Reconstitution of EBV-Specific T Cell Immunity in Solid Organ Transplant Recipients

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EBV-specific autologous CTL were grown in vitro from three adults (two liver transplant recipients and one patient on hemodialysis awaiting kidney retransplant). All CTL lines were TCR αβ, CD8 positive cells, EBV specific, and MHC class I restricted. The CTL lines were expanded in vitro and infused in three escalating doses ($5 \times 10^7$, $1 \times 10^8$, and $2 \times 10^8$) at monthly intervals. Weekly blood samples were collected following each infusion. EBV-specific CTL precursor cells in peripheral blood were quantitated by limiting dilution analysis, and their effect on EBV load in vivo was assessed by semiquantitative PCR. In all three patients, the numbers of CTL precursor cells increased during the weeks following the infusions and were highest after the third infusion. This level gradually declined but remained above the preinfusion levels for up to 3 mo. EBV genome copy number, on the other hand, dropped following the first infusion and became undetectable thereafter. The EBV DNA level remained lower than the pretransplant level in all patients for up to 3 mo after the last infusion. Our study shows that it is feasible to generate and expand EBV-specific CTL from pretransplant blood samples of solid organ transplant recipients, that these CTL can be stored and infused posttransplant, and that they remain cytotoxic and EBV specific in vivo. The aim of this study is to use these CTL for prevention and treatment of lymphoproliferative disease in solid organ transplant recipients. The Journal of Immunology, 1998, 160: 6204–6209.

Following primary infection, EBV persists as a low level, latent infection in circulating B cells and a replicative/permissive infection in the oropharynx (1–3). This persistent infection is kept at subclinical levels in immunocompetent individuals by EBV-specific CTL, which specifically recognize EBV Ags on the surface of the infected cells. These CTL are CD8 positive and MHC class I restricted (4, 5).

In transplant recipients, the use of immunosuppressive drugs to prevent graft rejection leads to suppression of CTL function, thereby generating an environment in which EBV-infected B cells can proliferate (6). In this setting, B cell lymphoproliferative disease (BLPD) develops in 1 to 10% of transplant patients and is associated with a high patient mortality (7). First line of treatment in most transplant centers is reduction or complete withdrawal of immunosuppressive therapy, which allows reactivation of EBV-specific CTL, resulting in partial or complete regression of the tumor in many cases (8). However, recurrences occur that exhibit decreased sensitivity to this conservative form of treatment, and cytotoxic drug therapy is then often required (9).

To date no drug-controlled trial has been performed to define an optimal BLPD treatment strategy, but a search for a nontoxic alternative to cytotoxic drug treatment has led to recent attempts at immunotherapy. In one trial, five allogeneic bone marrow transplant recipients with BLPD were treated with infusions of leukocytes from their EBV-seropositive donors. Regression of the tumor was noted in all cases, presumably due to infused CTL specific for EBV-infected targets, but fatal pulmonary complications occurred in two patients and graft-vs-host disease developed in all five (10). In this system, the beneficial contribution of a graft-vs-tumor effect on tumor regression could not be assessed.

A more recent approach to persistent herpes virus infection in the immunocompromised host is adoptive transfer of cellular immunity in the form of specific CTL. This has been pioneered by Riddell et al. (1994), using human cytomegalovirus-specific CD8 positive CTL clones generated in vitro from bone marrow transplant donor leukocytes. When infused into bone marrow recipients, short-term reconstitution of human cytomegalovirus-specific immunity was achieved (11). In a similar study, CD8 positive, EBV-specific CTL lines were generated in vitro from donor blood and used successfully to prevent and treat BLPD in bone marrow transplant recipients (12, 13). In these studies, donor CTL reconstituted EBV-specific immunity for as long as 18 mo.

