Persistently High Epstein-Barr Virus (EBV) Loads in Peripheral Blood Lymphocytes from Patients with Chronic Active EBV Infection

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Chronic active Epstein-Barr virus infection (CAEBV) is a severe illness with unusual EBV activation that persists for years, and its pathogenesis is largely unknown. After the creation of an accurate and reproducible polymerase chain reaction system to quantify EBV DNA, virus loads in peripheral blood lymphocytes (PBL) were determined in 54 children: 15 with CAEBV, 16 with infectious mononucleosis (IM), and 23 healthy children. Children with CAEBV and those with IM had high virus loads. Lower loads were detected in 47% of seropositive healthy donors. There were two distinct differences between children with CAEBV and those with IM: The former had greater viral replication (10^3–10^7 copies/2.5×10^5 PBL) than those with IM, and viral replication declined in children with IM whereas active replication persisted for years in subjects with CAEBV. Persisting high virus loads are a possible diagnostic criterion for CAEBV. EBV loads may enable classification and prognosis of EBV infections.

Epstein-Barr virus (EBV) is a causative agent of infectious mononucleosis (IM) [1] and of chronic active EBV infection (CAEBV). CAEBV is a severe illness with unusual EBV activation that may be fatal with multiple organ failure, and it is associated with malignant lymphoma without prior immunosuppression [2, 3]. The clinical symptoms of CAEBV and the serologic abnormalities corresponding to active EBV replication, such as markedly elevated anti–EA-DR antibody and absence of Epstein-Barr nuclear antigen (EBNA) antibody, persist for months to years. EBV replicates in NK [4], T [5], and B cells.

Once the infection is established, EBV usually resides in B lymphocytes for the host's lifetime without causing any clinical symptoms (designated as a latent infection). Therefore, not only the detection but also the quantification of EBV in affected tissues is essential to investigate EBV disease.

To clarify the status of EBV replication in persons with CAEBV, we used semiquantitative DNA polymerase chain reaction (PCR) to evaluate and compare virus loads in peripheral blood lymphocytes (PBL) in 3 groups of children: one with CAEBV, one with IM, and healthy controls. Our detection system was considered accurate and reliable for the quantification of EBV because we amplified a single-copy region of EBV. Most previous studies targeted the *Bam*HI-W region for which tandem repeat reiteration frequency is inconstant. We

believe this study is the first and largest scaled study of virus loads in PBL of children with CAEBV.

Materials and Methods

Patients and controls. We examined 16 children with IM (11 boys, 5 girls; ages, 0–8 years), 15 with CAEBV (11 boys, 4 girls; ages, 4–19 years), 19 immunocompetent EBV-seropositive healthy persons (10 boys, 9 girls; ages, 1–19 years), and 4 seronegative healthy children (3 boys, 1 girl; ages, 1–10 years). All subjects with IM presented with typical clinical manifestations (e.g., fever, tonsillitis, lymphadenopathy, hepatosplenomegaly, liver dysfunction, and atypical lymphocytosis). The diagnosis of IM was made on the basis of serologic findings of IgM antibody to viral capsid antigen (VCA) or seroconversion of VCA IgG and/or EA and negative EBNA antibody [6]. Twenty-two blood samples were collected from 16 children with IM 8 days to 8 months after disease onset (the initial symptom was fever in all patients). CAEBV was diagnosed by Rickinson's criteria [7]. Children with CAEBV had intermittent or persistent fever for >6 months, with hepatosplenomegaly, lymphadenopathy, and/or skin rash. The laboratory findings showed elevated transaminase levels, anemia, thrombocytopenia, and/or polyclonal hypergammaglobulinemia. Serologic examination revealed marked elevation of antibody titers to VCA and EA. Human immunodeficiency virus infection and rheumatic diseases were excluded. No subjects had undergone transplantation or had anticancer therapy.

Samples. Heparinized 2-mL peripheral blood samples were collected. PBL were isolated by ficoll-hypaque and lysed with 0.15 μg/μL proteinase K (Boehringer-Mannheim, Indianapolis); DNA was extracted with phenol-chloroform-ethanol precipitation. The extracted DNA was quantified by spectrophotometer, adjusted to 0.25 μg/μL DNA, and used as the PCR templates.

PCR sensitivity. EBV DNA were from three sources: Raji,

containing 50 copies of EBV per cell [8]; Akata, containing 20 copies per cell; and cloned *Bam*HI-L fragments, which were mixed with DNA from cord blood cells and used as a positive standard. To determine the sensitivity of the PCR system, serial 10-fold dilutions (10^0–10^4 copies of EBV) of the DNA extracted were subjected to PCR.

