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AUTOLOGOUS LYMPHOKINE-ACTIVATED KILLER CELL THERAPY OF EPSTEIN-BARR VIRUS-POSITIVE AND -NEGATIVE LYMPHOPROLIFERATIVE DISORDERS ARISING IN ORGAN TRANSPLANT RECIPIENTS[1]

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Lymphoreticular malignancies, collectively called posttransplant lymphoproliferative disorders (PTLD), eventually develop in 2–5% of organ transplant recipients. They frequently undergo regression when immunosuppression is reduced or stopped. This feature has been associated with a previous or de novo Epstein-Barr virus (EBV) infection. We herein describe immunotherapy with autologous lymphokine-activated killer (LAK) cells in seven patients with PTLD (four EBV-positive patients and three EBV-negative patients). Autologous peripheral blood mononuclear cells were obtained by leukapheresis, depleted of monocytes, and cultured in the presence of interleukin 2 for 10 to 11 days. A single dose of 5.2×10^9 to 5.6×10^{10} LAK cells was given intravenously. Systemic interleukin 2 was not administered.

The four patients with EBV^+ PTLD had complete tumor regression; two of them developed controllable rejection. Three patients are well 13–16 months after treatment; the fourth patient died of pneumonia 41 days after infusion. Three patients with EBV^- lymphomas had no response despite prior evidence that their tumors also were subject to immune surveillance. Two of these three patients died after being given other treatment, and the third patient has persistent tumor.

In conclusion, autologous LAK cell infusion was effective for treatment of four EBV^+ organ transplant recipients. LAK cell efficacy for three patients with EBV^- PTLD was not evaluable under the management circumstances in which this treatment was utilized.

B cell lymphomas make up most of the hematolymphopoietic tumors that collectively have been termed posttransplant lymphoproliferative disorders (PTLDs*). These usually are of host origin following organ transplantation (*1*), including transplantation of lymphoid-rich multivisceral grafts (*2*), but are most often of donor phenotype in bone marrow recipients (*1, 3, 4*). The frequent association of PTLD with the Epstein-Barr virus (EBV) (*3–7*) is similar to that seen with Burkitt's lymphoma and with more aggressive B cell lymphomas in patients with immune deficiency states (*8*).

The EBV^+ tumors' unusual susceptibility to immune surveillance was first demonstrated by involution of the PTLD in organ allograft recipients after reduction (or discontinuance) of immunosuppression (*9*) with its attendant risk of precipitating allograft rejection. The tacit assumption of most authors reporting such spontaneous tumor regression has been that it is dependent solely on the presence of the EBV epitopes. Some authorities have advocated early recourse to irradiation or chemotherapy (*10–13*). The argument has been that the benefit of affecting tumor regression is too unpredictable to justify risking allograft rejection by prolonged attenuation or discontinuance of immune suppression. Efforts to engineer host tumor surveillance by means other than lightening immunosuppression have met with limited success (*14*).

In contrast, restoration of EBV^+ tumor surveillance has been accomplished under the reverse circumstances, which occur in cytoablated EBV^+ bone marrow recipients: dominant donor immune system, donor origin of tumors, and therapeutic risk of graft-versus-host disease. In these patients, PTLD regression has followed the infusion of unmodified peripheral blood mononuclear cells or EBV-specific cytotoxic T cells obtained from the original donor (*4, 15, 16*) The unavailability of pretransplant recipient leukocytes has precluded direct application of this technology to the PTLDs developing after organ transplantation. However, we report here a strategy in which the recipients' peripheral leukocytes were "rearmed" in an interleukin (IL) 2-enriched culture. The resulting lymphokine-activated killer (LAK) cell infusate, which contained isologous cells with cytotoxic T lymphocyte

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* Abbreviations: EBER, Epstein-Barr virus early RNA; EBV, Epstein-Barr virus; CTL, cytotoxic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; LMP, latent membrane protein; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cells; PTLD, posttransplant lymphoproliferative disorder.

