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A Method of Limited Replication for the Efficient *In Vivo* Delivery of Adenovirus to Cancer Cells

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ABSTRACT

Replication-deficient viral vectors are currently being used in gene transfer strategies to treat cancer cells. Unfortunately, viruses are limited in their ability to diffuse through tissue. This makes it virtually impossible to infect the majority of tumor cells *in vivo* and results in inadequate gene transfer. This problem can be addressed by allowing limited viral replication. Limited viral replication facilitates greater penetration of virions into tissue and can improve gene transfer. We have developed a strategy of limited viral replication using AdRSVlaclys, a chemically modified E1-deleted adenovirus, to codeliver an exogenous plasmid encoding the adenovirus E1 region. This system allows one round of viral replication. We examined the effect of this limited adenovirus replication *in vitro* and *in vivo*. In culture, codelivery of virus and pE1 resulted in a large increase in infected cells when compared with control cells exposed to virus and pUC19. In experiments on nude mice bearing HeLa ascites tumors, intraperitoneal injection of AdRSVlaclys/pE1 resulted in a significantly higher percentage of infected HeLa cells as compared with the PBS controls ($p < 0.05$) or the AdRSVlaclys/pUC19 controls ($p < 0.01$). These data demonstrate that the transcomplementation of replication-deficient adenovirus with exogenous E1 DNA leads to limited replication, and this controlled replication enhances gene transfer efficiency of adenovirus *in vivo*.

OVERVIEW SUMMARY

Replication-defective viral vectors are limited in their ability to diffuse through tissue. This poses a problem for treating tumors *in vivo* using gene transfer. This article demonstrates that limited replication of adenovirus leads to greater gene transfer efficiency *in vitro* and *in vivo* without introducing additional safety concerns beyond traditional adenovirus administration. This has implications for the improvement of current gene transfer methods for treating cancer.

INTRODUCTION

VIRAL VECTORS are among the most efficient vehicles for gene transfer *in vitro* and *in vivo* (Mulligan, 1993). For this reason, replication-deficient viral vectors have been used in various gene transfer approaches to treat cancer cells (Crystal, 1995). Unfortunately, this approach is limited in that it is nearly impossible to infect the majority of tumor cells *in vivo* owing to physical constraints imposed by both the virus and tumor. Tumors *in vivo* are usually present as solid masses or sheets many layers thick, as opposed to the easily infected monolayers in cell culture. Typical virions are large enough to prevent significant diffusion through these cell layers. In addition, in some *in vivo* animal models the amount of virus administered is limited by the volume that can actually be physically injected.

The limitations in vector delivery present a serious problem in targeting cancerous cells *in vivo* using gene transfer. Delivery of a cytotoxic or tumor-suppressing virus may temporarily slow down tumor growth, but is doomed ultimately to fail if some tumor cells are left unharmed. Even strategies with a "bystander effect" (such as the herpes simplex virus thymidine kinase gene) require that a significant amount of tumor cells be infected, and most successful animal models of thymidine kinase delivery have involved the administration of retroviral producer cell lines to ensure that this is the case (Culver *et al.*, 1992; Takamiya *et al.*, 1992; Barba *et al.*, 1994).

It may be possible to circumvent viral delivery problems with controlled replication of a viral vector *in vivo* (Goldsmith,

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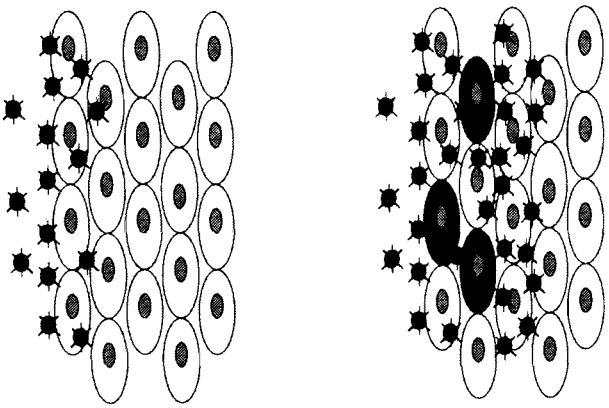

FIG. 1. Schematic diagram of limited replication *in vivo*. On the left, conventional techniques result in gene transfer only near the surface of tissue. On the right, limited replication allows virus diffusion to deeper areas in the tissue. Viral replication is occurring in dark cells.

1994). This could allow greater penetration of the virus beyond the first several cell layers (see Fig. 1). Limited replication would also increase the effective local viral titer. For controlled replication to occur, the genes necessary for viral replication must be codelivered with the viral vector. However, these genes must not be engineered into the viral genome or the result would be a fully replication-competent virus. We have developed a model of controlled *in vivo* replication using adenovirus. Adenovirus is relatively chemically stable and can be manipulated by adding polylysine without completely destroying infectious activity (Wagner *et al.*, 1992; Cristiano *et al.*, 1993; Fisher and Wilson, 1994). This allows exogenous DNA to bind to the modified adenovirus by electrostatic interactions and "piggyback" its way into infected cells. By using this method to codeliver a plasmid encoding the adenovirus E1 region, standard E1-deleted adenovirus can enter a round of replication. We will refer to this method as *limited replication*, because barring recombination events, all of the progeny of this process will be replication deficient, eliminating virus replication after one round. Adenovirus also has other favorable properties for use in this system. Adenovirus can be produced at high titers and infect a broad range of dividing and nondividing cells (Kozarsky and Wilson, 1993). In addition, adenovirus has a lytic life cycle, which lyses the cell after virus replication is complete and thus may expose more tumor surface area for further gene transfer.

Other viral vectors all have one or more deficiencies for use in such a system. Retrovirus integrates into the host genome and will continually produce virus for the life of the cell (assuming the transgene is not lethal). Both of these properties are safety concerns (Varmus, 1988). Retrovirus is also surrounded by a relatively unstable lipid bilayer that is difficult to modify chemically without completely abolishing biologic activity (Rosenberg *et al.*, 1997). Herpes simplex virus has a similar bilayer and presents possible toxicity concerns (Glorioso *et al.*, 1997). Adeno-associated virus (AAV) cannot replicate without adenovirus (Fisher *et al.*, 1997), which defeats the purpose of using AAV in the first place. Therefore, adenovirus appears to be the ideal choice for this system.

