EBV-specific cytotoxic T lymphocytes protect against human EBV-associated lymphoma in scid mice

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Abstract

scid mice develop human EBV-positive B cell tumors after injection with EBV-transformed human B cells or peripheral blood lymphocytes from EBV-seropositive donors. Injection of cytotoxic T cell (CTL) lines, selectively expanded in vitro to recognize autologous EBV-transformed B cells, protected mice against developing human lymphoproliferative disease. CTL protection required a single dose of polyethylene-glycol-conjugated IL2. Mice were not protected by CTL that did not specifically recognize EBV-transformed B cells or by mitogen-activated lymphocytes.

Keywords: Epstein-Barr virus (EBV); Cytotoxic T lymphocyte (CTL); Lymphoma; scid mice

1. Introduction

Epstein-Barr virus, a ubiquitous human herpes virus, establishes a lifelong, latent infection implicated in the pathogenesis of neoplasms, including Burkitt’s lymphoma, nasopharyngeal carcinoma, and B large cell lymphomas [1]. The frequency of EBV-related lymphomas has been increasing in conjunction with the AIDS epidemic and the increased use of transplantation. The occurrence of EBV-related lymphomas in the setting of T cell immunodeficiency suggests that immune surveillance in immunocompetent individuals is protective. Two groups have recently reported their efforts to restore immune surveillance by infusion of immunocompetent lymphocytes in the management of post-transplant EBV-associated lymphoproliferative disease. EBV-related B large cell lymphomas completely regressed in a small number of T cell-depleted allogeneic bone marrow transplant recipients after infusion of unmanipulated donor mononuclear leukocytes, although treatment was complicated by pulmonary compromise and GVHD [2]. Due to the heterogeneous mixture of cells infused, it was not possible to determine the protective cell type(s). In another study, donor-derived genetically marked T cell lines, enhanced for EBV-specific CTL activity, were infused prophylactically in two transplant recipients with rising peripheral blood EBV DNA and in one case of EBV-associated immunoblastic lymphoma. EBV DNA levels normalized and lymphadenopathy resolved with no evidence of GVHD [3]. Marked cells persisted for as long as 18 months [4]. These cell lines, although cytotoxic, were heterogeneous with regard to CD8/CD4 phenotype, and specific recognition of viral-encoded antigens was not determined.

EBV-infected human B cells undergo lymphoblastoid transformation and express latent viral proteins, some of which contain epitopes for CTL recognition [5]. scid mice injected with EBV-transformed B lymphoblastoid cell lines (B-LCL) or peripheral blood lymphocytes from EBV-seropositive donors develop B large cell tumors of human origin similar to those arising in patients after transplant or with advanced HIV infection [6–8], and represent a good small animal model for
therapeutic studies. Rencher et al. used this model to demonstrate that CD8+, rather than cytotoxic CD4+, T cells can protect against EBV-lymphomagenesis [9]. In this paper we demonstrate that transfer of EBV-specific CD8+ CTL to scid mice reconstituted with human B cells can protect against the development of EBV-associated human lymphoma but that CD8+ CTL that do not lyse EBV-transformed targets cannot.

2. Materials and methods

2.1. Scid mice

Six 12 week old C.B.17 scid mice (Taconic Farms), maintained in a sterile barrier facility, were injected i.p. with 35 x 10^6 PBMC from EBV-seropositive donors or with 10 x 10^6 B-LCL. At a different injection site, mice were injected i.p. with saline or varying numbers of T cells with or without 1 x 10^4 IU S.C. polyethylene-glycol-conjugated recombinant human IL2 (PEG-IL2; Cetus Oncology), which has a 10-fold increased biological half-life compared with IL2 [10]. Mice were followed for tumor development or death and by serial tail vein bleeds for human Ig by ELISA. Tumors present at autopsy were analyzed for phenotype and surface Ig expression and in some cases for immunoglobulin gene rearrangement and EBV genome by Southern blot.

2.2. Preparation of PHA-stimulated CTL

Non-adherent Ficoll density gradient-separated PBMC were stimulated with 2 µg/ml PHA in supplemented RPMI containing 15% FCS and 600 IU/ml rhuIL2 (Cetus Oncology). CTL were resuspended at 5 x 10^6 cells/ml twice weekly in fresh IL2-containing medium. After 10 days, cells for injection were depleted of CD4+ cells using an excess of Dynal Dynabeads M-450, and CD4-depletion was verified by flow cytometry to be > 99%.

