system (Applied Biosystems, Warrington, UK), as described (18). Briefly, DNA was purified automatically by the BIO ROBOT EZ1 (Qiagen, Hilden, Germany) from whole blood with the protocol and the reagents recommended by the manufacturer. Amplification of EBV DNA was carried out by the ABI Prism 7500 Real-Time PCR System (Applied Biosystems) with the EBV-specific primers derived from the conserved sequences in the BALF5 gene encoding the EBV DNA polymerase. PCR was carried out in a total volume of 25 μL, including 12.5 μL TaqMan Universal Master Mix, 0.2 μM of each primer, 0.1 μM of probe, and 0.1 μg of template DNA. Thermal cycling was performed in the following steps: denaturation and polymerase activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min. Standard solutions of Raji cell DNA at the concentrations of 10^5, 10^4, 10^3, and 10^2 EBV DNA copies/μg DNA were prepared and used to generate a standard curve. Peripheral blood EBV DNA load of $> 10^2$ copies/μg DNA was considered as significant elevation, because >95% of healthy EBV carriers had an EBV DNA load below this level in our preliminary study. Patients with EBV DNA load above this level were further examined by flow cytometry as described below.

Immunophenotype analysis and quantification of Epstein–Barr virus-specific T cells in the peripheral blood

PBMC were isolated by centrifugation on Lymphosepar I (Immuno-Biological Laboratories, Fujioka, Japan) and incubated for 30 min on ice with a mixture of appropriate combination of fluorescently labeled monoclonal antibodies and PE-labeled EBV-specific HLA-A*2402 tetramers presenting epitopes derived from the five EBV proteins LMP2, BFLF1, BMLF1, EBNA3A, and EBNA3B (Medical and Biological Laboratories, Nagoya, Japan). After washing, five-color flow cytometric analysis was carried out with the Cytomics FC500 analyzer (Beckman Coulter, Brea, CA, USA). The following directly labeled antibodies were used: PE-conjugated antibodies to CD7, CD26, CD45RA, CD8, and CD20, FITC-conjugated antibodies to CD4, CD8, CD23, CD28, and CD45RO, PC5 (PE-Cy5)-conjugated antibody to CD3 and CD56, PC7 (PE-Cy7)-conjugated antibodies to CD4, CD16, and CD19, and ECD-conjugated antibody to CD45 from Beckman Coulter; and FITC-conjugated antibody to HLA-DR from BD Pharmingen (Franklin Lakes, NJ, USA).

Reverse transcription PCR analysis of Epstein–Barr virus gene expression

Analysis of EBV gene expression by RT-PCR was carried out as previously described with the following primers (19). EBNA1: sense, gatgagcgtttgggagagctgattctgca; antisense, tcctcgtccatggttatcac. EBNA2: sense, agaggaggtggtaagcggttc; antisense, tgacgggtttccaagactatcc. LMP1: sense, ctctccttctcctcctcttg; antisense, caggagggtgatcatcagta. LMP2A: sense, atgactcatctcaacacata; antisense, catgttaggcaaattgcaaa. LMP2B: sense, cagtgtaatctgcacaaaga; antisense, catgttaggcaaattgcaaa. RT-PCR primers for β-actin were purchased from Takara (Osaka, Japan).

ELISPOT assay

ELISPOT assay was performed with the IMMUNOCYTO IFN-γ ELISPOT kit (Medical and Biological Laboratories) following instructions supplied by the manufacturer. Briefly, $CD8^+$ T cells were isolated from PBMC of transplant recipients with the IMag anti-human CD8 Particles-DM (BD Pharmingen). Mixture of these $CD8^+$ T cells and autologous lymphoblastoid cells immortalized by EBV were incubated with IL-2 in microplates coated with antibody to IFN-γ for 17 h. Captured IFN-γ was detected by biotinated antibody to IFN-γ and alkaline phosphatase-conjugated streptavidin, and visualized by reaction with the BCIP/NBT chromogen substrate.

Measurement of anti-Epstein–Barr virus antibody titers

Titers of EBV-specific antibodies were measured by SRL (Tokyo, Japan) using indirect immunofluorescence and/or enzyme immunoassay.