Here we demonstrate that transcomplementation of replication-deficient adenovirus with exogenous E1 DNA leads to limited viral replication. We have constructed a simple system in which adenovirus plasmid DNA complexes can be used to achieve this effect *in vitro*. Finally, we have extended this study to demonstrate enhanced gene delivery in a relevant *in vivo* model. This addresses a major problem in current approaches to cancer gene therapy.

MATERIALS AND METHODS

In vitro *limited replication of adenovirus using liposomes*

Construction of AdRSVlacZ has been described (Davidson *et al.*, 1994). The plasmid pE1, which contains the E1A and E1B regions of adenovirus (nucleotides 1–5778), was a gift from E. White (White and Cipriani, 1989). This plasmid, as well as all plasmids described hereafter, was purified by centrifugation through a CsCl–ethidium bromide gradient (Sambrook *et al.*, 1989). HeLa cells (human cervical carcinoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% P/S (penicillin [100 international units/ml] and streptomycin [100 μg/ml]). SKOV3 (human ovarian carcinoma) cells were maintained in McCoy's 5A medium containing 10% FBS and 1% P/S.

Cells were seeded in six-well dishes and allowed to reach 80% confluence. Lipofectamine (GIBCO-BRL, Gaithersburg, MD) was used to lipofect either 1 μg of pUC19 or 1 μg of pE1 into each well. Lipofection procedures were done as recommended by the manufacturer. After 24 hr at 37°C, the medium was replaced with 2 ml of fresh medium (2% FBS, 1% P/S) containing 1×10^6 AdRSVlacZ viral particles. Each well was fed with 2 ml of medium (2% FBS, 1% P/S) on day 3 and stained on day 6 or 8 with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Before staining, cells were fixed in 0.5% glutaraldehyde for 10 min and washed twice with phosphate-buffered saline (PBS)–1 m*M* $MgCl_2$. Stained cells were examined by light microscopy and photographed.

Preparation of adenovirus/polylysine complex

Polylysine was cross-linked to replication-deficient, recombinant adenovirus using a slightly modified version of a procedure described elsewhere (Fisher and Wilson, 1994). To maleimide-activate the virus, AdRSVlacZ was prepared and concentrated on a CsCl gradient. After centrifugation, this virus was immediately desalted on a 15-ml Sephadex G-50 column equilibrated with PBS, pH 7.0. The viral particle concentration was determined by absorbance at 260 nm, on the assumption that one absorbance unit equals 1×10^{12} viral particles/ml. The titered virus was diluted to approximately 5×10^{12} particles/ml and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL), was added to a final concentration of 25 m*M*. This reaction was rocked gently at room temperature for 7.5 min, then stopped by adding a one-tenth volume of 1 *M* Tris, pH 7.0.

While maleimide-activating the adenovirus, a thiol group

was added onto the amino terminus of poly-L-lysine (54 kDa; Sigma, St. Louis, MO) using 2-iminothiolane essentially as described (Fisher and Wilson, 1994). The 2-iminothiolane was removed on a 15-ml Sephadex G-25 column equilibrated with 100 m*M* Tris (pH 7.1 at 4°C), 100 m*M* NaCl, and 2 m*M* EDTA. The first fraction that contained virus as determined by absorbance at 220 nm was saved for further use.

The purified, thiolated polylysine was diluted to a volume equal to that of the maleimide-activated adenovirus mixture using 100 m*M* Tris (pH 7.1 at 4°C), 100 m*M* NaCl, and 2 m*M* EDTA. Then the maleimide-activated virus and modified polylysine were mixed and rocked gently at 4°C for 90 min. The polylysine is cross-linked to the virus at this step. Unreacted maleimide groups were blocked by adding a one-tenth volume of 1 *M* 2-mercaptoethylamine and incubating for 20 min at room temperature. To remove unincorporated polylysine and concentrate the virus, the polylysine-modified virus was then centrifuged on a CsCl gradient. The virus was desalted on a 15-ml Sephadex G-50 column. Storage was in 50 m*M* Tris (pH 7.1), 50 m*M* NaCl, 1 m*M* EDTA, and 50% glycerol at −80°C. This modified virus will be referred to as AdRSVlaclys.

Successful cross-linking was verified by a gel mobility shift assay. In a total volume of 100 μl of PBS, 200 μg of the plasmid pK7GFP (a gift from I. Macara; Casey *et al.*, 1996) was incubated with varying amounts of modified or unmodified virus for 30 min at room temperature. These samples were resolved by gel electrophoresis on a 1.5% agarose gel.

Transfection of exogenous DNA in vitro *using the adenovirus/polylysine complex*

We determined that the amount of cross-linked adenovirus necessary to infect more than 80% of HeLa cells was approximately 1×10^5 viral particles/cell. Therefore, this titer was used for the following infections. AdRSVlacZ or AdRSVlaclys was incubated with or without pK7GFP in a total volume of 100 μl of PBS for 20–30 min at room temperature. HeLa cells at 1×10^5 cells/well in 12-well dishes were washed once with PBS and 0.5 ml of DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were incubated at 37°C overnight. The next day, the medium was replaced with 1 ml of DMEM (10% FBS, 1% P/S) per well. Each experiment was performed in duplicate. One set was trypsinized, fixed, and stained with X-Gal to monitor infection efficiency. The other set was trypsinized, washed once with PBS, and visualized for green fluorescent protein (GFP) using a fluorescence microscope to monitor adenovirus-mediated transfection efficiency. The percentage of infected or transfected cells was determined with a hemacytometer and data represent the mean ± standard deviation of two experiments.

