Retroviral Immunotoxin Gene Therapy of Acute Myelogenous Leukemia in Mice Using Cytotoxic T Cells Transduced with an Interleukin 4/Diphtheria Toxin Gene

Daniel A. Valler,
Ni Jin, James M. R. Baldrica, Angela Panoskaltsis-Mortari, Si-Yi Chen, and Bruce R. Blazar

University of Minnesota Cancer Center, Departments of Therapeutic Radiology, Section on Experimental Cancer Immunology [D. A. V., N. J., J. M. R. B.], and Pediatrics, Division of Bone Marrow Transplantation [A. P.-M., B. R. B.], Minneapolis, Minnesota 55455, and Baylor College of Medicine, Houston, Texas 77030 [S.-Y. C.]

ABSTRACT

The potential benefit of immunotoxin (IT) for cancer therapy has mostly been limited by the fact that only a small portion of injected dose ever reaches the cancer target. Thus, we set out to determine whether antigen-specific CTLs would be suitable vehicles to deliver IT to the site of cancer cells in vivo. A retroviral vector was constructed for gene therapy with (interleukin 4) IL-4 positioned downstream of its 20-amino-acid leader sequence that permitted cotranslational protein synthesis of IT along with truncated diphtheria tox (DT). IL-4 was chosen as a ligand based on the expression of IL-4 receptor (IL-4R) on most acute myeloid leukemia cases. The first-time expression and secretion of a cytokine/DT fusion tox was accomplished in mammalian NIH.3T3 cells, and then a retroviral vector was assembled. The retroviral IT was used to transiently transduce T15, a CD8+ CTL T cell line that specifically recognized C1498 (a lethal murine acute myeloid leukemia cell line). Transduced T15 T cells expressed intracellular DT and IL-4 as determined by immunofluorescence. Secreted IT supernatants collected from T15 had enzymatic activity and may impart new anticancer defense mechanisms to antigen-specific T cells.

INTRODUCTION

T cells are critical immune effector cells in the defense against cancer and selectively recognize tumor antigens that are either specific to cancer cells or are overexpressed shared antigens (1, 2). This results in the destruction of tumors that threaten the host. However, T-cell immunotherapy has been only partially successful in destroying progressive cancer, in part, because they are often overwhelmed by large numbers of tumor cells. The use of gene therapy to introduce cytokines or antibody fragments to augment antitumor responses most often have been unsuccessful in adequately destroying neoplastic tissue because tumor cells are not adequately destroyed (3, 4). A solution to these problems would be to genetically arm T cells with a selective and potent toxin powerful enough to reduce tumor cell numbers and to rely on the T cells to deliver and secrete this IT at the site of cancer in vivo and to potent catalytic toxins such as DT.3 Intact DT contains two fragments, A and B. The fragment catalyzes the ADP-ribosylation of elongation factor 2 (EF-2) leading to protein-synthesis inhibition and cell death (5, 6). Although a single molecule of DT fragment A in the cytosol can be fatal to a cell, fragment A alone applied extracellularly is not highly toxic because the binding domain is located in fragment B. Thus, we included only the first 389 amino acids of DT that contained fragment A for our construct.

The major purpose of cytokine IT is to deliver therapy selectively to cancer cells that frequently overexpress cytokine receptors (7). Although these agents selectively bind and kill cancer cells, clinically their use has been limited by: (a) their failure to penetrate and localize in adequate concentrations in cancer target tissue; and (b) their localization in nontarget organs limiting the tolerated dose and narrowing the therapeutic window (8). We have explored a solution to this problem, reasoning that T cells that are highly suited for local cytokine delivery in the first place would be excellent vehicles for the delivery of cytokine fusion toxins if induced to secrete them by retroviral gene transduction. The secreted cytokine IT would preferentially destroy cancer cells displaying the appropriate cytokine receptor on their cell surface but would not kill normal tissue cells lacking the antigen. We chose a murine acute myeloid leukemia model because this leukemia is the most common form of adult leukemia (9). IL-4 was chosen as the ligand for our IT because IL-4R is expressed on over 90% of acute myeloid leukemia cases (10) and IL-4 IT has been previously used in experimental cancer therapy studies (11–13). DT was chosen as a toxin because it has extensive clinical use and has a history of use as a recombinant toxin (14–16).

These studies show for the first time that gene therapy can be used to manipulate antigen-specific T cells into secreting cytokine fusion toxins with selective activity against myeloid leukemia cells.

