The Development of Cellular Immunity to Epstein-Barr Virus After Allogeneic Bone Marrow Transplantation

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Epstein-Barr virus-induced lymphoproliferative disease (EBV-LPD) is a potentially lethal complication during the first 6 months after allogeneic bone marrow transplantation (BMT). To determine whether deficiencies of EBV-specific cellular immunity contribute to EBV-LPD susceptibility and distinguish patients at risk, we performed limiting dilution analysis to quantify anti-EBV cytotoxic T-lymphocyte precursor (CTLp) frequencies in 26 recipients of unmodified or T cell-depleted (TCD) grafts from EBV-seropositive donors. At 3 months post-BMT (n = 28), only five patients had EBV CTLp frequencies in the range of seropositive normal controls, irrespective of the type of transplant administered. By 6 months post-BMT, 9 of 13 patients tested had EBV CTLp frequencies within the normal range. The time period in which these patients had deficient cellular immunity to EBV corresponds to the period in which we have observed EBV-LPD in most prior patients. One patient with a low EBV CTLp frequency at 4 months post-BMT developed an EBV-LPD. Within 2 weeks of receiving an infusion of donor peripheral blood mononuclear cells (PBMC) providing less than 1,200 EBV-specific cytotoxic T-cell precursors, populations of EBV-specific CTL in the circulation were restored to levels detected in normal seropositive adults. Concurrently, the patient achieved a regression of the EBV-LPD, which has been sustained without further therapy. These studies indicate that recipients of both unmodified and TCD marrow grafts have profound deficiencies of EBV-specific T cell-mediated immunity early posttransplant, and that the period of risk for EBV-LPD closely corresponds to this interval of severe deficiency. Treatment of one patient with EBV-LPD with marrow donor-derived PBMC induced a rapid expansion of EBV-specific cytotoxic T-cell populations that occurred contemporaneously with the clinical regression of disease.

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Materials and Methods

Patients. The patients evaluated in this study included 26 children and adults who received allogeneic marrow transplants from EBV-seropositive donors between October 1993 and August 1994. The mean age was 33.5 years (range, 8 to 60 years). The majority of patients (n = 20) received transplants for leukemia; three patients received transplants for myelodysplastic syndrome, two for multiple myeloma, and one for non-Hodgkin’s lymphoma. Of the 26 patients, 10 received an unmodified marrow graft, and 16 received TCD marrow grafts, of which 11 were derived from HLA-identical sibling donors and five were from HLA-compatible unrelated donors. These studies were performed after informed consent was obtained under protocols approved by the Human Subjects Review Committee of Memorial Hospital (New York, NY).

Before transplantation, the patients were conditioned with cytoreductive regimens varying according to their diagnoses. Six patients were cytoreduced with busulfan and cyclophosphamide, either alone (n = 3) or with I-131 conjugated anti-CD33 monoclonal antibody.
bodies against CD3, CD4, CD8, CD20, CD56, CD57, and CD45 followed by magnetic separation. The T cell-enriched populations body-coated immunomagnetic beads (Dynal, Lake Success, NY) were analyzed for their immunophenotype by cytofluoroscopy after culture flasks (Coming, Coming, NY) for 1 hour at 37°C. The adherent monocytes and macrophages were harvested using a cell scraper (Becton Dickinson, San Jose, CA). The CD20+ and CD56+ cells bated with monoclonal antibodies directed against CD20 and CD56 cytodepleted peripheral blood lymphocytes (PBL) were then incu- and were subsequently irradiated and used as feeder cells. The mono- 
blasts from unrelated donors, and an additional 192 patients who received unmodified HLA-identical sibling transplants administered from January 1987 to March 1995. The titers of IgG antibodies specific for the viral capsid antigen (VCA) of the EBV virus were determined on all patients and donors before transplant using standard methodologies. Anti-VCA titers were also determined on a series of normal donors. Individuals with anti-VCA titers ≤1:10 were considered to be EBV-seronegative. All seropositive normal controls and all bone marrow donors had anti-VCA titers >1:40.

