Epstein-Barr Viral Load and Disease Prediction in a Large Cohort of Allogeneic Stem Cell Transplant Recipients


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Background. We wanted to determine the clinical significance and predictability of Epstein-Barr virus (EBV) infections among a large cohort of recipients of allogeneic, unselected stem cell transplants.

Methods. During 1988–1999, a total of 5479 consecutive serum samples obtained during 406 transplantations performed in Helsinki, Finland, were retrospectively analyzed by quantitative polymerase chain reaction for the presence of EBV DNA.

Results. Overall, EBV DNA was noted in at least 1 serum sample for 57 patients (14.0%), of whom 22 (5.4%) were found to have progressively increasing and ultimately high (>50,000 copies/mL) EBV DNA levels (median level, 179,000 copies/mL). In addition, 16 patients (4.0%) had low EBV DNA levels (median level, 3260 copies/mL) in isolated sera before death. Among the transplant recipients who survived, transient EBV DNAemia (median level, 3110 copies/mL), which apparently corresponded to asymptomatic EBV infection, was noted in 19 patients (4.7%).

Conclusions. Low-level EBV DNA positivity in serum occurs relatively frequently after stem cell transplantation and may subside without specific treatment. However, high EBV DNA levels (i.e., >50,000 copies/mL) are strong predictors for the development of posttransplantation lymphoproliferative disease, are not spontaneously reversible, and should be treated immediately. If the EBV DNA level is >50,000 copies/mL, the patient can be classified as having life-threatening EBV infection.

Posttransplantation lymphoproliferative disease (PTLD) comprises a wide spectrum of disorders that complicate immunosuppressive conditions of various forms. In the context of uncomplicated allogeneic stem cell transplantation (SCT), PTLD has been considered rare (incidence, ≤1%); however, because the risk factors are associated with severe immunosuppression, higher rates may be encountered [1–5]. Risk factors, such as HLA disparity, graft T cell depletion, severe graft-versus-host disease (GVHD) and receipt of antithymocyte globulin treatment, may increase the risk to as high as 15%–25% [1, 5–8]. Most cases of PTLD are observed during the first 6 months after SCT. Early diagnosis of PTLD is difficult. Quantitative PCR (qPCR) for detection of EBV DNA was developed in the past few years, and the initial experience has been encouraging [9–15]. With early diagnosis—even before the onset of symptoms—the disease can be treated promptly or preemptively [14, 16, 17]. In addition, the efficacy of PTLD treatment can be monitored with qPCR [14, 17–20]. The goal of the present study was to assess the diagnostic value of the real-time qPCR by testing serum samples for EBV infection and examining the development of EBV-associated PTLD.

MATERIALS AND METHODS

Patients. During 1988–1999, a total of 406 adult patients had undergone allogeneic SCT at Helsinki University Central Hospital. A total of 5479 serum samples were collected from these patients (range, 2–28 samples...
per patient). The reason for transplantation was malignant hematological disease for 398 patients and aplastic anaemia for 8 patients. Of the donors, 313 were siblings, and 93 were unrelated. All donors were HLA-A, HLA-B, and HLA-DR matched. Of the grafts, 374 were bone marrow, and 32 were blood stem cells. The grafts were unmanipulated. The clinical and transplantation data for the patients were described in detail elsewhere by Juvonen et al. [21].

**EBV in situ hybridization.** To detect EBV in the malignant cells, the tumor tissues were examined by in situ hybridization for EBV-encoded RNA (EBER 1 and EBER 2) [16, 22–24]. Fixed paraffin sections of the malignant tissues were permeabilized with proteinase K. In situ hybridization was performed using a fluorescence-labeled peptide nucleic acid probe complementary to EBER 1 and EBER 2 (Dako) and assayed using a Ventana ES automated slide stainer (Ventana Medical Systems). The probe was visualized with anti-fluorescein antibody (Boehringer Mannheim) and a Ventana DAB detection kit (Ventana Medical Systems). Hematoxylin was used as a counterstain. To confirm the preservation of RNA, separate slides for each sample were assayed with a positive control peptide nucleic acid probe (Dako) directed against glyceraldehyde 3-phosphate dehydrogenase RNA. A random peptide nucleic acid probe (Dako) was used as a negative control.