2.3. Preparation of EBV-specific CTL

Non-adherent Ficoll density gradient-separated peripheral PBMC were co-cultured with autologous δ-irradiated (400 cGy) B-LCL. Cells were resuspended twice weekly at 1 x 10^6 cells/ml in fresh medium containing supplemented RPMI with 15% FCS and 60 IU/ml rhuIL2. Stimulation was repeated once or twice at 10–14 day intervals. CD4+ cells were bead-depleted prior to cytotoxicity assay and injection. For some experiments cell lines were bead depleted for CD4, CD8 or CD16 expressing cells with an excess of specific Dynabeads and depletion (< 2% residual cells) was verified by flow cytometry.

2.4. Preparation of CTL clones

PBMC from donor EF were plated at 5 cells/well in supplemented RPMI containing 15% FCS and 750 IU/ml rhuIL2 (Cetus Oncology) into U-bottom microtiter plates with 1 x 10^4 cells/well of each autologous δ-irradiated (600 cGy) PBMC and EF-EFEBV. Plates were fed weekly with 1 x 10^4 cells/well of δ-irradiated EF-EFEBV and clones that grew out were tested for cell surface staining of CD3, CD4 and CD8 and for their ability to lyse EF-EFEBV. K562 and K562 (% specific lysis < 5% at an E/T ratio of 10:1) were used as control clones in mouse experiments.

2.5. EBV-vaccinia recombinant viruses

Vaccinia recombinants containing genes for EBV latent proteins were obtained from J. Stewart and M. Kurilla [11–13]: E1NX, deletion mutant of EBNA1; vE2A, type A EBNA 2; vE3a, EBNA 3a; vE3b, EBNA 3b; vE3c, EBNA 6(3c); vLMP, LMP 1; vTP, LMP 2. Because full-length EBNA 1 expressed from vaccinia is toxic, the E1NX mutant lacks the DNA binding region. Vaccinia virus vSC8 encodinglacZ was obtained from B. Moss [14]. Expression of EBV latent proteins by B cell blasts and adherent PBMC was verified by Western blot with immune sera.

2.6. Chromium release assays

Chromium release assays were performed according to the method of [15]. Targets were B-LCL, K562, or B cell blasts infected overnight with 10–20 pfu vaccinia recombinant virus. B cell blasts were prepared from density-separated PBMC by stimulation with Affigel 10 beads (Biorad) coupled to goat α-human IgM (Cappel). After culture in rhuIL4 50 U/ml (Schering Plough) and rhuIL2 240 IU/ml for 72 h, cells were maintained in IL2-containing medium for up to 4 weeks.

2.7. Proliferation assays

Adherent PBMC (2 x 10^4/well) in microtiter wells were infected with recombinant virus at 10 pfu/cell for 16 h. Plates were washed, UV-irradiated (1 foot for 20’) and δ-irradiated (400 cGy). CD8+ responder cells were added to triplicate wells in supplemented RPMI containing 10% human AB vaccinia-immune serum. After 40 h at 37°C, 1 µCi [3H]thymidine was added to each well and plates were harvested 8 h later onto Packard GF/C unifilter microplates and read on a Packard Topcount counter. Stimulation index was calculated from the ratio of the average cpm to cpm of wells containing vSC8-infected-adherent cells.
IT cells

Fig. 1. Scid mice injected with AB PBMC died of human B cell tumors. One mouse was treated with autologous mitogen-activated CD4-depleted T cells with (A) temporary resolution of serum monoclonal IgM and (B) longer survival (~-) than eight untreated mice (----).

2.8. Statistical analysis

Kaplan-Meier curves were calculated for each treatment group and the results analyzed by the logrank test [16].