(CTL) phenotype as well as an augmented natural killer (NK) cell portion, was given as a single dose without systemic IL-2 to four organ recipients with PTLD who were EBV positive and to three who were EBV negative.

MATERIALS AND METHODS

Patient and Case Material

The four male and three female patients were 41–64 years old and had received thoracic organ (n=3), liver (n=2), or renal allografts (n=2) from 2 months to 12 years earlier. LAK cell therapy was considered if there was an incomplete or no response of PTLD to reduction or discontinuance of immunosuppression plus antiviral therapy (acyclovir or ganciclovir) within a 3-week period. Other clinical and pathologic features of the seven cases are summarized in Table 1. The diagnosis of PTLD was made with established criteria (*17, 18*). Immunoglobulin gene rearrangement studies (*19*) demonstrated monoclonal cell populations in the three PTLD specimens in which they were performed (patients 2, 3, and 7, Table 1), and in a fourth patient, flow cytometry proved B cell monoclonality (patient 6, Table 1). Small specimen sizes or sampling problems precluded clonality determination in the other three cases.

In four patients (patients 1–4), evidence of intratumoral EBV was detected by immunoperoxidase staining for viral latent membrane protein (LMP)-1 and by in situ hybridization for EBV early RNA (EBER) (*20*). Both of these assays were negative in tumor samples from patients 5, 6, and 7. In addition, Southern blot analysis of tumoral DNA from patient 7 also was obtained and failed to demonstrate EBV sequences.

LAK Cell Preparation

Compassionate approval was obtained from the institutional review board for each patient in whom LAK cell therapy was considered. The infusates were prepared in the Cellular Adoptive Immunotherapy Laboratory at the University of Pittsburgh Cancer Institute (*21*) in accordance with the U.S. Food and Drug Administrations Guidelines for Good Laboratory Practice. Peripheral blood was collected by leukapheresis on a cell separator (Fenwal 3000; Fenwal). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque gradients, treated with phenylalanine methylester to remove cells of the monocyte-macrophage lineage, resuspended at a concentration of 2×10^6/ml in AIM-V medium (Gibco, Long Island, NY) supplemented with 6000 IU of recombinant human IL-2 (Chiron, Emeryville, CA), and cultured in an atmosphere of 5% CO_2 in air at 37°C for 10 to 11 days. On the day of LAK cell infusion, the cultures were centrifuged in sterile bottles. The cells were resuspended and washed in cold RPMI medium, centrifuged again, and pooled into a sterile 50-ml conical tube. The cells were then suspended in 5% solution of human albumin in 0.9% saline and counted. After aliquots for phenotypic and cytotoxicity assays were taken, the endotoxin-free cell suspension was injected into a transfer pack and given by intravenous infusion over 1 hr. The patients were not treated with IL-2.

Evaluation of LAK Cells

Phenotype. After 10 to 11 days of culture, the fresh PBMC and the LAK cells were studied with two-color flow cytometry, using fluorescein or phycoerythrin-labeled monoclonal antibodies purchased from Becton Dickinson (Mountain View, CA). The monoclonal antibody specificities were HLA-DR and anti-CD3, -4, -8, -16, -19, -25, -45RA, -45RO, and -56. Mouse IgG1 and IgG2a isotypes were used as controls in all assays.

Cytotoxicity. Activity of fresh PBMC and LAK cells at the end of their culture was measured in 4-hr ^{51}Cr release assays (*21*). Assays were performed in triplicate at four different effector-to-target ratios. One lytic unit was defined as the number of effector cells required for 20% lysis of 5×10^3 target cells. The number of lytic units present in 10^7 effector cells was calculated with a computer program, as described previously (*22*).

To assess major histocompatibility complex (MHC)-restricted versus nonrestricted anti-EBV cytotoxicity, quantitative assay of cytotoxicity against autologous or third-party EBV-infected B lymphoblastoid cell targets, respectively, was done. Calculation of lytic units could not be performed because of insufficient numbers of target cells for the computer program.