MATERIALS AND METHODS

Construction. A single-chain cytokine gene encoding 423 bp or 141 amino acids of the murine IL-4 gene including the 20-amino-acid signal peptide was fused using splice overlap extension with a truncated DT gene encoding the first 389 amino acids and devoid of its native binding region that renders the tox lethal to all eukaryotic cells (17). To determine whether mammalian cells can produce cytokine-directed toxins, this 1626-bp IT gene was ligated into the nonviral mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), as shown in Fig. 1A for transfection studies in NIH.3T3. For transduction, the target gene was ligated into the retroviral expression vector LNCX (Fig. 1b) or a modified LNCX in which a fragment encoding Neo was replaced with a gene fragment encoding human NGFR (18; Fig. 1C). Successful integration of this retrovirus resulted in the cell surface expression of NGFR, which could be used as a quantitative marker. To produce a purifiable IL-4 IT, a cytokine fusion toxin gene was assembled using DNA fragments encoding IL-4 without signal peptide (amino acids 20–140) spliced to DT190. The hybrid gene was ligated into a commercial pET expression vector, pET21d (Novagen, Madison, WI).

1 Received 9/14/99; accepted 12/14/99.
2 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
3 Supported in part by U34/CA57625, R01/CA82154, and R01/CA72669 awarded by the National Cancer Institute and the National Institute of Allergy and Infectious Diseases, Department of Health and Human Services, and by the Leukemia Research Fund.
4 To whom requests for reprints should be addressed, at University of Minnesota Cancer Center, Box 367 Mayo Building, Harvard Street at East River Road, Minneapolis, MN 55455. Phone: (612) 626-6664; Fax: (612) 624-3913; E-mail: Valle001@tc.umn.edu.

The potential benefit of immunotoxin (IT) for cancer therapy has mostly been limited by the fact that only a small portion of injected dose ever reaches the cancer target. Thus, we set out to determine whether antigen-specific CTLs would be suitable vehicles to deliver IT to the site of cancer cells in vivo. A retroviral vector was constructed for gene therapy with (interleukin 4) IL-4 positioned downstream of its 20-amino-acid leader sequence that permitted cotranslational protein synthesis of IT along with truncated diphtheria tox (DT). IL-4 was chosen as a ligand based on the expression of IL-4 receptor (IL-4R) on most acute myeloid leukemia cases. The first-time expression and secretion of a cytokine/DT fusion tox was accomplished in mammalian NIH.3T3 cells, and then a retroviral vector was assembled. The retroviral IT was used to transiently transduce T15, a CD8+ CTL T cell line that specifically recognized C1498 (a lethal murine acute myeloid leukemia cell line). Transduced T15 T cells expressed intracellular DT and IL-4 as determined by immunofluorescence. Secreted IT supernatants collected from T15 had enzymatic activity and may impart new anticancer defense mechanisms to antigen-specific T cells.

INTRODUCTION

T cells are critical immune effector cells in the defense against cancer and selectively recognize tumor antigens that are either specific to cancer cells or are overexpressed shared antigens (1, 2). This results in the destruction of tumors that threaten the host. However, T-cell immunotherapy has been only partially successful in destroying progressive cancer, in part, because they are often overwhelmed by large numbers of tumor cells. The use of gene therapy to introduce cytokines or antibody fragments to augment antitumor responses most often have been unsuccessful in adequately destroying neoplastic tissue because tumor cells are not adequately destroyed (3, 4). A solution to these problems would be to genetically arm T cells with a selective and potent toxin powerful enough to reduce tumor cell numbers and to rely on the T cells to deliver and secrete this IT at the site of cancer in vivo.

Cytokine fusion toxins are experimental pharmacological agents that are made by linking cytokines that specifically bind to cancer cells to potent catalytic toxins such as DT.3 Intact DT contains two fragments, A and B. The fragment catalyzes the ADP-ribosylation of elongation factor 2 (EF-2) leading to protein-synthesis inhibition and cell death (5, 6). Although a single molecule of DT fragment A in the cytosol can be fatal to a cell, fragment A alone applied extracellularly is not highly toxic because the binding domain is located in fragment B. Thus, we included only the first 389 amino acids of DT that contained fragment A for our construct.

The major purpose of cytokine IT is to deliver therapy selectively to cancer cells that frequently overexpress cytokine receptors (7). Although these agents selectively bind and kill cancer cells, clinically their use has been limited by: (a) their failure to penetrate and localize in adequate concentrations in cancer target tissue; and (b) their localization in nontarget organs limiting the tolerated dose and narrowing the therapeutic window (8). We have explored a solution to this problem, reasoning that T cells that are highly suited for local cytokine delivery in the first place would be excellent vehicles for the delivery of cytokine fusion toxins if induced to secrete them by retroviral gene transduction. The secreted cytokine IT would preferentially destroy cancer cells displaying the appropriate cytokine receptor on their cell surface but would not kill normal tissue cells lacking the antigen. We chose a murine acute myeloid leukemia model because this leukemia is the most common form of adult leukemia (9). IL-4 was chosen as the ligand for our IT because IL-4R is expressed on over 90% of acute myeloid leukemia cases (10) and IL-4 IT has been previously used in experimental cancer therapy studies (11–13). DT was chosen as a toxin because it has extensive clinical use and has a history of use as a recombinant toxin (14–16).