**EBV-transformed B lymphoblastoid cell lines (BLCL).** To assess EBV-specific T-cell responses, EBV-transformed lymphoblastoid cell lines were established from each of the bone marrow donors. Aliquots of 30 mL of heparinized blood were collected, and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. After washing, 10^9 PBMC were infected with EBV by incubating them for 48 hours in a medium containing one part of EBV containing supernatant from the marmoset cell line 95-8 and three parts RPMI 1640 containing 15% fetal calf serum (FCS) and 2 µg/mL phytohemagglutinin (PHA)-16 (Difco Laboratories, Detroit, MI). Thereafter, the cultures were centrifuged and transferred to the same medium without PHA-16 for 24 hours and then fed with RPMI 1640 supplemented with 15% FCS, 1% L-glutamine, 1% penicillin-streptomycin, and 1% tylosine (GIBCO, Grand Island, NY) daily until sufficient growth was appreciated. The actively growing EBV-BLCL were maintained in RPMI 1640 with 10% FCS and the same additives as described for the BLCL. T cells cultivated in 24-well plates were fed on days 3, 6, and 9 by replacing 0.5 mL of supernate with conditioned medium containing 2.5 U/mL recombinant interleukin-2 (IL-2) (Collaborative Biomedical Products, Bedford, MA).

Limiting dilution cultures were established using a modification of the methods of Bourgault et al.24 and Langhorne and Lindahl.25 Twenty-four replicate wells were used per dilution of T cells in 96-well, U-bottom plates (Nunc-Intermed, Kamstrup, Denmark) with 1,000 irradiated (3 Gy) adherent cells and 2.5 X 10^4 irradiated (10 Gy) donor BLCL per well, each well containing a total volume of 200 µL. Twenty-four wells of irradiated BLCL only were used as a control. Cultures were fed with IL-2 on days 3, 6, and 9 by removing 50 µL of culture supernate and replacing with the same conditioned medium containing 2.5 U/mL recombinant IL-2 as described above.

**Chromium release assay.** Bulk and limiting dilution cultures were assayed simultaneously on day 12 of culture. T cells from bulk cultures were collected from the 24-well plates and resuspended at 1.5 X 10^5/mL, 0.75 X 10^5/mL, and 0.38 X 10^5/mL in RPMI 1640 with 10% FCS. One hundred microliters of each dilution of bulk culture T cells was added in triplicate to 96-well, V-bottom plates (ICN Biomedical, Aurora, OH) for each target cell assayed.

To assay CTLp frequencies, the limiting dilution culture wells were mixed, and equal 35-µL volumes of cells were aliquotted into separate 96-well, V-bottom plates to assess cytotoxicity against each target. RPMI 1640 with 10% FCS was then added (65 µL) to make a total volume of 100 µL.

The following cells were used as targets: donor PHA blasts, donor BLCL, BLCL partially HLA-matched with the donor, allogeneic HLA-different BLCL, and K562, the latter to assess NK activity. Donor PHA blasts were generated 4 days before the cytotoxicity assay. Target cells (2 X 10^6) were each incubated with 250 µCi chromium 51^{51}Cr, New England Nuclear, North Billerica, MA) for 2 hours, washed with RPMI, and resuspended at 30,000/mL in RPMI 1640 with 10% FCS. Each target cell preparation (100 µL) was placed in each well containing T cells from either the bulk culture or the limiting dilution cultures for a total volume of 200 µL, and plates were spun at 2,000 rpm for 2 minutes. To determine total ^51Cr release, 100 µL of 5% Triton X was added to 100 µL of each ^51Cr-labeled target. Plates were incubated at 37°C and 5% CO2 for 4 hours. After the incubation with ^51Cr-labeled targets, 150 µL of supernate was harvested, transferred to microtiter tubes (Continental Labs, San Diego, CA), and counted in a gamma counter. Spontaneous and total release for each target was used to calculate percent specific release using the following formula:

\[
\% \text{ Specific Release} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100
\]

