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Gene Therapy for B-cell Lymphoma in a SCID Mouse Model using an Immunoglobulin-Regulated Diphtheria Toxin Gene Delivered by a Novel Adenovirus-Polylysine Conjugate

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Despite advances in conventional therapy, many lives continue to be lost to common forms of B-cell cancers, including leukemias, lymphomas and multiple myeloma. We propose a novel approach to therapy of such cancers using controlled expression of a diphtheria toxin gene (DT-A) to kill malignant cells. We have previously demonstrated selective killing of various cell types, in vitro and in vivo, by cell-specific, transcriptionally controlled expression of this gene. Organ-specific ablation in otherwise healthy transgenic mice has convincingly demonstrated the exquisite specificity achievable by this technique[1-5].

In the studies now described, DT-A was delivered in vitro and in vivo using a novel gene delivery system employing DNA physically attached to the exterior of adenovirus. After demonstrating the efficacy of gene delivery to Epstein-Barr virus transformed human B-cells in vitro, in vivo work was performed using a SCID mouse model for B-cell lymphoma, in which protection against tumor was observed. The concepts of tissue-regulated toxin gene therapy, and this novel adenovirus gene delivery system are discussed.

INTRODUCTION

Conventional cancer treatment is hampered by the narrow therapeutic window of many antineoplastic agents. Since most chemotherapy attacks DNA synthesis, effective therapy is severely limited by toxicity in normal replicating cell populations such as bone marrow and gut[6]. Restriction of the toxic effects of chemotherapy to malignant, rather than normal cells would be a great advance over therapies currently available. A convenient model for the study of cell-specific gene expression is represented by the B-cell malignancies. Regulatory nuclear proteins unique to B-cells bind transcriptional promoter and enhancer sequences associated with the rearranged immunoglobulin (Ig) heavy and light chain coding sequences, leading to transcription of mRNA for the various Ig isotypes[7]. However, this unique series of events has never been specifically exploited as a means of therapy for lymphomas, leukemias, or

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myeloma.

Despite conventional chemotherapy, 30 to 60% of patients with non-Hodgkin's lymphomas and more than 90% of myeloma patients relapse and succumb to their disease[8]. Infection with the Human Immunodeficiency virus (HIV) is currently associated with a large upsurge in B-cell lymphomas associated with Epstein-Barr virus (EBV) infection. This type of neoplasm is also seen in other settings associated with defective cell-mediated immunity. These tumors are likewise difficult to treat and often fatal[9-10].

Clinical use of natural toxins to kill tumor cells includes phase I/II trials in colon and breast cancers, melanoma, and B-cell malignancies such as chronic lymphocytic leukemia and non-Hodgkin's lymphomas and T-cell leukemias[11-13]. As an alternative means of exploiting a natural toxin for tumor cell killing, we have suggested the use of diphtheria toxin A chain (DT-A) gene as the therapeutic agent. We first demonstrated that cells could be induced to "commit suicide" by expressing *DT-A*, and that such activity could be enhanced in a specific cell type[14-15]. We subsequently developed Ig-regulated *DT-A* expression constructs effecting high level *DT-A* expression in murine and human B-cells, with little expression in non-B-cells[16-17], making them candidates as agents for B-cell-specific gene therapy.

In regard to gene delivery systems we demonstrated that addition of adenovirus to transfections using DNA/transferrin-polylysine complexes greatly enhanced the expression of heterologous DNA[18]. Next, we showed that if the DNA were physically linked to the adenovirus, heterologous gene expression was augmented by several orders of magnitude over levels achieved using unbound DNA. Strategies to link DNA to adenovirus include biotinylated adenovirus coupled to DNA/streptavidin-polylysine, biotinylated adenovirus coupled to anti-biotin antibody-polylysine/DNA, and anti-adenovirus antibody bridges[18-20].

This liganded adenovirus approach affords high-level heterologous gene expression in up to 67% of EBV transformed human B-cells (B-LCL) *in vitro*, for up to 17 days[20]. Anti-human Ig conjugates (which bind to the surface Ig of B-cells) induced up to three times more reporter gene expression in B-LCL than that induced when transferrin-polylysine or polylysine were used[20].