In vitro *limited replication of adenovirus using the adenovirus/polylysine complex*

AdRSVlacZ or AdRSVlaclys was incubated with either 2 μg of pUC19 or 2 μg of pE1 in a total volume of 200 μl of PBS for 20–30 min at room temperature. HeLa or SKNSH cells at 1.5×10^5/well in six-well dishes were washed once with PBS and 1 ml of DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were placed at 37°C overnight. The next day, medium was replaced with 2 ml of DMEM (2% FBS, 1% P/S). Cells were fed on days 3 and 6 with 2 ml of DMEM (2% FBS, 1% P/S). No medium was removed during this time. On day 8 postinfection, the cells were fixed and stained with X-Gal to monitor limited replication of the adenovirus. Staining was either done in the dish and subsequently photographed or done in suspension and the percentage of cells infected was determined with a hemacytometer.

In vivo *limited replication of adenovirus*

Nude mice (CD-1 *nu/nu*; Charles River, Wilmington, MA) were injected intraperitoneally with 1×10^7 HeLa cells. Four days later the mice were injected with a virus/DNA mixture in 100 μl of PBS, using a Hamilton syringe. Each injection contained 7×10^9 particles of AdRSVlaclys with either 2 μg of pUC19 or 2 μg of pE1. Two mice were injected with PBS only. These injections were repeated every other day for a total of four injections.

Eight days after the final injection (to allow time for limited replication) mice were sacrificed. The peritoneal cavity was washed twice with 1.5 ml of PBS and the resulting cell suspension was removed and saved. These samples were centrifuged at $350 \times g$ in a microcentrifuge for 2 min, washed once with PBS, and recentrifuged. The resulting pellets of HeLa and blood cells were resuspended in 1 ml of DMEM (10% FBS, 1% P/S) each and placed in six-well dishes in a total volume of 2 ml of medium. These dishes were cultured at 37°C for 2 days in order to allow most of the blood cells to die or lyse. The dishes were then washed six times with PBS and the remaining HeLa cells were fixed and stained with X-Gal for 48 hr at 37°C. Cells were examined by light microscopy and photographed. The percentage of infected cells was determined for each sample. Groups were compared using a one-sided Student *t* test.

Detection of the adenovirus hexon protein

Twenty-four hours after infection with the chemically modified AdRSVlaclys and 2 μg of either pUC12 or pE1, HeLa cells were harvested and attached to microscope slides. Immunohistochemistry using a fluorescein isothiocyanate (FITC)-conjugated anti-hexon IgG (Chemicon, Temecula, CA) was done using the protocol recommended by the supplier. Microscopy was performed with a Zeiss fluorescence microscope.

RESULTS

Independent codelivery of the pE1 plasmid and adenovirus in vitro

To test whether exogenous E1 plasmid DNA could transcomplement replication-deficient adenovirus *in vitro*, an independent plasmid lipofection was followed by AdRSVlacZ infection of HeLa or SKOV3 cells. A low titer of AdRSVlacZ was used for these experiments in order to make viral replication easily detectable. Over the course of the next 6 to 8 days, cells initially transfected with pE1 showed a substantially

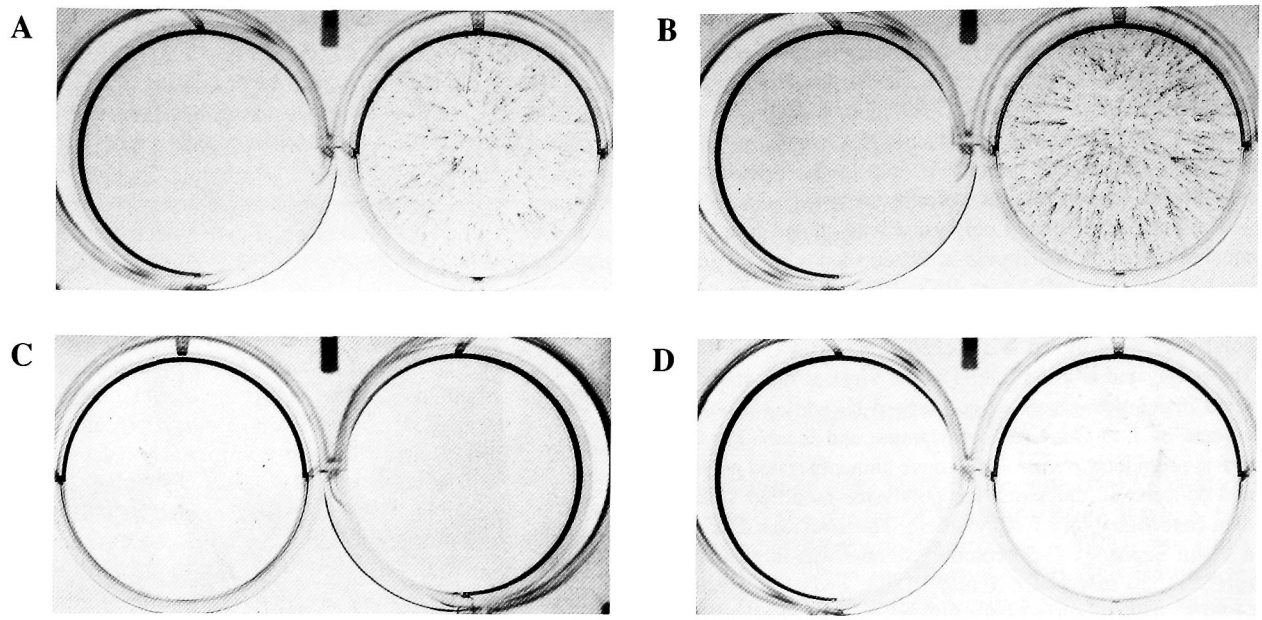

FIG. 2. Lipofection of plasmid DNA followed by adenoviral infection. Cells were lipofected with 1 μg of pUC19 (left) or pE1 DNA (right), then infected with AdRSVlacZ. X-Gal staining is shown at 6 and 8 days. (A) HeLa cells at 6 days; (B) HeLa cells at 8 days; (C) SKOV3 cells at 6 days; (D) SKOV3 cells at 8 days.

greater proportion of infected cells than those initially transfected with the pUC19 control (Fig. 2A). The staining pattern on E1-transfected cells was present in clusters, indicating areas where limited replication and subsequent cell lysis occurred. There was a greater amplification of the adenovirus in the HeLa cells. This may be due to either greater amplification of virus in HeLa cells or greater susceptibility to adenovirus infection of HeLa cells. This verifies our hypothesis that codelivery of pE1 will support viral replication.