Limiting dilution wells were scored as positive if release exceeded 10%. CTLp frequencies were calculated by the method of Taswell using a computer program provided by Dr Y. Kawanishi, Medical College of Wisconsin (Milwaukee, WI). Antibody blocking studies

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were performed as described by Flomenberg et al using the monoclonal antibody OKT3 (anti-CD3). This antibody was generated from mouse ascites and diluted 1:10 with RPMI. Fifty microliters of blocking antibody was placed into each of the respective wells, for a final antibody dilution of 1:40. Blocking was considered present when there was greater than 20% inhibition of cytotoxicity compared with the wells with responder and target cells alone.

Biostatistics. Correlations of results of different assays of cell-mediated and humoral EBV-specific immunity were computed using the Pearson correlation coefficient. A nonparametric 95% confidence interval (CI) for each population correlation coefficient was generated using the bootstrap resampling procedure. The probability of developing EBV-LPD was calculated after an adjustment for competing risks.

RESULTS

The CTLp frequencies detected for T cell-enriched PBL derived from normal EBV-seropositive and -seronegative individuals against autologous EBV-BLCL, autologous PHA blasts, and allogeneic EBV-BLCL are presented in Fig 1. The range of frequencies for CTLp reactive against autologous EBV-BLCL for EBV-seropositive controls was 1/177,900 to undetectable. The mean CTLp frequency against autologous EBV-BLCL for normal EBV-seropositive controls was 1/14,600 (range, 1/1,960 to 1/42,790). While 8 of 10 seropositive donors had CTLp frequencies against allogeneic EBV-BLCL that were less than or equal to 1/100,000, two seropositive donors had values greater than 1/100,000 for fully allogeneic EBV-BLCL. Both EBV-seropositive and -seronegative donors had CTLp frequencies against autologous PHA blasts that were less than 1/100,000.

The lower portion of Fig 2 depicts the cytotoxicity results of bulk cultures of T cells from seropositive and seronegative controls against autologous and allogeneic BLCL targets. The mean percentage of specific lysis directed against the autologous BLCL for EBV-seropositive donors was 39% ± 16% (range, 24% to 70%), which differed significantly from the mean of 4.5% ± 4.2% (range, 0 to 10%) for EBV-seronegative donors \( P < .001 \). The highest level of T cell-mediated cytotoxicity observed on EBV-BLCL targets that did not share HLA antigens with the responder cells was 13%. This cut-off point was, therefore, selected for defining positive and negative responses.

The top portion of Fig 2 depicts the log CTLp frequency of normal donors against autologous EBV-BLCL (ordinate) and the percent specific lysis of autologous BLCL (abscissa). There was a strong correlation between log CTLp frequency and the percent specific lysis from bulk cultures \( r = .80; 95\% \) CI, .57 to .95. The responses of T cells from four normal EBV-seropositive controls and four BMT patients were analyzed after the effector cells were incubated with medium containing a monoclonal antibody against CD3. EBV-specific cytotoxic responses were largely or completely inhibited by treatment of each individual’s effector cells with anti-CD3. The mean inhibition by anti-CD3 in EBV-seropositive normal donors was 78% ± 17% (range, 54% to 93%) and 82% ± 14% (range, 68% to 100%) in patients.

The log of the EBV CTLp frequencies in BMT patients at 3, 6, and 9 months posttransplant are displayed graphically in Fig 3. Twenty-six patients were studied at 3 months after BMT. Of this group, five (19%) had CTLp frequencies in the seropositive range, and six (23%) were in an intermediate range, between that detected for the seropositive and seronegative controls; 15 patients (58%) were in the seronegative range. Of patients (23%) were in an intermediate range, between that detected for the seropositive and seronegative controls; 15 patients (58%) were in the seronegative range. Of patients whose CTLp frequencies were in the seropositive range at 6 months continued to have CTLp frequencies in this range when studied at 9 months post-BMT. The in vitro cytotoxicity against the autologous donor EBV-BLCL in these experiments was virus-specific, because CTLp frequencies for autologous
PHA blasts were negligible in the patient and control specimens analyzed.