Results

Epstein–Barr virus DNA load in the peripheral blood of pediatric liver transplant recipients

Among the 123 patients listed in Table 1, EBV DNA load exceeded 10^2 copies/μg DNA at any one occasion in 63 recipients, while in the remaining 60 patients, the EBV DNA level stayed below this level throughout the observation period of 2–61 months. The number of recipients who had EBV DNA load of $> 10^2$ copies/μg at least once in relation to the EBV serostatus of the donor and the recipient is shown in Table 1. In accordance with previous reports (9, 20), a chi-square test revealed that transplantation from an EBV-seropositive donor to a seronegative recipient (D^+/R^- cases) comprises a risk factor for the rise of EBV DNA load to $> 10^2$ copies/μg DNA ($p < 0.01$). Among the 123 patients, 61 were < 1 yr old, and in this particular age range, the risk for a rise in EBV DNA load in D^+/R^+ cases was as high as in D^+/R^- cases (data not shown), probably reflecting the presence of maternally transferred antibodies that interfere with correct determination of EBV serostatus. The average peak level of EBV DNA load was significantly higher in patients under the age of one yr (average 3.0×10^4 (s.d. = 3.6×10^4) copies/μg DNA) than in those above one yr old (average 1.1×10^4 (s.d. = 2.2×10^4) copies/μg) ($p < 0.01$ by Student's t test).

Changes in Epstein–Barr virus DNA load and lymphocyte marker expression in response to reduction of immunosuppression

Among the 140 patients, 63 showed an increase in EBV DNA load to $> 10^2$ copies/μg DNA and further examined periodically by flow cytometry for the expression of lymphocyte functional markers. Expression of CD3, CD4, CD8, CD19, CD20, CD23, and HLA-DR was monitored

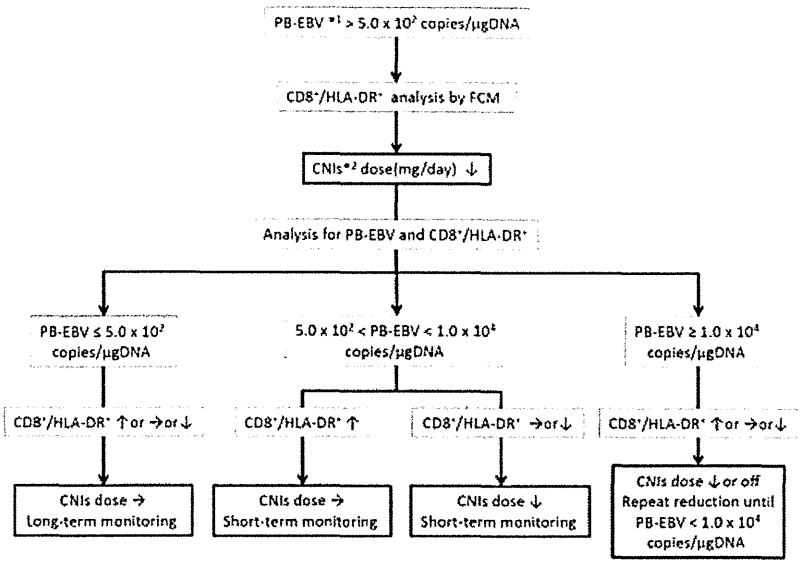

Fig. 1. An example algorithm for the management of EBV infection after pediatric liver transplantation. *1 PB-EBV: peripheral blood EBV (copy/μgDNA), *2 CNIs Calcineurin Inhibitors.