RESULTS

LAK Cell Characteristics

Cell quantity and composition. The number of infused LAK cells ranged from 0.52×10^{10} to 5.6×10^{10} (Table 1). Their phenotype during the 10–11 days of culture shifted toward $CD56^+$ cells in all cases compared with the naive PBMC (Table 2): a threefold increase in mean number of $CD3^-CD56^+$ (NK cells) and a sevenfold increase in $CD3^+CD56^+$ (T cells with NK-like activity) was seen (Table 2).

Cytolytic function. Marked increases in cytotoxic activity against Daudi and K562 targets occurred during the culture period (Table 2). Cytotoxicity of aliquots of LAK cells against autologous (three of three cases) and third-party (HLA-unrelated; two of two cases) EBV-infected lymphoblastoid cells also was demonstrated in all cases in which the tests were done (Table 3).

Clinical Outcome

EBV^+ patients. The PTLD lesions involuted in all four cases, coincident with allograft rejection 3 days and 3 weeks after LAK cell infusion in patients 1 and 2, respectively. The rejection episodes were easily controlled with prednisone therapy. The antitumor effect was particularly obvious in patient 2, whose lower-extremity immunoblastic lymphoma nodules, which had developed 6 years after cardiac transplantation, could be seen to shrink almost overnight.

At 12 years after cadaveric renal transplantation, patient 3 presented with renal failure (15 ml/min creatinine clearance) due to chronic rejection. The diagnosis of PTLD was made by CT scan and shown by needle biopsy to be a B cell lymphoma with Hodgkin features. Five months later, when allograft nephrectomy was performed, tumor was demonstrated in biopsies of the iliac lymph nodes. LAK cell treatment was given a few days after nephrectomy. Nine months after this, all imaging studies were normal. No residual neoplasm was present at the time of successful renal retransplantation a few days later. The patient remains tumor-free 5 months after the retransplantation.

Patients 1, 2, and 3 are well with no evidence of residual tumor 13–16 months after LAK cell treatment (it has been 5 months since patient 3's renal retransplantation). Neither chemotherapy nor radiotherapy has been utilized in any of these patients. Patient 4, a double-lung recipient whose polymorphic PTLD of undetermined clonality originated in the allograft, died of *Pseudomonas* pneumonia 41 days after infusion. No tumor was found at the autopsy, which was limited to the thorax.

EBV^- patients. Examination of several specimens each from patients 5, 6, and 7 failed to disclose evidence of EBV. In patient 5, LMP-1 stains on tissue from the retroperitoneal tumor (day 678), chest wall nodule (day 708), and pleural fluid (consistent with PTLD, day 910) were all negative. The

TABLE 1. Patient population and outcomea

Patient	Allograft	Age	Tumor diagnosis (POD)	STLD site(s)	Histology of PTLD	Monoclonal	LAK infusion (POD)	No. of LAK cells	Outcome
EBV^+									
1	Heart/lungs, BM	51	62	Allograft lung	Polymorphic PTLD	ND	105	5.2×10^9	Resolved; NED 16 mo PI
2	Heart	64	2138	Legs	IBL	+	2184	1.5×10^{10}	Resolved; NED 14 mo PI
3	Kidney	57	4276	Retroperitoneal nodes	IBL/HD	+	4475	5.6×10^{10}	Resolved; NED 11 mo PI
4	Double lung	41	353	Allograft lung	Polymorphic PTLD	ND	373	6.4×10^9	NED at autopsy 41 days PI; pneumonia
EBV−									
5	Liver	63	656	Pelvis, thorax, extremities	LCNC	ND	727	1.1×10^{10}	Chemoherapy started 4 days PI; patient died 188 days PI
6	Kidney + BM	52	1098	Abdomen, cervical nodes	LCNC	+	1133	3.8×10^{10}	Radiotherapy (50 Gy) started 14 days PI, chemotherapystarted 60 days PI; patient alive with tumor 9 mo PI
7	Liver	59	1591	Paraspinal mass, intestines, liver	LCNC	+	1819	1.5×10^{10}	Chemotherapy started 14 days PI; patient died 16 days PI