These studies show for the first time that gene therapy can be used to manipulate antigen-specific T cells into secreting cytokine fusion toxins with selective activity against myeloid leukemia cells.
Successful cloning of the designed gene was confirmed by sequencing. Expression was induced and a $M$, 60,000 recombinant protein was purified by ion exchange chromatography as described previously (19).

**Cells, Cell Lines, and Antibodies.** C1498 is an IL-4R$^+$ spontaneously occurring C57BL/6 myeloid leukemia, which is lethal to mice in 20–30 days when injected at doses greater than 10$^3$ cells (20, 21). C1498 cells were cultured in RPMI 1640/10% FCS and 1% penicillin/streptomycin (Life Technologies, Inc., Grand Island, N.Y.). T15 is a MHC class I-restricted CD8$^+$ cytotoxic T-cell line produced by hyperimmunizing C57BL/6 mice with an irradiated subline of C1498 cells with enhanced costimulatory activity due to its transduction with B7–2. Previous studies showed T15 responds against C1498 in vitro and in vivo (22). T15 cells were maintained by culturing in RPMI 1640/10% FCS supplemented with 100 units/ml IL-2 (Cetus Corp., Emeryville CA) and stimulated every 2–3 weeks with irradiated C1498 as described previously (22). NIH.3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in DMEM/10% FCS with antibiotics. For studies requiring neutralization of IL-4 fusion toxin, a rat antimouse IgG1 anti-IL-4 antibody (from clone 11B11; 23) was used. Anti-Ly5.2 (from clone A20 –1.7, generously provided by Dr. Uli Hammerling, Sloan-Kettering Institute for Cancer Research, NY), which is not reactive with either IL-4 or C1498 cells, was used as a control for the blocking studies.

**Genomic PCR.** To detect the DT provirus integrated into genomic host DNA, DNA from transduced T15 cells was studied by PCR using Taq polymerase (Perkin-Elmer, Foster City, CA) and primers 5'-GGCGTGATGATGGTTGTTGA3' and 5'-AAAAATGTGTGGTTTTATG-3'. Amplification in a DNA thermal cycler (Perkin-Elmer, 30 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 120 s), produced an 1170-bp product.

**Transfection, Harvesting Viral Supernatants, and Viral Transduction.** For transfection of IT genes into mammalian cells, target genes were cloned into pcDNA.3 mammalian expression vector designed for constitutive transcription from mammalian enhancer-promoter sequences, and was then transfected into NIH.3T3 cells. A, sigIL4DT$_{390}$/pcDNA.3 encodes 1.7-kb NgoI-XhoI gene fragment consisting of the IL-4 leader sequence (aa 1–19) followed by the murine IL-4 gene (aa 20–140), a flexible EASGGPE linker, and a downstream fragment encoding DT$_{390}$ (the first 389 aa of DT devoid of the native binding region). The SOE gene product was ligated into pcDNA.3, a eukaryotic expression vector designed for constitutive transcription from mammalian enhancer-promoter sequences, and was then transfected into NIH.3T3 cells. B, sigIL4DT$_{390}$/LNCX encodes sigIL4DT390 described in A, cloned into the retroviral vector LNCX and then transfected into the PA317 packaging line to generate viral supernatant. C, sigIL4DT$_{390}$/LNCX.NGFR is identical to sigIL4DT$_{390}$/LNCX except neo was replaced with the gene encoding human NGFR to provide a selectable marker for assessing transduction levels and isolating stable transductants.

For transductions, viral supernatants were collected from packaging cells and were used to transduce T15 cells. T15 cells were transiently transduced prior to each in vivo injection. One ml of supernatant was diluted in an equal volume of media and added to cells in 24-well plates plus 8 μg/ml polybrene, 100 units/ml IL-2. The mixture was centrifuged at 2500 rpm at 32°C for 1.5 h and then was incubated at 32°C for 5 h. Cells were then transferred to a 100-mm dish and incubated in RPMI 1640/10% FCS for 24 or 48 h. Transduction frequency was quantitated by the cytometric analysis of NGFR-expressing transduced cells. Typically, 38–40% of cells expressed NGFR cell-surface marker after this transduction procedure. In one experiment, T15 cells transduced with sigIL4DT$_{390}$/LNCX were selected in selection media containing G418 [600 μg/ml (Life Technologies, Inc. Co., Gaithersburg, MD)]. Cells were fed with new selection media every 3 days.

