A virus-associated hemophagocytic syndrome is characterized by high fever, liver dysfunction, coagulation abnormalities, pancytopenia, and a benign histiocytic proliferation with prominent hemophagocytosis in bone marrow, lymph node, spleen, and liver. We describe six Japanese children with fatal Epstein-Barr virus (EBV)–associated hemophagocytic syndrome. Five of the six patients had serologic evidence of primary EBV infection at the onset of their diseases. EBV genomes were detected in all the patients by Southern blot hybridization or the polymerase chain reaction. Furthermore, clonality analysis of the EBV genome showed that EBV-infected cells proliferated monoclonally or bidirectionally in three examined patients. In situ hybridization study using EBV-encoded RNA 1 (EBER1) showed that EBER1 was detected in one of two examined liver tissues, which localized in hepatocytes.

**Materials and Methods**

**Subjects.** Six Japanese children (3 boys and 3 girls) with fatal VAHS, who were 1 to 12 years of age, were studied (Table 1). These patients had neither an underlying immunodeficiency nor a family history. All patients had fever, hepatosplenomegaly, liver dysfunction, hypofibrinogenemia, pancytopenia, hyperferritinemia, hypertriglyceridemia, and a benign histiocytic proliferation with hemophagocytosis in bone marrow (BM; Table 2). All attempts at treatment of this disease with a wide variety of agents were unsuccessful. All patients died within 3 months after the onset. The median survival after the onset of the disease was 48 days (range, 18 to 73 days). The major cause of death was related to bleeding, multiple organ failure, or opportunistic infection secondary to severe neutropenia (Table 3).

Human DNA samples from EBV genome-positive Raji cells, EBV genome-negative Molt-4 cells, and four organs (liver, spleen, lung, and kidney) of three children with EBV-unrelated diseases were used as controls. Human embryonic fibroblast cells infected with herpes simplex virus type-1 (HSV-1), varicella-zoster virus (VZV), and human cytomegalovirus (CMV) and human herpes virus-6 (HHV-6)-infected cord blood mononuclear cells were used for this study.

**EBV serology.** Serum samples were tested for antibodies to viral capsid antigen (VCA), early antigen (EA), and EBV-determined nuclear antigens (EBNA). VCA-IgG and -IgM antibody titers were measured by indirect immunofluorescence using P3HR-1 cells. EA-IgG antibody titers were measured by indirect immunofluorescence using Raji cells superinfected with P3HR-1 EBV in the presence of cytomeòbin (20 μg/mL). EBNA antibody titers were measured by anticomplement immunofluorescence using Raji cells.

**EBNA staining.** Frozen sections 5-μm thick of two liver tissue samples (patients no. 1 and 2) and histologically normal liver tissue were fixed with acetone:methanol (1:1) at 4°C for 1 minute. They were incubated with serum positive reference serum or seronegative reference serum at 37°C for 45 minutes. Thereafter, they were washed with 0.1 mol/L phosphate-buffered saline (PBS; pH 7.2). Subsequently, they were reacted with fluorescein-isothiocyanate-conjugated goat antihuman C3c (Dakopatts, Copenhagen, Denmark) at 37°C for 30 minutes.

**Detection of EBV DNA.** DNA samples taken from different sites in the six children were assessed for detection of EBV DNA. DNA was extracted from frozen tissues, heparinized peripheral blood mononuclear cells (PBMCs), and heparinized BM aspirates. The DNA samples were digested with BamHI, electrophoresed through 0.6% agarose gel, and transferred onto nitrocellulose membrane filters. Each filter was hybridized with 32P-labeled cloned fragment BamHI-W of the EBV genome for 48 hours at 41°C in 1× standard saline citrate (SSC; 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and heat-denatured salmon sperm DNA (100 μg/mL). After hybridization, the filters were washed three times at room temperature (RT) in 0.1× SSC and 0.1% SDS and then incubated for 1 hour at 50°C three times. Filters were dried and exposed to X-ray film (Tokyo, Japan) at −80°C. Furthermore, the EBV genome-positive DNA samples by Southern blot hybridization were digested with EcoRI and hybridized with 32P-labeled cloned fragment EcoRI-Dhet of the EBV genome to examine clonality of EBV-infected cells.