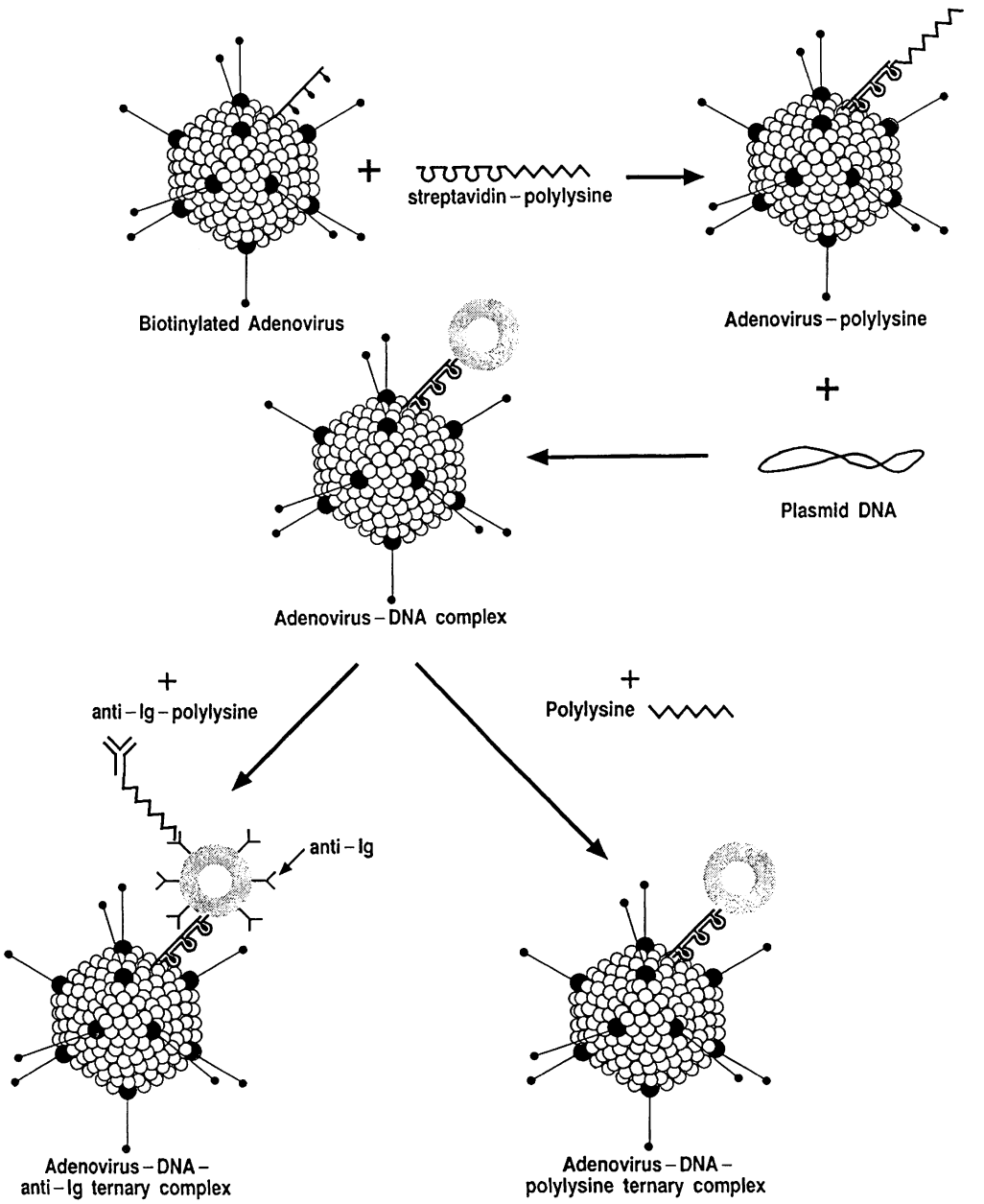
Encouraged by these *in vitro* results, we used these conjugates to assess this novel adenovirus system for delivery of Ig-regulated DT-A to B-LCL *in vivo* in a SCID mouse model. SCID mice injected with B-LCL develop tumor ascites, and limited metastases[21]. By increasing the tumor cell inoculum from the order of 10^6 cells previously reported, to 10^7 cells, we were able to reproduce widely metastatic tumors in SCID mice. These tumors share a number of similarities with EBV-associated human immunoblastic lymphomas[22-23]. Treatment of these tumors using an Ig-regulated DT-A construct delivered by this adenovirus system apparently protected SCID mice from a lethal tumor challenge in the single such experiment performed to date.

METHODS

Conjugate Synthesis

Biotinylated adenovirus dl312 and streptavidin-polylysine conjugates were all prepared as described[24]. Biotinylation of adenovirus does not significantly affect viral titer (Wagner *et al.*, unpublished observations). Anti-human Ig-conjugates were prepared by conjugation through disulfide bridges after modification with succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia, Uppsala, Sweden) using goat anti-human Ig (Southern Biotechnology Associates, Inc., Birmingham, AL) and poly(L)lysine (with an average chain length of 300 monomers) at a molar ratio of 1:2 in analogous fashion as described[25]. Before use, antibodies were subjected to gel filtration (Sephadex G25; 150 mM NaCl, 20 mM HEPES buffer, pH 7.3). Ig conjugates prepared in this fashion are uncontaminated by free Ig or free components (our unpublished observations). UV-psoralen inactivation of virus was performed as described[18].

Figure 1. Schematic representation of binary and ternary adenovirus-DNA-ligand complexes.



Complex Formation

Binary DNA complexes were prepared as follows: biotinylated adenovirus dl312 (3×10^{10} particles) in 50 µl HBS (20 mM HEPES, pH 7.3 and 150 mM NaCl) was mixed with 800 ng streptavidin-polylysine in 100 µl HBS. After a 30 minute incubation at room temperature, a

solution of 12 μg plasmid DNA in 200 μl HBS was added, and after an additional 30 minute incubation at room temperature, a solution of 5.1 μg poly(L)lysine (average chain length 450 amino acids) in 150 μl HBS was added. A schematic of the final ligands is depicted in Figure 1.

