Activation and adoptive transfer of Epstein–Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease

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ABSTRACT The treatment of Epstein–Barr virus (EBV)-associated lymphoproliferative disease (PTLD) in EBV-seronegative solid organ transplant recipients who acquire their EBV infection after engraftment poses a considerable challenge because of underlying immunosuppression that inhibits the virus-specific cytotoxic T cell (CTL) response in vivo. We have developed a protocol for activating autologous EBV-specific CTL lines from these patients and show their potential use for immunotherapy against PTLD in solid organ transplant patients. Peripheral blood mononuclear cells from a panel of solid organ transplant recipients with and without active PTLD were used to assess EBV-specific memory CTL responses. The activation protocol involved cocultivation of peripheral blood mononuclear cells with an autologous lymphoblastoid cell line under conditions that favored expansion of virus-specific CTL and hindered the proliferation of allospecific T cells. These CTL consistently showed (i) strong EBV-specificity, including reactivity through defined epitopes in spite of concurrent immunosuppressive therapy, and (ii) no alloreactivity toward donor alloantigens. More importantly, adoptive transfer of these autologous CTLs into a single patient with active PTLD was coincident with a very significant regression of the PTLD. These results demonstrate that a potent EBV-specific memory response can be expanded from solid organ recipients who have acquired their primary EBV infection under high levels of immunosuppressive therapy and that these T cells may have therapeutic potential against PTLD.

Posttransplant lymphoproliferative disease (PTLD) that arises in organ transplant patients is an increasingly important clinical problem (1–3). Histological analysis of PTLD shows a quite complex clonal diversity ranging from polymorphic B lymphocyte hyperplasia to malignant monoclonal lymphoma. This condition is clearly associated with the proliferation of EBV-infected B cells whose expansion in normal healthy immune individuals is restricted by cytotoxic T lymphocytes (CTL) (4). The nature of the immunosuppressive therapy needed to maintain the engrafted organ inhibits these specific CTL and results in an expansion of the pool of EBV-infected B cells and the emergence of the clinical problems associated with PTLD (5). The importance of CTL in controlling these B cell expansions has been dramatically demonstrated in the case of PTLD in bone marrow transplant patients transfused with EBV-specific CTL (6, 7). These CTLs, which were derived by activating donor lymphocytes in vitro and subsequently were adoptively transferred into bone marrow transplant recipients, resolved the PTLD.

It is important to point out that, in the case of bone marrow transplantation, PTLD are exclusively of donor origin whereas PTLD in solid organ transplant patients are usually of recipient origin (1), although exceptions to this principle have been reported (6). While the idea of applying a similar rationale of adoptively transferring EBV-specific CTL to resolve PTLD arising in solid organ recipients is attractive, there are fundamental differences between bone marrow and solid organ transplantation. These include (i) the potential problem of activating a CTL response in vitro from an individual receiving high levels of immunosuppressive drugs, (ii) the risk of expanding allospecific-specific CTL that will threaten the integrity of the transplanted organ when adoptively transferred, and (iii) the efficacy of adoptively transferred CTL in the face of high levels of immunosuppression in vivo (1). Taken together, these factors have led to the perception that adoptive immunotherapy in these patients is both unlikely to be successful and poses a significant risk to the engrafted organ.

In this report, we describe a protocol for expanding EBV-specific CTL from a panel of solid organ transplant recipients with and without PTLD who were EBV-seronegative at the time of engraftment. Furthermore, we illustrate the potential efficacy of these CTL in resolving these lymphomas in one of these patients after adoptive transfer.

MATERIALS AND METHODS

Establishment and Maintenance of EBV-Transformed Cell Lines. Lymphoblastoid cell lines (LCLs) were established from a panel of solid organ recipients with PTLD and from healthy EBV-seropositive individuals by exogenous virus transformation of peripheral B cells by using QIMR Wil (8) and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin plus 10% FCS (growth medium).