[a] Abbreviations used in table: BM, bone marrow; HD, Hodgkin's disease-like; IBL, immunoblastic lymphoma; LCNC, largecell noncleaved lymphoma; ND, not determined; NED, no evidence of disease; PI, post-infusion; POD, postoperative day.

TABLE 2. Phenotypic and functional studies of leukapheresed peripheral blood cells both before and after ex vivo culture in the presence of recombinant human IL-2 (mean ± SEM) (n=7)[a]

	Pre-culture	Post-culture	P
Cell phenotype (%)			
$CD3^+$ 56^-	78±4.5	50±5.6	0.002[b]
$CD56^+$			
$CD3^+$	2.6±0.6	18±6.0	<0.001[c]
$CD3^-$	8.9±27	29.3±5.8	0.008[b]
DR^+	4.4±1.2	34.4±5.4	<0.001[c]
Cytotoxicity ($LU_{20}/10^7$ cells)			
Daudi	43±40	2614±954	0.002[c]
K562	25±10	5388±1596	0.001[c]

[a] Data are expressed mean ± SEM. LU, lytic units.
[b] By *t* test.
[c] By Mann-Whitney rank-sum test.

TABLE 3. Cytotoxicity of LAK cell preparations against autologus and third-party EBV^+ lymphoblastoid targets[a]

Patient	E:T	Autologous EBV^+LCL (% specific killing)	Third-party EBV LCL (% specific killing)
1	40:1	21	19
	20:1	25	16
2	80:1	25	ND
	40:1	23	ND
	20:1	19	
3	80:1	41	5
	40:1	10	7

[a] Abbreviations used in table: E:T, effector-to-target ratio; EBV^+ LCL, Epstein-Barr virus-transformed, lymphoblastoid cell line; ND, not done.

latter two samples were also negative for EBER by in situ hybridization. In patient 6, in situ hybridization for EBER on a biopsy of retroperitoneal tumor on day 1099 was negative, and LMP-1 stain on a neck mass biopsy performed on day 1193 was also negative. Both LMP-1 and EBER studies were negative on paraspinal mass biopsies from patient 7, performed on days 1591 and 1600. Southern blot analysis for EBV was performed on the latter biopsy, and this was negative for EBV sequences.

All three recipients (two liver recipients and one kidney recipient) had large-cell noncleaved lymphomas. LAK cell infusion did not precipitate rejection in any of the patients, but it had no effect on the tumors that were subsequently treated (also unsuccessfully) with chemotherapy (n=3) or irradiation (n=1). One patient is alive with tumor 9 months after infusion, and the other two patients died after 16 and 188 days (Table 1).

The circumstances of LAK cell treatment in these three patients precluded evaluation of efficacy. Dramatic regression of a large multifocal lymphoma had occurred in patient 5 after discontinuance of immunosuppression, 2 years after liver transplantation. The decision to give LAK cell therapy was prompted by a small, residual skin lesion on the thorax. When the skin tumor did not disappear by the fourth day after LAK cell infusion, multiagent chemotherapy was begun, and repeated in 21- to 28-day cycles until the patient's death 6 months later (Fig. 1). Although conventional immunosuppression was never restarted, the patient remained rejection-free throughout this period.

In the other EBV^- liver recipient (patient 7), massive tumor regrowth following regression was associated with resumption of immunosuppression in response to transaminase elevations, which were ascribed to a rejection. The patient, who was moribund by the time LAK cells were given, died 16 days later, 2 days after chemotherapy was started.