Although this shows that the codelivery concept is feasible, the methodology used for these pilot experiments is not practical for general *in vivo* use. Liposomes have been shown to deliver genes *in vivo*, but the efficiency varies dramatically depending on cell type/animal model and would probably need to be extensively optimized for most scenarios (Gao and Huang, 1995). Thus, other methods were explored for the codelivery of pE1.

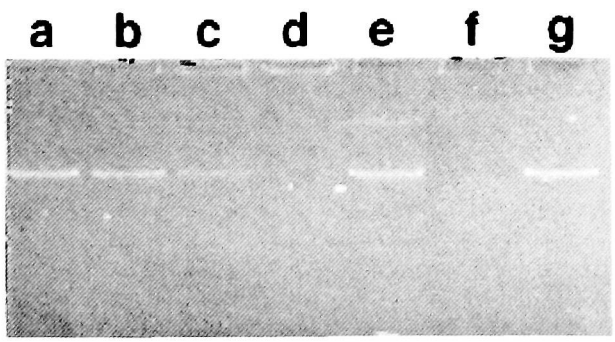

FIG. 3. Gel mobility shift of polylysine-modified adenovirus. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with 200 μg of plasmid DNA. Attachment of DNA to AdRSVlaclys results in a large complex with neutralized charge that does not run into the gel. Lane a, 2.5×10^9 particles of AdRSVlaclys plus DNA; lane b, 5×10^9 particles of AdRSVlaclys plus DNA; lane c, 1×10^{10} particles of AdRSVlaclys plus DNA; lane d, 2.5×10^{10} particles of AdRSBlaclys plus DNA; lane e, 2.5×10^{10} particles of AdRSVlacZ plus DNA; lane f, 2.5×10^{10} particles of AdRSVlacZ (no DNA); land g, DNA only.

Codelivery of plasmid DNA with adenovirus/polylysine complex

Polylysine-modified adenovirus has been shown to complex with and transfect plasmid DNA (Wagner *et al.*, 1992; Cristiano *et al.*, 1993; Fisher and Wilson, 1994). We planned to use such a virus to codeliver the pE1 plasmid. Our method of cross-linking polylysine to AdRSVlacZ was based on the procedure of Fisher and Wilson. This previously reported method essentially modified the entirety of the adenoviral capsid. The modified virus, AdRSVlaclys, would bind to plasmid DNA (Fig. 3).

We aimed next to demonstrate that AdRSVlaclys could transfect plasmid DNA without the assistance of tertiary substrates (such as a polylysine-modified cellular ligand). This would simplify infection procedures for future *in vivo* experiments. Approximately 15% of HeLa cells infected with AdRSVlaclys also introduced the pK7GFP plasmid when examined by fluorescence microscopy (Fig. 4). Because fluorescence microscopy to detect GFP is not terribly sensitive, we expect that the actual percentage of transfected cells is higher. In contrast, cells exposed to plasmid alone or plasmid with AdRSVlacZ exhibited no detectable GFP expression. Thus, the polylysine attached to adenovirus allows codelivery of plasmid DNA.

On the basis of these experiments, we predicted that delivery of the pE1 plasmid complexed with AdRSVlaclys would result in significantly enhanced gene transfer owing to a round

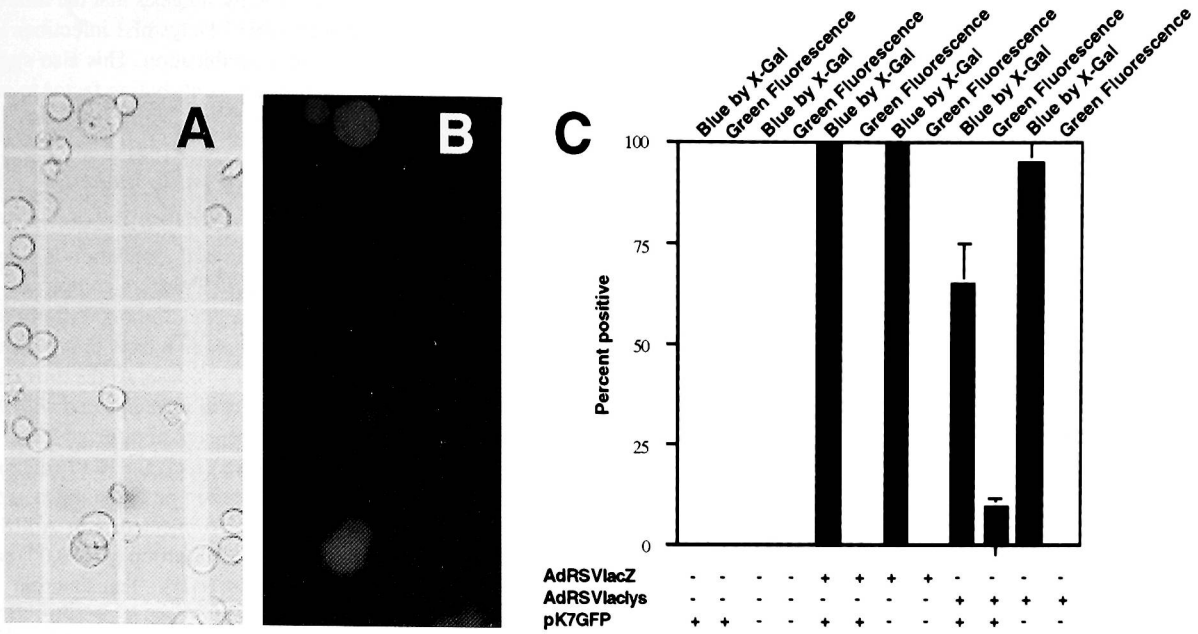

FIG. 4. Transfection of plasmid DNA with AdRSVlaclys. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with pK7GFP before infection of HeLa cells. (A) AdRSVlaclys-infected cells under light; (B) AdRSVlaclys-infected cells using fluorescence microscopy; (C) the infection and transfection efficiencies of various combinations of virus and DNA on HeLa cells. (A and B) magnification: ×200. Addition of plasmid DNA caused a slight decrease in infectivity. This is most likely due to reduced cell membrane binding because of the negatively charged DNA.