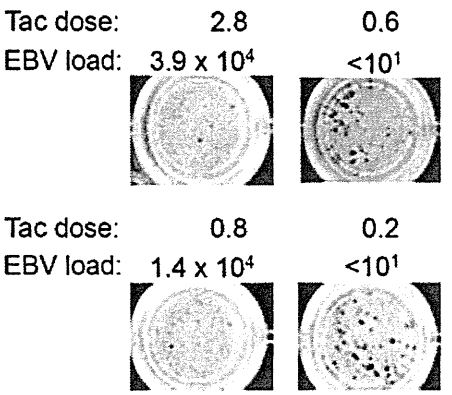

Fig. 2. Comparison of the frequency of EBV-specific T cells before and after reduction of immunosuppression. $CD8^+$ T cells were isolated before (left) and after (right) reduction of immunosuppression from two LDLT recipients (upper and bottom), and the frequency of EBV-specific cells was determined by ELISPOT assay. The dose of tacrolimus (mg/day) and EBV DNA load (copies/μg DNA) are shown for each time point.

regularly in these patients. Further analysis on the expression of CD7, CD16, CD26, CD28, CD45RO, CD45RA, and CD56 was made in selected patients. Once high levels of EBV DNA load (more than 5.0×10^2 copies/μgDNA) were detected in pediatric LT patients, we attempted to minimize CNIs. An average CNI reduction rate was 26.6% (range, 10–50%). An example strategy is outlined in Fig. 1. Patients were followed for an unpredictable and dynamic clinical course that may require customized immunosuppression therapy based on their status of rejection, infection, PTLD, and other complications after liver transplantation. Reduction in the dose of immunosuppressive drugs resulted in increase in the number of EBV-specific T cells as shown by ELISPOT assay (Fig. 2) and decrease in EBV DNA load in most cases. So far no clinically apparent PTLD has been diagnosed in the 140 patients. Among the 63 patients who experienced a rise in EBV DNA load, two showed signs of acute rejection and treated mainly with steroids. No patients suffered from chronic rejection or de novo autoimmune hepatitis. The peripheral EBV DNA load and various immunological markers changed in response to the reduction in the dose of immunosuppressive drugs in various manners depending on individual patients. We present three typical cases in which EBV monitoring and flow cytometric analysis in combination provided useful information in the control of EBV infection. Fig. 3 shows a patient (patent 1, D^+/R^- case) who had elevated EBV DNA load that persisted for more than two yr. He showed a sudden increase in EBV DNA load on the POD 48 (Fig. 3a), and flow cytometry indicated the CD4/CD8 ratio of 1.9, 3.8% frequency of $HLA\text{-}DR^+CD8^+$ T cells (i.e., activated $CD8^+$ T cells) among total lymphocytes, and 15.2% frequency of $CD23^+CD19^+$-activated B cells among total lymphocytes (Fig. 3b). A fraction of these activated B cells were considered to be EBV-transformed cells. As tacrolimus was reduced from 2.4 to 0.2 mg/day, EBV DNA gradually declined. During this decline of EBV DNA (POD 337, 555,