To date, all EBV-specific CTL therapy has been conducted in bone marrow transplant recipients where blood from the healthy bone marrow donors is available for the generation of CTL. A similar strategy in solid organ transplant recipients, who have a higher incidence of BLPD, but where the donors are generally unavailable, would require recipient CTL to be grown from pretransplant blood samples taken before the start of immunosuppressive therapy. We report a pilot study undertaken to test the feasibility of EBV-specific CTL therapy in solid organ graft recipients. CD8 positive CTL lines generated from pregraft samples of three solid organ (two liver, one kidney) recipients were successfully expanded in vitro and, following transplantation, infused into the
PBMC were plated at 2 \times 10^6 cells/well in tissue culture medium in 24-well plates with 5 \times 10^5 autologous LCL that had been x-irradiated at 40 Gy to prevent proliferation (40:1 PBMC:LCL ratio; Ref 17). After 10 days the cells were subcultured at a concentration of 1 \times 10^6 cells/well, and a further 2.5 \times 10^5 x-irradiated autologous LCL (T cell:LCL ratio, 4:1) were added. After 14 days 20 U/ml of rIL-2 (Eurocetus U.K., Middlesex, U.K.) was added to each well. Cultures were maintained routinely in tissue culture medium with the addition of 20 U/ml of rIL-2 every 2 to 3 days and restimulated with autologous LCL (4:1 ratio) every 7 days. When sufficient numbers of CTL had been obtained (usually after 2 to 3 mo of culture), aliquots were analyzed for cytotoxic function and cell surface phenotyping.

Materials and Methods

Patient Details

Ethical approval for this study was obtained from Ethical Committees at the Royal Free Hospital and the London School of Hygiene and Tropical Medicine, and patients gave informed consent before entry into the study. CTL lines were grown from six EBV-seropositive adults (age range 29 to 61 years; HLA types shown in Table I), and three were subsequently infused with CTL. The age of three patients was adult (ages 29 and 51 and 54 years). Two patients had liver transplants (5 and 6 mo previously) and no EBV-associated disease, whereas the third (patient 3) had received a kidney transplant 9 years earlier but underwent a transplant nephrectomy after 3 mo, when the patient developed BLPD and immunosuppression was discontinued. He has since been maintained on regular hemodialysis. At the time of study, one liver transplant patient was receiving Tacrolimus (FK506) at 2 mg in the morning and 3 mg at night and the other Neoral (emulsified cyclosporin) at 75 mg twice daily. None of the patients received any corticosteroids or mycophenylate therapy.

Peripheral blood (50 ml) was taken pretransplant and used to generate autologous EBV-positive B lymphoblastoid cell lines (LCL). These LCL were used as stimulators to grow autologous EBV-specific CTL that were then infused into three patients at three escalating doses of 5 \times 10^5, 1 \times 10^6, and 2 \times 10^6 at monthly intervals. Blood samples were taken before and at weekly intervals following each infusion and were assayed for EBV CTL precursor frequency by limiting dilution analysis and for EBV DNA by PCR.

Generation of LCL

PBMC were separated from whole blood by Ficoll-Hypaque density gradient (14). PBMC (1 \times 10^7) were incubated with 100 \mu l of supernatant cultur medium (RPMI-1640 (Life Technologies, Paisley, U.K.) containing 100 IU/ml penicillin, 100 \mu g/ml streptomycin, 10\% v/v FCS (HyClone, Logan, UT)), and seeded in flat-bottom microtiter wells at a concentration of 1 \times 10^6 cells/well in 200 \mu l tissue culture medium. The cultures were maintained by weekly feeding, and the resulting LCL were expanded to 25 cm^2 flasks. LCLs were maintained in culture medium containing acyclovir (100 \mu M; Glaxo-Wellcome, Kent, U.K.) to prevent lytic replication. Regular testing by indirect immunofluorescence for viral capsid Ag (VCA) showed no evidence of lytic Ag expression (16).

Patient 1 A2, A19
Patient 2 A9, A19
Patient 3 A2, A3
Patient 4 A11, A19
Patient 5 A26, A31
Patient 6 A2, A32

ND, not done.

Table I. HLA types of the patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>HLA Class I Types</th>
<th>HLA Class II Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>A2, A19</td>
<td>B12, B51, Bw4</td>
</tr>
<tr>
<td>Patient 2</td>
<td>A9, A19</td>
<td>B7, B40, Bw6</td>
</tr>
<tr>
<td>Patient 3</td>
<td>A2, A3</td>
<td>B5, B8</td>
</tr>
<tr>
<td>Patient 4</td>
<td>A11, A19</td>
<td>B12, B22, Bw4, Bw6</td>
</tr>
<tr>
<td>Patient 5</td>
<td>A26, A31</td>
<td>B8, B51</td>
</tr>
<tr>
<td>Patient 6</td>
<td>A2, A32</td>
<td>B7, B27, Bw4, Bw6</td>
</tr>
</tbody>
</table>

ND, not done.