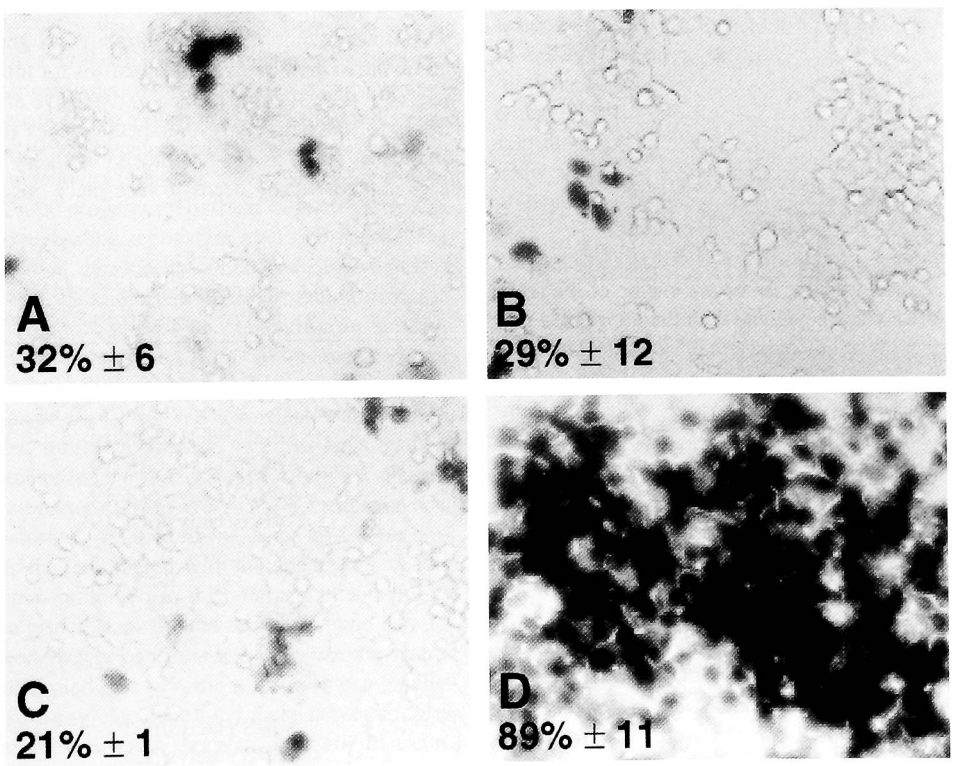

FIG. 5. Limited replication of AdRSVlaclys in HeLa cells. (A) AdRSVlacZ preincubated with pUC19; (B) AdRSVlacZ preincubated with pE1; (C) AdRSVlaclys preincubated with pUC19; (D) AdRSVlaclys preincubated with pE1. Original magnification ×200. Percent infected ± sSD is indicated for (A)–(D).

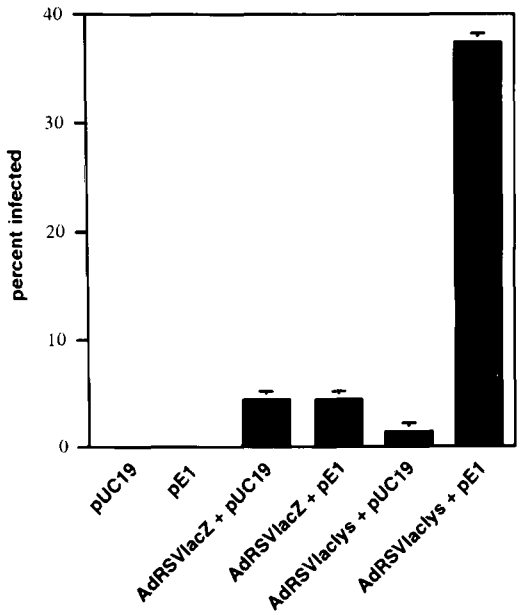

FIG. 6. Limited replication of AdRSVlaclys in SKNSH cells. The experiment described in Fig. 5 was extended to SKNSH cells.

of viral replication. Transfection of only 15% of infected cells translates into a great deal of additional virus when one considers that the typical adenovirus life cycle results in 10^4 viral progeny (Shenk, 1996). When AdRSVlaclys and pE1 were used to infect HeLa cells, a large increase in infected cells was observed by X-Gal staining when compared with the control AdRSVlaclys/pUC19 sample (Fig. 5). Once again, clusters of blue cells indicated areas of viral replication. As expected, infection of HeLa cells with AdRSVlacZ resulted in the same amount of gene transfer regardless of preincubation with pE1 or pUC19. This experiment generated analogous results when repeated with SKNSH (human neuroblastoma) cells, indicating that this effect is not specific to HeLa cells (Fig. 6).