Solid Organ Transplant Patients. A panel of five solid organ transplant recipients was included in this study (details in...
Table 1. List of solid organ transplant patients assessed for EBV-specific CTL response

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age/sex</th>
<th>Transplanted organ (indication of Tx)</th>
<th>EBV status of the recipient</th>
<th>EBV status of the donor</th>
<th>PTLD (months after transplant)</th>
<th>Pathological description of PTLD</th>
<th>HLA type of the donor</th>
<th>HLA type of the recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM 51 yr/F</td>
<td>Single lung (lymphangioleiomyomatosis)</td>
<td>Pre-Tx: –ve; Post-Tx: +ve (12 months)</td>
<td>–ve to +ve</td>
<td>B cell lymphoma of donor origin; EBV status of lymphoma: inconclusive (24 months)</td>
<td>B cell mixed large and small lymphocytes, polymorphic LMP1-negative</td>
<td>HLA A3, A31, B7, B35, DR7, DR11, DQ2, and DQ3</td>
<td>HLA A2, A30, B7, B13, DR1, DR7, DRw53, DQ1, and DQ2</td>
<td></td>
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<tr>
<td>KVM 40 yr/M</td>
<td>Bilateral lung (emphysema)</td>
<td>Pre-Tx: –ve; Post-Tx: +ve (4 months)</td>
<td>–ve to +ve</td>
<td>B cell lymphoma of recipient origin EBV positive (5 months)</td>
<td>B cells mixed large and small lymphocytes, polymorphic LMP1-positive</td>
<td>HLA A2, A25, B8, B35, DR3, and DR4</td>
<td>HLA A2, A3, B51, B57, DR1101, DR1103, and DQ3</td>
<td></td>
</tr>
<tr>
<td>LF 20 yr/M</td>
<td>Heart (hypertrophic cardiomyopathy)</td>
<td>Pre-Tx: –ve; Post-Tx: +ve (4 months)</td>
<td>–ve to +ve</td>
<td>B cell lymphoma of recipient origin EBV positive (24 months)</td>
<td>Differentiated large B cell lymphoma, monomorphic LMP1-negative LMP2A-positive by PCR EBER positive</td>
<td>HLA A1, A25, B8, B35 DR3, and DR4</td>
<td>HLA A1, A2, B7, B60, DR13, and DR15</td>
<td></td>
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<tr>
<td>MD 36 yr/M</td>
<td>Bilateral lung (cystic fibrosis)</td>
<td>Pre-Tx: –ve; Post-Tx: +ve (2 months)</td>
<td>–ve to +ve</td>
<td>No PTLD</td>
<td>NA</td>
<td>HLA A2, B44, B56, DR1, and DR4</td>
<td>HLA A2, B44, B51, Bw4, Cw1, Cw5, DR1501, and DR1301</td>
<td></td>
</tr>
<tr>
<td>TT 26 yr/M</td>
<td>Kidney/pancreas (type 1 diabetes)</td>
<td>Pre-Tx: –ve; Post-Tx: +ve (20 months)</td>
<td>–ve to +ve</td>
<td>No PTLD</td>
<td>NA</td>
<td>HLA A3, A11, B7, B47, DR4, and DR15</td>
<td>HLA A2, A3, B14, B49, DR1, and DR4</td>
<td></td>
</tr>
</tbody>
</table>

M, male; F, female; Tx, transplant; –ve, negative; +ve, positive; EBER, Epstein–Barr virus-encoded RNA.
Quantitation of EBV-Specific CTL After Adoptive Transfer. The level of EBV-specific CTL was estimated by limiting dilution analysis (LDA) in a patient who underwent adoptive immunotherapy. This analysis was conducted before adoptive therapy and subsequently 6 weeks after the initial adoptive immunotherapy. In brief, peripheral blood mononuclear cells were distributed in graded numbers (2-fold dilutions) from 6.25 × 10^3 to 5 × 10^4 cells per well in round-bottomed microtiter plates. Approximately 5 × 10^4 γ-irradiated (2,000 rads) peptide-sensitized (1 μg/ml) peptide transporter (TAP)-negative B × T hybrid cell line 174 × CEM.T2 (referred to as T2 cells) (13) were added to give a total volume of 100 μl. Twenty-four replicates were used at each concentration in each experiment. Cultures were fed on days 4 and 7 with 50 μl of medium supplemented with 20 units of rIL-2 and 30% (vol/vol) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into two replicates and was used as effectors in a standard 5-h 51Cr-release assay against autologous PHA blasts precoated with peptide epitopes from latent membrane protein 1 (LMP1) or left uncoated. Wells were scored as positive when the percent of specific 51Cr-release for untreated control wells by 3 SDs. LDA was performed by the method of maximum likelihood estimation (10). Data from all experiments were compatible with the hypothesis of single-hit kinetics (P > 0.4), and precursor estimates are given with 95% confidence limits.