Surface marker analysis

The phenotype of the CTL lines was analyzed by FACS analysis after staining with mAbs against TCR \alpha \beta, TCR \gamma \delta, CD4, CD8, CD16, CD25, CD26, CD38, CD45RA, CD45RO, CD54, CD57, and monomorphic anti-HLA class II (DR, DP, and DQ), as described previously (19).

Safety measures taken before the infusion of CTL

Rigorous precautions were taken during the culture period to maintain sterility of the cell lines. The laboratory strain of EBV, B958, used to generate autologous LCL was screened for mycoplasma regularly using a commercially available detection kit (Gen-Probe, San Diego, CA) and for type D retrovirus (Ref 20; tests kindly conducted by Ms. E. Grogan, Yale University Medical School, New Haven, CT). All LCL were grown in medium containing the antiviral agent acyclovir, which inhibits the EBV late lytic cycle and thereby prevents virus production (21). The LCL were constantly negative for EBV late lytic cycle Ag, VCA, by indirect immunofluorescence techniques. Additionally, to prevent viable LCL cells used as stimulators from contaminating the T cell infusions, tests were performed to obtain an optimum dose of irradiation (40 Gy) that was sufficient to prevent their proliferation. To exclude other infectious contaminants, CTL cultures were subjected to a full microbiologic screen for bacteria, fungi, and mycoplasma (conducted at University College, London, U.K.) before being stored frozen. Similarly, to exclude the possibility that the cell lines had been contaminated with allogeneic cells during the culture period, HLA typing was conducted before freezing. All CTL lines were found to consist of the respective recipient’s HLA type only (HLA typing was conducted at the Anthony Nolan Trust, The Royal Free Hospital, London, U.K.).

Infusion of CTL

Three escalating doses of CTL (5 \times 10^5, 1 \times 10^6, and 2 \times 10^6) were infused at four weekly intervals. Frozen CTL were thawed at 37°C, washed once in HBSS containing 10% human serum albumin (BioProducts, Harfordshire, U.K.), freezing at 1 \times 10^6 cells/vial in autologous serum containing 10% DMSO (tissue culture grade, Sigma), and storing in the vapor phase of liquid nitrogen.

Chromium release assay for EBV-specific cytotoxicity

Each T cell line was tested for cytotoxic activity against the autologous LCL, an HLA-mismatched LCL, and K562, a NK cell-sensitive target (18). Cells (3 \times 10^5) from each target cell line were labeled with \textsuperscript{51}Cr (100 \mu Ci; ICN Pharmaceuticals, Costa Mesa, CA) for 1 h and plated with CTL at effector:target ratios of 40:1, 20:1, 10:1, and 5:1. After 4 h, the release of \textsuperscript{51}Cr from lysed cells was measured on a gamma counter (Wallac, Milton Keynes, U.K.). Percent specific lysis was calculated using the formula: (test sample − spontaneous release)/(maximum release − spontaneous release) \times 100.

Limiting dilution analysis (LDA) for EBV-specific CTLp frequency

PBMC were seeded into 96-well, round-bottom microtiter plates in doubling dilutions at concentrations ranging from 20,000 to 625 per well. Twenty four replicate wells were plated at each concentration, and 5000
irradiated (40 Gy) autologous LCL in 200 μl culture medium were added to each well. rIL-2 (5 U/ml) was added on days 3 and 7. The cytotoxic activity was assessed after 10 to 14 days by the standard 4-h chromium release assay. The contents of each well were split into two and tested against HLA-mismatched LCL targets. Any well giving chromium release (cpm) greater than 10% specific lysis (always more than three SD above the spontaneous release) was taken as positive. CTLp frequency was estimated by using a Poisson distribution slope where the relationship between the responding cell number and the logarithm of the percentage of nonresponding (negative) cultures was plotted (22, 23).

