulation in CD5-deficient mice [27], down-regulation of CD5 (for which the reasons remain unclear) might contribute clonal proliferation of EBV-infected CD8+ T cells.

In support of these findings, monoclonality in T cells and massive expansion of CD8+ T cells with down-regulation of CD5 were recently described in 2 EBV-HLH patients by other groups [28, 29]. However, increased subpopulations of CD5−/dim CD8+ T cells have been also reported in patients with other clinical conditions. These rare conditions include allogeneic bone marrow transplantation [30]; human immunodeficiency virus type 1 infection [31]; infection with acute herpes viruses, including EBV and varicella zoster virus [32]; and peripheral T cell neoplasms [33]. In addition, a 17-day-old patient with familial HLH who carried a homozygous mutation in the Perforin gene was reported to exhibit uncontrolled reactive proliferation of CD8+ T cells that lacked CD5 expression [34]. Although clonal expansion of αβ-T cells with skewed Jδ1 usage may exist in familial HLH [35], there was no evidence of monoclonality as assessed by TCR-γ gene rearrangement in this familial HLH case. Accordingly, the appearance of CD5−/dim CD8+ T cells is a likely more general consequence of the dysregulated proliferation of CD8+ T cells, rather than a specific event in EBV-HLH. However, our simultaneous assessment of the TCR VB repertoire, which differs from the methods of previous reports, may point to a characteristic component of the immune dysregulation in EBV-HLH. Additional studies are necessary to address this issue and to elucidate the underlying mechanisms. It is worth noting that the percentages of CD5−/dim HLA-DR+ CD8+ T cells correlated with serum markers, such as the ferritin level, over the course of the treatments, indicating that our approach would be a valuable tool for detecting and monitoring abnormal clones infected by EBV in the acute phase of EBV-HLH.

Limitations of this study include the small number of patients, the absence of patients with other forms of HLH as control patients, and a homogeneous group of EBV-HLH patients who were all previously healthy Japanese children. In most cases, EBV-HLH developed in apparently immunocompetent individuals; however, it can also occur in patients with familial HLH, immunodeficiency such as X-linked lymphoproliferative syndrome, CAEBV, and natural killer cell leukemia [7]. Moreover, in contrast to our observation and other studies from Asia [9, 29], it has recently been reported that EBV was not only detected in T cells but also in B cells with comparable EBV copy numbers in non-Asian children suffering from EBV-HLH, although the immunophenotype of lymphocyte subsets and the clonality of EBV were not well characterized in those cases [36]. Therefore, additional investigation will be required to assess the immunophenotypic features of EBV-infected cells in a variety of patients with EBV-HLH with different underlying conditions, including genetic and ethnic backgrounds and EBV tropism.

In summary, our studies demonstrate the immunophenotypic characteristics of EBV-infected CD8+ T cells in EBV-HLH, provide important insights into the biology of the disease, and offer valuable perspectives on the early diagnosis of EBV-HLH.

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References


