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

RAJIV KHANNA*†, SCOTT BELL†‡, MARTINA SHERRITT*, ANDREW GALBRAITH‡, SCOTT R. BURROWS*, LEE RAFTER‡, BELINDA CLARKE‡, RICHARD SLAUGHTER‡, MICHAEL C. FALK§, JO DOUGLASS¶, TREVOR WILLIAMS¶, SUZANNE L. ELLIOTT*, AND DENIS J. MOSS*||

*Epstein–Barr Virus Unit, Queensland Institute of Medical Research and University of Queensland Joint Oncology Program, Brisbane 4006, Australia; ‡Queensland Heart and Lung Transplant Unit, Prince Charles Hospital, Brisbane 4032, Australia; §Princess Alexandra Hospital, Brisbane 4102, Australia; and ¶Department of Allergy and Clinical Immunology and Department of Respiratory Medicine, Alfred Hospital and Monash University, Prahran, Melbourne 3181, Australia

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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 Epstein–Barr virus (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

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Abbreviations: EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocytes; PTLD, posttransplant lymphoproliferative disease; LCL, lymphoblastoid cell line; CSA, cyclosporin; PHA, phytohemagglutinin; LDA, limiting dilution analysis; LMP1, latent membrane protein 1; CT, computerized tomography.

†R.K. and S.B. contributed equally to this work.

||To whom reprint requests should be addressed at: Joint Oncology Program, Epstein–Barr Virus Unit, Queensland Institute of Medical Research, Post Office, Royal Brisbane Hospital, Qld 4029, Australia. E-mail: denisM@qimr.edu.au.

Table 1). All of these patients were EBV-seronegative at the time of transplant and received a graft from a seropositive donor. These patients received standard triple immunosuppressive therapy, which included prednisolone (initially starting at 15 mg/kg/day and then reduced to 0.2–0.3 mg/kg/day by day 28 posttransplant), azathioprine (2.0 mg/kg/day), and cyclosporin (CSA) (250–350 nmol/liter by HPLC). One patient (TT) received cytolytic therapy (OKT3, 5 μg/day) for 10 days 3 weeks after transplantation for acute rejection. All recipients had evidence of seroconversion within 4–20 months, three developed PTLD (Table 1), and one of these received adoptive CTL therapy. All studies on these patients were approved by the institutional ethics committee, and the clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

***In Vitro* Expansion of EBV-Specific CTLs.** Peripheral blood (10 ml) from these patients and spleen from two donors were collected and used to establish LCLs and phytohemagglutinin (PHA) blasts, respectively, as described (9). A subsequent blood sample (40 ml) from these patients 3–4 weeks after seroconversion was used to generate EBV-specific polyclonal CTLs as follows. Approximately 4–8 $\times$ 10^6 peripheral blood mononuclear cells were recovered from 40 ml of blood sample. These peripheral blood mononuclear cells (2 $\times$ 10^6 per ml) were cocultivated with irradiated autologous LCLs at a responder-to-stimulator ratio of 25:1 for 7 days in a 24-well Linbro plate. Cultures were restimulated with autologous LCL on day 7 and 14 in the absence of exogenous IL2, and, on day 21, culture medium was supplemented with recombinant IL2 (Proleukin, 20 units/ml). T cell cultures were subsequently restimulated weekly in the presence of IL2 for 4–6 weeks and were screened for EBV specificity by using a standard ^{51}Cr-release assay (9). A total of 35–50 $\times$ 10^6 activated T cells were recovered 4–6 weeks after the first stimulation. This protocol for activation and expansion differs from that used previously by Rooney *et al.* (6) in that (*i*) a higher responder-to-stimulator ratio (25:1) was required to generate specific T cells, (*ii*) Ficoll gradient isolation of cultured CTLs was avoided, and (*iii*) addition of IL2 was delayed until day 21 (rather than day 14) to ensure the deletion of potential alloreactive T cells. Polyclonal T cell cultures were rigorously tested for specificity against a panel of targets including donor PHA blasts and recipient LCLs and PHA blasts. In addition, HLA-matched and -unmatched allogeneic LCLs and PHA blasts sensitized with EBV CTL peptide epitopes also were included in the assay.

