Longitudinal Dynamics of Epstein-Barr Virus–Specific Cytotoxic T Lymphocytes during Posttransplant Lymphoproliferative Disorder

Kiyotaka Kuzushima,1 Hiroshi Kimura,2 Yo Hoshino,2 Ayami Yoshimi,2 Ikuya Tsuge,2 Keizo Horibe,2 Tsuneo Morishima,1 Tatsuya Tsurumi,1 and Seiji Kojima 2

1Division of Virology, Aichi Cancer Center Research Institute, and Departments of 2Pediatrics/Developmental Pediatrics and 3Health Science, Nagoya University School of Medicine, Nagoya, Japan

Epstein-Barr virus (EBV)–associated lymphoproliferative disorder (LPD) is a serious complication after allogeneic bone marrow transplantation (BMT). Dynamics of EBV-specific cytotoxic T lymphocytes (CTL), which are important in controlling EBV during the LPD, have not been fully elucidated. A patient with Wiskot-Aldrich’s syndrome was diagnosed as suffering from LPD on day 47 after BMT. Fluorescence-activated cell sorter (FACS) analysis for interferon-γ production revealed that 70% of the patient’s CD8+ T cells were EBV specific. The patient’s lymphocytes were directly cytotoxic to donor-derived EBV-positive lymphoblastoid cells, which was blocked by an anti-class I antibody. EBV-specific CD8+ T cell counts declined in parallel with EBV genome load, and full recovery of LPD was obtained with relaxation of immunosuppressive drugs. The results illustrate longitudinal dynamics of EBV-specific CTL during the posttransplant LPD; they also illustrate the advantages of using FACS analysis for EBV-specific CTL to make decisions about treatment.

Epstein-Barr virus (EBV) is closely associated with lymphoproliferative disorder (LPD) seen in immunocompromised hosts [1–3]. Major histocompatibility complex (MHC) class I–restricted, virus-specific CD8+ cytotoxic T lymphocyte (CTL) responses play an important role in controlling the virus both during primary infection and in the long-term carrier state [4].

In patients with primary EBV infection, the notion of antigen-driven expansion of CD8+ T cells has become widely accepted because of technologies that allow antigen-specific T cells to be detected with high sensitivity [5, 6]. Direct staining of antigenic peptide–specific T cells by tetrameric MHC–peptide complexes showed that T cells specific for an EBV peptide comprised 44% of the total CD8+ T cells in peripheral blood mononuclear cells (PBMC) of a HLA-B8–positive patient [5]. Measurement of interferon (IFN)-γ production in antigen-responding T cells at the single-cell level by fluorescence-activated cell sorter (FACS) also disclosed that 30%–60% of activated CD8+ T cells in PBMC are EBV specific [6].

The importance of EBV-specific T cells for control of latently infected B cells has been re-emphasized by observations in patients with LPD after bone marrow transplantation (BMT); adoptive transfer of EBV-specific CTLs or un fractionated donor lymphocytes can result in remission of the LPD [7, 8]. Withdrawal of immunosuppressants may cause regression of EBV-associated LPD in some patients after BMT [9, 10].

To understand the precise roles of specific cellular immunity to EBV and to construct an effective and practical strategy for the management of posttransplant LPD, details of EBV-specific T cell responses need to be elucidated. However, as far as we know, there have been no reports of the dynamics of EBV-specific T cells in such cases in light of the sensitive assays described above.

We here present a case of post-BMT LPD in which viral load and EBV-specific CD8+ T cell frequencies were closely monitored in a real-time manner. We discuss the clinical significance of such a strategy against serious infectious disease in immunocompromised hosts.

Materials and Methods

Case report. A 1-year-old boy suffering with Wiskot-Aldrich’s syndrome received bone marrow from an unrelated female donor whose class I HLA was mismatched at 1 locus with the patient (patient HLA typing, A24/2603, B52/4006; donor HLA typing, A24/2602, B52/4006; DR0901/1502). Conditioning regimen consisted of cyclophosphamide, anti-thymocyte globulin,
and total-body irradiation, and prophylaxis against graft versus host disease (GVHD) of tacrolimus and short-term methotrexate. On day 47, a biopsy of cervical lymph nodes was performed, to find a cause of the patient’s fever and general swelling of the lymph nodes. The pathological diagnosis was polyclonal (mixed κ and λ chains) B cell hyperplasia (EBV-associated LPD). On the basis of the strong EBV-specific cellular immune responses in the patient’s PBMC at that time, we watched the clinical course with slight relaxation of tacrolimus by 25%. He became afebrile on day 62 and was discharged from the hospital on day 123 after BMT.