3. Results

In a preliminary experiment, nine scid mice were injected i.p. with 3.5 × 10⁶ PBMC from EBV-seropositive donor AB (Fig. 1). Within 11 days after injection, all mice developed serum human immunoglobulin, demonstrating engraftment of human PBMC. Eight otherwise untreated mice died of human B cell immunoblastic lymphomas from 10 to 26 weeks later. One mouse, whose serum Ig developed into a serum human monoclonal IgM spike at 15 weeks, was injected at 21 weeks i.p. with 50 × 10⁶ autologous PHA-stimulated CD8+ T cells (≥ 85% CD8+, < 1% CD4+, < 1% CD14+, < 1% CD20+). After CTL injection, the IgM spike decreased dramatically, suggesting tumor regression. This CTL line showed only minimal specific killing of autologous EBV-transformed B-LCL in vitro (10% specific cytotoxicity at an effector/target (E/T) ratio of 100:1). Ten weeks after CTL injection IgM levels again began to increase, consistent with tumor regrowth and the mouse died. Autopsy revealed intraperitoneal human B cell tumors, with two clonal populations identified by surface light chains and immunoglobulin gene rearrangement, associated with two distinct EBV episomal forms (data not shown). No human T cells were found, consistent with reports that adoptively transferred T cells do not survive in scid mice more than several days in the absence of exogenous IL2 [17]. However, this mouse, which developed human Ig at a comparable rate to the other mice, survived longer than the eight mice who did not receive T cells.

PHA-stimulated cell lines, while predominantly CD8+, contain a mixture of CD4-cells, including nonspecific LAK-like T cells and NK cells. To test the hypothesis that the anti-tumor effects were due to EBV-specific CD8+ cells, rather than to nonspecific CTL or NK cells, we developed a CD8+ CTL line (98% CD3+, 97% CD8+, < 1% TcRζβ+, < 1% CD4+, 1% CD16+) from donor CD (HLA A2, A28, B38, B55) which recognized a specific EBV latent protein. At an E/T ratio of 25:1 this CTL line lysed autologous B cell blasts expressing LMP2 with 23% specific lysis and killed autologous B-LCL with 65% specific lysis (Fig. 2a). It also proliferated to autologous adherent cells infected with vaccinia-LMP2 [10-12] with a stimulation index of 136 and proliferated to autologous B-LCL with a stimulation index of 31 (Fig. 2b). In this and subsequent experiments, CTL were cultured in concentrations of IL-2 that do not elicit significant LAK-like activity (specific cytotoxicity vs K562 1.8% at E/T ratio of 9:1). Lysis of CDEBV by CDCTL was mediated by CD8+CD4−CD16− effector cells since lysis was unaffected by depletion of CD4 or CD16-expressing cells but was abrogated by
depletion of CD8 cells (Fig. 2c). The lysis was MHC-restricted since only B-LCL that share at least one class I allele were lysed by the CD EBV-specific CTL line (Fig. 2d).

To test protection by this LMP2-specific CTL line, 12 scid mice were injected i.p. with $10^7$ autologous B-LCL (CDEBV) and s.c. with $1 \times 10^4$ IU PEG-IL2 (Fig. 2c). Four of these mice were injected concurrently at a separate i.p. site with the autologous LMP2-specific T cell line ($10^7$ cells). All eight mice with no T cells developed serum human IgM detectable by ELISA and died 6–23 weeks later. Six of eight developed human B cell tumors and one mouse developed high levels of serum IgM but died of a murine thymic tumor (to which scid mice are prone [18]). Treatment with PEG-IL2 alone did not affect lymphomagenesis since tumors developed within the same time period (at 6 and 23 weeks) in two additional mice that received CDEBV without PEG-IL2. Of the four mice injected with T cells, only one developed serum human IgM and none developed human tumors. One of these mice also died after 27 weeks from a murine thymic tumor. The absence of human tumors was verified at autopsy in the remaining mice who were sacrificed at 31 weeks. The survival advantage of mice treated with T cells was statistically significant ($P < 0.025$).