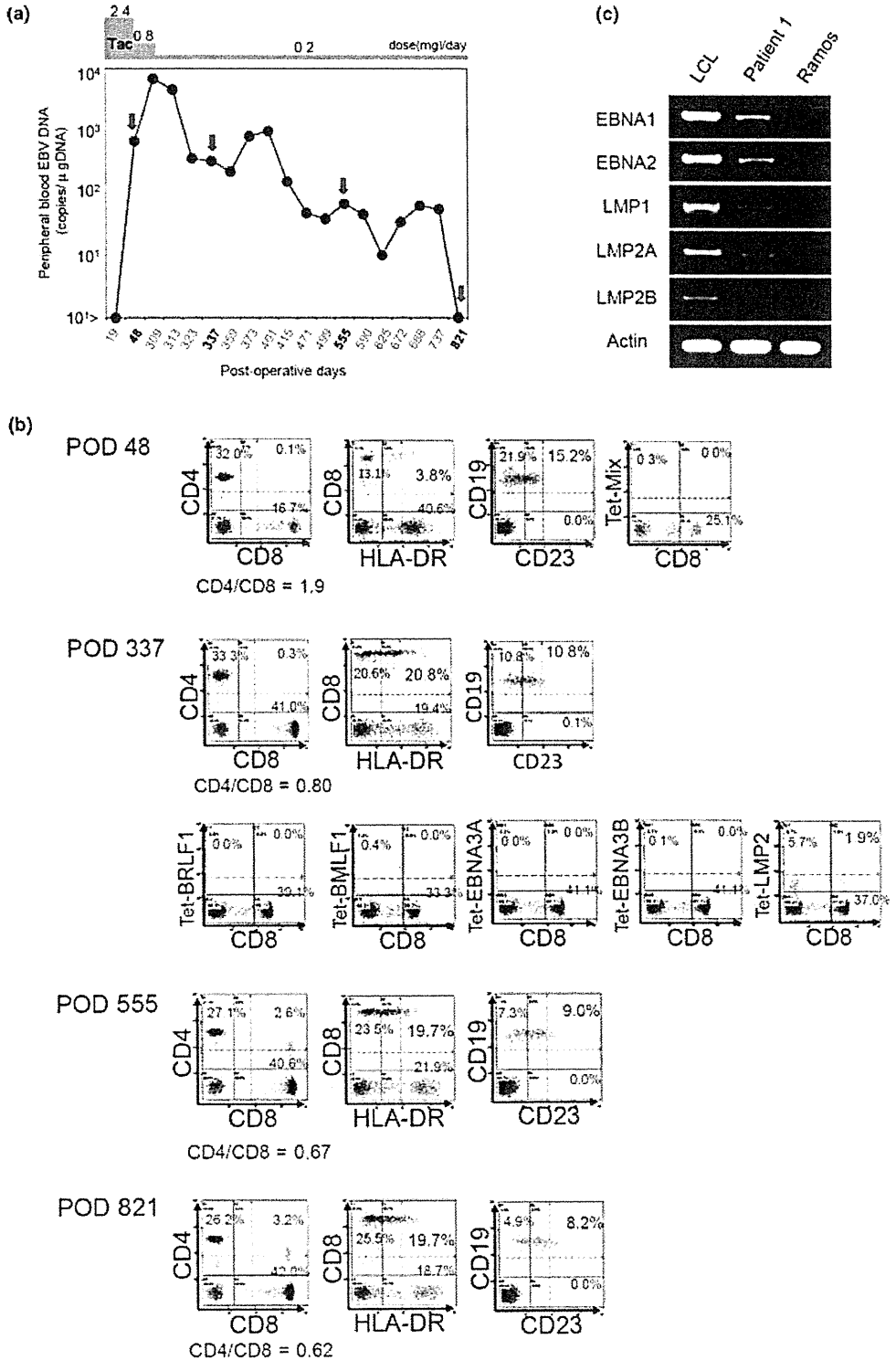

Fig. 3. EBV DNA load, lymphocyte phenotypes, and EBV gene expression in the liver transplantation patient 1. (a) Change of peripheral blood EBV DNA load in relation to the dose of tacrolimus (Tac). Arrows indicate the time points when immunological analyses shown in b were performed. (b) Flow cytometric analyses on the expression of lymphocyte markers and recognition by EBV-specific tetramers. Lymphocyte fractions gated by the side scatter, the forward scatter, and staining with anti-CD45 antibody were further analyzed for the expression of the indicated lymphocyte surface markers and for the recognition by EBV-specific tetramers. These analyses were carried out on POD 48, 337, 555, and 821. (c) Expression of EBV-encoded genes in the peripheral blood of the patient 1. Total RNA was prepared from PBMC of this patient on POD 337 and analyzed for the transcription of the indicated EBV genes by RT-PCR. RNA from an EBV-transformed LCL was analyzed as a positive control, and that from an EBV-negative Burkitt lymphoma cell line Ramos was used as a negative control.