Adoptive Transfer of CTL and Clinical Monitoring. For adoptive transfer, CTL lines were subsequently tested for microbial contamination and were prepared for intravenous infusion as follows. CTLs were washed twice in GMP grade normal saline supplemented with 10% autologous serum (infusion solution) and finally were resuspended in 20 ml of infusion solution. Cells were infused intravenously over a period of 20 min. The patient received four CTL transfers and was monitored clinically with serial computerized tomography (CT) scans of the abdomen.

Table 1. List of solid organ transplant patients assessed for EBV-specific CTL response

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

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 $\times$ T hybrid cell line 174 $\times$ 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 ^{51}Cr-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 ^{51}Cr-release for peptide-sensitized target cells exceeded the mean release from 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 *Bam*H1 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 αI 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 large 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 Scedosporidium 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 through out this period. Serial CT scans of the abdomen demonstrated regression of the liver nodules (25 mm to not detectable; 20 mm to not detectable; 8 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 (*A*) 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 $\approx$ 8 ml) to 1.4 cm (volume $\approx$2 ml). Based on this observation, a fourth infusion was administered (60×10^6 CTL) 2 weeks later. Four days after this

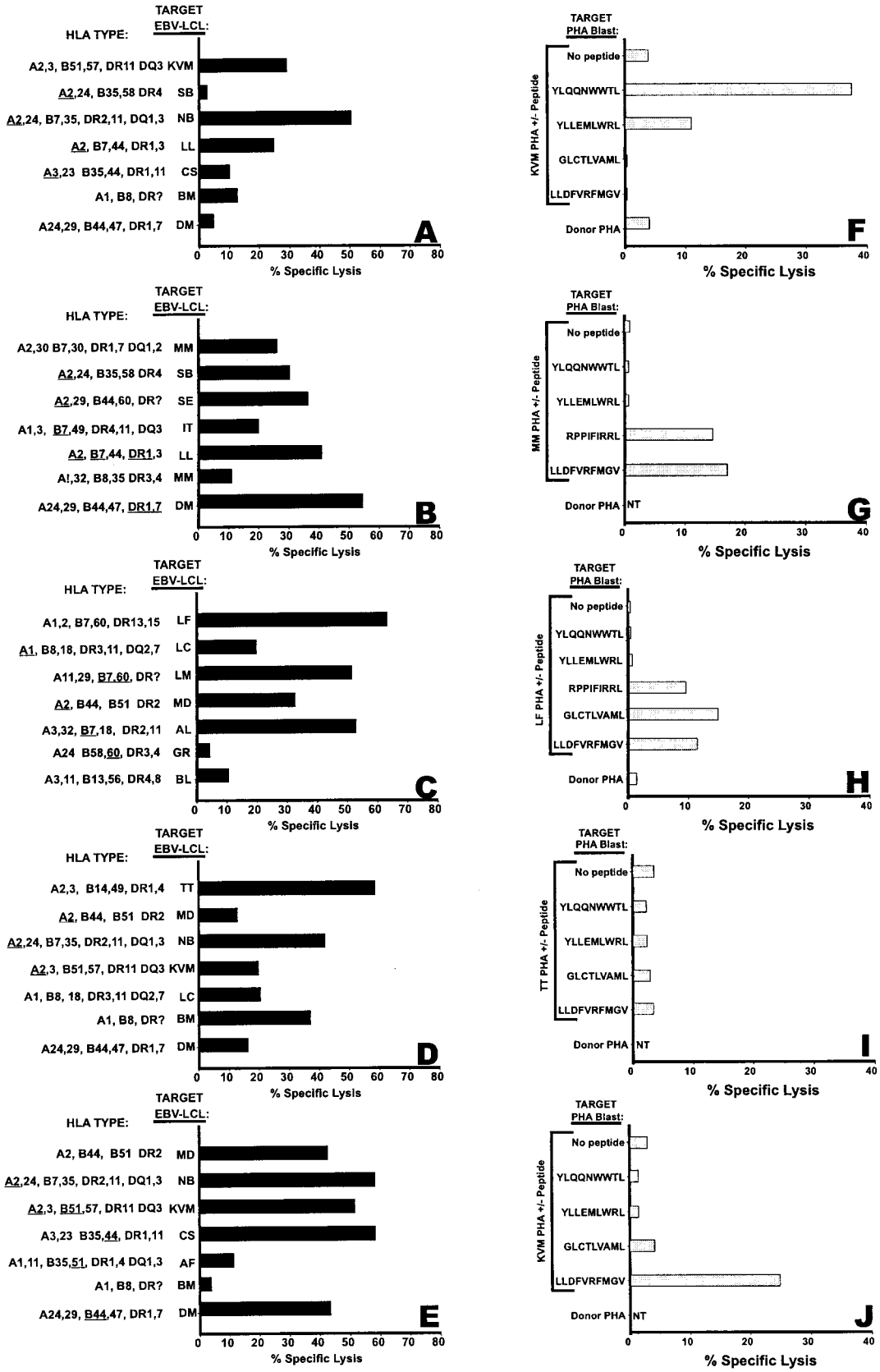

FIG. 1. EBV specificity of T cell lines from five different solid organ transplant patients with or without PTLD. A detailed description of these patients is presented in Table 1. *A–E* show the CTL recognition of autologous and HLA-matched and -unmatched allogeneic LCLs while *F–J* show peptide epitope 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: YLQQNWWTL (HLA A2; LMP1; ref. 10), YLLEMLWRL (HLA A2; LMP1; ref. 10), RPPIFIRRL (HLA B7; EBV nuclear antigen 3; ref. 11), GLCTLVAML (HLA A2; *Bam*H1 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.

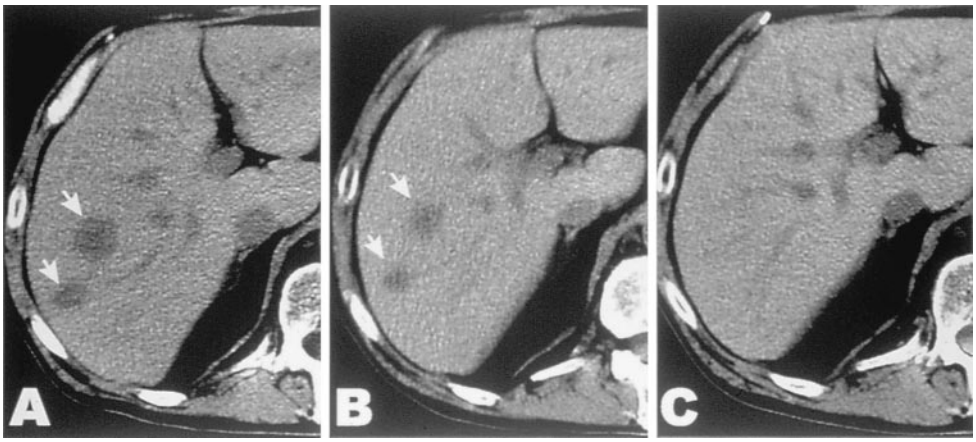

FIG. 2. (*A*) CT scans of liver before initial CTL therapy showing two of the three lymphomas. (*B*) CT scan of liver 2 weeks after first CTL infusion. (*C*) CT scan of liver 20 weeks after the first infusion and before the fourth CTL infusion revealed complete regression of PTLD.

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.

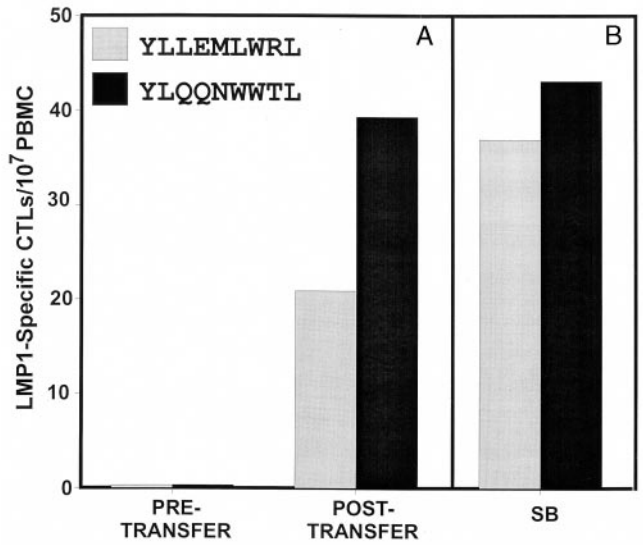

FIG. 3. Quantitation of EBV-specific T cells from patient KVM pre- and postinfusion. The frequency of LMP1-specific CTLs in the peripheral blood of patient KVM was determined by LDA. The precursor frequency for two HLA A2-restricted CTL epitopes (YLLEMLWRL and YLQQNWWTL) is presented in *A*. Also illustrated in *B* is the precursor frequency for the epitopes in a healthy EBV seropositive donor, SB.

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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