**PCR**

DNA was extracted from 5 × 10^6 PBMC using a commercially available DNA extraction kit (Invitrogen, San Diego, CA). The B cell lines B95-8 and Ramos were used as EBV-positive and-negative controls, respectively (24). Sterile distilled water was always included as a template-free control. All samples were routinely amplified using primers specific for the human β-globin gene as an internal control (25). PCR was conducted using a primer set that amplifies a sequence (116 bp long) of the EBV genome within the EBNA 2 gene (26). PCR products (10 μl) were run on a 2% agarose gel and Southern hybridized using [α-32P]dCTP-labeled EBNA 2-specific plasmid pBanHI-H2 (27). With every PCR the sensitivity of the EBNA 2 primer set was determined using Namalwa, an EBV-positive Burkitt lymphoma cell line known to carry 1 to 2 integrated EBV genome copies per cell (28). Tenfold dilutions of Namalwa cells (10^5 - 10^2) were mixed with 10^6 Ramos cells. Total DNA was extracted and subjected to PCR. Our PCR system was capable of detecting EBV signal from one Namalwa cell (1-2 EBV genome) in a mixture of 10^6 Ramos cells (data not shown). The density of the gel-electrophoresed test bands was determined using a densitometer (Sharp, Oxford, U.K.), compared with the standard curve obtained from Namalwa dilutions and the EBV genome copy number calculated.

**Statistical analysis**

The CTL-mediated lysis of HLA-mismatched LCL and anti-HLA class I Ab-coated LCL was compared with that of autologous LCL by paired t test. Spearman’s rank correlation was used to compare the numbers of CTLp and EBV genome copies in each patient. Values for each variable were ranked separately (omitting the pretransplant values) in ascending order of magnitude, and Spearman’s rank correlation was calculated (with two-tailed p values).

**Results**

**Phenotype of in vitro generated and expanded CTL lines**

The immunophenotype of the six CTL lines was determined by FACS analysis using mAbs against different T, B, and NK cell surface Ags (Table II). The CTL lines consisted mainly of T cells expressing TCR αβ surface markers, and, in five of six cases, virtually all cells were cytotoxic cells (CD8 positive) with only a very few remaining CD4-positive cells (range 0.1–7%). The sixth CTL line (patient 6) contained 80% of CD4-positive T cells. Most cells expressed HLA-DR Ag, and a variable proportion expressed the costimulatory molecule, CD28 (16.8–41%; data not shown). No CD19-positive B cells and very few CD16/CD7+ NK cells (0–1.7%) were detected.
The effect of CTL infusions on EBV DNA levels in peripheral blood was determined using semiquantitative PCR (Figs. 1 and 2). In patients 1 and 2, EBV DNA was undetectable in the pretransplant PBMC; however, EBV genome copy number was high (2.1 and 1.7 \times 10^4 per 10^6 PBMC, respectively) in blood samples taken before the CTL infusions (at 5 and 6 mo posttransplant, respectively). Following the first CTL infusion, the number of viral genome copies decreased, and gradually became undetectable thereafter. In patient 1, EBV DNA remained undetectable in PBMC for 2.5 mo after the third infusion, and, in patient 2, it remained lower than the preinfusion level for 2.5 mo after the last infusion (Figs. 1A and 2A; Figs. 1B and 2B). Patient 3 had detectable EBV DNA in the preinfusion sample, which decreased gradually following CTL infusions and became completely undetectable following the last CTL infusion. The EBV DNA was detected 3 mo following the last infusion, albeit at a lower level than before the infusion (Figs. 1C and 2C). A significant, inverse correlation was observed between the numbers of CTLp and EBV genome copies in peripheral blood in two patients (patients 2 and 3) using Spearman’s rank correlation test (two-tailed p values < 0.04 and 0.0125, respectively).

Discussion

This study investigates the feasibility of using cytotoxic T cell infusion as a treatment strategy in solid organ transplant recipients. The study was conducted on EBV-seropositive transplant recipients who did not have EBV-related disease. One patient had previously developed BLPD following a renal transplant and underwent complete long-term remission after withdrawal of immunosuppressive therapy and removal of the graft. At the time of infusion of CTL, this patient was supported on regular hemodialysis and had been free of BLPD for 9 years.

As indicators of the function of the infused CTL, the levels of EBV DNA and CTLp in peripheral blood were monitored. The results show that functional CTLp could be detected immediately after the first infusion and that they remained above preinfusion levels for up to 3 mo after the last CTL infusion. Each infusion increased the CTLp level to above that seen before the infusion. 