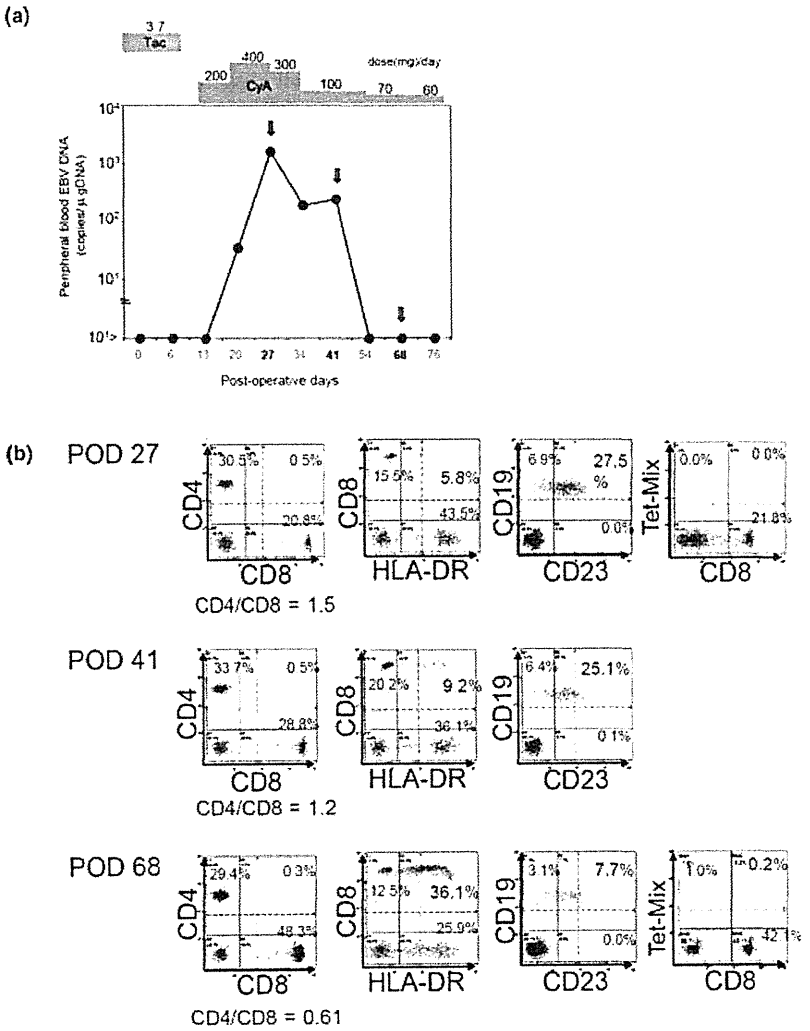

Fig. 4. EBV DNA load and lymphocyte phenotypes in the liver transplantation patient 2. (a) Change of peripheral blood EBV DNA load in relation to the dose of tacrolimus (Tac) and cyclosporine A (CyA). Arrows indicate the time points when immunological analyses shown in b were performed. (b) Flow cytometric analyses on the expression of lymphocyte markers and recognition by EBV-specific tetramers. Lymphocyte fractions gated by the side scatter, the forward scatter, and staining with anti-CD45 antibody were further analyzed for the expression of the indicated lymphocyte surface markers and for the recognition by EBV-specific tetramers. These analyses were carried out on POD 27, 41, and 68.

and 821), the CD4/CD8 ratio was gradually reversed (0.80, 0.67, and 0.62, respectively); the rate of $HLA\text{-}DR^+CD8^+$ T cells increased (20.8%, 19.7%, and 19.7%, respectively); and $CD23^+CD19^+$ cells decreased (10.8%, 9.0%, and 8.2%, respectively) (Fig. 3b). As HLA-DR is an activation marker of T cells, these changes were interpreted as a general increase in activated $CD8^+$ cytotoxic T cells and consequent reduction in the EBV-infected B cells. EBV-specific cytotoxic T cells were measured by HLA tetramers that bind TCRs specific to either of the five EBV-encoded proteins LMP2, BRLF1, BMLF1, EBNA3A, or EBNA3B. On POD 48, when the increase in EBV DNA load was noted, no $CD8^+$ T cells specific to these EBV proteins were detected, whereas on POD 337, when EBV DNA load was declining, $CD8^+$ T cells that recognize the IYVLVMLVL epitope common to LMP2A and LMP2B were detected at 1.9% (Fig. 3b). $CD8^+$ T cells specific to the other four EBV proteins remained undetectable. RT-PCR analysis demonstrated the expression of LMP2A and LMP2B in the PBMC isolated from this patient (Fig. 3c). These results suggest that LMP2-specific T cells were actually involved in the elimination of EBV-infected cells in this patient. Patient 2 (D^+/R^- case) (Fig. 4) showed a short course of increase in the EBV DNA load. When the EBV DNA load was at its peak level