Preparation of PBMC, lymph node cells, and lymphoblastoid cell lines (LCLs). PBMC were separated by centrifuging heparinized blood on density gradients. Single-cell suspensions of lymphocytes were obtained by mincing biopsied cervical lymph nodes with a surgical blade. LCLs were prepared by transforming lymphocytes with B95-8 cell culture supernatant as described elsewhere [11] and expanded in RPMI 1640 (Gibco, Grand Island, NY) supplemented with penicillin, streptomycin, 2-mercaptoethanol, and 10% fetal calf serum (referred to as culture medium).

Analysis of surface antigens on lymphocytes. PBMC were incubated with fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)–labeled monoclonal antibodies (MAbs) specific to CD3, CD4, CD8, or CD19 antigens (Coulter, Miami). Stained cells were analyzed by FACScan (Becton Dickinson, San Jose, CA).

Detection of IFN-γ–producing CD8+ T cells in response to LCLs or antigenic peptides by FACS. EBV-specific CD8+ T cell frequencies were measured as described elsewhere [8]. Briefly, $1 \times 10^6$ PBMC or cells from minced lymph nodes were mixed with $1 \times 10^6$ donor-derived LCLs in 1 mL of culture medium. For detection of peptide-specific T cells, EBV peptides presented by HLA-A24 molecules [4, 12] were synthesized by Sawady Technology (Toshima-ku, Tokyo). Each peptide was added at a final concentration of 10 μg/mL. These cell suspensions were incubated at 37°C for 6 h in the presence of 10 μg/mL brefeldin A (Sigma, St. Louis). For blocking experiments, anti-HLA class I MAb (clone W6/32, Cedarlane, Ontario, Canada) or isotype-matched control MAb was added at a final concentration of 50 μg/mL. The percentage of specific lysis was calculated as described elsewhere [11].

Y chromosome–specific polymerase chain reaction (PCR) anal-

Figure 1. Dynamics of Epstein-Barr virus (EBV) DNA copy numbers and subpopulations of peripheral blood lymphocytes during regression of lymphoproliferative disorder (LPD). A biopsy (arrow) of cervical lymph nodes was performed, and a diagnosis of posttransplant LPD resulted. BMT, bone marrow transplantation.
Figure 2. Dynamics of Epstein-Barr virus (EBV)-specific (top) and EBV-derived peptide-specific (bottom) CD8\(^+\) T cells during regression of lymphoproliferative disorder. Peripheral blood mononuclear cells were stimulated with donor-derived lymphoblastoid cells or EBV nuclear antigen 3A peptide (RYSIFFDY) presented by HLA-A24 molecules. The frequency of CD8\(^+\) T cells producing interferon (IFN)-\(\gamma\) is shown as a percentage of the total CD8 bright cells. The days after bone marrow transplantation (BMT) at which samples were taken are indicated.

DNA was extracted from CD4, CD8, or CD19 lymphocyte subpopulations that had been isolated with immunomagnetic beads (Dynal, Oslo, Sweden). The details of Y chromosome-specific PCR for distinguishing donor versus recipient cells have been described elsewhere [13]. A 50-ng aliquot of DNA was used for the PCR reaction for each sample.

Real-time quantitative PCR. Real-time quantitative PCR assays with a fluorogenic probe were performed as described elsewhere [14]. DNA extracted from PBMC was used for PCR reactions of 50 cycles of 15 s at 95°C and 1 min at 62°C with a 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). A threshold cycle value for each sample was calculated by determining the point at which fluorescence exceeded 10 times the standard deviation of the baseline. For a positive control, a plasmid pGEM-BALF5 containing the BALF5 gene was used [14]. The threshold cycle values for clinical samples were plotted on the standard curve, and copy numbers were calculated and expressed per milligram of DNA.

Results

Donor-derived lymphoproliferation in association with elevation of EBV DNA copy numbers in PBMC. We decided to monitor EBV genome load in PBMC of the patient because of the risk factors associated with EBV LPD. One such risk was primary immunodeficiency. Another was HLA mismatch; in this patient’s case, the donor was unrelated. A third risk was use of anti-thymocyte globulin [2, 3]. The EBV DNA was first detected on day 41, followed by a peak titer on day 46 accompanied by a sharp elevation of the peripheral lymphocyte counts, as shown in figure 1. Serial FACS analyses indicated the presence of lymphocytosis with predominant CD3 and CD8 subpopulations. CD4 and CD19 counts elevated and decreased in parallel with the CD8 count, albeit in smaller magnitudes. When we sorted with the aid of magnetic beads (data not shown), we found that EBV DNA was largely detected in the CD19 subpopulation.

There was no amplification of Y chromosome genes that used the DNA extracted from CD4, CD8, and CD19 lymphocyte subpopulations sampled on day 49 after BMT. The PCR reaction can detect at least 1% of the recipient DNA (data not shown); thus, at that time, >99% of peripheral CD4, CD8, and CD19 lymphocytes were of donor origin.