PCR. A nested double PCR was designed to amplify the conserved region encoding gp220 (within the *Bam*HI-L fragment) [9]. The outer primer pair amplifies a 302-bp fragment, by using the oligonucleotides 5′-GCGAACTGGTGGACACATGA and 5′-AAGTCCACAGGCAAATGCCA, corresponding to positions 2870–3171 of B95-8 (GenBank M10593). The inner pair amplifies a 239-bp fragment [10]. The PCR was performed in a 50-μL reaction containing 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM dNTP, 0.5 μM of each primer, 2.5 U of Taq DNA polymerase (Boehringer-Mannheim), and 4 μL of sample DNA. The products amplified were run on 2% agarose and stained with ethidium bromide, and Southern blotting was used to confirm the specificity using the *Bam*HI-L fragment as a probe [10]. Each PCR reaction was done in triplicate and included the negative control to exclude cross-contamination. To confirm the specificity of the PCR, DNA was analyzed from herpes simplex virus types 1 and 2, varicella-zoster virus, human cytomegalovirus, and human herpesviruses-6 and -7. No specific amplification was noted.

Quantification of EBV genome. The quantity of EBV DNA in PBL was determined by limiting dilution experiments. Serial 10-fold dilutions (1–10^{-6} μg) of genomic DNA extracted from each sample DNA was subjected to the nested PCR as described above, and the minimal limit for the positive result was converted into the virus load (copies/2.5×10^5 cells).

Results

The sensitivity of the PCR system was carefully determined by 10-fold dilutions of the three sources of EBV DNA: Raji and Akata, both containing constant copy numbers of EBV DNA per cell, and the cloned *Bam*HI-L fragment. Raji contains 50 copies of EBV genome per cell [8], and 1 μg of genomic DNA from Raji cells equals that extracted from 2.5×10^5 cells: hence, 1.25×10^7 copies of EBV DNA. The DNA from Raji was diluted appropriately, and DNA containing 10^n copies (serial dilutions of 10^0–10^4 copies of the virus) was subjected to amplification. The minimal limit for a positive PCR result was examined. Repeated examinations revealed that the sensitivity was 100 copies per reaction with reproducibility. Results were the same using Akata DNA and with the dilution experiment using 10^n copies of *Bam*HI-L fragments mixed with 10^5 μg, 10^2 μg, and no cord blood cell DNA.

All PBL from subjects with IM and CAEBV were positive for the EBV genome. Nine (47%) of 19 samples from EBV-seropositive healthy persons were positive. All PBL from seronegative persons were negative.

EBV DNA in PBL was quantified and compared for EBV-seropositive healthy controls, children with IM, and children with CAEBV (figure 1). In healthy EBV-seropositive children,
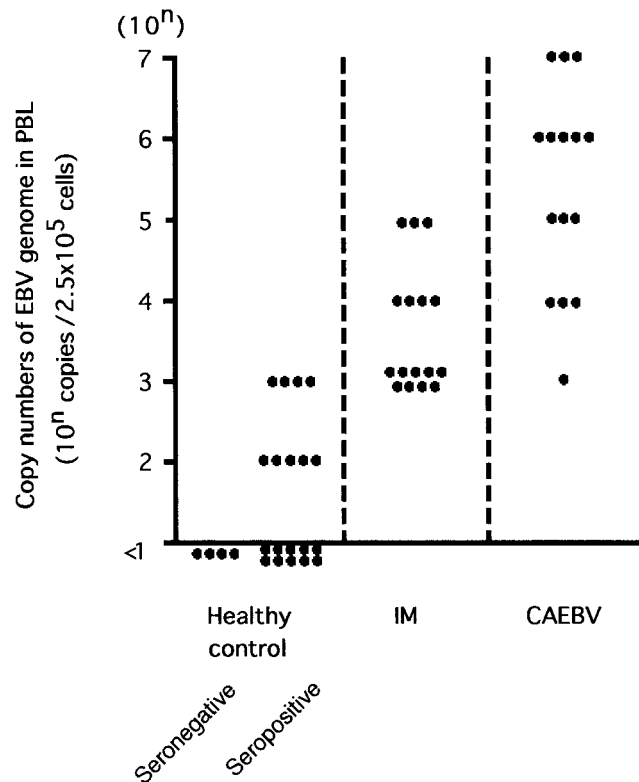

Figure 1. EBV DNA in peripheral blood lymphocytes (PBL) at diagnosis in healthy controls (4 EBV-seronegative, 19 -seropositive), 16 children with infectious mononucleosis (IM), and 15 patients with CAEBV infection. In 9 of 19 healthy EBV-seropositive children, EBV DNA was detectable (100–1000 copies/2.5×10^5 PBL). Larger quantities of EBV genome were detected in PBL from patients with EBV disease.

the virus loads were 100–1000 copies per 2.5×10^5 PBL. In PBL from children with IM, the lowest limits for positive PCR results ranged from 10^0 μg (2.5×10^5 PBL) to 10^{-3} μg (2.5×10^2/PBL) of genomic DNA, indicating that the virus load was 100–100,000 copies/2.5×10^5 PBL. Three blood samples collected within a month after the disease onset harbored higher loads (1000–100,000 copies/2.5×10^5 PBL). The PBL collected >1 month after onset of IM tended to contain decreased quantities of the virus. We followed the virus loads in 4 IM patients (figure 2A). In all 4, the virus loads decreased during the clinical course but were higher (100–10,000 copies/2.5×10^5 PBL) than in the seropositive healthy hosts.