**Flow Cytometric Analysis.** To assess the percentage of cells transduced with target gene, transduced and nontransduced T15 cells were stained with mouse anti-NGFR primary antibody (Boehringer Mannheim, Indianapolis, IN), diluted 1:1000 for 15 min at room temperature, and FITC-labeled with antimouse IgG secondary antibody diluted 1:100 (Chemicon, Temecula, CA). The mixture was incubated for 15 min at room temperature, and samples were analyzed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) as described previously (24). Forward- and side-scatter settings were gated to exclude red cells and debris. Cells (7,000–10,000) were analyzed for each determination. Irrelevant monoclonal antibody control values were subtracted from values obtained with relevant monoclonal antibodies.

**Staining for Intracellular IT.** Cells were cultured on coverslips and transduced with sigIL4DT$_{390}$/pcDNA.3. After 30 h, coverslips were washed twice with PBS and fixed with 95% ethanol/5% acetic acid at −20°C for 5 min. Fixed cells were washed with PBS, incubated with primary 1H11 Moab, diluted 1:50 in 5% BSA/PBS, and then incubated 1 h at room temperature. For DT staining, cells were incubated with primary polyclonal rabbit anti-DT (generously provided by Dr. S. Ramakrishnan, University of Minnesota, Minneapolis, MN) and secondary FITC-labeled antibody. Coverslips were washed and incubated with secondary FITC-rabbit antimouse IgG (Sigma, St. Louis, MO) diluted 1:500 for 45 min at room temperature. Coverslips were washed again and then mounted using a SlowFade Light Antifade Kit (Molecular Probes, Eugene, OR). The cells were observed and digitally photographed using a
Fig. 2. Activity and selectivity of recombinant IL-4 IT. To determine whether bacterial IL-4 IT selectively killed target cells in vivo, A) C1498 cells were incubated with various concentrations of the IL-4 fusion toxin for up to 72 h. At 24-h intervals, individual wells were sampled, and aliquots were stained with trypan blue and counted. B) C1498 cells were cultured with IT plus the neutralizing anti-IL-4 antibody (11B11) or C irrelevant control antibody, anti-Ly5.2 (which did not bind either cells or IT). Error bars, SE. Student’s t test indicated that responses of cells treated with IL-4 DT were significantly (P < 0.0001) different from the responses of untreated cells. However, there was no difference between responses of treated and untreated cells when blocking anti-IL-4 antibody was added.

Nikon fluorescent microscope with Spot software (Diagnostic Instruments, Ann Arbor, MI).

Viability Assays. To assess retroviral IT killing, IL-4R C1498 cells were plated at 2 x 10^5/well in 24-well plates (Costar). One ml of filtered supernatant from cultured transfected or transduced T15 cells was added to each well. Wells were sampled at 24, 48, and 72 h and diluted in trypan blue dye/PBS solution, and the number of surviving cells was determined. To assess selectivity, supernatants were simultaneously tested on IL-4R EL4 cells.

CTL Assay. Cytotoxicity was measured by a modified JAM assay in which target cell proliferation is assessed by thymidine incorporation (25). Briefly, 2 x 10^5 C1498 or EL4 target cells are pulsed for 3.5 h with 10 µCi tritiated thymidine (Amersham Corp., Arlington Heights, IL), washed, and then added to LAK or T15 effector cells in 96-well U-bottomed plates (Costar) at E:T ratios of 100, 50, 25, 12.5, 6.2, 3.1, and 1.5 to 1. Plates were centrifuged and incubated for an additional 3.5 h at 5% CO2/95% air 37°C and then were harvested and counted by standard scintillation counting techniques. Cytotoxicity was calculated.

In Vivo Studies. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene.

In Vivo Studies. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene. Two million C1498 cells were injected s.c. into the shaved flank of C57BL/6 mice (5–6-week-old females purchased from The Jackson Laboratory, Bar Harbor, ME) housed in a specific pathogen-free facility at the University of Minnesota. Mice were given i.v. injections (via caudal vein) of M anti-IL-4 monoclonal antibody (11B11) or irrelevant control antibody, anti-Ly5.2, for an IL-4R gene.