In vitro transfections

DNA complex solutions were added to 3 x 10^5 to 1 x 10^6 EBV-transformed B-cells (B-LCL) in 24-well plates in 1 ml of RPMI-1640 plus 2% FCS. After 2 hours, 1 ml of RPMI-1640 plus 20% FCS was added. Further cell culture procedures were as described[42].

Plasmids

pRSVß-gal[26], pCMVL[27], pRSVL[28], have been previously described. pTHA81 is an Ig-regulated DT-A expression plasmid containing an Ig kappa promoter and intragenic sequences, which confer efficient *DT-A* expression in murine B-cells[17]. The corresponding control plasmid pTHA71ΔDT contains a *DT-A* frameshift mutation.

Cell lines

B-LCL were made as described[29]. Briefly, peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, and incubated with supernatant from the EBV producer cell line B95-8. Resultant B-LCL were maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10-15% heat-inactivated fetal calf serum (Fisher, Pittsburgh, PA), glutamine 2 m*M*, Hepes buffer 10 m*M*, penicillin 50 U/ml and streptomycin 50 μg/ml. Cells were diluted with fresh medium the day prior to animal inoculation to obtain exponential growth at the time of challenge. For the animal work described, the B-LCL line DN[20] was used.

Assays for reporter gene expression

Assays for luciferase[27] (luc) and ß-galactosidase[30] (ß-gal) activity were performed as described. The luc inhibition co-transfection assay was performed as described[16] except that the luc reporter was assayed on day 3 following transfection, as we had previously shown this to be the day of maximal reporter gene expression[20]. In addition, to account for the potentially toxic effects of biotinylated adenovirus or chimeric proteins, for assays in which less than 6 μg DNA was used, biotinylated adenovirus (free of DNA) was added in excess of that needed to deliver DNA to make all transfections contain the identical amount of virus and chimeric proteins.

SCID mouse experiments

SCID mice of either sex, 6 to 10 weeks old were purchased from Taconic. Animals were housed in microisolator cages with three to five animals per cage, and fed sterile water and autoclaved food pellets *ad libitum*. Prophylactic antibiotics were not used. Animals were inoculated with 3 x 10^7 B-LCL by intraperitoneal injection. 12 μg plasmid DNA was conjugated to adenovirus using polylysine in the final incubation and injected into the animals i.p. immediately after tumor challenge, on the side contralateral from the tumor inoculation. Separate syringes were used for cell and DNA injections, and no mixing of cells or gene transfer reagents occurred *ex vivo*. UV-psoralen inactivated virus was used for all animal work. All dying animals were necropsied and tissues were examined using standard histologic techniques to assess for the presence of tumor. This protocol was approved by the Animal Care Committee of the University of Colorado Health Sciences Center.

RESULTS

Non-specific toxicity of virus/DNA conjugates in cell culture

We first tested for non-specific toxic effects of adenovirus/DNA conjugates on B-LCL using pRSVLuc, a plasmid known not to be toxic for these cells. Transfection of B-LCL with conjugates made from replication defective adenovirus dl312 rapidly resulted in death of the cell culture. Conjugates made from

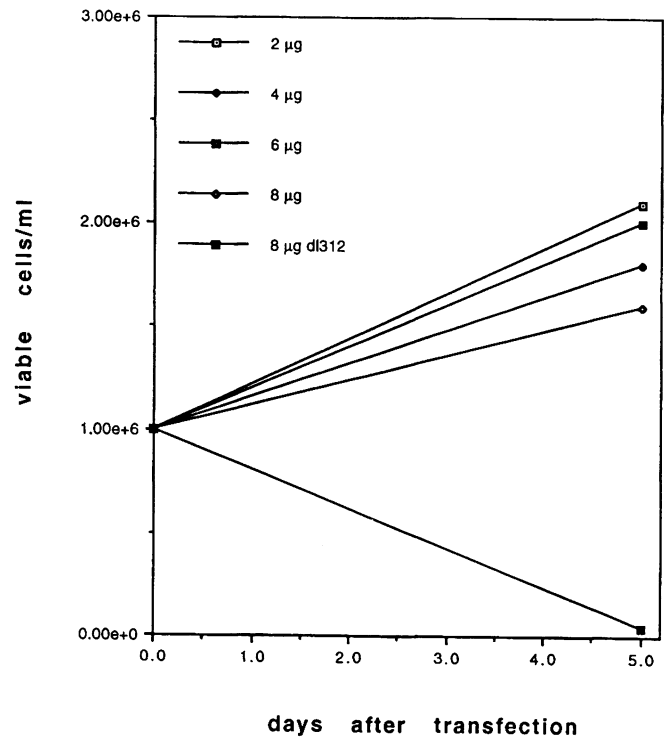


Figure 2. Uv psoralen-inactivated adenovirus used in the production of DNA conjugates did not affect the growth of transduced B-LCL. Micrograms refer to the quantity of transduced pRSVLuc. The solid squares with the caption "8 µg dl312" refer to conjugates delivered with replication-defective, but not UV-psoralen inactivated adenovirus.