It is possible that the replication observed in AdRSVlaclys/pE1 infected/transfected cells was due to recombination of the replication-defective AdRSVlaclys genome with the transfected pE1 plasmid to generate replication-competent helper virus. This presents a potential safety concern for future *in vivo* applications of this method. To address this concern, the AdRSVlaclys/pE1 infection was repeated on HeLa cells. These infections were harvested at 4 days postinfection, freeze–thawed, and the supernatant of this lysate was used to infect a new monolayer of HeLa cells in a 35-mm dish. These cells were monitored over the next 14 days for the appearance of cytopathic effect (CPE). As a positive control, HeLa cells were incubated with various dilutions of sub360, which is both the original adenovirus strain used to generate AdRSVlacZ (Davidson *et al.*, 1994) and the predicted recombination product of pE1 and AdRSVlaclys. Dilutions ranged from 10^4 viral particles (the amount of virions generated from one viral life cycle) to 10^8 viral particles/well. All dilutions of sub360 produced CPE within the 2-week time period, as evidenced by completely detached, swelled, or lysed cells. Monolayers from HeLa cells cultured with the AdRSVlaclys/pE1 infection extract were intact after 14 days and showed no signs of CPE (data not shown). This strongly suggests that the limited viral replication observed with AdRSVlaclys/pE1 infections is due to transcomplementation, not recombination. This also suggests that helper virus is not a significant contributing factor to any of the results presented in this study.

Enhanced gene transfer in vivo *using limited replication of AdRSVlaclys*

To demonstrate the ability of limited replication to improve transduction efficiency *in vivo*, AdRSVlaclys complexes were injected into mice bearing HeLa ascites tumors. After a series of four injections, the virus was allowed 8 days to replicate, infect more cells, and express β-galactosidase. At the time of sacrifice, there was no apparent toxicity to mice injected with virus. X-Gal staining revealed a low percentage of infected HeLa cells in mice injected with PBS or AdRSVlaclys/pUC19 (data summarized in Table 1). A higher infection percentage was apparent in HeLa cells isolated from mice injected with AdRSVlaclys/pE1 when compared with the PBS group ($p < 0.05$) or the AdRSVlaclys/pUC19 group ($p < 0.01$). Transduction efficiency reached close to 90% in one of the mice (Fig. 7C). *In vivo*/virus replication was confirmed. E1 transfected cells infected with the *lacZ* recombinant adenovirus, but not the pUC19-transfected cells infected with the same recombinant virus, produced the adenovirus late hexon protein (Fig. 8). These data provide evidence that limited replication results in a significantly higher proportion of tumor cells infected *in vivo*.

DISCUSSION

Viral vectors are extremely attractive as gene transfer vehicles owing to their evolved mechanisms for introducing genetic material into cells. This has led to extensive efforts in adapting viruses for the delivery of therapeutic genes (Miller, 1992). In some cases (such as cystic fibrosis), delivery of the therapeutic gene theoretically needs to take place only in a small pro-

TABLE 1. EFFICIENCY OF GENE TRANSFER BY *in Vivo* LIMITED REPLICATION

Mouse number	*Treatment*	*Percent HeLa cells blue*
1	AdRSVlaclys/pUC19	14.0
2	AdRSVlaclys/pUC19	23.0
3	AdRSVlaclys/pUC19	9.4
4	AdRSVlaclys/pUC19	15.3
		15.425 ± 4.89
5	PBS	6.5
6	PBS	3.5
		5 ± 1.5
7	AdRSVlaclys/pE1	21.3
8	AdRSVlaclys/pE1	66.4
9	AdRSVlaclys/pE1	84.6
10	AdRSVlaclys/pE1	69.6
		60.475 ± 23.64

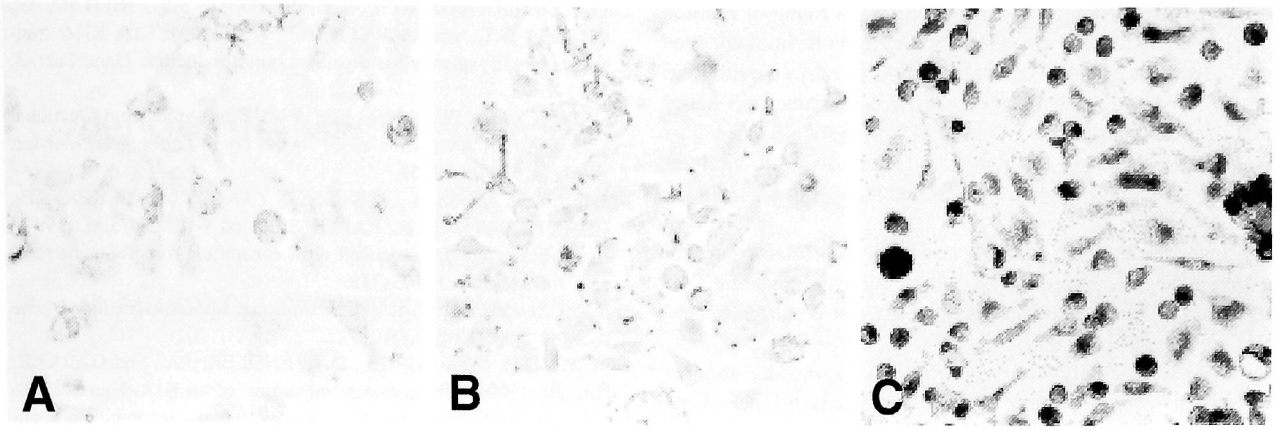

FIG. 7. Examples of limited replication of AdRSVlaclys *in vivo*. Ascites were injected, harvested, and stained as described in Materials and Methods. (A) PBS (mouse 5); (B) AdRSVlaclys with pUC19 (mouse 6); (C) AdRSVlaclys with pE1 (mouse 9). Original magnification: ×200.

portion of affected cells in order to produce significant benefits. However, in other cases, infection of the majority of the target cells may be necessary. For example, the proliferative nature of cancer dictates that it is necessary to infect the majority, if not all, transformed cells in order to achieve true clinical benefit from gene transfer. This problem is exacerbated by the extreme difficulty of transducing the majority of targeted cells *in vivo*, since viruses can diffuse only for limited distances in solid tissues.