BUN, Creatinine, and ALT Assays. All three of these assays were performed on Kodak EKTACHEM clinical chemistry slides on a Kodak EKTACHEM 950 by the Fairview University Medical Center-University Campus (Minneapolis, MN). Mice were killed, individual serum samples collected, and analysis was performed in a coded fashion on the undiluted samples. Minimum specimen volume was 11 µl for each assay. The BUN assay is read spectrophotometrically at 670 nm. The creatinine assay was read at 670 nM. The ALT assay is read at 670 nm. The creatinine assay was read at 670 nM. The ALT assay was expressed and purified. IL-4R C1498 leukemia cells were cultured in the presence of various concentrations of DT 390 IL-4. At 24, 48, and 72 h, surviving cells were counted. Fig. 2A shows that inhibition was dose-dependent and 1.0 nM killed all of the cells by 48 h. Fig. 2B shows that 20 µM anti-IL-4 monoclonal antibody blocked C1498 killing at DTIL-4 concentrations of 1 and 10 nM. The addition of anti-Ly5.2, an irrelevant control antibody, did not alter activity (Fig. 2C). In independent experiments, we found that DTIL-4 was selectively toxic for another IL-4R murine myeloid leukemia B162, for an IL-4R glioma, and IL-4R neuroblastoma (data not shown). It did not kill the IL-4R T cell thymoma EL4. Together, these data indicate that IL-4 IT killing was selective and attributed to the IL-4 moiety of the hybrid protein.

Expression of the sigIL-4-DT 390 Gene in Mammalian Cells. To determine the feasibility of producing cytokine fusion ITs intracellularly, IL-4-DT 390 with the 20-amino-acid leader sequence was assembled and cloned into the mammalian expression vector pcDNA.3. The correct assembly of these and all of the constructs was confirmed by DNA sequencing. Thirty h after transfection of NIH.3T3 cells with sigIL4DT 390/pcDNA.3, indirect immunofluorescence (IF) staining with anti-IL-4 or anti-DT revealed definitive intracellular presence of both IL-4 and the DT moiety of the hybrid protein (data not shown). No positive staining was observed when cells transfected with the target gene were stained with FITC-labeled secondary antibody without primary antibody, which indicated that secondary antibody was selectively binding. No positive staining was observed in controls transfected with the empty pcDNA.3 vector and stained with anti-IL-4 or anti-DT.

To determine whether expressed protein was secreted, supernatants were collected from an aliquot of these same transfected cells. Fig. 3 shows that supernatants collected from cultured NIH.3T3 cells transfected with the target gene killed C1498 cells but not control EL4 cells. Control supernatants from NIH.3T3 cells transfected with empty vector did not inhibit either cell. Control recombinant DTIL-4 at a concentration of 10^-8 M inhibited C1498 in a similar manner, as did supernatants from cells transfected with target gene.
Together, these data show that transfection with target gene results in the secretion of functional fusion toxin that is selectively toxic.

In Vitro Selectivity Studies with T15. To be useful for retroviral production of DTIL-4, T15 cannot be susceptible to killing by DTIL-4. Fig. 4 shows that T15 was not inhibited (Fig. 4B) by concentrations of DTIL-4 that otherwise killed C1498 (Fig. 4A). Also, the fibroblast packaging line PA317 (Fig. 4C) was not killed by DTIL-4, which rendered them acceptable hosts for packaging virus containing the sigDTIL-4 target gene. The in vitro growth of T15 is dependent on IL-2 and antigen stimulation by irradiated C1498 cells every 3 weeks. IL-2-dependent proliferation of T15 cells measured by thymidine uptake was not inhibited by the addition of DTIL-4 and other recombinant cytokine fusion toxins described in other studies (17, 19), including DT390mIL-3, DT390mGM-CSF, and DT390 (data not shown). Proliferation was inhibited by DT390IL-2. Fig. 5 shows that T15 is specific in its cytolytic activity because it kills C1498 leukemia but has minimal activity against EL4 leukemia, although killing of EL4 was measured at high E:T ratios in this experiment.

In Fig. 3, supernatants (collected from transfected cells) killed target cells selectively; now we asked whether T15 T cells could be transduced with viral supernatants that would induce them to express and secrete IL-4 IT. T15 cells were transduced with viral supernatants from PA317 packaging cells electroporated with the sigIL4DT390/LNCX.NGFR retroviral vector. Fig. 6 shows T15 cells visualized by fluorescent microscopy that expressed IL-4 (Fig. 6A) or DT (Fig. 6C) only after being transduced with target gene. About 40–50% of cells were positive in this experiment 30–40 h after transduction. Non-transduced T15 cells were not positive for IL-4 or DT. As a tool for studying transduction frequency, T15 cells were transduced with an LNCX vector in which neo had been replaced with NGFR. Transduction with a gene encoding NGFR results in the cell surface expression of the human NGFR protein that can be detected with labeled anti-
body by flow cytometry and, thereby, used to measure the transduction frequency. Transduction with viral supernatants from sigIL4DT390/LNCX.NGFR-treated packaging cells resulted in cell surface expression of NGFR on T15 cells (data not shown).