The 3-year posttransplant recipient of a cadaver kidney and adjunct bone marrow (patient 6) presented with epigastric pain and a large left retroperitoneal mass which had shrunk to half of its original volume during 6 weeks off all immunosuppression, preceding LAK cell infusion. Fourteen days after the infusion, radiotherapy was started because of

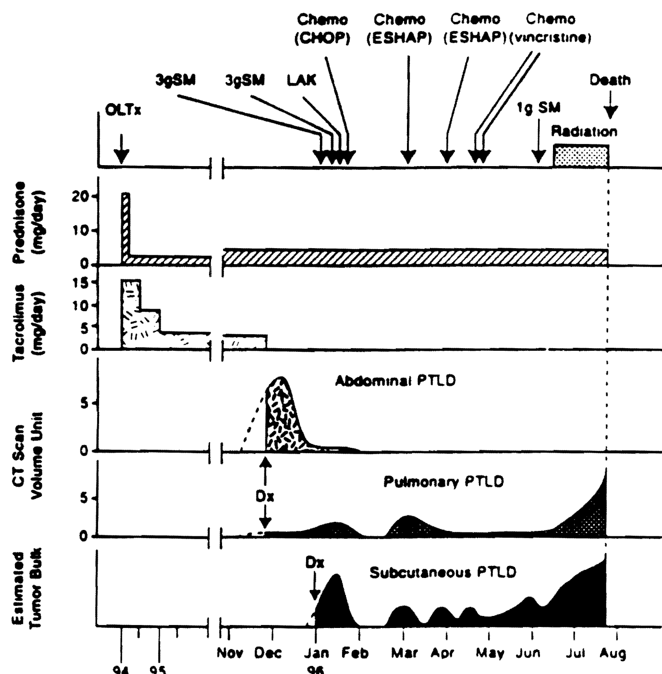

FIGURE 1. Clinical course of EBV⁻ PTLD in patient 5. The main tumor mass shrunk coincident with discontinuation of tacrolimus. LAK cell therapy was administered, but any potential benefit was obscured by the initiation of chemotherapy beginning 4 days thereafter.

continued pain. This was given to the left flank mass in 17 fractions over a 22-day period (total 3060 rads) involving a target field of 20×22 cm that included the T10-L5 vertebral bodies, spleen, liver, and entire suprapelvic abdomen. After a rest of 13 days, 2000 more rads were delivered over 5 days to a separate vertebral field that included T7-T10. Although less extensive than total lymphoid irradiation, the size of the field(s) plus scatter implied a significant immunosuppressive effect (Dr. John Flickinger, radiotherapist, University of Pittsburgh). Sixty days after LAK cell infusion (6 days after completion of the second course of irradiation), CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) was begun (six cycles). Although conventional immunosuppression has never been restarted, the patient has good renal function. A CT scan, which followed six cycles of CHOP therapy (7 months after LAK cell infusion) showed persistence of the malignancy.

DISCUSSION

In bone marrow recipients, for whom drug withdrawal usually is not an option, tumor surveillance has been restored with regression of EBV^+ PTLD of donor phenotype by infusing unmodified peripheral blood lymphocytes or CTLs obtained from the original donor (*4*, *15*, *16*). Analogous cellular immune modulation of the mirror-image PTLDs of host phenotype that develop after organ transplantation has not been possible because of the unavailability of naive recipient leukocytes. This need could be met in three ways: (1) cryopreservation of the patient's pretransplant PBMC proposed by Todo et al. (*23*) for high-risk intestinal candidates, (2) the use of a surrogate leukocyte donor HLA identical to that of the recipient (as reported by Emanuel et al. [*24*]), or (3) the infusion of functionally resurrected autologous cells. The first option is impractical, and the second is inconvenient, even if an HLA-identical donor can be found, as in Emanuel's case (*24*). Evaluation of the third option was our objective, using LAK cells.