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The reaction mixture consisted of 200 pmol of each deoxyribonucleotide primer, and 1 mmol/L of Tris-HCl (pH 8.3), 1.5 mmol/L of magnesium chloride, 0.01% (vol/wt) of gelatin, 20 pmol of each oligonucleotide primer, and 1 μg of DNA in a volume of 100 μL. Samples were then subjected to 35 cycles of PCR, each consisting of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 1.5 minutes of polymerization at 72°C by a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). The oligonucleotide primers used to amplify a 410-bp sequence in the region within the EBV BamHI-W fragment were 5'-GTGACTTCACCAAAGGTCAG-3' and 5'-TTA-ACCTTACCTCTG-3'. The primers amplify and detect a 1.8% agarose gel. The gel was stained with 1 pg/mL of ethidium bromide for 5 minutes, and the DNA bands were visualized under UV light. To assess the specificity of the technique, the PCR-amplified products were confirmed by Southern blot hybridization using 32P-labeled cloned fragment BamH1-W.

In situ hybridization studies of EBV RNA were performed with an alkaline phosphatase (APase)-linked 40-base oligonucleotide within the EBV-encoded RNA 1 (EBER1) gene, a region of the EBV-infected Raj cells but did not detect EBV RNA in the T-cell samples (patients no. 1 and 2) were available in this study. Histologically normal liver tissue was also examined as a negative control. Table 2 shows the clinical features and laboratory findings in six patients with fatal VAHS. All the EBV genome-negative DNA samples by Southern blot hybridization were reexamined by the polymerase chain reaction (PCR) to determine the presence of genetic EBV information. The PCR was performed according to a previously described method.13 The reaction mixture consisted of 200 μmol of each deoxyribonucleotide, 2.5 U of Taq DNA polymerase, 50 nmol/L of potassium bromide, 10 mmol/L of Tris-HCl (pH 8.3), 1.5 mmol/L of magnesium chloride, 0.01% (vol/wt) of gelatin, 20 pmol of each oligonucleotide primer, and 1 μg of DNA in a volume of 100 μL. Samples were then subjected to 35 cycles of PCR, each consisting of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 3 minutes of polymerization at 72°C by a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). The oligonucleotide primers used to amplify a 410-bp sequence in the region within the EBV BamHI-W fragment were 5'-GTGACTTCACCAAAGGTCAG-3' and 5'-TTA-ACCTTACCTCTG-3'. The primers amplify and detect a 1.8% agarose gel. The gel was stained with 1 pg/mL of ethidium bromide for 5 minutes, and the DNA bands were visualized under UV light. To assess the specificity of the technique, the PCR-amplified products were confirmed by Southern blot hybridization using 32P-labeled cloned fragment BamH1-W.

Table 2. Clinical Features and Laboratory Findings in Six Patients With Fatal VAHS

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cause of Death</th>
<th>Laboratory finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bleeding, infection (60)</td>
<td>Fever +/+/+/+/+</td>
</tr>
<tr>
<td>2</td>
<td>Steroid, ACV, VP-16, γ-globulin, plasmapheresis</td>
<td>Hepatomegaly +/+/+/+/+</td>
</tr>
<tr>
<td>3</td>
<td>Steroid, ACV, γ-globulin</td>
<td>Splenomegaly +/+/+/+/+</td>
</tr>
<tr>
<td>4</td>
<td>Steroid, VP-16, cyclophosphamide</td>
<td>Lymphadenopathy +/+/+/+</td>
</tr>
<tr>
<td>5</td>
<td>ACV, IL-2, IFN-α, G-CSF</td>
<td>Pancreatitis +/+/+/+/+</td>
</tr>
<tr>
<td>6</td>
<td>Steroid, ACV, γ-globulin, plasmapheresis</td>
<td>Liver dysfunction +/+/+/+/+</td>
</tr>
</tbody>
</table>

Table 3. Summary of Causes of Death and Treatments in Six Patients With Fatal VAHS