While the numbers of patients in each group are too few for statistical analysis, the proportion of transplant recipients developing EBV-specific CTLp frequencies in the intermediate or seropositive range by 3 months after an unmodified graft from a matched sibling (4 of 10) or a TCD graft from a matched sibling (5 of 11) or an unrelated donor (two of five) were comparable. Furthermore, as illustrated in Fig 3, the frequencies of EBV-specific CTLp developed by 7 of 16 patients at 3 months and 8 of 11 studied at 6 months after TCD grafts were similar to those detected in recipients of unmodified marrow grafts at the same time period.

Figure 4 correlates patient EBV CTLp frequencies from limiting dilution cultures on the ordinate and 51Cr release from bulk culture T cells on the abscissa. Bulk culture data were available for all patients except one recipient of an unrelated, TCD graft at 3 months. At 3 months post-BMT, the four patients with CTLp frequencies in the seropositive range also had positive cytotoxicity in bulk culture against the donor BLCL. Cells from two of the six patients with CTLp frequencies in the intermediate range exhibited EBV-specific cytotoxicity in bulk culture, while four lacked detectable cytotoxicity in bulk culture. None of the patients with EBV-CTLp frequencies in the seronegative range exhibited detectable lysis of the donor BLCL. At 6 months post-BMT, T cells from all patients with CTLp frequencies in the seropositive and intermediate range exhibited EBV-specific cytotoxicity; the three patients with CTLp in the seronegative range also lacked cytotoxicity in bulk culture. The degree of cytotoxicity for the donor EBV-BLCL correlated with the log of the EBV-CTLp frequency (at 3 months, r = .60; 95% CI, 0.35 to 0.86). Too few observations were recorded at 6 months posttransplant to permit informative analyses for that time point.

To determine whether and to what degree the posttransplant interval associated with severe deficiencies of EBV-specific cellular immunity observed early posttransplant coincided with the period of risk for developing EBV-LPD, we examined the medical records of 655 patients who received allogeneic bone marrow transplants for leukemia at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) between January 1, 1987, and March 1, 1995; of whom, 28 developed EBV-associated lymphomas (4.3%). In this series, the cause-specific estimates of EBV-LPD in recipients of SBA`E' TCD grafts from related or unrelated donors treated with ATG and/or steroids were 6.7% and 18.3%, respectively. Among those patients who received SBA`E' grafts who were not treated with ATG or steroids, the cause-specific estimate was 0.8%. There were no EBV lymphomas in recipients of unmodified transplants. As shown in Fig 3 (bottom panel), all cases of EBV-LPD developed between 1 and 7.4 months posttransplant, with the peak being at 3 months post-BMT.
One recipient of a TCD, HLA-identical sibling BMT developed an EBV lymphoma at 4 months posttransplant. As shown in Fig 5A (top panel), T cells isolated from this patient before donor leukocyte infusion did not induce lysis of donor EBV-BLCL in bulk culture; by limiting dilution analysis (Fig 5A, bottom panel), the frequency of EBV-specific CTLp was 1/119,150, which is in the intermediate range. As shown in Fig 5B, 2 weeks after a donor leukocyte infusion of 0.5 \times 10^6 CD3+ cells per kilogram, T cells from this patient grown in bulk culture exhibited 35% specific lysis against the donor BLCL. Limiting dilution analysis performed at the same time revealed an EBV CTLp frequency of 1/10,970, which is in the seropositive range. T cells isolated from the blood of this patient’s bone marrow donor had an EBV-specific CTLp frequency of 1/42,790, with 24% specific lysis of autologous BLCL from bulk culture. Based on the donor’s T lymphocyte count, unique patient number (UPN) 1461 received 818 EBV-specific CTLp at the time of the leukocyte infusion. Based on preinfusion CTLp frequencies in this patient, and assuming that the blood volume is approximately 8% of body weight and that lymphocytes in the peripheral blood constitute 2% of total body lymphocytes, \(^{30}\) UPN 1461 had a total of 1.64 \times 10^6 EBV CTLp before infusion and \(17.9 \times 10^6\) EBV-specific CTLp at 2 weeks postinfusion. This indicates that there was an 10.9-fold increase in the number of EBV CTL generated in this patient within 2 weeks of the donor leukocyte infusion. By 2 months after PBMC infusion (Fig 5C), the EBV CTLp frequency was 1/1,580. At this time, there was 81% specific lysis of the autologous BLCL and some cytotoxicity against the unrelated BLCL target JL, which shared the HLA antigens A26, B14, and DR7 with this patient. The cytotoxicity against the donor EBV-transformed autologous BLCL and the BLCL JL could be largely inhibited by pretreatment of effector cells with anti-CD3.