UV-psoraleninactivated adenovirus showed no significant toxicity to cell culture using up to 8 µg total DNA (Fig. 2). Thus, the adenovirus itself, when inactivated and the corresponding chimeric proteins and DNA were not themselves toxic to B-LCL in the dose range of 2 to 8 µg DNA. Unless noted otherwise, only UV-psoralen inactivated virus was used further in the formation of conjugates for the work reported here.

Specific toxicity of pTHA81 for B-LCL

600 ng of pTHA81 (Ig-regulated, DT-A encoding) was sufficient to effect 95% reduction in *luc* expression in the co-transfection assay (Fig. 3). By contrast, pTHA71ΔDT effected no detectable luc inhibition at a dose of up to 2 µg DNA, although at higher doses, some inhibition was observed (Fig. 2, and data not shown). The toxicity at higher doses likely relates to the non-specific toxicity of adenovirus proteins, even when heat-killed (our unpublished data). Therefore, as expected, the plasmid encoding the functional *DT-A* gene showed significant toxicity for B-LCL even when low doses were used in this co-transfection assay.

Adenovirus/DNA conjugates mediate gene transfer in vivo

We had previously shown that this adenovirus system delivered a ß-gal reporter gene to B-LCL *in vivo*[20]. For that work, we did not assess for reporter gene expression in mouse tissues. To extend those observations, two tumor-bearing animals were inoculated with 12 µg of ß-gal reporter gene as described[20] using an adenovirus-polylysine conjugate. ß-gal activity was detected in peritoneal serosa, spleen cells and liver, but not in control animals which were not inoculated with adenovirus conjugates (not shown). High background activity precluded adequate assessment of Fallopian tubes and kidneys.

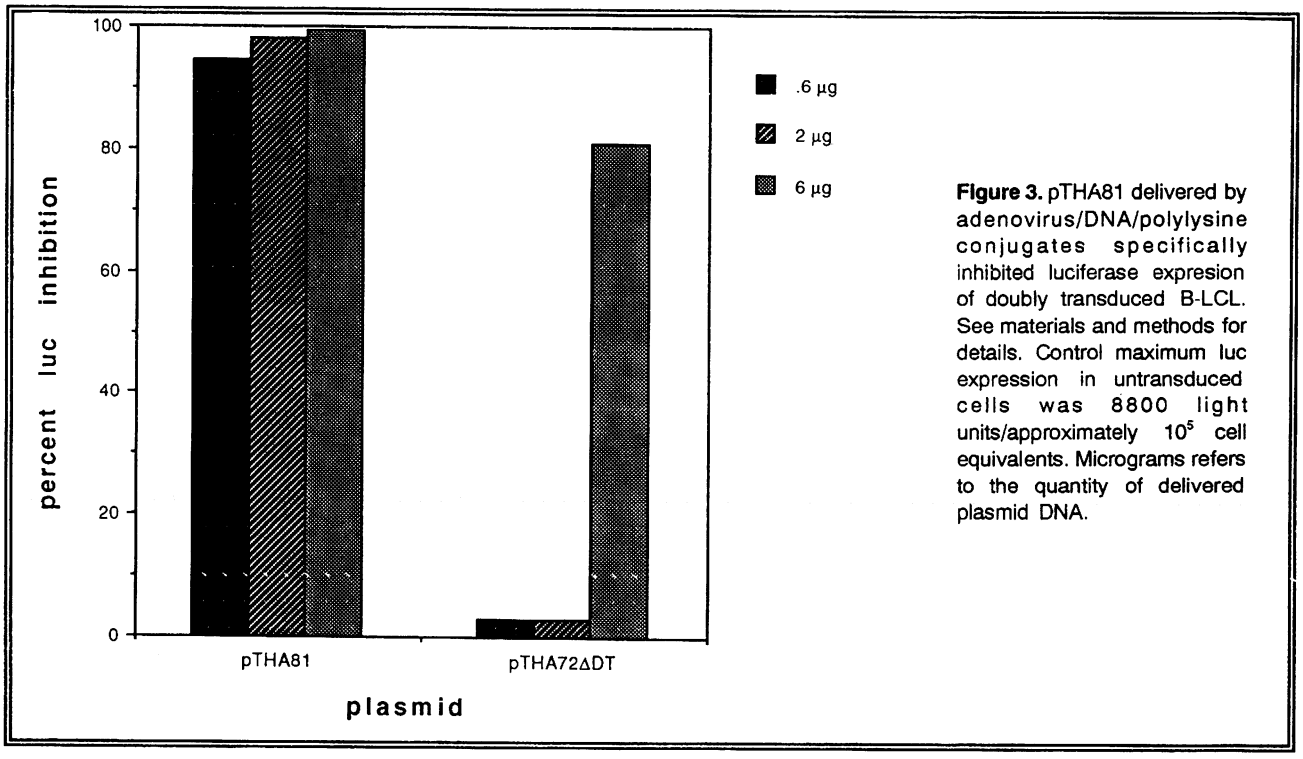


Figure 3. pTHA81 delivered by adenovirus/DNA/polylysine conjugates specifically inhibited luciferase expresion of doubly transduced B-LCL. See materials and methods for details. Control maximum luc expression in untransduced cells was 8800 light units/approximately 10^5 cell equivalents. Micrograms refers to the quantity of delivered plasmid DNA.