RESULTS
Assessment of EBV-Specific Memory CTL Response in Solid Organ Transplant Patients. The activation and maintenance of an EBV-specific response in the face of strong immunosuppression is a central question in the development of immunotherapeutic protocols for PTLD in solid organ transplant patients who acquire an EBV infection after engraftment. To address this issue, we developed a CTL activation protocol (see Material and Methods) that was specifically designed to favor the expansion of virus-specific T cells and hinder the proliferation of allospecific T cells. This protocol was applied to five solid organ transplant patients (Table 1) who were on continuous triple immunosuppressive therapy including prednisolone, azathioprine, and CSA (or tacrolimus). Fig. 1 illustrates the EBV specificity of these T cell lines. Fig. 1 A–D shows CTL recognition of autologous and HLA-matched and unmatched allogeneic LCLs while F–J shows the peptide epitope specificity of T cell lines from each of these patients. All CTL lines showed strong lysis of the autologous and HLA-matched LCLs. An analysis of these cell lines revealed that it was possible to assign EBV peptide specificity in CTLs from 4/5 patients (the exception being patient TT). Most of the lysis appeared to be restricted through the HLA A1, A2, B7, B44, and/or B60 alleles, and the peptide reactivity was identified toward HLA A2- and HLA B7-restricted epitopes within LMP1, EBV nuclear antigens 3 and 6, and BamHI fragment M left forward. Furthermore, in two instances, these T cells showed no reactivity against donor PHA blasts (Fig. 1 F and H). Although it was not possible to test the reactivity of T cells toward donor PHA blasts in the other three instances, a similar analysis revealed no reactivity against allogeneic PHA blasts sharing MHC class I alleles with the donor (data not shown). A fluorescence-activated cell sorter analysis indicated that these cell lines were >90% CD3 positive, 70–80% CD8 positive, 10–20% CD4 positive, and 5–8% CD56 positive (data not shown).