Limited viral replication represents a strategy for improving the efficiency of transducing target cells *in vivo*. The feasibility of limited replication has been demonstrated here and by others *in vitro* with several cell lines, using a model system consisting of an E1-deleted adenoviral vector that could introduce exogenous E1 sequences (on a plasmid), permitting replication (Goldsmith *et al.*, 1994). Because these DNA sequences were separate from the adenovirus genome and thus were not incorporated into adenoviral progeny, all of the resultant virions were replication defective, ending replication after one round. Minimal helper virus was produced, which indicates that this method may be as safe as traditional adenovirus administration. Adenoviral thymidine kinase-mediated regression of subcutaneous tumors in nude mice arising from cells transfected with E1A *in vitro* was greater than regression of tumors arising from parental cells (Dion *et al.*, 1996). We have also extended these results to show that limited replication of adenovirus leads to enhanced gene transfer *in vivo*. The potential of the E1 plasmid with the adenovirus vector to form a replication-competent adenovirus could be further minimized by using an E1 plasmid with no sequences that are shared with the adenovirus vector.

The model system presented in this study is intended only to demonstrate the principle of limited replication and can obviously be improved. Safety concerns regarding the transforming potential of integrated pE1 plasmid sequences can be alleviated by engineering a suicide gene such as the herpes simplex virus thymidine kinase gene into the plasmid. Cells that contain the pE1 plasmid can then be purged from the body, using gancyclovir after the course of treatment. Furthermore, adenovirus cross-linking procedures that result in greater transfection and infection activity have been reported, and would likely lead to better *in vivo* results (Cristiano *et al.*, 1993). Ideally, though, chemical modification of the adenovirus capsid would not take place because it invariably decreases infectability. A plausible alternative would have the E1 sequences packaged inside the adenovirus capsid (but not in the genome), which would

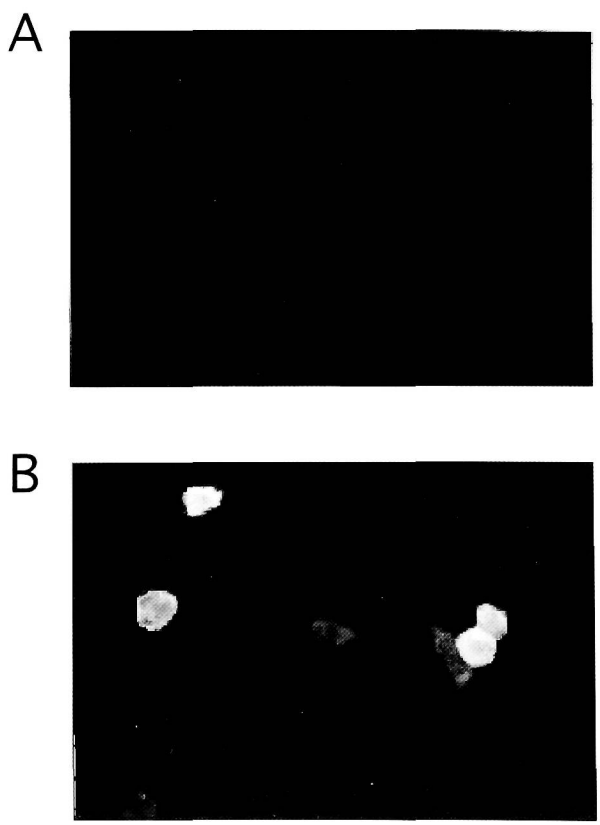

FIG. 8. *In vivo* expression of the adenovirus hexon protein in HeLa cells. Immunohistochemistry showing expression of the adenovirus hexon protein in HeLa cells infected with AdRSVlaclys conjugated with either pUC19 (A) or pE1 (B). Original magnification: ×200.

result in 100% of infected cells undergoing a round of replication, provided the cells are permissive. Current technology does not allow us to do this. Another possible scenario would be to place the E1 sequences in the adenovirus genome, but under tumor-specific promoters. This could be difficult since it is likely that leaky E1 expression would occur owing to viral enhancers present throughout the adenovirus genome (Shenk, 1996).

Limited replication could be especially useful for cancers arising in cavities where adenovirus is easily administered. Two examples of this are bladder and ovarian cancer. Bladder cancers are superficial tumors that arise focally, or diffusely, initially penetrating only a few cell layers of the bladder lumenal epithelium. Ovarian cancer initially spreads in the peritoneal cavity. In both of these cases, it may be possible to infect all transformed cells using limited replication of adenovirus. This would also be a probable improvement on current gene transfer methods in solid tumor masses, although it is still unlikely that all the cells of a large tumor mass can be transduced. In summary, we have shown that limited replication of adenovirus is safe and results in enhanced gene transfer both *in vitro* and *in vivo*. This addresses the problem of inadequate gene transfer in cancer gene therapy and may eventually lead to potential clinical benefits in the future.

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REFERENCES

BARBA, D., HARDIN, J., SADELAIN, M., and GAGE, F.H. (1994). Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. Proc. Natl. Acad. Sci. U.S.A. **91,** 4348–4352.

CASEY, K.L., RICHARDS, S.A., LOUNSBURY, K.M., and MACARA, I.G. (1996). Evidence using a green fluorescent protein–glucocorticoid receptor chimera that the RAN/TC4 GTPase mediates an essential function independent of nuclear protein import. J. Cell. Biol. **133,** 985–996.

CRISTIANO, R.J., SMITH, L.C., KAY, M.A., BRINKLEY, B.R., and WOO, S.L. (1993). Hepatic gene therapy: Efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus–DNA complex. Proc. Natl. Acad. Sci. U.S.A. **90,** 11548–11552.

CRYSTAL, R.G. (1995). Transfer of genes to humans: Early lessons and obstacles to success. Science **270,** 404–410.

CULVER, K.W., RAM, Z., WALLBRIDGE, S., ISHII, H., OLDFIELD, E.H., and BLAESE, R.M. (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science **256,** 1550–1552.

DAVIDSON, B.L., DORAN, S.E., SCHEWACH, D.S., LATTA, J.M., HARTMAN, J.W., and ROESSLER, B.J. (1994). Expression of *Escherichia coli* β-galactosidase and rat HPTR in the CNS of *Macaca mulatta* following adenoviral mediated gene transfer. Exp. Neurol. **125,** 258–267.