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cause of Death (survival in days)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bleeding, infection (60)</td>
<td>Steroid, ACV, VP-16, γ-globulin, plasmapheresis</td>
</tr>
<tr>
<td>2</td>
<td>Steroid, ACV, γ-globulin</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>3</td>
<td>Steroid, VP-16</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>4</td>
<td>Liver failure (73)</td>
<td>ACV, IL-2, IFN-α, G-CSF</td>
</tr>
<tr>
<td>5</td>
<td>Steroid, ACV, γ-globulin</td>
<td>Plasmapheresis</td>
</tr>
<tr>
<td>6</td>
<td>Multiple organ failure (68)</td>
<td>Steroid, ACV, Ara-A, VP-16, IL-2, G-CSF, plasmapheresis</td>
</tr>
</tbody>
</table>

Abbreviations: ACV, acyclovir; VP-16, epipodophyllotoxin VP-16; Ara-A, adenine arabinoside.
RESULTS

The six patients were EBV-seropositive, and five of the six patients were EBNA antibody-negative. Of these five patients, two patients were positive for VCA-IgM antibody. At the onset of the disease, only one had antibody to EBNA (Table 2). IgM antibodies to CMV were negative in all six patients.

Southern blot hybridization assays detected EBV DNA in DNA samples from three of the six patients using the EBV BamHI-W fragment. Southern blot hybridization with the EcoRI-Dhef probe, EBV terminal regions, showed one molecular weight band in patient no. 1 and two molecular weight bands in patients no. 2 and 3. Furthermore, the bands were the same sizes in DNA digests from different sites of patients no. 1 and 2 (Table 1 and Fig 1).

The PCR-amplified genomic Raji-cell DNA was confirmed on ethidium bromide-stained gel and by Southern blot hybridization. When DNA samples from cells infected with HSV-1, VZV, human CMV, and HHV-6 were used as templates in the PCR reaction, no amplification was noted by direct gel analysis or Southern blot hybridization. DNA samples from EBV genome-negative Molt-4 cells and four organs of three control children were also PCR-negative.

Even by means of the PCR, EBV DNA was not detected in PBMCs and BM aspirate from patient no. 4 during the course of the disease. However, EBV DNA was detected in the liver and spleen from patient no. 4 obtained at autopsy. DNA samples in PBMCs and BM aspirate from patient no. 5 and in BM aspirate from patient no. 6 were PCR-positive (Table 1 and Fig 2).

The in situ hybridization of two liver tissue samples with the EBER I antisense probe showed the EBER I in the hepatocytes of patients no. 1 and 2. Signals in the hepatocytes were strong in patient no. 2 (Fig 3). However, signals in the hepatocytes of patient no. 1 were too weak to be resolved from background. The hepatocytes were negative with the EBER I sense probe. Control liver tissue had no detectable EBER I by the in situ hybridization with the EBER I antisense and sense probes. Furthermore, a percentage of the hepatocytes in patient no. 2 was EBNA-positive by seropositive reference serum (Fig 4).

DISCUSSION

Initially, the VAHS described by Risdall et al was associated with a systemic viral infection mainly in immunosuppressed adult patients. However, the term VAHS has also been used in children without any prior immunosuppressive therapy at all. The latter condition is often clinically indistinguishable from FHL and HMR. He reported that the major criteria distinguishing VAHS from HMR are the presence of cytologically neoplastic histiocytes and the relative infrequency of erythrophagocytosis that is observed in HMR. However, subsequent reports indicate this differentiation is not always obvious. Su et al have reported young patients from Taiwan diagnosed as having HMR who presented a proliferation of EBV-infected atypical histiocytes. They suggested that cases assigned the conventional diagnosis of HMR may have a fatal form of EBV infection.

FHL may be an autosomal recessive disorder. However, the hereditary nature of the disease is rarely obvious. The onset is usually in early infancy, and the disease typically follows a fulminant course with a fatal outcome. Furthermore, in some cases the first manifestations in FHL seem to be triggered by an infection that is often of a viral nature. Therefore, the distinction between FHL and VAHS is not always easy. No conclusive clinical, laboratory, or histopathologic method is available to distinguish between VAHS and sporadic cases of FHL.