To confirm these results, an EBV-specific CD8 + CTL line was developed from donor EF (HLA A11, A28, Bw52, Bw56) (100% CD3+, 100% TcRβ+, 97% CD8+, <1% CD4, <1% CD16+). This line was highly cytotoxic against autologous EBV-transformed B-LCL (EFEBV) with 73% specific lysis at E/T of 9:1 (Fig. 3a), but its specificity could not be defined with the panel of recombinants expressing EBV latent proteins. It also did not proliferate to autologous monocytes expressing EBV latent proteins or to autologous EBV-transformed B cells (data not shown). The cytotoxicity was mediated by CD8 + CD4 --
Fig. 3. (A) EFCTL lyse autologous B-LCL EFEBV (■) and allogeneic CDEBV (□) which share expression of HLA A28. Lysis was mediated by CD8 T cells (B) and was MHC restricted (C). (D) Survival of mice injected with EFEBV plus ( - - ) nothing, (*** ) PEG-IL2, (- - - ) nonspecific CTL clones and PEG-IL2, (--- ) specific CTL without IL2, and (-----) specific CTL with PEG-IL2 is shown. Only the mice treated with specific CTL and IL2 had statistically significant prolonged survival.
CD16− cells (Fig. 3b) and MHC-restricted with an A28-restricted component (Fig. 3c). There was no detectable NK-like killing against K562 (% specific lysis = 0.3% at E/T ratio of 9:1).

Mice injected with 1 × 10⁷ EF B-LCL with or without PEG-IL2 (4 mice in each group), developed serum human immunoglobulin and tumors within 3–6 weeks, more rapidly than mice injected with CDEBV (mean time to tumor 32 vs. 105 days). None of four mice that received 1 × 10⁷ EFCTL developed human serum Ig, two mice died of human tumors and two mice were free of tumors when they were sacrificed after 12 weeks (Fig. 3b). Two mice treated with 1 × 10⁷ EFCTL without PEG-IL2 developed tumors and died 5 weeks, as did 12 mice receiving smaller numbers (1 × 10⁶ to 7.5 × 10⁶) of EFCTL (data not shown).

To determine whether viral-specific CTL were required for protection, nine mice were injected with equal numbers (1 × 10⁷ cells) of EFEBV and autologous cytotoxic CD8+ T cell clones which did not recognize or lyse EFEBV, but were capable of lectin-mediated cytolysis (data not shown). Eight different non-EBV-specific CTL clones were tested, some in combination. All nine mice developed human B cell lymphomas with only a minor delay in death (mean 38 ± 5 days vs. 32 ± 8 days for untreated mice), indicating that the protective effect seen with EBV-specific CTL was not due primarily to non-specific cytokine release. The survival advantage conferred by specific CTL with IL2 was statistically significant when compared to infusion of nonspecific CTL (P < 0.05) and when compared to all the treatments without specific CTL (P < 0.05).

4. Discussion

Adoptive transfer of autologous EBV-specific cytotoxic T cells can prevent the development of EBV-associated human B cell lymphoma in scid mice. Because of incomplete cross-reactivity of mouse and human adhesion and homing receptors, the scid model does not completely reflect the trafficking of human immune cells in vivo. Because of this, we injected both the B-LCL and effector cells into the peritoneal cavity, albeit at separate sites.

Viral-specific T cells are likely to be important in immune surveillance against some virus-associated tumors (e.g. human papilloma virus-cervical and squamous cell carcinomas, EBV-Burkitt's and large cell lymphomas), as demonstrated by the increased incidence of these malignancies in patients with AIDS or iatrogenic immunosuppression. The recent report of a new herpes virus in the Kaposi's sarcoma lesions of AIDS patients also suggests a role for T cell antiviral immunity in this cancer [19]. Previous studies have demonstrated that transfer of viral-specific CTL can prevent the development of lethal consequences of viral infection in animals, including adenovirus, influenza, LCMV, and RSV [20–23]. Development of murine leukemias and lymphomas induced by murine retroviruses can also be prevented by the adoptive transfer of viral-specific CTL [24]. Here we demonstrate protection from virus-associated human cancer by transfer of viral-specific CTL.

Specific CTL were required for protection in these experiments. Although in a pilot experiment nonspecifically activated T cells appeared to delay death from tumor in a single mouse, estimates of the frequency of EBV-specific precursor CTL in the PBMC of normal donors of about 1 in 10⁴ PBMC [25] suggest that this mouse probably received about 10⁴ EBV-specific CTL. This was enough to delay but not prevent death, which required larger numbers of specific CTL in an experiment with varying numbers of specific CTL. Neither non-specific cytotoxic T cell clones nor non-specific autologous lymphocytes prevented lymphomagenesis when transferred at equivalent numbers. This implies that specific T-cell receptor recognition plays a significant role in immune surveillance and that secretion of soluble factors by non-specifically activated CTL may be less important. Infusion of greater numbers of non-specific CTL or PBMC than we used might be protective in this model.

