

Replicative retroviral vectors for cancer gene therapy

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Poor efficiency of gene transfer into cancer cells constitutes the major bottleneck of current cancer gene therapy. We reasoned that because tumors are masses of rapidly dividing cells, they would be most efficiently transduced with vector systems allowing transgene propagation. We thus designed two replicative retrovirus-derived vector systems: one inherently replicative vector, and one defective vector propagated by a helper retrovirus. *In vitro*, both systems achieved very efficient transgene propagation. In immunocompetent mice, replicative vectors transduced >85% tumor cells, whereas defective vectors transduced <1% under similar conditions. It is noteworthy that viral propagation could be efficiently blocked by azido-thymidine, *in vitro* and *in vivo*. In a model of established brain tumors treated with suicide genes, replicative retroviral vectors (RRVs) were approximately 1000 times more efficient than defective adenoviral vectors. These results demonstrate the advantage and potential of RRVs and strongly support their development for cancer gene therapy.

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The main drawback of currently utilized gene transfer vectors is their low efficacy of target cell transduction.¹ This problem is most acutely apparent in cancer gene therapy. Indeed, one-shot delivery systems, based on nonviral or defective viral vectors, cannot efficiently transduce a continuously growing malignant target tissue.^{2,3} We thus reasoned that vectors that can propagate should be better suited to reach satisfying transduction of growing target tissues. For this approach, a replicative vector system that is safe for the patient and which does not pose a threat of dissemination in the environment is needed.

Already in the 1950s, wild-type (wt) oncolytic viruses were used for “virotherapy” in cancer patients, showing some antitumor activity but also significant side effects.^{4–7} Later, in the 1990s, the field was rekindled by studies conducted with replicative herpes-, adeno-, or Newcastle disease viruses. Results of experimental studies conducted with these viruses^{8–11} as well as initial clinical trials^{12,13} were encouraging. More recently, recombinant replicative viruses harboring therapeutic genes have been designed and are now under development.^{14–20}

Retroviruses harbor several interesting properties as backbones for replicative vectors for cancer gene therapy. Firstly, they are efficient gene transfer vectors²¹ transmitted

mainly by blood or sexual exposure, which limits dissemination.²² Secondly, oncoretroviruses can only be expressed in dividing cells.²³ This restriction provides a desirable specificity towards rapidly growing tumors invading organs consisting mainly of more slowly dividing or quiescent cells. Thirdly, the existence of efficient antiretroviral drugs, such as azido-thymidine (AZT), provides the possibility to block retroviral replication for safety reasons.²⁴ Replicative retroviral vectors (RRVs) have already been designed for gene transfer studies or more recently for gene therapy.^{25–27} However, their therapeutic potential has not yet been evaluated in a relevant model for cancer gene therapy, i.e., treatment of established tumors in immunocompetent animals. Here, we compared the transduction potential of newly developed murine leukemia virus (MLV)-derived replication-competent vector systems to conventional defective retroviral vectors (DRVs), *in vitro* and *in vivo*. The gene for enhanced green fluorescence protein (EGFP) was used as a reporter to compare individual transduction rates of these vectors and, furthermore, a fusion between the EGFP and equine herpes virus type 4 thymidine kinase (EHV4-TK)²⁸ genes was then used to investigate their respective therapeutic potentials in experimental rat glioma.

Materials and methods

Vectors

Two infectious proviral DNA plasmids were used. pNCA, kindly provided by S Goff,²⁹ contains the entire genome of

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the ecotropic wt Moloney (Mo) MLV. In pBSK-A, kindly provided by FL Cosset, the MoMLV envelope is replaced by the amphotropic 4070A envelope.³⁰ RRV-SA was constructed from pNCA by introducing, downstream of the *env* sequence, the *NdeI/XbaI* 0.36-kb region of *pol* containing the splice acceptor (SA) site, followed by the *EGFP* gene (Clontech, Palo Alto, CA). RRV-IRES was constructed from pNCA by replacing the ecotropic by the amphotropic envelope from pBSK-A and by introducing downstream of the *env* sequence the internal ribosomal entry site (IRES) of the encephalomyocarditis virus obtained from pCITE (Novagen, Madison, WI), followed by the *EGFP* gene.