DION, L.D., GOLDSMITH, K.T., STRONG, T.V., BILBAO, G., CURIEL, D.T., and GARVER, R.I., JR. (1996). E1A RNA transcripts amplify adenovirus-mediated tumor reduction. Gene Ther. **3,** 1021–1025.

FISHER, K.J., and WILSON, J.M. (1994). Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer. Biochem. J. **299,** 49–58.

FISHER, K.J., JOOSS, K., ALSTON, J., YANG, Y., HAECKER, S.E., HIGH, K., PATHAK, R., RAPER, S.E., and WILSON, J.M. (1997). Recombinant adeno-associated virus for muscle directed gene therapy. Nature Med. **3,** 306–312.

GAO, X., and HUANG, L. (1995) Cationic liposome-mediated gene transfer. Gene Ther. **2,** 710–722.

GOLDSMITH, K.T., CURIEL, D.T., ENGLER, J.A., and GARVER, R.I., JR. (1994). *Trans* complementation of an E1A-deleted adenovirus with codelivered E1A sequences to make recombinant adenoviral producer cells. Hum. Gene Ther. **5,** 1341–1348.

GLORIOSO, J.C., GOINS, W.F., SCHMIDT, M.C., OLIGINO, T., KRISKY, D.M., MARCONI, P.C., CAVALCOLI, J.D., RAMAKRISHNAN, R., POLIANI, P.L., and FINK, D.J. (1997). Engineering herpes simplex virus vectors for human gene therapy. Adv. Pharmacol. **40,** 103–136.

KOZARSKY, K.F., and WILSON, J.M. (1993). Gene therapy: Adenovirus vectors. Curr. Opin. Genet. Dev. **3,** 499–503.

MILLER, A.D. (1992). Human gene therapy comes of age. Nature (London) **357,** 455–460.

MULLIGAN, R.C. (1993). The basic science of gene therapy. Science **260,** 926–932.

ROSENBERG, A.R., DELAMARRE, L., PIQUE, C., PHAM, D., and DOKHELAR, M.C. (1997). The ectodomain of the human T-cell leukemia virus type 1 TM glycoprotein is involved in postfusion events. J. Virol. **71,** 7180–7186.

SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T., editors. (1989). In *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) pp. 1.21–1.52.

SHENK, T. (1996). *Adenoviridae:* The viruses and their replication. In *Fields Virology.* B.N. Fields, D.M. Knipe, P.M. Howley, *et al.*, editors. (Lippincott-Raven Publishers, Philadelphia, PA) pp. 2111–2148.

TAKAMIYA, Y., SHORT, M.P., EZZEDDINE, Z.D., MOOLTEN, F.L., BREAKEFIELD, X.O., and MARTUZA, R.L. (1992). Gene therapy of malignant brain tumors: A rat glioma line bearing the herpes simplex virus type 1-thymidine kinase gene and wild type retrovirus kills other tumor cells. J. Neurosci. Res. **33,** 493–503.

VARMUS, H. (1988). Retroviruses. Science **240,** 1427–1435.

WAGNER, E., ZATLOUKAL, K., COTTEN, M., KIRLAPPOS, H., MECHTLER, K., CURIEL, D.T., and BIRNSTIEL, M.L. (1992). Coupling of adenovirus to transferrin–polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. Proc. Natl. Acad. Sci. U.S.A. **89,** 6099–6103.

WHITE, E., and CIPRIANI, R. (1989). Specific disruption of intermediate filaments and the nuclear lamina by the 19-kDa product of the adenovirus E1B oncogene. Proc. Natl. Acad. Sci. U.S.A. **86,** 9886–9890.

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2. Makoto Sunamura, Masaru Oonuma, Fuyuhiko Motoi, Hisashi Abe, Yukoh Saitoh, Toru Hoshida, Shigeru Ottomo, Akira Horii, Seiki Matsuno. 2002. Gene Therapy for Pancreatic Cancer Targeting the Genomic Alterations of Tumor Suppressor Genes using Replication-selective Oncolytic Adenovirus. *Human Cell* **15**:3, 138-150. [CrossRef]
3. Vera Braiden , Akira Ohtsuru , Yujo Kawashita , Fumio Miki , Takahiro Sawada , Masahiro Ito , Yu Cao , Yasufumi Kaneda , Takehiko Koji , Shunichi Yamashita . 2000. Eradication of Breast Cancer Xenografts by Hyperthermic Suicide Gene Therapy under the Control of the Heat Shock Protein PromoterEradication of Breast Cancer Xenografts by Hyperthermic Suicide Gene Therapy under the Control of the Heat Shock Protein Promoter. *Human Gene Therapy* **11**:18, 2453-2463. [Abstract] [PDF] [PDF Plus]
4. Ruben Hernandez-Alcoceba , Michael Pihalja , Max S. Wicha , Michael F. Clarke . 2000. A Novel, Conditionally Replicative Adenovirus for the Treatment of Breast Cancer That Allows Controlled Replication of E1a-Deleted Adenoviral VectorsA Novel, Conditionally Replicative Adenovirus for the Treatment of Breast Cancer That Allows Controlled Replication of E1a-Deleted Adenoviral Vectors. *Human Gene Therapy* **11**:14, 2009-2024. [Abstract] [PDF] [PDF Plus]
5. Wahiba Oualikene , Linda Lamoureux , Joseph M. Weber , Bernard Massie . 2000. Protease-Deleted Adenovirus Vectors and Complementing Cell Lines: Potential Applications of Single-Round Replication Mutants for Vaccination and Gene TherapyProtease-Deleted Adenovirus Vectors and Complementing Cell Lines: Potential Applications of Single-Round Replication Mutants for Vaccination and Gene Therapy. *Human Gene Therapy* **11**:9, 1341-1353. [Abstract] [PDF] [PDF Plus]