Adoptive Transfer of EBV-Specific CTLs into a PTLD Patient. Having established the conditions for activation of a strong EBV-specific CTL response from individuals receiving high levels of immunosuppression, we then assessed their in vivo efficacy against an EBV-positive PTLD in a solid organ transplant patient. This patient was a 39-year-old EBV seronegative male with α1 antitrypsin deficiency-induced emphysema who received a bilateral lung transplant from an EBV seropositive donor in September, 1997. The patient experienced two episodes of moderate rejection at week 4 and week 8 posttransplant. Each rejection episode was treated with pulses of methylprednisolone (750 mg daily for 3 days). Immunosuppression was changed from CSA to a tacrolimus-based regimen. At 3 months posttransplant, the patient developed profound lethargy, and serological tests confirmed EBV seroconversion (IgG response to EBV viral capsid antigen). Subsequently, the patient presented with an acute abdomen, and, at laparotomy, there was evidence of perforation of the distal ileum. Histopathology indicated PTLD (B cell mixed lobe and small lymphocyte lymphoma, which was LMP1-positive) of recipient origin (confirmed by DNA typing for class II MHC). Multiple nodules of lymphomas were seen through out large and small bowel. A CT scan of the abdomen demonstrated three discrete nodules, and a percutaneous biopsy confirmed a necrotic lymphoma in the liver. A CT scan of the thorax and fiberoptic bronchoscopy did not demonstrate any evidence of tumor within the lung. In view of the extent of the disease, tacrolimus was ceased, the dose of azathioprine was reduced, prednisolone was continued, and gancyclovir treatment (10 mg/kg/day) was commenced. His clinical course was complicated by the development of a colonic perforation, a pelvic abscess requiring colectomy, and a cutaneous Scedosporium apiospermum infection requiring wide excision. Transbronchial lung biopsies 6 days after the cessation of tacrolimus confirmed moderate allograft rejection (A3B2). The patient was treated with methylprednisolone and CSA aiming for a CSA level of 150–175 nmol/liter by HPLC. In view of the cutaneous fungal infection, recurrent peritonitis, pelvic abscess, and PTLD, Ethics Committee approval was sought and received to infuse in vitro-expanded autologous EBV-specific CTLs. The EBV-specificity of these adoptively transferred CTLs is illustrated in Fig. 1 A and F. Two separate infusions of these EBV-specific CTLs (35 × 10^6 cells for each infusion) 14 days apart were delivered intravenously into the patient, and each was well tolerated. Two months after the second infusion, there was no clinical evidence of lymphoma and no further gastrointestinal symptoms. Immunosuppressive therapy was maintained at constant level throughout this period. Serial CT scans of the abdomen demonstrated regression of the liver nodules (25 mm to not detectable; 20 mm to not detectable) (Fig. 2). The level of EBV-specific CTL before and after adoptive immunotherapy was estimated by LDA. Because the adoptively transferred CTL showed reactivity toward EBV epitopes included in LMP1 (YLLEMLWRL and YLQQNWWTL), the LDA analysis was based on an estimation of the precursor (CTLp) frequency of these epitopes. As shown in Fig. 3, there was no detectable CTLp in this assay specific for either of these epitopes before transfer (4) whereas 1 month after the second infusion, CTL specific for these LMP1 epitopes were at levels comparable to those seen in a healthy seropositive individual (A and B).

Ten weeks after the second infusion, the patient developed a secondary PTLD within the wall of the right lower lobe bronchus and within the lung parenchyma of left lower lobe. Immunohistological and DNA analysis indicated that this PTLD was of recipient origin, expressed LMP1 expression, and displayed an identical cellular morphology to that seen initially in the liver and bowel. Patient was reinfused twice with EBV-specific CTLs (60 × 10^6 per infusion). Three weeks later, there was evidence of PTLD regression within the lung. The maximum diameter of the left lower lobe lesion decreased from 2.5 cm in diameter (volume ~ 8 ml) to 1.4 cm (volume ~ 2 ml). Based on this observation, a fourth infusion was administered (60 × 10^6 CTL) 2 weeks later. Four days after this
FIG. 1. EBV specificity of T cell lines from five different solid organ transplant patients with or without PTLD. A detailed description of these patients is presented in Table 1. A–E show the CTL recognition of autologous and HLA-matched and -unmatched allogeneic LCLs while F–J show peptide epitope specificity of these CTL lines tested on PHA blasts. Also illustrated in F and H is the CTL recognition of PHA blasts from the organ donor. The HLA class I restriction and antigen location for the peptide epitopes used in the CTL assays is as follows: YLQQNWWT1 (HLA A2; LMP1; ref. 10), YLLEMLWRL (HLA A2; LMP1; ref. 10), RPPIFIRRL (HLA B7; EBV nuclear antigen 3; ref. 11), GLCTLVAML (HLA A2; BamHI fragment on left forward 1; ref. 12), LLDFVRFMGV (HLA A2; EBV nuclear antigen 6; ref. 4). Data from patient KVM is shown in A and F, from patient MM in B and G, from patient LF in C and H, from patient TT in D and I, and patient MD in E and J. The data is presented as percent specific lysis.
infusion, the patient collapsed at home and was unable to be resuscitated. At autopsy there was minimal residual PTLD within the bronchus of the right lower lobe. Histological examination showed massive necrosis that also involved the wall of a moderate sized vein in direct communication with the bronchus. Within the left lower lobe there was a minimal residual viable PTLD (0.1 cm in diameter) associated with extensively necrotic tumor. Histological assessment of the tumor in the right lower lobe showed no difference in the proportion of inflammatory cell infiltrate to that seen in any of the previous biopsies of the tumor. In addition, tumor samples stained for T and B cell markers showed no marked difference in the number of infiltrating T cells. There was no evidence of PTLD within the liver or the small or large bowel.