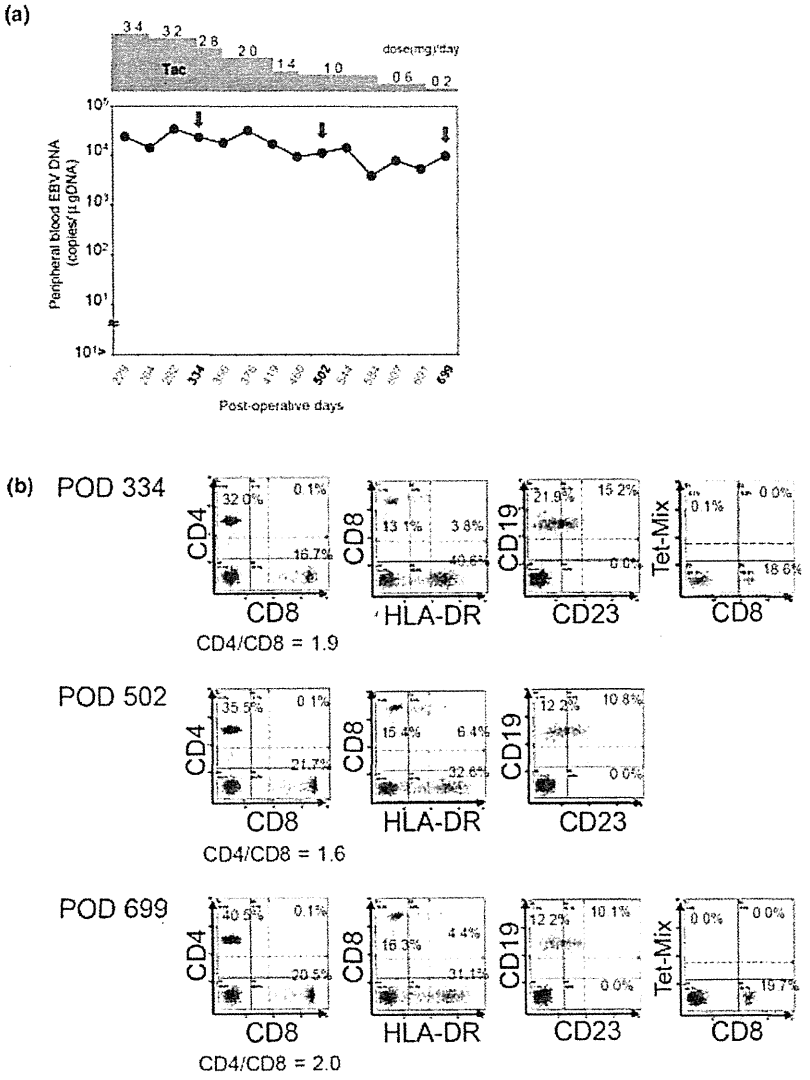

Fig. 5. EBV DNA load and lymphocyte phenotypes in the liver transplantation patient 3. (a) Change of peripheral blood EBV DNA load in relation to the dose of tacrolimus (Tac). Arrows indicate the time points when immunological analyses shown in b were performed. (b) Flow cytometric analyses on the expression of lymphocyte markers and recognition by EBV-specific tetramers. Lymphocyte fractions gated by the side scatter, the forward scatter, and staining with anti-CD45 antibody were further analyzed for the expression of the indicated lymphocyte surface markers and for the recognition by EBV-specific tetramers. These analyses were carried out on POD 334, 502, and 699.