Effect of pTHA81 treatment on survival of mice bearing B-LCL tumors

Five SCID mice were inoculated i.p. with 1 x 10^7 B-LCL followed immediately by 12 µg pTHA81. Five control mice were inoculated with B-LCL and treated with the control plasmid pTHA71Δ DT in identical fashion. All dying animals were necropsied. The four control mice, and the pTHA81 treated mouse that died on day 70 had widely metastatic tumor involving peritoneal cavity, liver, spleen, lymph nodes and other organs (not shown). The pTHA81 treated mouse that died on day 230 had no tumor detected by standard histologic techniques, although more sensetive detection methods such as polymerase chain reaction were not performed. pTHA81-treated animals had apparently longer survival than controls (Fig. 4), but statistical analysis was not performed owing to the small number of animals involved.

DISCUSSION

We have previously demonstrated the feasibility of delivering foreign genes to human B-cell tumors using a novel adenovirus system[20]. We now extend these observations to demonstrate that this system also mediates *in vitro* and *in vivo* delivery of an Ig-regulated DT-A expression construct to human B-cell tumors which is toxic to them. The conjugates (made with UV-psoralen inactivated virus) were found to be non-toxic for SCID mice as well, demonstrating that this model will be useful for further studies. Conjugates made from non-inactivated virus likewise were not harmful to SCID mice at doses of up to 100 µg DNA, corresponding to 3 x 10^{11} viral particles (not shown).

The ability to deliver genes *in vivo* by intravenous injection with this system has not yet been unequivocally demonstrated, owing to inactivation of the conjugates in blood (unpublished observations). Nontheless, this delivery system may be applicable to non-blood compartments such as the peritoneal cavity, bladder epithelium or the central nervous system in its present form. Our finding of ß-gal reporter gene expression in distant organs (spleen and liver) following i.p. inoculation was unexpected, and may relate to diffusion of complexes in the peritoneal fluid or to ß-gal expression from transduced B-LCL that had migrated to those areas. ß-gal expression in these tissues owing to

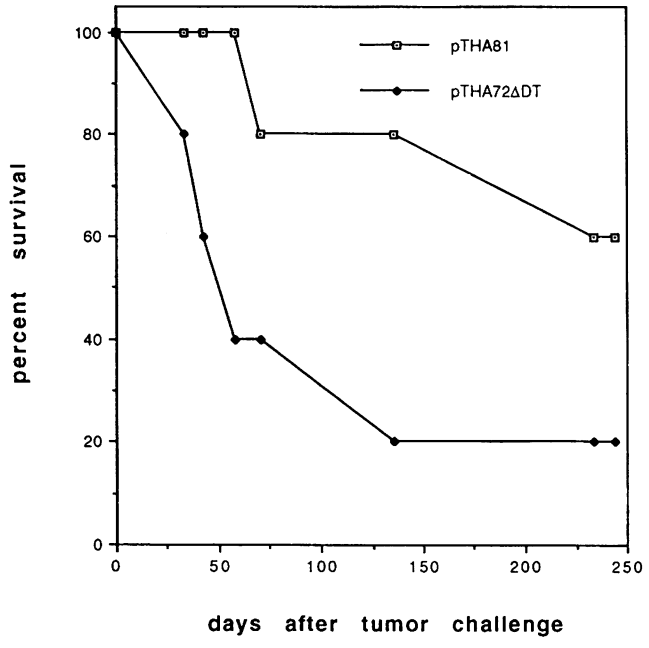


Figure 4. pTHA81 delivered by adenovirus/DNA/polylysine conjugates to B-LCL bearing SCID mice apparently afforded a survival advantage. n = 5 for each group of animals tested.

small amounts of complex surviving in blood is unlikely, based on our prior experience with intravenous delivery. Experiments to assess these possibilities are in progress.

Many experiments *in vivo* in transgenic animals have clearly demonstrated that sufficient regulation of toxin gene expression may be achieved to produce animals with foreign gene expression only in targeted tissues even though all cells of the transgenic animal are transduced. We have also previously demonstrated[16,17,55-58] the exquisite specificity of regulated DT-A expression systems *in vitro*. Due to this high level of specificity, it is possible to exploit the efficiency of relatively non-specific gene delivery pathways, including the transferrin receptor, polycationic lipids, and the charge-dependent polylysine ligand used here.

These experiments were performed with a B-cell-specific plasmid, but a B-cell-specific ligand for cell targeted delivery was not used. Using these non-tissue specific ligands, we demonstrated ß-gal reporter gene expression in numerous mouse tissues. The fact that tumor-bearing mice did not experience clinical or histologic signs of conjugate-associated toxicity suggests that the Ig regulation of *DT-A* expression must have been sufficiently stringent to have afforded an acceptable therapeutic index. These data therefore further confirm the potential advantage of a genetically controlled toxin expression system. We have already demonstrated that anti-immunoglobulin-polylysine ligands efficiently mediate heterologous gene delivery to B-LCL *in vitro*[20]. The B-cell specificity of these, and other ligands, and means to decrease non-specific interactions of the polylysine, adenovirus and other components of the conjugates are in progress. Nonetheless,we acknowledge that B-cell specific complexes will bind to normal as well as malignant B-cells, thus potentially diluting their therapeutic effect.