Effects of CTL infusions on EBV DNA levels in peripheral blood

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The CTL infusions produced rapid drop of EBV genome copies in PBMC to undetectable levels in all three patients after the first infusion and the maintenance of a level lower than that seen in the pretransplant blood samples for 3 mo after the last infusion. Taken together, these data suggest that even the lowest dose of CTL used in this study (5 x 10^4), given every 3 to 4 wk, would be sufficient to prevent or treat BLPD. This estimate is similar to those deduced from other studies in bone marrow transplant recipients (12, 13).

In this study, EBV-specific CTL were grown from six patients, and, in three cases, autologously reinfused without deleterious effect. Clinical and biologic testing clearly demonstrates that it is possible to generate EBV-specific CTL from pretransplant recipient blood samples, that these can be grown up to high cell numbers in vitro, and that they can be stored frozen without loss of function. Of the six CTL lines generated (including the three that were infused) showed a CD8-positive, activated T cell phenotype, with less than 2% contaminating CD19-positive NK cells and no detectable CD19-positive B cells. One (patient 6) of the six T cell lines generated contained 80% CD4-positive T cells (CTL not infused), but all six lines gave significant specific killing of the autologous LCL when compared with the HLA-mismatched target. No attempt was made to clone the CTL or to direct the antigenic specificity of the lines generated, on the basis that polyclonal lines, containing cells with specificities for a number of different EBV epitopes, would be more efficient than single clones at recognizing and eliminating EBV-carrying cells.

CTL infusion is an experimental approach to treat EBV-associated BLPD. It has evolved as a refinement to the present approach in which reduction in immunosuppressive therapy presumably promotes an increase in CTL activity and leads to tumor regression in many cases. The problem inherent with reducing immunosuppression is the risk of cell-mediated graft rejection from a concomitant increase in alloreactive CTL. This conservative approach is more acceptable in renal transplant patients where, if necessary, the graft can be sacrificed and the patient supported on hemodialysis until the tumor has regressed. However, in liver, heart, and lung recipients, this approach is more precarious, and it often proves difficult to maintain a balance between tumor destruction and graft rejection, made worse by the fear of not being able to replace the function of the failing organ. Furthermore, recurrences of BLPD commonly develop and display decreased sensitivity to conservative therapy. Cytotoxic drugs are often required at this stage, and mortality is high (7).

CTL immunotherapy has been used successfully to prevent and treat BLPD in bone marrow transplant recipients (12, 13), but the present study is the first to report potential CTL infusion therapy in solid organ transplant recipients. In this situation, the donor is usually not available to provide a starting population of T cells. Thus, autologous CTL must be grown from recipient blood samples taken before transplant, since, afterward, T cells are inactivated by continuous immunosuppressive therapy.

The accumulated data from studies similar to our own now warrant a randomized controlled trial to compare CTL therapy for BLPD with the standard treatment. If CTL therapy proved the better therapy, then, for each patient undergoing transplantation, LCL and CTL would have to be stored before engraftment. However, this would require levels of funding, laboratory facilities, and workforce that are outside the reach of most transplant centers. An alternative strategy is to generate a large panel of well-characterized EBV-specific CTL from EBV-seropositive healthy individuals that would be available for use in BLPD patients on a best match basis. This strategy has the advantage of treating recipients who are EBV seronegative before transplant, and from whom CTL cannot therefore be generated, but who are at higher risk of developing BLPD if they later experience primary EBV infection.

The main risks involved in using allogeneic CTL are the induction of graft-vs-host disease and graft rejection caused by the infused CTL. However, these risks can be minimized by ensuring that the CTL are entirely EBV specific with no detectable alloreactivity in vitro testing. In addition, in certain circumstances, a graft-vs-host effect may be beneficial to tumor regression. Recognition and destruction of the infused foreign CTL by the host immune system would probably be delayed because of the immunosuppressive drugs taken to prevent graft rejection. The proposed trial will determine not only whether CTL immunotherapy is the treatment of choice for BLPD but also whether it is worth developing as a more widespread treatment option for opportunistic viral infections.

Acknowledgments

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