**EBV qPCR.** The EBV qPCR assay that we used was described elsewhere by Aalto et al. [16]. In brief, DNA was purified from a 200-μL serum aliquot by proteinase K digestion (0.5 mg/mL in 10 mmol/L Tris-buffer [pH, 8.0] containing 10 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% SDS) overnight at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The purified DNA was diluted in 50 μL, of which 5 μL was used as the qPCR template [25]. The primers amplifying a conserved sequence of viral DNA polymerase (BALF5) gene and a fluorogenic probe for this area have been described by Kimura et al. [9]. The probe was synthesized by PE Applied Biosystems (Foster City, CA). Five microliters of purified DNA was added to a PCR mixture containing 1× TaqMan Universal PCR Master Mix (PE Applied Biosystems), 0.3 μmol/L of each primer, and 0.2 μmol/L of fluorogenic probe. After undergoing reaction times of 2 min at 50°C for AmpErase UNG enzyme activity and 10 min at 95°C for activation of AmpliTaq Gold DNA polymerase, we subjected the product to 40 cycles of 15 s at 95°C and 1 min at 60°C in a 7700 Sequence Detector (PE Applied Biosystems).

Real-time fluorescence was measured, and the threshold cycle value for each sample was calculated [25]. For each PCR run, a standard curve was prepared by sequential logarithmic dilutions of EBV DNA extracted from 6.00 × 10⁶ EBV particles, strain B95-8 (Advanced Biotechnologies). The dilutions corresponded to 10⁻¹⁰–10² EBV particles per reaction. The curve was created with ABI 7700 Sequence Detection System software by plotting the threshold cycle values against the known EBV DNA concentration. The detection limit for DNA positivity was 500 copies per milliliter of serum. All samples were tested in duplicate, and the mean value was used as the DNA copy number. In addition to a standard curve obtained with EBV control DNA, each run included several negative and nontemplate controls, as well as a positive control containing EBV DNA of a known copy number.

The serum specimens were assayed retrospectively in pools of 5, each comprising sequential serum specimens obtained from the same patient, if possible. If the pool yielded EBV-positive qPCR results (detection limit for DNA positivity, 500 EBV copies/mL), its constituent serum specimens were reexamined individually. Samples with positive results were reexamined. Before the commencement of this study, we carefully designed the pooled-sample qPCR approach by investigating a large number of serum samples from 12 patients with PTLD that had a known, variably high EBV DNA level (data not shown) [16].

**RESULTS**

Altogether, EBV DNA was present in at least 1 serum sample obtained from 57 (14.0%) of 406 patients. A total of 105 (1.9%) of 5479 samples studied were positive for EBV DNA.

Twenty-two patients (5.4%) had high EBV DNA levels (median level, 179,000 copies/mL). In all 22 patients, the EBV DNA level either increased progressively, with peak levels occurring shortly before death (for 14 patients) or was already high in the first EBV-positive sample (8 patients). In the first EBV-positive sample for all 22 recipients, the median EBV DNA level was 39,000 copies/mL (range, 600–660,000,000 copies/mL). The last EBV-negative and first EBV-positive samples were obtained a mean of 21 days apart (range, 5–52 days). The median EBV DNA level in the last sample was 1,623,000 copies/mL (range, 50,100–1,090,000,000 copies/mL); these last samples were obtained a mean of 8 days before death (range, 1–61 days before death). EBV DNA was first detected a mean of 63 days after transplantation (range, 24–330 days after transplantation) and a mean of 18 days before death (range, 4–84 days before death) (figure 1). In 1 patient who experienced relapse of the hematological malignancy, the EBV DNA appeared 10 months after transplantation and after the patient had experienced donor lymphocyte infusion–induced GVHD. All 22 patients died; the diagnosis of EBV PTLD was confirmed for 15 cases by autopsy, which revealed disseminated infiltrates of EBER-containing lymphocytes.