To determine whether sigIL-4DT was secreted from transduced T15 cells, supernatants were collected from transduced cells and tested on either IL-4R⁺ C1498 cells or IL-4⁻ EL4s. C1498 were inhibited by supernatant from transduced but not from nontransduced cells (Fig. 7A). EL4s continued to proliferate despite exposure to supernatants from transduced or nontransduced T15 (Fig. 7B). Control DTLIL-4 inhibited C1498 but not EL4. Together, Figs. 3, 6, and 7 showed that the IT gene, when either transfected into mammalian embryonic cells (NIH.3T3) or transduced into T15 T cells, induced the expression and secretion of IL-4 IT that could selectively destroy IL-4R-expressing target cells.

Selecting Clones with Stably Integrated Provirus. To determine whether the IT was stably integrated into the host genome as a provirus, we performed a genomic PCR to determine the presence of the DT390 gene. A subline of T15 was produced following transduction with sigIL4DT390/LNCX and a 2-week selection in G418. Fig. 8 shows the presence of the 1.2-kb DT390 gene in cultured T15 cells.

Inhibition of Tumor Growth in Vivo. In vitro data showed that retroviral IT delivery was indeed feasible; therefore, we constructed a murine model to determine whether T15 T cells could deliver retroviral IT to C1498 target cells in vivo. We chose an in vivo model whereby tumor cells were s.c. injected so that their growth could be quantitated on a daily basis. Mice were given s.c. inoculations of C1498 cancer cells on day 0 to induce tumors (Fig. 9). C1498-specific T15 T cells were transiently transduced with sigIL4DT390/LNCX-NGFR virus and then administered i.v. to tumor-infected mice. The transduction frequencies of the T15 cells at the time of injection were 38–40% by FACS analysis and the viability of the transduced cells was 91–95%. Because the cytolytic activity of T15 T cells is IL-2-dependent, these mice were given 20,000 units of mIL-2 i.p.
with the T15 cells given i.v. All of the groups, including controls, received mIL-2. Twenty-seven $3 \times 10^6$ T15 cells were injected on day 5, $18 \times 10^6$ cells were injected on day 10, and $30 \times 10^6$ cells were injected on day 16 after C1498 injection. Tumor size steadily increased in groups of mice given nontransduced T15 cells or in groups of tumor mice that were not given T15. In contrast, the rate of tumor growth remained very low until day 18 in groups of mice injected with transduced T15. After day 18, growth began to steadily increase. These data show that transduced T cells inhibited tumor growth, whereas nontransduced cells did not. Lack of activity of nontransduced T15 could relate to inadequate amounts of IL-2 or high tumor volume. Mice given transduced cells ultimately developed tumor and metastases, which indicated that a greater level of IT delivery is needed in vivo.

Next, we set out to determine whether systemic i.p. therapy with recombinant IL-4 IT would be as effective as retroviral IT therapy in Fig. 9. In a separate experiment, C1498 cells were administered s.c. in a manner identical to that in Fig. 9. Groups of mice were given the maximum tolerated dose of IT (a total dose of 0.5/μg/day or 1.0 μg/day given b.i.d.) that was determined in a separate experiment.4 Fig. 10 shows that systemic administration of recombinant IL-4 IT,

---
4 N. Jin, unpublished data.
which was previously shown to be selectively toxic in nanomolar concentrations in vitro (Fig. 2), had no effect on the growth of C1498 cells in vivo. Three of five of the mice in the group given 1.0 mg/day died of toxicity on day 9. There were no toxic deaths in mice given 0.5 mg/day. Controls were not injected with IT.

Renal and Hepatic Toxicity in Mice Given Transduced T15 Cells. Renal and hepatic damage have been previously reported in mice given recombinant DT IT (26–28) and examination of H&E-stained tissues from those mice showed the presence of infiltrates and glomerular changes in kidney and fatty changes in the liver. Therefore, serum levels of BUN and creatinine were measured as indications of renal damage, and ALT levels were measured to determine liver damage in a separate cohort of mice from Fig. 9 (n = 3/group). Fig. 11 shows that there were no significant differences in BUN or creatinine levels in groups of mice given transduced T15 cells, which indicates that retroviral IT did not damage organs despite its effect on C1498 cells. Tissue from mice given transduced T cells was collected between the second and third administration of transduced T cells (days 10–15). Also, there was no difference in ALT levels, which indicates that hepatic activity also was unaffected. These findings were confirmed by histological analysis of tissues.