The requirement for injection of subcutaneous PEG-IL2 for protection could possibly be bypassed by the infusion of larger numbers of viral-specific CTL. However in a recent study [26] scid mice injected with B-LCL survived longer when EBV-specific CTL were adoptively transferred without IL2 but eventually died of lymphoproliferation despite the fact that they received four times as many T cells per B cell as we administered. Their result supports our finding that exogenous IL2 or some form of T₉₉ support is necessary for effective prolonged CTL function. Rencher et al. found a protective effect of CD8+ T cells in the absence of IL2, albeit using a less tumorigenic model (50% tumors in untreated mice) [8]. However CTL were not phenotyped or CD4-depleted immediately prior to injection and could have contained small numbers of contaminating CD4+ cells. Although CD8+ T cells persist for up to a month in scid mice reconstituted with human PBMC [27], they do not survive long when injected in the absence of CD4 T cells [17]. The enhanced protection we observed provided by PEG-IL2 plus CTL suggests that some CTL persist long enough after PEG-IL2 administration to provide adequate surveillance. We did not find prolonged survival in mice given only PEG-IL2, although IL2 itself has been reported to protect against EBV-lymphomagenesis in scid mice when given daily, probably via enhancement of endogenous murine NK function [28]. Daily dosing
differs from the single dose we administered, which would have been largely cleared within 24 h. In addition, we injected EBV-transformed B cells rather than PBL, which is a more malignant model.

CDCTL completely protected scid mice from human tumors while EFCTL only protected half the mice. Although the numbers of treated mice were small, the difference could be due to differences in the CTL or in the tumor. CDCTL recognized LMP2, which contains a previously identified A2-restricted epitope [29]. EFCTL did not recognize an identifiable viral protein, despite a described immunodominant A11-restricted epitope encoded by EBNA 4 [30]. This CTL may recognize an epitope contained in one of the latent EBNA proteins that is not properly presented by our vaccinia recombinants, another viral protein, or a non-viral protein which is induced by EBV infection. Although CTL that recognize different viral antigens may differ in their protective ability, the greater cytolytic activity of EFCTL compared to CDCTL at lysing the autologous B-LCL injected into the scid mice would have suggested that it should have provided better protection. EBV-transformed B cell lines have variable tumorigenic potentials in scid mice which may have been the critical difference in the two experiments [31,32]. EFEBV displayed a more malignant phenotype than CDEBV in untreated mice with death from tumor occurring much earlier. These variations in malignant phenotype may have a parallel in human EBV infection as suggested by Picchio et al. [33].

Interpretation of experiments with polyclonal cell lines, as described here, are less clear-cut than inference of clones, due to possible contaminating non-specific T cells and NK cells. Although the characterization of these cells is limited, they had the phenotype of CD3+TcRab+CD8+ T cells, their lysis was mediated by CD8+CD4−CD16− T cells and was MHC restricted and they had no detectable NK-like activity. In addition, we were unable to demonstrate tumor protection by non-specific CTL clones. Thus, we attribute the tumor protection to CTL which are specific for EBV-transformed B cells. Our results need to be verified by experiments with cloned CTL. However, clinical treatment with polyclonal cell lines may make more sense to minimize chances of tumor escape.

**Note added in proof**

Similar results, demonstrating tumor protection by EBV-specific CTL in the scid mouse model, were described by Lacerda et al. [34] after the submission of this work.

**Acknowledgements**

We thank M. Kurilla, J. Stewart, and B. Moss for recombinant vaccinia, Cetus Oncology for PEG-IL2, Schering-Plough for IL4, and R.S. Schwartz and D. Thorley-Lawson for helpful discussions. This work was supported by NIH K11CA59452, by the Carter-Wallace Foundation, and the National Immunotherapy Cancer Research Foundation (RB), and by NIH K08CA01449, U19AI36611 and a Pew Scholar Award in the Biomedical Sciences (JL).

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