**DISCUSSION**

EBV-induced lymphomas have recently emerged as a significant complication of HLA nonidentical related and matched unrelated marrow allografts, particularly when the allografts are administered after selective depletion of T cells...
REGENERATION OF EBV-SPECIFIC T CELLS POST-BMT

Fig 5. A recipient of a TCD BMT from an HLA-identical sibling (UPN 1461) developed EBV-LPD at 40 months posttransplant. (A) Bulk culture cytotoxicity and limiting dilution data from patient T cells before donor leukocyte infusion and 4 months after BMT are presented. Note a lack of killing against any target from bulk culture (top) and a CTLp frequency of 1/119,150 (below). (B) By 2 weeks postinfusion, the patient has detectable lysis of the donor BLCL and a normal CTLp frequency.

with certain monoclonal antibodies or application of more intensive T cell-targeted immunosuppressive regimens to ensure engraftment or prevent GVHD. These EBV-LPD most often present as high-grade diffuse large cell malignant B-cell lymphomas which are oligoclonal or monoclonal. These lymphomas express not only EBNA-1 but also EBNA-2, EBNA-3 and LMP-1. Their clinical course is fulminant and usually fatal.

A striking feature of the EBV lymphomas emerging in marrow allograft recipients is that they are almost invariably of marrow donor rather than host origin. Furthermore, as illustrated in Fig 3, these lymphomas arise only in the period between the initiation of engraftment (approximately 1 month posttransplant) and 6 to 8 months posttransplant, at which time, early reconstitution of T- and B-cell populations of donor origin is usually appreciated. The majority of these patients also have profound deficiencies of EBV-specific cytotoxic T cells at 3 months after transplant. However, EBV-specific cytotoxic T-cell populations, as measured by levels of EBV-specific cytotoxicity...
detected in standard $^{51}$Cr assays and quantitation of EBV-specific CTLp frequencies, approximate those in normal seropositive adults by 6 months posttransplant. Using standard assays of cell-mediated cytolysis, Crawford et al. also found that in recipients of unmodified BMT, 7 of 10 patients studied had defective killing of the autologous BLCL at 3 months posttransplant, while all 10 patients had normal killing by 6 months post-BMT.

As shown in Fig 3, the interval during which profound deficiencies of EBV-specific cytotoxic T cells are observed closely corresponds with the time period in which patients are at risk for EBV-LPD. Given the suggested contribution of a more profound immunodeficiency posttransplant to EBV-LPD susceptibility in recipients of SBA $^\text{E}^-$ TCD grafts, we were surprised to find that deficiencies of EBV CTLp observed in recipients of unmodified marrow grafts at 3 months posttransplant were as frequent and quantitatively as severe as those detected in recipients of SBA $^\text{E}^-$ TCD grafts.