(POD 27), the CD4/CD8 ratio was highest (1.5), the percentage of HLA-DR$^+$CD8$^+$ T cells was lowest (5.8%), the percentage of CD23$^+$CD19$^+$ B cells was highest (27.5%), and CD8$^+$ T cells specific to the five EBV proteins were not detected. As the dose of cyclosporine A was reduced and the EBV DNA decreased dramatically, these parameters also changed significantly: on POD 41, when EBV DNA load started to decrease, the CD4/CD8 ratio was 1.2, the frequency of HLA-DR$^+$CD8$^+$ T cells was 9.2%, and that of CD23$^+$CD19$^+$ B cells was 25.1%; and on POD 68, when EBV DNA was undetectable, the CD4/CD8 ratio was 0.61, the frequency of HLA-DR$^+$CD8$^+$ T cells was 36.1%, that of CD23$^+$CD19$^+$ B cells was 7.7%, and EBV-specific CD8$^+$ T cells recognizing either of the five EBV proteins described above were detected at 0.2%. Patient 3 (D^+/R^- case) (Fig. 5) is a case in whom no significant reduction in EBV DNA load was observed in spite of reduction of the dose of tacrolimus from 3.4 mg/day to 0.2 mg/day. In this patient, the CD4/CD8 ratio, the percentage of HLA-DR$^+$CD8$^+$ T cells, and the percentage of CD23$^+$CD19$^+$ B cells did not change significantly following the reduction in the drug, and EBV-specific CD8$^+$ T cells were not detected

throughout the observation period. Careful clinical examinations, however, did not detect any signs and symptoms of PTLD in this patient. Southern blot analysis of the terminal repeat region of EBV DNA indicated polyclonal lymphoproliferation in this patient. A total of 22 patients (all < 1 yr old) were identified with this type of refractoriness to the reduction in immunosuppressive drugs. The increase in the frequency of HLA-DR$^+$CD8$^+$ cells following the reduction of immunosuppression was less evident in the patients who retained high EBV DNA load than in those with significant decrease in viral load (Fig. 6).

Discussion

Successful transplantation of both solid organs and hematopoietic stem cells requires effective control of EBV infection. Previous studies have given evidence that molecular EBV monitoring is effective in finding patients with high risk of developing PTLD (14–17). However, it has become also evident that molecular EBV monitoring alone is not sufficient to achieve complete prediction and hence prevention of PTLD (21–23). Some researchers propose that monitoring of humoral immune parameters, including serum immunoglobulin concentrations and mono-/oligoclonal gammopathy, in combination with EBV monitoring, is valuable in identifying high-risk patients (24, 25). In the present study, expression of lymphocyte markers was analyzed in LDLT recipients who experienced significant increase in the EBV DNA load. The increase in the number of CD8$^+$ T cells and HLA-DR$^+$CD8$^+$ T cells as well as the decrease in the CD4/CD8 ratio following the reduction of immunosuppression was considered to reflect the recovery of T-cell functions because these changes occurred in parallel with the decline in EBV DNA load (Figs. 3 and 4). Among the three parameters described above, we speculate the number of HLA-DR$^+$CD8$^+$ T cells is the most useful in estimating the host's capacity of immunosurveillance against EBV, because its increase was more prominent in those patients in whom EBV load decreased to an undetectable level following the reduction of immunosuppression than in those who retained high viral load (Fig. 6). This hypothesis is in accordance with the generally accepted fact that the main force in the immunological control of EBV is CD8$^+$ cytotoxic T cells (2). We prepared an example algorithm for the management of EBV infection after pediatric liver transplantation based on the monitoring of EBV DNA load and the number of HLA-DR$^+$CD8$^+$ T cells (Fig. 1).