On the other hand, serum samples obtained from 19 patients (4.7%) revealed transient EBV DNA positivity. At follow-up, 10 of these 19 patients had only 1 sample that contained EBV DNA (the initial EBV-positive sample), 7 patients had 2 EBV-positive samples, and 2 had 3 EBV-positive samples. The viral
DNA was first detected a mean of 98 days after transplantation (range, 24–537 days after transplantation). The EBV DNA level (median, 3110 copies/mL; range, 700–36,200 copies/mL) remained <50,000 copies/mL in all patients. During follow-up, none of these patients was found to have developed PTLD. Of these 19 patients, 7 are still alive.

In addition, 16 patients (3.9%) had PCR results positive for EBV with a low viral load (median level, 3260 copies/mL; range, 800–17,650 copies/mL) observable in only 1 or 2 samples before death (15 patients) or in the last sample available (1 patient who lived) (table 1). These terminal samples had been obtained a mean of 94 days after transplantation (range, 18–534 days after transplantation) and a mean of 69 days before death (range, 3–444 days before death). Five of these patients had histologically confirmed PTLD in the postmortem study. Of these 5 patients, 4 had diffuse PTLD; their last serum samples had been obtained several weeks or months before death (mean, 100 days before death; range, 20–230 days before death), possibly accounting for the relatively low EBV DNA levels in these samples. The fifth patient's last sample (EBV DNA level, 15,000 copies/mL) had been obtained 4 days before death; autopsy revealed that this patient had PTLD of a localized form, involving only the spleen. The causes of death for the other 10 patients were GVHD (6 patients), other infections (1 patient), relapse of the original malignancy (2 patients), and veno-occlusive disease (1 patient).

In total, 349 patients (86%) had no detectable EBV DNA in serum specimens. During follow-up, only 2 of these patients were known to have developed PTLD, beyond sampling for this study, after DLI-induced severe GVHD.

**DISCUSSION**

Our goal was to determine the number, clinical significance, and predictability of EBV infections and their relationships with EBV-associated PTLD in a large cohort of allogenic, unselected SCT recipients. Altogether, EBV infections after SCT were relatively common; 14% of our patients had detectable EBV DNA in at least 1 serum sample; this is in concordance with others’ observations. Cesaro et al. [17] reported reactivation of EBV infection (as determined by increasing viral load noted by qPCR) in 53% of pediatric allogenic SCT recipients. The higher rate of EBV DNA positivity in that study may have had at least 2 reasons: the patients were children, and PBMCs were used for samples. The only risk factor significantly associated with reactivation of EBV infection was the use of antithymocyte globulin. In an earlier study, van Esser et al. [26] analyzed plasma samples obtained from adult SCT recipients by qPCR and reported an incidence of reactivation of EBV infection of 28%. In that study, none of the recipients of unmanipulated SCTs developed PTLD. Clave et al. [15] detected EBV DNA in PBMCs obtained from 28 (50%) of 56 recipients of allogenic SCTs. Plasma EBV DNA was detected in 9 (16%) of 56 recipients, who also displayed the highest cellular viral loads [15].
Table 1. Findings of Epstein-Barr virus (EBV) quantitative PCR for the last serum samples obtained from 16 patients with a low-level terminal EBV-PCR finding, in relation to time after transplantation.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time, months</th>
<th>EBV DNA level, copies/mL</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>After death</td>
<td>0.6</td>
<td>7700 VOD</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>14.6</td>
<td>3220 Relapse</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>1.3</td>
<td>8910 Relapse</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>3.9</td>
<td>4190 PTLD</td>
</tr>
<tr>
<td>5</td>
<td>2.9</td>
<td>1.6</td>
<td>3080 Infection</td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>0.2</td>
<td>1270 GVHD</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
<td>7.7</td>
<td>3410 PTLD</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>0.7</td>
<td>8340 GVHD</td>
</tr>
<tr>
<td>9</td>
<td>5.1</td>
<td>1.1</td>
<td>800 PTLD</td>
</tr>
<tr>
<td>10</td>
<td>17.9</td>
<td>...</td>
<td>2060 ...</td>
</tr>
<tr>
<td>11</td>
<td>2.0</td>
<td>0.4</td>
<td>1355 GVHD</td>
</tr>
<tr>
<td>12</td>
<td>2.2</td>
<td>0.2</td>
<td>4990 GVHD</td>
</tr>
<tr>
<td>13</td>
<td>2.0</td>
<td>0.9</td>
<td>800 GVHD</td>
</tr>
<tr>
<td>14</td>
<td>2.4</td>
<td>0.1</td>
<td>15,000 PTLD</td>
</tr>
<tr>
<td>15</td>
<td>3.0</td>
<td>0.8</td>
<td>3200 GVHD</td>
</tr>
<tr>
<td>16</td>
<td>3.0</td>
<td>0.7</td>
<td>17,650 PTLD</td>
</tr>
</tbody>
</table>

NOTE. GVHD, graft-versus-host disease; PTLD, posttransplantation lymphoproliferative disease; VOD, veno-occlusive disease.