Conventional DRVs were constructed from pMTK26 by replacing the *HSV1-TK* gene by either *EGFP* or *TK-EGFP* engineered by an in-frame fusion of *EGFP* to *EHV4-TK*.³¹ The recombinant adenovirus, RAdhCMV/*HSV1-TK* (RAd128), has been described in detail previously.^{32,33} Production of high-titer stocks, purification with the fluorocarbon compound Arklone P (Basic Chemical, High Wycombe, Bucks, UK), and titration of RAd128 (hCMV/*HSV1-TK*) were carried out as described previously.³⁴ Viruses were purified using double cesium chloride gradients. Titers of 10^{10} – 10^{11} infectious units (IU)/mL and particle/plaque-forming unit ratios of 30 were obtained.

Cell lines

The NIH3T3 murine fibroblast and DHDK12 rat colon carcinoma were previously described.^{35,36} The rat glioma cell line CNS-1 was provided by W Hickey (Dartmouth Medical Center, Department of Pathology, Lebanon, NH).³⁷ The B16-F10 murine melanoma cell line was purchased from ATCC (ATCC CRL-6475; Manassas, VA). All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DHDK12, B16-F10, and CNS-1 cells) or 10% newborn calf serum (NIH3T3 cells), 2 mM glutamine, 100 mM nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin sulphate (Gibco, Invitrogen, Cergy-Pontoise, France).

In vitro experiments and VPC generation

For assessing replicative vectors, 1×10^5 NIH-3T3 cells were grown in six-well plates and transfected with 3 μ g/well of each plasmid DNA formulated in polyethyleneimine (ExGen 500; Euromedex, Munbolsheim, France) according to the manufacturer's recommended procedure. Culture medium was changed and cells were split twice a week. During each passage, cell aliquots were analyzed for EGFP expression using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA).

The dependence of propagation on the release of viral particles was analyzed by transfer of 0.45- μ m filtered supernatant (SN) from infected cell cultures onto naive cells. Target NIH3T3 cells were plated at 1×10^5 cells/well in a six-well plate. The next day, cells were transduced with SN containing Polybrene (8 μ g/mL; Sigma, St. Quentin Fallavier, France). Three days later, flow cytometric analysis was performed to determine the percentage of EGFP⁺ cells. For inhibition experiments, 10 μ M AZT (Sigma) was added in the culture medium.

For titer determination, a total of 5×10^5 NIH3T3 cells/well were seeded in a six-well plate. Twenty-four hours later, the medium was replaced with 0.5 mL of serially diluted viral SN containing Polybrene (8 μ g/mL). After overnight incubation, SN was replaced with fresh medium. Forty-eight hours later, transduced cell numbers (% EGFP⁺ cells) were analyzed by FACS, and the titer was calculated from the volumes corresponding to the linear slope of the curve according to the following formula:

Viral titer

$$= \frac{\% \text{ EGFP}^+ \text{ NIH3T3 cells} \times \text{NIH3T3 cells at initial exposure}}{\text{Volume of SN (mL)}}$$

$$= \text{particles/mL}$$

Titers on NIH3T3 cells are: RRV, 4.5×10^6 ; DRV, 3.3×10^5 ; and DRV+wt, 6×10^6 p/mL.

Replicative VPCs were cloned by limiting dilution from these propagation experiments. Controls were conventional VPCs releasing defective viruses expressing EGFP or TK-EGFP, respectively.