Cellular immune modulation of PTLD in organ transplant patients has been based largely on the assumption that efficacy required an EBV^+ target (*25*, *26*). Because control of EBV replication in vivo is primarily by EBV-specific CTL (*8*, *25*, *26*), and since NK cells have also shown effector activity against these cells in vitro (*27*, *28*), the presence of both phenotypes in the LAK cell infusate was reassuring. A therapeutic benefit was seen in all four EBV^+ cases. Although undesirable consequences of cellular immunotherapy (rejection in our organ recipients and graft-versus-host disease after bone marrow transplantation [4]) have been manageable, these might be preventable by isolation with limiting dilution techniques of epitope-specific CTL, as has already been reported in bone marrow recipients at high risk for cytomegalovirus infection (*29*) or for complications of EBV (*16*). However, before this prediction can be made with confidence, more data are needed on the extent of cross-reactions between EBV and class I MHC epitopes (*30*, *31*). In the present series, a disproportionate number of patients lacked evidence of intratumoral EBV. This is not representative of our entire PTLD patient population, in which such lesions are exceptional.

The results in the EBV^+ patients fit well within the context of PTLD and its EBV association that has evolved over the last 15 years (*25*, *26*). However, acceptance of this association as an immutable precondition for LAK cell-responsive PTLD was an underlying factor in treatment decisions in the patients with EBV^- tumors. By the time LAK cell therapy was started, all three of the EBV^- patients had proved their ability to restore tumor surveillance with no other management change than discontinuance of immunosuppression (see Fig. 1). In our opinion, this capability was interdicted by premature intervention with high-dose irradiation in one case, and with a highly immunosuppressive chemotherapeutic lymphoma protocol in all three.

Early recourse to chemotherapy has been recommended as initial therapy for PTLD (whether EBV positive or negative) with increasing frequency during the last dozen years (*10–13*). In contrast, our opinion is that management must begin with reduction or discontinuance of immunosuppression (*9*) and that chemotherapy (or multiregional radiotherapy) should be the last step, if it is used at all, in the treatment of PTLD. Retardation of immunologic recovery by chemotherapy or high-dose radiotherapy was contraindicated in two, and probably all three, of our EBV^- patients, just as in those who were EBV^+. Because this conventional antitumor therapy worked at cross-purposes with the strategy of surveillance restoration, it remains to be determined whether EBV^- tumors can be treated with LAK cells. Furthermore, we emphasize that LAK cell therapy is only a bridge to the ultimate objective of natural surveillance and that repeat infusions can be given. Future studies should seek to evaluate donor or recipient origin of the tumors in order to document whether MHC restriction is a prerequisite for PTLD remission in the setting of LAK cell administration. Finally, in all cases, a variable period of reduced immunosuppression preceded LAK cell therapy. Failure of the tumors to regress was the

impetus for LAK cell administration. We cannot discount a contributory effect of lowered or reduced immunosuppression on tumor regression, and we do not recommend at this time that LAK cell therapy be undertaken in the absence of a trial of lowered immunosuppression.

PTLD is estimated to occur in 2–5% of organ allograft recipients and in 1% of bone marrow recipients (*32, 33*). It has been viewed as an enigmatic complication of transplantation. However, 3 years before PTLD were first reported in humans (*34–36*), Schwartz, Beldotti, and co-workers (*37, 38*) described similar tumors in an F_1 hybrid mouse model in which lesions indistinguishable from clinical PTLD regularly developed after the induction of low-level asymptomatic donor leukocyte chimerism. Although Schwartz concluded that the tumors resulted from a graft-versus-host reaction (*38*), the relevance of these tumors to human PTLD could be appreciated after the discovery a quarter century later of donor leukocyte microchimerism in organ recipients (*39, 40*), and of a residual host leukocyte population (*41, 42*) in all bone marrow recipients previously thought to have complete donor leukocyte chimerism (*43*). Then, it was evident that all organ allografts are potentially "lymphomagenic," particularly when the cofactors of T cell-directed immunosuppression and viral infection including EBV are added (*43*).

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