**DISCUSSION**

The major contribution of this work is the unique accomplishment of intracellular expression and subsequent secretion of intact cytokine fusion toxin from mammalian cells by infecting them with a retrovirus encoding mIL-4/DT390. The administration of antigen-specific T cells transiently transduced with this virus resulted in a significant in vivo anticancer effect against a lethal myeloid leukemia. A further interesting component of these studies was that in vivo retroviral IT delivery via antigen-specific T cells resulted in less systemic toxicity and more efficacy than systemic delivery of the same IT. This implies that this mode of delivery may have a future for diminishing the toxicity associated with the administration of certain biological agents.

An additional strength of the studies is the use of a syngeneic murine model to determine the feasibility of using antigen-specific T-cell lines for retroviral IT delivery in vivo. The CD8⁺CD4⁻ MHC class I restricted T-cell line, T15, was produced by hyperimmunizing C57BL/6 mice with irradiated C1498 cells. Previous studies showed that T15 elicited an anticancer effect in C1498-infected mice (22). In this report, transduction of T15 with sigIL4DT390 resulted in a significant (P < 0.05) anticancer effect in mice with s.c. tumors. Tumor growth was steadily held in check after injection of 18–30 × 10⁶ transduced cells and then began to increase in size after 18 days. In contrast, the nontransduced control had no effect on the growing tumor. These findings, in which i.v. injected transduced T cells are used to treat s.c. tumors, indicate that T cells and therapy are finding their in vivo targets. Nontransduced T15 did not contribute an anti-C1498 effect as it did in past studies. This might be explained by the fact that we did not administer enough IL-2 to drive IL-2-dependent T15. In future studies, in vivo survival of injected T15 might be enhanced by the continuous administration of IL-2 injections or an additional transduction with IL-2 gene.

At the same time, these findings raise important issues. For example, although a significant anti-C1498 effect was measured, we do not
know why efficacy was incomplete. Perhaps, more cells, stimulated to higher levels of cytotoxicity and higher transduction frequencies may be necessary for improved efficacy. It is also possible that only a small portion of i.v. injected T15 cells cleared the lungs and other internal organs and arrived at the tumor site.

This is also the first reported use of DT, a powerful inhibitor of protein synthesis (5) for retroviral IT production. Retroviral delivery of ITs has been theoretically limited by “cell suicide” expected to result from the delivery of the catalytic toxin into the cytosol. Although posttranslational protein transport in yeast does indeed permit translated toxins to react with their target, most mammalian cells undergo cotranslational protein transport in which the signal peptide directs the single-chain protein through the ER membrane directly into the ER lumen (29, 30). Data in this report indicate that toxin separation between the intracellular ER lumen and cytosolic compartments is remarkably conserved so that even minute amounts of toxin are prevented from gaining access to ribosomes. It is known that leader sequences are responsible for the passage of proteins into or through membranes, and retroviral ITs assembled in our laboratory without a leader sequence positioned upstream from the ligand were lethal to transfected cells. The leader peptide permits recognition by the signal recognition particle (SRP) and translocation of IT polypeptide directly into the ER lumen where it is sequestered (reviewed in 30). Whether this separation is maintained indefinitely is not known, but our data—in which transduced T15 cells, selected with G418, and then later examined for the presence of genomic DT<sub>390</sub>—indicate that cells are capable of surviving at least 2 weeks despite stable genomic integration of a lethal toxin provirus.

Another issue is the duration of secretion. Studies are in progress to measure retroviral IT production in vitro and in vivo. Although it will be useful to establish a correlation between retroviral IT levels and efficacy, it will be important to determine whether stable transductants can be generated in which provirus is stably integrated and retroviral IT is secreted. Currently, T15 must be transduced transiently prior to each injection, an approach that is neither labor-efficient nor cost-effective. The further generation of stable transductants via neo selection or flow cytometry sorting of NGFR<sup>+</sup> transductants may add this point. Another issue to be considered is whether systemic leukemia will be an appropriate target for retroviral IT therapy. In these studies, C1498 was administered s.c. so that we could monitor tumor size on a daily basis. If C1498 is administered i.v., it will disseminate systemically with multiple growth sites. The production of retroviral IT at multiple sites by transduced T15 T cells may enhance the risk of systemic toxicity. Fortunately, all of these issues can be addressed in this model, and studies are currently underway.