Massive expansion of EBV-specific CD8\(^+\) T cells at the peak of EBV genome load. To analyze EBV-specific CD8\(^+\) T cell response quantitatively, we performed serial FACS analyses for detecting IFN-\(\gamma\) production with autologous (donor-derived) LCL and EBV nuclear antigen 3A peptide (RYSIFFDY) [12] as stimulators. Strikingly, >70% of peripheral CD8\(^+\) T cells on day 46 after BMT were EBV specific (figure 2, upper lane). EBV-specific IFN-\(\gamma\) production of any given samples was almost completely blocked by anti-HLA class I MAb (data not shown). Unstimulated, <0.5% of the CD8\(^+\) T cells produced IFN-\(\gamma\) (data not shown).

When the EBV-specific CD8\(^+\) T cell response was at its peak on day 46, the frequency of the peptide-specific CD8\(^+\) T cells was 2.09% (figure 2, lower lane), adding epitope-based evidence for the reaction, although the peptide was not an immunodominant epitope in the patient. We also tested peptides derived from EBV nuclear antigen 3B and from latent membrane protein 2A, both of which are known to be presented by HLA-A24 molecules [4]. However, the frequencies of CD8\(^+\) T cells specific for these were <0.1% even at the peak (data not shown). Thus the peptide RYSIFFDY was used for the serial study.
The frequencies of EBV-specific CD8+ T cells, as a whole or for RYSIFFDY, fell to half the peak frequency over the first 3 days and then gradually decreased further during the observation period. Analysis of the data provided in figures 1 and 2, taken in combination, demonstrates that total CD8+ T cell count on day 46 after BMT was boosted by the massive expansion of the EBV-specific CD8+ T cells, and the subsequent rapid disappearance of the EBV-specific population greatly contributed to the contraction of the total CD8+ T cell counts.

**PBMC were directly cytotoxic to donor-derived LCLs in a class I restricted manner.** To confirm the cytotoxic activities against donor-derived LCLs, cytotoxic assays were performed with PBMC on day 49 as effector cells. Specific lysis of donor-derived LCLs at an effector:target cell ratio of 20:36.2%, which was specifically blocked by anti-HLA class I MAb (10.6%).

**EBV-specific CD8+ T cells infiltrated in lymph nodes during LPD.** Finally, we examined whether EBV-specific CD8+ T cells had infiltrated in lymphoid organs. Biopsied lymph nodes on day 47 were minced, and single-cell suspensions were used as effector cells. When stimulated with the donor-derived LCLs, 39.6% of CD8+ cells in the suspension produced IFN-γ. The IFN-γ-producing population was reduced (1.26%) with anti-HLA class I MAb but not with isotype-matched MAb (45.9%). The cells were directly cytotoxic to donor-derived LCLs, which was again specifically blocked by the anti-HLA class I MAb (data not shown). These results indicate that the class I-restricted, EBV-specific CTLs expanded in both peripheral blood flow and local lymphoid organs.

**Discussion**

The present quantitative analysis of both EBV genome load and EBV-specific CD8+ T cell responses in a patient during regression of EBV LPD after BMT—the first time, to our knowledge, that such a study has been undertaken—demonstrated a massive expansion of EBV-specific CD8+ T cells, to >70% of total CD8+ T cells at its peak. Donor versus recipient chimeric study showed residual recipient-derived CD8+ T cells were <1% after BMT. Thus, these IFN-γ-producing populations in response to autologous (donor-derived) LCLs are considered to be EBV specific but not allospecific. Interestingly, the peak of the T cell expansion was synchronized with that of EBV DNA copy numbers in the PBMC, a finding also observed in primary EBV infection [6].

The successful management of the patient whose case we present was based on 2 strategies. The first is prospective monitoring of EBV DNA copy numbers by use of a real-time PCR method in patients at high risk for EBV LPD. Consequently, the DNA could be detected in an early phase of the disease, when B cell proliferation is mostly polyclonal. We usually obtain initial positive results when patients are febrile only—or even without symptoms. At this very early stage of disease, we could optimize the doses of immunosuppressants. Second, precise EBV-specific CD8+ T cell frequencies are assayed in real time. As a result, the decision not to perform donor lymphocyte transfusion, which might have added another risk of GVHD, but rather to watch the patient carefully, could be made. Kern et al. [15] pointed out the clinical usefulness of monitoring of human cytomegalovirus-specific T cell frequencies (besides EBV) in immunocompromised hosts.

Taken together, our data underscore the notion that EBV-specific CD8+ T cells are responsible for regression of posttransplant LPD by graphically illustrating the dynamics of the population. Our findings also feature substantial advantages of both real-time PCR assays for EBV-DNA quantification and FACS analysis for EBV-specific T cells for early diagnosis and decision making for treatments of some cases of EBV-associated posttransplant LPD.

**References**