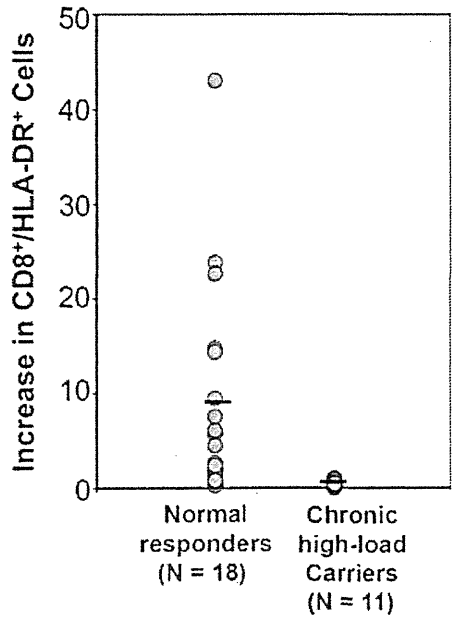

Fig. 6. The frequency of HLA-DR$^+$CD8$^+$ T cells in chronic high EBV load carriers and normal responders. The increase in the frequency of HLA$^+$CD8$^+$ T cells following reduction of immunosuppression, was defined as the maximum percentage of HLA$^+$CD8$^+$ T cells among total lymphocytes observed following reduction of immunosuppression minus the percentage of HLA$^+$CD8$^+$ T cells among total lymphocytes observed just before reduction of immunosuppression/the percentage of HLA$^+$CD8$^+$ T cells among total lymphocytes observed just before reduction of immunosuppression, was compared between those who showed decline of EBV DNA load to $< 10^1$ copies/μg DNA after reduction of immunosuppression (N = 18) and those who retained EBV load $> 10^3$ copies/μg DNA (N = 11). The difference was significant by the Mann–Whitney test (U = 13.5, p < 0.01).

EBV is an oncogenic virus and has a potential to cause systemic lymphoproliferation if the immunosurveillance mechanism of the host fails. In previous studies of EBV-specific T-cell responses following allogeneic hematopoietic stem cell transplantation, the number of EBV-specific T cells as measured by MHC class I tetramer assay was shown to be a reliable marker for the prediction of patients at high risk of PTLD (26–28). Although such studies on solid organ transplantations are limited, similar value of tetramer-based measurement of EBV-specific T cells has been reported (29). Among the nine viral proteins expressed in LCLs, EBNA3s are considered to be the most immunodominant proteins in the latent phase of EBV infection in that T cells reacting to the epitopes derived from these proteins are most frequently detected in the peripheral blood of EBV carriers (2). However, the exact role of T cells recognizing individual

EBV epitopes has not been characterized. In the patient 1 described in this study, no $CD8^+$ T cells recognizing immunodominant epitopes derived from the five EBV proteins, LMP2, BFLF1, BMLF1, EBNA3A, and EBNA3B were found when EBV DNA load was at high levels. When EBV DNA load started to decrease as the dose of tacrolimus was reduced, the frequency of LMP2-specific $CD8^+$ T cells increased dramatically, while those specific to the other four EBV proteins remained undetectable. Thus, these results document for the first time that an increase in the number of a particular population of $CD8^+$ T cells recognizing a specific EBV epitope is accompanied by a decrease in peripheral blood EBV DNA load, suggesting that this population of T cells is actually involved in the elimination of EBV-infected lymphoblastoid cells in this patient. Further study is necessary to elucidate the exact role of LMP2-specific T cells in the control of EBV infection.

The 22 patients who did not respond to the reduction of immunosuppression appear to meet the published criteria for "chronic high EBV load carriers" (30–32). Recently Macedo and others described signs of cellular exhaustion (programmed death 1^+/$CD127^-$ and decline in IFN-γ release) in EBV-specific T cells of these chronic high-load carriers (33). A significant difference between the previous study and the present study is the age of the patients at transplantation; it was 10.8 ± 5.9 yr (mean ± s.d.) in the former and one yr or less in the latter. We speculate that the chronic high-load carriage in our patients probably reflects immaturity of immune system rather than cellular exhaustion. Weak responses of CD8+T cells in the chronic high-load carriers (Fig. 6) is in accordance with this hypothesis.

The average age of EBV seroconversion is shifting to the elder direction in certain countries including Japan (34), and it is expected that we are going to have more EBV-seronegative recipients with a higher risk of PTLD in pediatric transplantation. The importance of proper management of EBV infection will be highlighted more in the future. As the average incidence of EBV-associated PTLD in pediatric liver transplant recipients is 8–10% (6–8), the result in this study, no PTLD development in 140 consecutive recipients, strongly suggests that our practice of molecular EBV monitoring coupled with flow cytometric characterization of lymphocyte phenotypes is effective in the prevention of EBV-related PTLD. In this study, we set a borderline at 10^2 EBV DNA copies/μg DNA, a relatively low level in this type of practice, and started rigorous care when this level was exceeded. This cautious approach may have helped improve our results of EBV control. The overall five-yr survival rate in our patients is 88.9%.

Acknowledgments

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