Identification of Epstein–Barr virus-infected CD27+ memory B-cells in liver or stem cell transplant patients

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To analyse the phenotype of Epstein–Barr virus (EBV)-infected lymphocytes in EBV-associated infections, cells from eight haematopoietic stem cell/liver transplantation recipients with elevated EBV viral loads were examined by a novel quantitative assay designed to identify EBV-infected cells by using a flow cytometric detection of fluorescent in situ hybridization (FISH) assay. By this assay, 0.05–0.78 % of peripheral blood lymphocytes tested positive for EBV, and the EBV-infected cells were CD20+ B-cells in all eight patients. Of the CD20+ EBV-infected lymphocytes, 48–83 % of cells tested IgD positive and 49–100 % of cells tested CD27 positive. Additionally, the number of EBV-infected cells assayed by using FISH was significantly correlated with the EBV-DNA load, as determined by real-time PCR (r2 = 0.88, P < 0.0001). The FISH assay enabled us to characterize EBV-infected cells and perform a quantitative analysis in patients with EBV infection after stem cell/liver transplantation.

Epstein–Barr virus (EBV) is a ubiquitous virus that infects humans worldwide. Acute infectious mononucleosis (AIM) is the primary EBV infection (Rickinson & Kieff, 2006), and current understanding of primary EBV infection is primarily based on studies of patients with AIM. EBV infects naïve B-cells in the tonsils and activates them; these infected B-cells can differentiate through a germinal centre in lymphoid tissue, becoming resting memory B-cells (Thorley-Lawson & Gross, 2004). Alternatively, EBV may infect memory cells directly (Kurth et al., 2000).

EBV-related post-transplant lymphoproliferative disorder (PTLD) is a life-threatening disease following haematopoietic stem cell or solid organ transplantation, and EBV infects B-lymphocytes in most cases (Rickinson & Kieff, 2006). EBV also causes mild/moderate symptomatic diseases other than PTLD. Recently, serial EBV load monitoring following solid organ transplantation identified a population of recipients who subsequently developed and maintained very high EBV loads in the absence of clinical symptoms (Bingler et al., 2008; D’Antiga et al., 2007; Green et al., 2009). In these cases, EBV also infected B-cells in peripheral blood (Gotoh et al., 2010). Characterizing the EBV-infected lymphocytes in these different EBV-associated infections may provide a better understanding of the pathophysiology of EBV-related disorders.

We recently established a novel quantitative assay to identify EBV-infected cells that uses flow cytometric detection of fluorescent in situ hybridization (FISH) (Kimura et al., 2009). With a fluorescein-conjugated probe that specifically hybridizes to the EBV-encoded small RNA (EBER), both nuclear EBER and surface lymphocyte antigens can be stained. This assay can be used with peripheral blood to characterize EBV-infected lymphocytes. With this FISH assay, we analysed peripheral blood in patients with EBV-associated T/NK-cell lymphoproliferative disease. The EBER-positive cells were found to be CD3+CD4−CD8−TCRγδ+/− T-cells in patients with hydroa vacciniforme-like lymphoproliferative disease, which is an EBV-positive cutaneous T-cell lymphoma (Kimura et al., 2009), thus providing new insight into EBV-associated T/NK-cell lymphoproliferative disease.

In this study, we applied the FISH assay to peripheral blood from eight haematopoietic stem cell/liver transplantation recipients with increasing amounts of EBV DNA in their peripheral blood to characterize the EBV-infected lymphocytes. The number of EBER-positive cells in peripheral
blood, as quantified by the FISH assay, was also compared with the EBV-DNA load, as determined by quantitative PCR.

Four haematopoietic stem cell transplant recipients and four liver transplant recipients were enrolled in the study. A quantitative PCR assay detected a large amount of EBV DNA in the peripheral blood of all of the patients. A prospective analysis of EBV load in peripheral blood is routinely performed in our department to provide an early diagnosis of EBV-associated diseases in patients after stem cell and liver transplantation. Blood samples for the FISH assay (described below) were obtained from: four haematopoietic stem cell transplantation recipients with suspected symptomatic EBV infection; four liver transplant recipients without clinical symptoms but with the continuous presence of high EBV viral loads; and five healthy volunteers with a history of EBV infection. PBMCs were separated by density gradients from whole blood. Informed consent was obtained from all participants or their parents. This study was approved by the University of Nagoya Institutional Review Board.