Recipients of SBA $^\text{E}^-$ TCD grafts only receive total clonable T-cell doses ranging between $2 \times 10^6$ and $10 \times 10^6$ T cells per kilogram, doses that are 500- to 1,000-fold lower than those transferred in an unmodified graft. Based on the frequencies of EBV CTLp detected in normal seropositive donors, the number of EBV-specific CTLp infused with an SBA $^\text{E}^-$ TCD graft would be expected to be quite small. As a result, the capacity of such patients to generate an adequate response to an EBV oligoclonal proliferation may be markedly inferior to that of a recipient of an unmodified graft and may be even more profoundly compromised by posttransplant immunosuppression. Despite this, 8 of 11 recipients of SBA $^\text{E}^-$ BMT in this series developed frequencies of EBV CTLp by 6 months posttransplant that were well in the range of seropositive normals. This suggests that despite the limited number of EBV CTLp transferred in the graft, most recipients of either unmodified or SBA $^\text{E}^-$ TCD grafts are able to generate strong virus-specific responses by 6 months posttransplant that are likely to be effective in controlling or suppressing EBV-induced lymphoproliferations.

Prospective analyses of large populations of patients transplanted with unmodified or TCD marrow will be required to determine definitively whether or not the dose of EBV-specific CTLp administered in a graft, the rapidity of recovery of EBV-specific immunity or the level of EBV-specific immunity achieved in recipients of unmodified grafts differs significantly from that of recipients of TCD grafts and whether such deficiencies are correlated with susceptibility to EBV-LPD. Nevertheless, while the number of patients evaluated in this study is too small for statistical analysis, the lack of any apparent difference between the two groups in the frequency of EBV-specific cytotoxic T-cell precursors or their virus-specific cytotoxic activity at 3 months or 6 months posttransplant suggests that other factors must also be considered to explain the differential susceptibility to EBV-LPD observed. For example, during the acute phase of infectious mononucleosis, both MHC-nonrestricted and MHC-restricted EBV-reactive T cells, as well as NK cells, have been implicated in the initial cellular immune response to the EBV virus.

Previous studies from our group have shown that recovery of IL-2-activated NK cell populations and their lytic activity against susceptible targets such as K562 occur as early as 3 weeks posttransplantation and is equivalent in recipients of unmodified and SBA $^\text{E}^-$ TCD marrow grafts. However, it is possible that NK cells emerging after an SBA $^\text{E}^-$ graft might not be as effective in limiting the expansion of EBV-BLCL emerging early in the posttransplant period. Analyses to address this issue are planned. Alternatively, the more prolonged deficits in IL-2 production by T cells that we have demonstrated in recipients of SBA $^\text{E}^-$ grafts might limit the capacity of the transplant recipient to activate NK cells or expand EBV-reactive T-cell populations sufficiently to induce lysis and thereby control the proliferation of EBV-transformed targets.

The specific features of host, donor, or viruses that instigate the development of oligoclonal or monoclonal EBV-LPD in a subset of these immunodeficient patients early posttransplant remain a conundrum. It is possible that TCD transplants from donors without prior exposure to EBV or who maintain only a low level of residual EBV-specific T-cell immunity do not confer populations of EBV-reactive
T cells sufficient to mount a response of the magnitude necessary to limit proliferation of EBV-transformed B cells emerging early posttransplant. In support of this possibility are studies of patients developing EBV-LPD after organ allografts, which have demonstrated an increased risk of EBV-LPD among EBV-seronegative transplant recipients.\textsuperscript{35} It is also possible that lymphomagenesis occurs only in patients exposed to more lymphomagenic EBV strains\textsuperscript{39,40} or to high doses of lytic virus\textsuperscript{41} as a result of transfusions administered in the early posttransplant period. Similarly, it is possible that coinfection with another facilitating virus contributes to lymphomagenesis, as has been suggested by recent studies of EBV+ lymphomas in AIDS patients who have herpes simplex virus-8.\textsuperscript{32}

That EBV-specific T cells can eradicate established EBV-induced lymphomas was initially suggested by our studies demonstrating that infusions of small doses of PBMC from a patient's EBV-seropositive marrow donor could consistently induce prompt and durable remissions of EBV-induced mononclonal lymphomas developing in recipients of TCD marrow grafts.\textsuperscript{15} Direct demonstration of the capacity of adoptively transfused EBV-specific cytotoxic T cells to induce regressions of disease has subsequently been demonstrated in murine xenograft models of EBV-LPD by Boyle et al\textsuperscript{19} and Lacerda et al\textsuperscript{14} in a human EBV-LPD xenograft model in the SCID mouse, and by Rooney et al\textsuperscript{45} and Servida et al,\textsuperscript{46} who administered small numbers of in vitro-expanded, genetically marked, EBV-specific T cells and thereby induced regressions of EBV-LPD in human marrow allograft recipients.