* Patient is alive.

Altogether, in the present retrospective study, 22 SCT recipients (5.4%) had high EBV DNA levels (i.e., >50,000 copies/mL). The EBV DNA levels for these patients either increased progressively, with the peak levels occurring shortly before death, or were already elevated in the first EBV-positive serum sample. The prognosis of these patients was sinister; all cases were fatal. In addition, 19 patients (4.7%) had only transient EBV DNA positivity, the mean time of which was during the first 3 months after transplantation. The EBV DNA levels remained significantly lower for these patients than for those with fatal EBV infection; only 1 of the former patients had a moderately high EBV DNA level (36,200 copies/mL). Importantly, for all of these patients, EBV disappeared without clinical intervention, and none developed EBV PTLD during follow-up. Therefore, our data demonstrate that low EBV DNA levels in serum specimens occur frequently and may resolve spontaneously.

It is interesting to note that 16 patients (4.0%) had low EBV DNA levels (median level, 3260 copies/mL) in the last available samples before death. In 5 of these patients, the postmortem diagnosis was PTLD. To understand the EBV DNA levels in these patients, it is important to note that 3 of these final samples had been obtained several months before the manifestation of PTLD. The other 2 patients had, in their last samples, moderately high EBV DNA levels (17,560 and 15,000 copies/mL in samples obtained 20 and 4 days before death, respectively). Autopsy revealed that the latter patient’s PTLD was of the localized form; in contrast, PTLD was diffuse in the other patients. Therefore, the finding of moderately high EBV DNA levels warrants very close follow-up to monitor for increases in the EBV DNA level.

Presently, no consensus exists as to which sample should be used for EBV qPCR: PBMCs, unfractioned blood, serum, or plasma. Clave et al. [15] used qPCR to study both cellular and plasma samples obtained from allogenic SCT recipients. EBV DNA was noted in 50% of the PBMC samples and in 16% of the plasma samples. The patients with EBV-positive plasma samples also had the highest cellular viral loads [15]. In our study, EBV DNA appeared in serum in all patients with severe EBV infection. Niesters et al. [10] reported very high plasma EBV DNA levels in patients with PTLD (mean level, 540,000 copies/mL), moderately high levels in patients with infectious mononucleosis (mean level, 6400 copies/mL), and low levels in transplant recipients without PTLD (mean level, 440 copies/mL). Kullberg-Lindh et al. [27] used qPCR analysis of serum samples obtained from pediatric liver transplant recipients and found significantly higher EBV DNA levels in patients with primary symptomatic infection (mean level, 65,500 copies/mL) than in those with primary asymptomatic infection (mean level, 3700 copies/mL). In terms of sampling, PBMCs may be difficult to obtain from cytopenic patients, and plasma or serum specimens can be considered the samples of choice in situations in which cell counts are low. This is often the case when immune reconstitution is delayed or during administration of anti-CD 20 antibody therapy [13, 15, 19]. Of note, the actual volume of serum (or plasma) required for the qPCR assay in our study is small (200 μL).

For improved management of EBV-associated PTLD, the availability of universal laboratory standards for EBV qPCR is crucial. Such standards would facilitate correlation between viral loads and disease, would enhance dissemination of qPCR results and treatment guidelines between researchers and clinicians, and, in general, are a prerequisite for universal qPCR-based criteria for diagnosis and treatment of this severe disease.

In conclusion, low serum EBV DNA levels occur relatively frequently after SCT and may subside without specific treatment. On the other hand, high EBV DNA levels (i.e., >50,000 copies/mL) are diagnostic of PTLD, are not spontaneously reversible, and warrant immediate treatment.

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