Many different non-viral systems to accomplish gene transfer have also been developed, including $CaPO_4$ co-precipitation[31], liposomes[32], and direct DNA injection[33]. These methods are ultimately membrane perturbing, and thus may be associated with significant cytotoxicity. In addition, because most non-viral vectors do not possess a specific mechanism to facilitate gene transfer events distal to cell membrane transition, they may be extremely inefficient. *In vivo*, gene transfer efficiencies with these agents

may be further compromised by lack of cell-specific tropism and clearance by reticuloendothelial mechanisms[34].

Design of recombinant viral vectors may be limited if tissue-specific expression is desired or if the introduced foreign gene requires regulation of expression. The obligatory co-introduction of elements of the genome of the parent virus poses significant safety hazards. To circumvent these limitations, methods have been developed to deliver DNA by the receptor-mediated endocytosis pathway[35-48], the merits of which have been described elsewhere[49-50].

Adenovirus was chosen as the "backbone" for our conjugates for several reasons. The entry pathway of adenovirus is analogous to that of the conjugate vector in certain respects. Like the molecular conjugate vector, the adenovirus has an efficient internalization mechanism via a cellular internalization pathway. Unlike the molecular conjugate vector, however, after entry the adenovirus possesses a specific mechanism to escape from the cell vesicle system and thus avoid lysosomal degradation.

Despite the extreme toxicity of diphtheria and ricin toxin, the genes encoding the A-chains of these toxins can be placed under specific transcriptional regulation with sufficient stringency to ablate specific tissues (such as exocrine pancreas, eye lens or pituitary somatotropes) during development without other adverse effects on the transgenic animals[1-5]. We chose to deliver a regulated toxin gene using our liganded adenovirus delivery system. Others have exploited similar strategies in the development of chimeric proteins delivering a toxin by means of the specificity of the conjugated receptor[12,13].

While targeted toxin genes may be used in therapy of various forms of cancer, leukemias (especially of lymphoid cells), lymphomas and multiple myeloma represent particularly appropriate diseases for developing and applying such a therapeutic approach for reasons stated above. Since transient expression of small amounts of toxin would suffice to achieve cell death, tumor ablation may be more readily achievable than in most systems in which long-term expression of substantial amounts of gene product is required[51]. For the present, our efforts are directed towards efficient ablation of any B-cells, rather than only malignant B-cells. This does not necessarily negate the therapeutic usefulness of this approach, bearing in mind that conventional chemotherapy is still less specific. Furthermore, in the unlikely event all B-cells were to be ablated by this method, one would expect regrowth from bone marrow progenitor cells in a few weeks[7]. Finally, it is unlikely that any one therapeutic approach to the treatment of these cancers will be effective. Thus, if these therapies serve to decrease tumor mass, they may be combined with other therapies to afford an outcome superior to the use of either alone.

It is important to point out both the potential advantages and problems associated with this type of gene therapy. While targeting tissue-specific gene expression is a radical departure from most previous methods, tissue-specific targeting is far from cancer-specific. However, it is also true that many tissues are not required for survival, and that chemotherapy is also not cancer-specific. Furthermore, targeting Ig synthesis should provide a therapeutic window which might actually spare normal stem cells capable of reconstituting the immune system, and would potentially be less toxic than the highly immunosuppressive combination chemotherapy regimens now employed. Other possible disadvantages include resistance to therapy by down-regulation of conjugate receptors on cell surfaces, antibody formation against conjugates and resistance to the effects of DT-A (such as development of mutant elongation factor 2). Some of these disadvantages are shared with monoclonal antibodies and chemotherapy.

Because it is likely that patients treated with adenovirus conjugates will develop neutralizing antibodies leading to rapid clearance of viral particles, virus particles may be engineered with sufficiently different capsid proteins to avoid neutralization during repeated treatments. Nonetheless, in a study of application of recombinant adenoviruses to respiratory epithelium, efficient gene delivery was accomplished despite the development of high-titer local anti-adenovirus IgA antibody[52]. The multiple adenovirus strain approach has been

used successfully in a primate model[53]. Furthermore, compared with other gene therapy efforts which will generally require long-term, stable expression of large amounts of protein, our approach theoretically requires only transient expression of small amounts of protein to be successful. Thus, the number of required treatments may be minimized. Moreover, the specific targeting of protein synthesis by DT-A already abrogates the known gene-mediated multidrug resistance which develops rapidly in B-cell neoplasms[54].

In summary, these studies demonstrate the efficacy of B-LCL growth inhibition in vitro, and apparently *in vivo* as well using an Ig-regulated DT-A gene delivered by a novel adenovirus system. Although these data suggest efficacy in an animal model, further work will be necessary to confirm these results, and to gain a clearer understanding of the mechanisms by which these conjugates afford protection. In addition, the conjugates are complex, with much potential for non-specific interactions which may ultimately hinder or possibly negate their use as cell-specific delivery agents. Nevertheless, we feel these experiments serve as tentative steps towards elucidation of clinically useful gene therapy strategies directed at B-cell (and possibly other) malignancies. Thus, toxin gene therapy for B-cell malignancies may ultimately be feasible. Further work will focus on the use of B-cell specific ligands, such as anti-immunoglobulin-polylysine, and on the production of conjugates stable by intravenous injection.