In each of our cases successfully treated by adoptive transfer of donor-derived PBMC or cultured EBV-specific T cells, the onset of regressions of EBV-induced lymphomas has been detected by clinical and radiologic criteria as early as 2 to 4 weeks after PBMC infusion. As early as 7 days postinfusion of donor PBMC providing as few as 0.5 \( \times 10^6 \) to 1.0 \( \times 10^6 \) T cells per kilogram, tumor cells may be almost completely eradicated from biopsied tumors and involved lymph nodes.\textsuperscript{19} The residual cells in these tissues consist of histologically normal stromal elements and T lymphocytes. Because of the rapidity and extent of the clinical and pathologic responses observed, we were particularly interested in quantitating the effects of donor PBMC infusions on EBV-specific cell-mediated immune responses during this time period. As shown in one patient treated for an EBV lymphoma (Fig 5A and B), an infusion of donor PBMC providing 0.5 \( \times 10^6 \) T cells per kilogram and a calculated dose of 818 (95% CI, 625 to 1,182)\textsuperscript{26} EBV-specific T-cell precursors led to the development of large populations of HLA-restricted EBV-specific T cells within 2 weeks of their infusion. During this interval, the frequency of EBV CTLp detected in the circulation increased more than 10-fold. If this increment was based solely on the expansion of the EBV CTLp infused with the donor PBMC, this would represent a 20,000-fold amplification of the adoptively transferred EBV CTLp. To distinguish between the contributions of the adoptively transferred EBV CTLp and EBV-reactive T cells that may be recruited from T-cell populations developing within the transplanted host, it will be necessary to genetically mark the adoptively transferred EBV CTLp and assess, quantitatively, the proportion of adoptively transferred cells in the EBV-specific T-cell populations that emerge after their transfer. Strategies using retroviral vectors for transfection of in vitro-generated EBV-specific T cells have been developed by Servida et al\textsuperscript{46} and Rooney et al\textsuperscript{45} and are currently being used by Servida et al\textsuperscript{46} to address this issue.

While the determination of EBV CTLp frequencies using limiting dilution analysis is a sensitive measure of EBV cytotoxic capacity, the use of bulk cytotoxicity assays provides useful information and is well correlated with CTLp frequency as a measure of the presence or absence of anti-EBV immunity in normal seropositive and seronegative individuals (Fig 2). As demonstrated in Fig 3, patients with CTLp frequencies in the range of seronegative donors also had an absence of EBV-specific cytotoxicity in bulk culture, while patients with CTLp in the range of seropositive donors also were positive in bulk culture assays. Four patients with CTLp in the intermediate range lacked bulk culture cytotoxicity, while the remaining four patients had positive bulk culture cytotoxicity. If the bulk culture assay for EBV immunity was to be used as the only assay for prediction of emerging cellular immunity to EBV, we would have classified the four patients with intermediate CTLp but negative bulk culture cytotoxicity as patients at risk, while the four patients with intermediate CTLp and positive bulk culture would be classified as patients with cellular immunity to EBV. It is interesting to note that the one patient in this study group who developed an EBV-LPD during the study period, in fact, had CTLp in the intermediate range (1/119,150) but lacked detectable bulk culture cytotoxicity. Thus, in high-risk BMT patients, the latter assay might be sufficient to determine which patients have clinically significant deficiencies of cellular immunity to EBV. LDA may be a useful assay to perform on bone marrow donors before transplant to anticipate which patients are receiving fewer EBV-specific CTL with their graft. Such patients may be candidates for prophylactic leukocyte or EBV-CTL infusions. LDA is a useful assay for monitoring the effects of these donor leukocyte infusions in vitro.

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