LAK cells might be considered for retroviral IT delivery because they are more easily obtained than antigen-specific CTL. Investigators used transduced LAK cells to deliver an sFv IT recognizing Her-2/neu-expressing human breast cancer in severe-combined immunodeficient mice (31). Because LAK cells are not antigen-specific, they may not be the best choice. Also, we transduced LAK cells in a manner identical to CTL and typically found that transduction of LAK cells with target gene resulted in at least half the frequency obtained with antigen-specific T15 cells. This might be attributed to the highly activated nature of T15 undergoing both antigen-stimulation and IL-2 expansion. Postmitotic cells cannot be transduced because mitosis is required for entry of the viral integration complex into the nucleus (32, 33), and perhaps there is a higher frequency of postmitotic cells in the LAK population. Certainly, differing transduction frequencies in the two populations are not strictly related to IL-2 because both T15 and LAK cells were transduced in the presence of IL-2. Perhaps, antigen-sensitized CTLs express a higher number of ectotropic receptors, and retroviral infection is dependent on virus binding to these specific receptors on the cell surface. The literature generally prefers the use of CTLs over LAK cells because antigen-specific T cells are more likely to penetrate to the site of tumor antigen (34, 35), and clinical adoptive immunotherapy studies with LAK cells have been limited by low response rates (36).

Delivering IT using T cells may have advantages in reducing toxicity. The secretion of retroviral IT at the site of tumor may result in a higher saturation of target cells and less IT escaping to nontarget locations. Our data indicate that T-cell delivery resulted in efficacy combined with no hepatic or renal toxicity. In contrast, systemic delivery of IT at the maximum tolerated dose was not efficacious. Others have shown that the dose-limiting toxicity of IL-4 IT is due to hepatic toxicity (37), and histological examination of tissues from our treated mice also revealed damaged livers (data not shown). Taken together, these data suggest that T cells are locally delivering IT at the site of tumor, and this is reducing systemic toxicity.

One appealing aspect of the retroviral IT approach is that T cells could be recruited as vehicles to deliver any cytokine fusion toxin. Although any cytokine could be used as a ligand for toxin delivery, we chose IL-4 because IL-4 receptors have been reported on most myeloid leukemias (10), various lymphoid malignancies (11), and non-lymphoid tumors (12). Also, recombinant IL-4 fusion toxins have been effective in animal models because complete remission of human IL-4R-bearing carcinoma can be induced in nude mice by administering IL-4 IT (11). Although expression of IL-4R on committed hematopoietic cells may be problematic, not all T cells express IL-4R as evidenced by our findings with transduced T15, and systemic exposure may be limited by local delivery to cancer cells. Also, although only a single molecule of toxin in the cytosol is capable of killing a cell, toxin must survive the endosomal journey to the cell’s interior, which may require the binding of hundreds or thousands of ITs and the avoidance of lysosomal compartments. Thus, low receptor expression and subsequent binding of low numbers of ITs may not result in cell killing. Normal hematopoietic cells that express low IL-4R levels may be spared. Other cytokine fusion toxins might work better than IL-4 IT. For example, studies show that IL-3 IT, which has been limited by systemic toxic effects, are highly selective and capable of destroying IL-3-receptor-expressing leukemias (18). Because these studies show that IL-3 kills committed but not uncommitted bone marrow progenitor cells and IL-3 receptor is expressed on greater than 90% of myeloid leukemias, it is also an excellent candidate for the retroviral IT approach. IL-2 or any other cytokine with high level receptor expression on T cells are undesirable candidates for retroviral IT because the secretion of IL-2 IT, for example, would immediately result in the suicide of T cells expressing IL-2R.

In conclusion, we have used retroviral gene therapy of cancerspecific CTLs to deliver a cytokine fusion toxin to C1498 cancer cells in vivo. Retroviral transduction of T cells resulted in a significant, albeit temporary, antitumor effect with no organ toxicity. In contrast, systemic administration of this same IL-4 IT was not efficacious and was highly toxic. Delivering ITs using T cells in this manner could overcome the unfavorable physiology, vascularization, and high-tumor interstitial pressures that have limited the penetration of biologicals to cancer sites in vivo (38).

ACKNOWLEDGMENTS

We thank Naomi Fujioka for assistance in the histological studies and Dr. Patricia A. Taylor for helpful comments.

---

REFERENCES


Retroviral Immunotoxin Gene Therapy of Acute Myelogenous Leukemia in Mice Using Cytotoxic T Cells Transduced with an Interleukin 4/Diphtheria Toxin Gene


Cancer Res 2000;60:976-984.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/4/976

Cited articles
This article cites 36 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/4/976.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/60/4/976.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.