Viral load was examined in the PBMCs of all patients. Viral DNA was extracted from $1 \times 10^6$ PBMCs by using QIAamp DNA blood kits (Qiagen). The real-time quantitative PCR assay was performed as previously described (Kimura et al., 1999; Wada et al., 2007). The FISH assay was also performed as previously described (Kimura et al., 2009). Briefly, for surface marker staining, $5 \times 10^5$ PBMCs were stained with phycoerythrin cyanin 5 (PC5)-labelled anti-CD20 mAb (clone B-Ly1, DakoCytomation), and phycoerythrin (PE)-labelled anti-IgD mAb (clone IgD26, MACS) or biotin-labelled anti-CD27 (clone O323, Biologend) mAb, followed by streptavidin–PC5 (eBioscience), for 1 h at 4 °C. The cells were then fixed with 1% acetic acid in 4% paraformaldehyde/PBS (pH 7.4) for 40 min at 4 °C. After washing, cells were permeabilized in 50 μl 0.5% Tween 20/ PBS at room temperature. The cells were resuspended in 45 μl of hybridization solution (6% dextran sulphate, 10 mM NaCl, 17.5% formamide, 0.061% sodium pyrophosphate, 0.12% polyvinylpyrrolidone, 0.12% Ficoll, 5 mM Na$_2$EDTA, 50 mM Tris/HCl, pH 7.4) containing 12 nM of the EBER PNA Probe/FITC (Y5200, DakoCytomation). Hybridization was carried out for 1 h at 56 °C. Then, the cells were washed twice and an Alexa Fluor 488 Signal Amplification kit (Molecular Probes) was used to enhance fluorescence and photostability. The stained cells were analysed using a FACSCalibur and CellQuest software (BD Biosciences). Up to 50 000 events were recorded for each analysis. The lymphocytes were gated by standard forward- and side-scatter profiles. Dead cells were not excluded. To determine the lower detection limit of the FISH assay for EBV$^+$ cells, we mixed EBV$^+$ Raji and EBV$^-$ BJAB cells in various ratios and quantified them by using the FISH assay (Kimura et al., 2009). EBV-positive cells could be quantified, as the Raji/BJAB ratio was between 0.1 (EBV-positive cells were 10%) and 0.0001 (0.01%), although the population of CD19$^+$EBER$^+$ cells was less clear at a ratio of 0.0001. Therefore, the detection limit of the FISH assay was considered to be 0.01–0.1%.

Statistical analyses were performed with SPSS for Windows version 18.0 (SPSS). Regression analysis (Pearson product-moment correlation coefficient) was used to compare the FISH assay and the real-time PCR. Values of $P<0.05$ were considered statistically significant.

The characteristics of each patient are shown in Table 1. Based on the FISH assay, EBER-positive lymphocytes were detected in all eight patients and they ranged from 0.05 to 0.78% of lymphocytes (Table 1). The mean and SEM of the percentage of the EBER-positive cells was 0.27 ± 0.13% in haematopoietic stem cell transplantation recipients and 0.38 ± 0.15% in liver transplantation recipients. The phenotypes of each patient are also shown in Table 1. The percentage of the total of CD20$^+$ lymphocytes varies among patients, particularly in patients with haematopoietic stem cell transplant. In contrast, the percentages in the five healthy volunteers were within the normal range (14, 15, 19, 22 and 25). The EBV-infected cells were CD20$^+$IgD$^+$ or CD20$^+$CD27$^+$ cells in most patients. Representative results of the dual staining are shown in Fig. 1. In patient 2, EBER-positive lymphocytes were detected in 6% of CD20$^+$ lymphocytes, and EBV-infected cells were mostly IgD$^+$ or CD27$^+$ cells. In patient 4, EBER-positive lymphocytes were detected in 2% of CD20$^+$ lymphocytes, and EBV-infected cells were detected in approximately 50% of IgD$^+$ or CD27$^+$ cells. The main EBV-infected cells were identified as being CD20$^+$IgD$^+$ or CD20$^+$CD27$^+$ cells in other patients. In contrast, EBER-positive cells were not detected in any of the five healthy volunteers (representative results are also shown in Fig. 1c, d), and the mean percentage of IgD$^+$ cells or CD27$^+$ cells within the sample of CD20$^+$ lymphocytes was 66% (range, 56–78%) and 48% (range, 33–59%), respectively.

Next, we compared the results of the FISH assay with those from the real-time quantitative PCR. The number of EBER-positive cells assayed by FISH was significantly correlated with the EBV-DNA load determined by real-time PCR ($r^2=0.88, P<0.0001$; Fig. 2).

The percentage of the total of CD20$^+$ cells in the five healthy volunteers was within the normal range, suggesting that the FISH assay was well performed. In contrast, these percentages vary among patients, particularly in patients with haematopoietic stem cell transplants (from 10 to 66%, Table 1). Myeloablative chemotherapy followed by haematopoietic stem cell transplantation is associated with substantial B- and T-cell immunodeficiency for a period of up to several years (Douek et al., 2000; Storek et al., 1993). As the reconstitution of B- and T-cells was not parallel, and this reconstitution is influenced by various factors, such as graft-versus-host disease (Storek et al., 2001), the ratio of these cells may vary in patients for prolonged periods after transplantation. B-cells play a large role in the humoral immune response. Naïve B-cells proliferate and differentiate to yield memory
Table 1. Characteristics of haematopoietic stem cell/liver transplantation recipients with elevated EBV loads