The VAHS reported by Risdall et al was associated with EBV infection in only 2 of 19 patients. We described six patients with fatal VAHS who fulfilled both clinical and laboratory criteria for VAHS: fever, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, pancytopenia, hyperferritinemia, and histiocytic proliferation with benign cytologic features and marked hemophagocytosis. Under-
lying immunologic abnormalities and a previous family history were absent in our patients. Primary CMV infections were not found in our patients by serologic test for CMV. Ansbacher et al.\textsuperscript{10} reported hypertriglyceridemia was probably a useful marker of FHL to distinguish it from VAHS. However, the levels of serum triglyceride were high in all our patients. Many types of treatment were attempted, including steroid, antiviral drugs, cytotoxic drugs, γ-globulin, interferon-α (IFNα), interleukin-2 (IL-2), granulocyte-colony-stimulating factor (G-CSF), and plasmapheresis. However, all the treatments failed to stem the rapid course of the disease. Ambruso et al.\textsuperscript{20} reported that epipodophyllotoxin (VP-16) appeared to be an effective agent in the treatment of lymphohistiocytic reticulosis with phagocytosis.\textsuperscript{20} However, VP-16 had no effect on the course of our three patients. Death was usually associated with bleeding, multiple organ failure, and sepsis.

All six patients were EBV-seropositive, and five of the
patients were completely EBNA antibody-negative, indicating serologic evidence of primary infection. Of the five patients, two were positive for VCA-IgM antibody, which was again strongly indicative of primary infection. However, the five patients did not have high antibody titers to EA and VCA, suggesting that EBV infection may be nonpermissive in fatal VAHS. Only patient no. 3 was positive for EBNA antibody, indicative of past infection. The patient had high antibody titers to replicative antigens of EBV. However, the diagnosis of EBV-associated hemophagocytic syndrome (EBV-AHS) could not be made serologically in patient no. 3; the serologic tests were inadequate to detect the association of EBV with VAHS.

Southern blot hybridization assay using the EBV BamHI-W fragment detected EBV DNA in DNA samples from three of the six patients. EBV DNA is a 172-kb double-stranded linear molecule with homologous direct-tandem repeats of approximately 500 bp at each end. The linear termini are joined intracellularly to form closed episomal DNA. The variation in length of the EBV-terminal region is caused by variation in the number of reiterations of the terminal repeat sequence within the circularized episome. The DNA configuration of intracellular EBV-terminal regions is a virus-specific clonotypic marker. Southern blot hybridization using the EBV EcoRI-Dhet fragment containing the right terminal repeats of the EBV genome showed one band in patient no. 1 and two bands in patients no. 2 and 3. Furthermore, the bands were the same sizes in DNA digests from different sites of patients no. 1 and 2. This result showed that EBV-infected cells proliferated monoclonally or biclonally and that EBV-AHS might be a neoplastic or preneoplastic disorder in the three examined patients. Therefore, it was more difficult to differentiate fatal EBV-AHS from HMR. EBV DNA could not be detected by Southern blot hybridization in the DNA samples from three of the six patients. However, the PCR procedure in this study was 10 to 100 times more sensitive than Southern blot analysis without prior DNA amplification. EBV DNA was detected in DNA samples from the three patients by means of the PCR. These findings suggest that molecular biologic studies, especially the PCR, are necessary for diagnosis of EBV-AHS.

EBV RNA was identified by the in situ hybridization in hepatocytes of one patient, which is a novel and very significant finding. EBV entry into B lymphocytes is believed to be critically dependent on the expression of a specific EBV/C3d receptor. However, in recent years, the range of host cells known to be susceptible to EBV infection has steadily expanded to include epithelial cells, T lymphocytes, and hepatocytes. The EBV receptor has not yet been documented on hepatocytes. The mechanism of the infection of the hepatocytes by EBV remains unknown.

In conclusion, we described six patients with fatal VAHS. Virologic (serologic and molecular biologic) studies suggest that EBV is implicated in the pathogenesis of fatal VAHS; it is considered to be one of the etiologic agents in fatal VAHS in Japan. All the therapy for EBV-AHS was disappointing; therefore, new therapies are needed for fatal EBV-AHS.

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Fatal Epstein-Barr virus-associated hemophagocytic syndrome

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