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REFERENCES

1. Breitman ML, Rombola H, Maxwell IH, Klintworth GK and Bernstein A. Genetic ablation in transgenic mice with an attenuated dephtheria toxin A gene. *Mol Cell Biol* 1990;10:474.
2. Breitman ML, Clapoff S, Rossant J, Tsui LC, Glode LM, Maxwell IH and Bernstein A. Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. *Science* 1987;238:1563.
3. Palmiter RD, Behringer RR, Quaife CJ, Maxwell F, Maxwell H and Brinster RL. Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* 1987;50:435.
4. Landel CP, Zhao J, Bok D and Evans GA. Lens-specific expression of recombinant ricin induces developmental defects in the eyes of transgenic mice. *Genes Dev* 1988;2(9):1168.
5. Behringer RR et al. Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes and Development* 1988;2:453.
6. Glode LM. Dose limiting medullary toxicity of high dose chemotherapy. *Exp Hematol* 1979;7 Suppl 5:265.
7. Cooper MD. Current concepts. B lymphocytes. Normal development and function. *N Engl J Med* 1987;317(23):1452.
8. Vose JM, Armitage JO, Bierman PJ, Weisenburger DD, Hutchins M, Dowling MD, Moravec DF, Sorensen S, Okerbloom J, Bascom G et al. Salvage therapy for relapsed or refractory non-Hodgkin's lymphoma utilizing autologous bone marrow transplantation. *Am J Med* 1989;87(3):285.
9. Beral V, Peterman T, Berkelman R and Jaffe H. AIDS-associated non-Hodgkin lymphoma. *Lancet* 1991;337(8745):805.
10. Houle AM, McLoire GA, Churchill BM, Khoury AE, Karvey E and Herbert D. Rapid development of an immunoblastic lymphoma and death in children following cadaveric renal transplant. *J Pediatr Surg* 1992;27(5):626.
11. FitzGerald D and Pastan I. Targeted toxin therapy for the treatment of cancer. *J Natl Cancer Inst* 1989;81(19):1455.
12. Ghetie M-A, Tucker K, Richardson J, Uhr JW and Vitetta ES. *Blood* 1992;9(1):2315.
13. Jansen B, Vallera DA, Jaszcz WB, Nguyen D and Kersey JH. *Cancer Res* 1992;52:1314.
14. Maxwell IH, Maxwell F and Glode LM. Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: a possible strategy for inducing cancer cell suicide. *Cancer Research* 1986;46:4660.
15. Maxwell F, Maxwell IH and Glode LM. Cloning, sequence determination, and expression in transfected cells of the coding sequence for the tox 176 attenuated diphtheria toxin A chain. *Mol Cell Biol* 1987;7(4):1576.
16. Maxwell IH, Glode LM and Maxwell F. Expression