EBV, EBV-encoded small RNA.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Transplant</th>
<th>Clinical symptoms</th>
<th>Time post-transplant (months)</th>
<th>Time post testing EBV positive (months)</th>
<th>EBV DNA in PBMCs [copies (μg DNA)^-1]</th>
<th>CD20^+ lymphocytes (% of total)</th>
<th>EBER^+ lymphocytes (% of total)</th>
<th>Percentage of CD20^+ EBER^+ lymphocytes</th>
<th>Percentage of CD20^+ EBER^+ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>7</td>
<td>Bone marrow</td>
<td>Fever, cough, diarrhea</td>
<td>1</td>
<td>1</td>
<td>41,175</td>
<td>40.63</td>
<td>0.63</td>
<td>56.0</td>
<td>80.0</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>1</td>
<td>Bone marrow</td>
<td>Fever, diarrhoea, rash</td>
<td>3</td>
<td>1</td>
<td>3,730</td>
<td>15.00</td>
<td>0.10</td>
<td>83.0</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>6</td>
<td>Bone marrow</td>
<td>Malaise, cough, hiliar lymphadenopathy</td>
<td>10</td>
<td>1</td>
<td>2,441</td>
<td>10.00</td>
<td>0.05</td>
<td>58.0</td>
<td>100.0</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>48</td>
<td>Cord blood</td>
<td>Hepatomegaly</td>
<td>37</td>
<td>6</td>
<td>10,500</td>
<td>66.00</td>
<td>0.28</td>
<td>48.0</td>
<td>49.0</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>1</td>
<td>Liver</td>
<td>Asymptomatic</td>
<td>9</td>
<td>8</td>
<td>13,392</td>
<td>29.00</td>
<td>0.39</td>
<td>81.0</td>
<td>99.0</td>
</tr>
<tr>
<td>6</td>
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<td>Liver</td>
<td>Asymptomatic</td>
<td>15</td>
<td>15</td>
<td>4,729</td>
<td>12.00</td>
<td>0.15</td>
<td>79.0</td>
<td>99.0</td>
</tr>
<tr>
<td>7</td>
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<td>Asymptomatic</td>
<td>15</td>
<td>15</td>
<td>8,302</td>
<td>23.00</td>
<td>0.19</td>
<td>83.0</td>
<td>98.0</td>
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<tr>
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<td>Asymptomatic</td>
<td>96</td>
<td>96</td>
<td>16,438</td>
<td>36.00</td>
<td>0.78</td>
<td>68.0</td>
<td>83.0</td>
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Fig. 1. Characterization of EBV-infected lymphocytes in representative patients. PBMCs were stained with mAbs for surface markers, fixed, permeabilized and hybridized with an EBER probe. The lymphocytes were gated by standard forward- and side-scatter profiles, and plotted on the quadrant that is at the centre of each panel for each patient or control. CD20⁺EBER⁺ lymphocytes (red) and CD20⁺EBER⁻ lymphocytes (blue) were gated and the expression of IgD or CD27 is shown in each histogram. (a) Patient 2; (b) patient 4; (c) control 1 (a healthy volunteer with a history of EBV infection); (d) control 2.
were measured by using a real-time PCR assay. PBMCs from all eight patients were analysed by using the FISH assay. Amounts of EBV DNA in PBMCs from all eight patients were measured by using a real-time PCR assay.

Fig. 2. Correlation between the percentage of EBER-positive lymphocytes, as measured by FISH, and the EBV-DNA load, as measured by using real-time PCR. EBER-positive lymphocytes in PBMCs from all eight patients were analysed by using the FISH assay. Amounts of EBV DNA in PBMCs from all eight patients were measured by using a real-time PCR assay.

demonstrated a correlation between the number of EBER-positive cells and the EBV-DNA load, as measured by FISH, and the EBV-DNA load, as measured by real-time PCR. As the number of EBERs is not correlated with the number of EBV genomes in infected cells, detection of EBERs does not provide a precise estimate of the number of EBV genomes present per cell. However, the number of EBV-genome equivalents can be calculated if the number of EBV genomes is equal in EBER-positive cells. Looking at the data in Fig. 2, 10^4 copies μg^-1 of DNA points to the result that the percentage of EBER-positive cells is approximately 0.3%. Because 1 μg DNA appears to be extracted from 2 × 10^6 PBMCs (Kimura et al., 1999), 10^4 copies μg^-1 of DNA is equivalent to 10^4 copies per 2 × 10^6 PBMCs, that is, five copies per 100 cells. If infected cells represent 0.3% of cells, 16.7 copies of the EBV genome are in a single infected cell. Additionally, in a separate study we demonstrated a correlation between the number of EBER-positive cells, as measured by FISH, and the amount of EBV DNA in EBV-associated T- or NK-cell lymphoproliferative diseases, in which EBV infects T- or NK cells (unpublished data). The number of EBV genomes was estimated to be one tenth of that observed in the memory B-cells in this study (data not shown). These data are consistent with the results of Calattini et al. (2010). Characterizing the phenotype of EBV-infected cells by the FISH assay combined with a real-time PCR assay enables a quantitative analysis of EBV-associated diseases.

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