The PBL from children with CAEBV had more EBV DNA than did samples from children with IM. Four CAEBV patients were followed for >6 months (figure 2B illustrates the findings for 2 patients). Two other patients had unchanged virus loads over 2–3 years (10^5 and 10^6 copies/2.5×10^5 PBL, respectively). All of these patients had persistently high virus loads, unlike the findings with IM. When we examined the relationship be-

A

B

Figure 2. *A,* EBV loads and times from disease onset to blood collection. 12 blood samples collected within 1 month after onset of infectious mononucleosis (IM) harbored large quantities of EBV (1000–100,000 copies/2.5×10^5 cells). Peripheral blood lymphocytes (PBL) collected $\geqslant 1$ month after onset of IM tended to less virus. 4 IM patients were followed to test virus loads. In all 4, EBV DNA declined during clinical course of disease. *B,* Temporal change of EBV loads in PBL of 2 patients (UT, YK) with CAEBV. Large quantities of EBV DNA were consistently detected in PBL.

tween the clinical manifestations and the EBV loads, only fever was correlated with the virus load. There was no correlation with hepatosplenomegaly, lymphadenopathy, exanthema, mosquito allergy, liver dysfunction, anemia, thrombocytopenia, or serum antibody titers against VCA, EA-DR, and EBNA.

Discussion

The PCR quantifying EBV DNA demonstrated that the PBL from children with CAEBV or IM had high virus loads, in-

dicating active EBV replication in the lymphocytes. The viral replication was considered more active in CAEBV than in IM; in the IM group the replication gradually declined, whereas active replication persisted for years in the CAEBV patients.

Our PCR system amplifies EBV-specific sequences encoding gp220, which is a single copy gene in a virus, whereas the previous studies targeted the internal repeating long located in the *Bam*HI-W region. Since in polyclonal EBV proliferation the repeating number of this region is inconstant, our system

is considered more accurate for quantifying EBV, though less sensitive for detection. The defect of low sensitivity was overcome by use of the nested PCR. The sensitivity was 100 copies per reaction, compared with 10 copies in the previous report [11]. We quantified EBV genomes in PBL by the serial dilution of extracted DNA. Our system quantifying EBV DNA by serial dilutions of extracted DNA was shown to be reproducible and reliable.

The PBL collected from children with IM and with CAEBV harbored larger quantities of the EBV genome than did PBL from healthy seropositive controls. Of the latter, 47% had detectable EBV DNA ($100–1000/2.5 \times 10^5$ PBL). EBV in 2 subjects was detected at higher levels than in the previous studies ($1–50/10^6$ PBL) [11]. Since we studied children, the shorter periods after primary infection may contribute to the higher EBV loads than seen in previous studies of adults, reminding us that, especially in children, positive PCR results include asymptomatic latent EBV infection.

The children with IM had large quantities of EBV. EBV loads peaked within a month after onset (1000–100,000 copies/ 2.5×10^5 PBL) and then gradually decreased but remained high several months ($\leqslant 8$) after onset, compared with levels in healthy controls. Even when the clinical manifestations disappeared, relatively high levels of EBV replication continued in the PBL.

The patients with CAEBV had persistently large quantities of EBV, suggesting that chronic unregulated active replication of EBV in PBL results in severe illness with a poor prognosis. The factors and mechanisms leading to the surprisingly active viral replication remain unclear. Various heterogeneous deficiencies in host defense systems of both humoral and cellular immunity have been described [2, 3] and might allow active viral replication. High virus load in PBL ($>300,000$ EBV/10^5 PBL) has been shown in EBV-associated postransplant lymphoproliferative disease (PTLD), a complication after transplantations [12, 13], although the virus loads decreased within a year in parallel with the recovery from immunosuppression. A quantitative difference in circulating EBV load was correlated with EBNA antibody level in PTLD [12] but not in CAEBV. Therefore, the behavior of EBV in PTLD was considered to be both similar to and different from that in CAEBV. In view of the clinical manifestations, CAEBV seems similar to X-linked lymphoproliferative (XLP) syndrome [14], for which the responsible gene was recently identified. However, unlike XLP syndrome, CAEBV is not a hereditary disease, and females can be affected. Further immunologic analysis of CAEBV is necessary to clarify predisposing factors for the viral activation.

Acyclovir, interferon-α, and interleukin-2 have been used to treat CAEBV—with unsatisfactory results [2, 3]. Our results indicate that cytotoxic drugs that induce immunosuppression

and enhance the viral activation should be chosen carefully, although the clinical features are similar to those of hematologic malignancies. Persisting high EBV loads in PBL are a possible diagnostic criterion for CAEBV and may be useful for monitoring the disease activity, for classifying the disease, and for predicting the prognosis.

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