**DISCUSSION**

Although adoptive immunotherapy has been used to treat and prevent PTLD in bone marrow transplant patients, the application of this technology to the treatment of these lymphomas in solid organ transplant patients has remained a significant challenge. Although a previous study has demonstrated that it is possible to expand EBV-specific CTLs from seropositive solid organ transplant patients (14), there has been a strong belief that such an expansion would be improbable in solid organ transplant patients who seroconvert after engraftment. In particular, it might be anticipated that immunosuppressive therapy in this group of patients at greatest risk may hinder CTL activation in vitro and their effector function in vivo. Another significant consideration is that there is a potential risk of activating an allospecific response directed toward the engrafted tissue. However, the data presented in this study clearly demonstrate that it is indeed possible to activate a potent CTL response from solid organ transplant patients who receive high levels of immunosuppression and seroconvert after engraftment. These CTLs showed strong EBV specificity and reactivity toward previously defined peptide epitopes within the viral latent proteins. The in vitro activation of a strong CTL response to EBV in these patients indicate the continued presence of a memory response, albeit at a low level.

Because of underlying immunosuppression, this memory CTL response fails to expand as an effector population to control the outgrowth of the EBV-infected B cells in vivo (15–18). The protocol established in this study overcomes this limitation and allows expansion of EBV-specific CTLs in vitro in the absence of any obvious reactivity toward donor alloantigens. It is important to mention here that the methodology described for the expansion of CTL from bone marrow transplant recipients (19) yielded a CTL profile that showed strong reactivity toward donor alloantigens when applied to this patient (data not shown). To avoid this potential risk, we have devised a modified activation protocol that includes the delayed addition of exogenous IL2. This procedure presumably favors the deletion of non-EBV-specific T cells from the expanded T cell population.

Having established the EBV specificity of these expanded CTL, Ethics Committee approval was received for adoptive transfer of these T cells into one of these patients who developed PTLD. It is interesting that these T cells showed reactivity toward LMP1 CTL epitopes and that immunohistochemical staining of the biopsy from this patient demonstrated strong LMP1 expression. Two separate CTL infusions were given intravenously, and disease regression was monitored by CT scan of the abdomen. After adoptive transfer, there was a dramatic decrease in the PTLD mass over a period of 8 weeks and no clinical evidence of any further gastrointestinal symptoms. Additionally, there was no evidence of any deleterious effect on the transplanted organ.

A quantitative analysis revealed that epitope-specific T cells could be readily detected by LDA after adoptive transfer 2 months after the first infusion. Although the patient showed no
clinical symptoms of PTLD for 6–8 weeks, new lesions of PTLD emerged. This PTLD was treated with two separate infusions resulting in a reduction in the tumor mass. This reemergence of the PTLD raises the question of the longevity of the infused T cells in solid organ transplant patients. The data presented in this study suggest that the levels of immunosuppression used in these patients may hinder the long-term survival of T cells. This is in contrast to the observations in bone marrow transplant patients in whom infused EBV-specific T cells can survive up to 3 years in vivo (7). Evidence of vascular invasion with necrosis and hemorrhage of a pulmonary vein at autopsy emphasizes that caution is required when administering EBV-specific T cells to patients with bulky visceral disease. This is reinforced by earlier reports by Papadopoulos et al. (18) and Rooney et al. (20) in which respiratory compromise occurred after lymphocyte infusions. Rooney reports a case of PTLD after bone marrow transplantation in which the patient developed airway obstruction requiring prolonged mechanical ventilation after adoptive therapy. Overall, these results suggest that, for solid organ transplant patients, a higher frequency of infusions may be required to maintain a therapeutic level of EBV-specific CTLs. It will be necessary to infuse autologous CTL by using the protocol described in this study in a larger panel of patients to confirm the benefit of adoptive immunotherapy in the treatment of PTLD in solid organ transplant patients.

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