of the diphtheria toxin A-chain coding sequence under the control of promoters and enhancers from immunoglobulin genes as a means of directing toxicity to B-lymphoid cells. *Cancer Res* 1991;51:4299.
17. Maxwell IH, Glode LM and Maxwell F. Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. *Leukemia and Lymphoma* 1992;7:457.
18. Wagner E, Zatloukal K, Cotten M, Kirlappos H, Mechtler K, Curiel DT and Birnstiel ML. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc Nat Acad Sci* 1992;89(13):6099.
19. Curiel DT, Wagner E, Cotten M, Birnstiel ML, Agarwal S, Li C-M, Loechel S and Hu P-C. High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. *Human Gene Ther* 1992;3(2)147.
20. Curiel TJ, Cook D, Bogedain C, Jilg W, Harrison GS, Cotton M, Curiel DT and Wagner E. Efficient foreign gene expression in Epstein-Barr virus transformed human B-cells. *Virology* 1994;(In Press).
21. Cannon MJ, Pisa P, Fox RI and Cooper NR. Epstein-Barr virus induces aggressive lymphoproliferative disorders of human B-cell origin in SCID/hu chimeric mice. *J Clin Invest* 1990;85:1333.
22. Purtilo D, Falk K, Pirruccello SJ et al. SCID mouse model of Epstein-Barr virus induced lymphomagenesis of immunodeficient humans. *Int J Cancer* 1991;47:510.
23. Rowe M, Young LS, Crocker J, Stokes H, Henderson S and Rickinson AB. Epstein-Barr virus (EBV)-associated lymphoproliferative disease in the SCID mouse model: implications for the pathogenesis of EBV-positive lymphomas in man. *J Exp Med* 1991;173:147.
24. Wagner E, Cotten M, Mechtler K, Kirlappos H and Birnstiel ML. DNA-binding transferrin conjugates as functional gene delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. *Bioconjugate Chem* 1991;2:226.
25. Wagner E, Zenke M, Cotten M, Beug H and Birnstiel ML. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci* 1990;87:3410.
26. deWet JR, Wood KV, Deluca M, Helsinki DR and Subramani S. *Mol Cell Biol* 1987;7:725.
27. Plank C, Zatloukal K, Cotten M, Mechtler K and Wagner E. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem* 1992;3:533.
28. deWet JR, Wood KV, Deluca M, Helsinki DR and Subramani S. *Mol Cell Biol* 1987;7:725.
29. Walls EV and Crawford DH. Klaus GGB. ed. 1989 Oxford Press. p.149.
30. Lim K and Chae C B. *BioTechniques* 1989;7:576.
31. Loyter A, Scangos GA and Ruddle FH. Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc Natl Acad Sci* 1982;79:422.
32. Mannino RJ and Gould-Fogerite S. Liposome mediated gene transfer. *Biotechniques* 1988;6:682.
33. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A and Felgner PL. Direct gene transfer into mouse muscle in vivo. *Science* 1990;247:1465.
34. Brigham KL, Meyrick B, Christman B, Magnuson M, King G and Berry LC Jr. Rapid communication: In vivo transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle. *Am J Med Sci* 1989;298:278.
35. Wu GY and Wu CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem* 1987;262:4429.
36. Wu C, Wilson J, and Wu G. Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. *J Biol Chem* 1989;264:16985.
37. Cotten M, Langle-Rouault F, Kirlappos H, Wagner E, Mechtler K, Zenke M, Beug H, and Birnstiel ML. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: Stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc Natl Acad Sci* 1990;87:4033.
38. Curiel DT, Agarwal S, Romer N, Wagner E, Cotten M, Birnstiel ML and Boucher RC. Gene transfer to respiratory epithelial cells via the receptor-mediated endocytosis pathway. *Am J Respir Cell Mol Biol* 1992;6:247.
39. Huckett B, Ariatti M and Hawtrey AO. Evidence for targeted gene transfer by receptor-mediated endocytosis. *Biochem Pharmacol* 1990;40:253.
40. Rosenkranz AA, Yachmenev SV, Jans DA, Serebryakova NV, Murav'ev VI, Peters R and Sobolev AS. Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Exp Cell Res* 1992;199:323.
41. Testa U. Transferrin receptors: Structure and function. *Curr Top Hematol* 1985;5:127.
42. Zenke M, Steinlein P, Wagner E, Cotten M, Beug H and Birnstiel ML. Receptor-mediated endocytosis of transferrin-polycation conjugates: An efficient

way to introduce DNA into hematopoietic cells. *Proc Natl Acad Sci* 1990;87:3655.
43. Wu GY and Wu CH. *J Biol Chem* 1988;263:14621.
44. Wu GY, Wilson JM, Shalaby F, Grossman M, Shafritz DA and Wu CH. *J Biol Chem* 1991;266:14338.
45. Wilson JM, Grossman M, Wu CH, Chowdhury NR, Wu GY and Chowdhury JR. *J Biol Chem* 1992;267:963.
46. Wilson JM, Grossman M, Cabrera JA, Wu CH and Wu GY. *J Biol Chem* 1992;267:11483.
47. Cotten M, Wagner E and Birnstiel ML. *Methods Enzymol* 1993;217:618.
48. Zatloukal K, Wagner E, Cotten M, Phillips S, Plank C, Steinlein P, Curiel DT and Birnstiel ML. *N Y Acad Sci* 1992;(In Press)
49. Testa U. Transferrin receptors: Strucure and function. *Curr Top Hematol* 1985;5:127.
50. Newman R, Schneider C, Sutherland R, Vodinelich L, and Greaves M. *Trends Biochem Sci* 1982;7:397.
51. Friedmann T. The evolving concept of gene therapy. *Hum Gene Ther* 1990;1(2):175.
52. Crystal RG. Gene Therapy Meeting, Washington, D.C. 1992.
53. Juillard V, Godfrin D, Villefroy P, Ragot T, Perricaudet M, Guillet J-G and Venet A. Engineered Vaccines for Cancer and AIDS. San Francisco, CA 1993. Meeting abstract no. 13.
54. Armentano D, Yu SF, Kantoff PW, von Ruden T, Anderson WF and Gilboa E. *J Virol* 1987;61(5):1647.
55. Harrison GS, Maxwell F, Long CJ, Rosen CA, Glode LM and Maxwell IH. Activation of a diphtheria toxin A gene by expression of human immunodeficiency virus-1 Tat and Rev proteins in transfected cells. *Human Gene Therapy* 1991;2:53.
56. Harrison GS, Long CJ, Maxwell F, Glode LM and Maxwell IH. Inhibition of HIV production in cells containing an integrated, HIV-regulated diphtheria toxin A chain gene. *Aids Res and Human Retrov* 1992;8:39.
57. Harrison GS, Long CJ, Curiel TJ, Maxwell F and Maxwell IH. Inhibition of human immunodeficiency virus-1 production resulting from transduction with a retrovirus containing an HIV-regulated diphtheria toxin A chain gene. *Human Gene Therapy* 1992;3:461.
58. Curiel TJ, Cook D, Wang Y, Ghosh S, Hahn B and Harrison G. Long-term inhibition of clinical and laboratory human immunodeficiency virus strains in human T-cell lines containing and HIV-regulated diphtheria toxin A chain gene. *Human Gene Therapy* 1993;4:741.