In vivo experiments

The two subcutaneous flank solid tumor models were generated either in immunodeficient Swiss nude mice (nu/nu Swiss; Iffa Credo, L'Arbresle, France) for the DHDK12 tumor model or in immunocompetent C57Bl6 mice (Elevage Janvier, le Genest St. Isle, France) for the B16-F10 tumor model. Figure 3, A and C shows that tumors were generated by the injection of 1.6×10^7 cells, representing a mixture of 98% DHDK12 or 1×10^6 B16-F10 tumor cells and 2% VPCs. Figure 3, B and D shows that 1.6×10^7 DHDK12 or 1×10^4 B16-F10 tumor cells were injected, and on days 8–10, tumor-bearing mice received intratumoral injections of 1.6×10^6 RRV VPCs, 3×10^6 DRV+wt, or DRV VPCs in the DHDK12 tumor model or 5×10^6 RRV VPCs in the B16-F10 tumor model. In experiments assessing EGFP propagation, animals were sacrificed 2 weeks after VPC injections. Tumors were resected and weighed. After dissociation, they were then digested at 37° for 1 hour in HBSS supplemented with 0.1 mg/mL CaCl₂, 0.14 mg/mL MgSO₄, and 100 U/mL collagenase D (Boehringer Mannheim, Meylan, France). Digests were filtered through a 70- μ m Falcon Cell Strainer (Becton Dickinson, Franklin Lakes, NJ) and separated on a Percoll (Amersham Pharmacia Biotech, Orsay, France) gradient (75%, 50%, 40%) at 2000 rpm for 20 minutes. The fraction banding between the 75% and 50% cushions was collected and analyzed by FACS.

Anesthetized male Lewis rats (Charles River Laboratory, Margate, UK) weighing 200–250 g, were injected, as described in Dewey *et al*,³³ with 5×10^3 CNS-1 cells in 3 μ l of phosphate-buffered saline into the left striatum using a 10- μ l syringe fitted with a 26-gauge needle. The coordinates were: anterior +1 mm, lateral +3 mm, ventral +5 mm. Injection of cells was performed over a period of 3 minutes and the needle was left in place for 5 minutes before

retraction. Three days post-CNS-1 implantation, viruses in 3 μ l of phosphate-buffered saline were injected into the same lateral and anterior coordinates but at the following ventral coordinates: +5.5 mm, +5 mm, and +4.5 mm. The weight of each rat was recorded immediately after surgery. Twenty-four hours postviral injection, the rats received ganciclovir (GCV) treatment (Cymevene; Roche Products, Welwyn Garden City, UK). Each rat was injected intraperitoneally with 25 mg GCV/kg body weight twice daily for a period of 7 days. Control groups received saline twice daily for 7 days ($n=6$ /group).

Histological analysis of replicative VPC-treated tumors

Animals with established DHDK12 tumors were treated once by intratumoral injections of 5×10^6 irradiated DRV or RRV-*IRES* VPCs. After 3, 5, or 7 days, animals were sacrificed and tumors were excised, postfixed 2 hours in paraformaldehyde (2%), and embedded in BSA/gelatin.³⁸ Vibratome sections of 50 μ m were analyzed for EGFP fluorescence.

Real-time quantitative PCR

For biodistribution analysis, nu/nu Swiss and Balb/C (Iffa Credo) mice were used. Retroviral particles were administered to animals by injection of 0.1 mL of VPC SN (diluted in HBSS medium) into the retro-orbital sinus veins. Two days before injection and then continuously until the end of the experiments, AZT (1 mg/mL) was added to mouse drinking water.

Genomic DNA was extracted from various organs of test mice or from 10×10^6 mononuclear fibroblastic cells using Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions.

To detect integrated retroviral vector, quantitative PCR was performed targeting the 4070A envelope gene, using the ABI Prism 7700 sequence detector (TaqMan; PE Applied Biosystems, Branchburg, NJ). Primers and probes (PE Applied Biosystems) were chosen with the assistance of the computer programs Oligo TM 4.0 (National Biosciences) and Primer Express 3.0 software (PE Applied Biosystems).

For detection of the 4070A envelope gene: forward primer 5'-ACCCTCAACCTCCCCTACAAG-3', reverse primer 5'-GTAAAGCGCCTGATAGGCTC-3', and fluorogenic probe 5'-(6FAM)-AGCCACCCCAAGGAAGTGGAGATAGA-(TAMRA)-3'. For precise amounts and qualities of input genomic DNA, we also quantified an internal control gene, *apolipoprotein B* (*ApoB*), in each reaction: forward primer 5'-CACGTGGCTCCAGCATT-3', reverse primer 5'-TCACGATCATTCTGCCTTTG-3', and fluorogenic probe 5'-(6FAM)-CCAATGGTCGGGCACTGCTCAA-(TAMRA)-3'.

TaqMan amplification reactions were carried out in 50 μ l of solution, using the components supplied in the TaqMan PCR Core Reagent Kit (PE Applied Biosystems). Each sample was analyzed in triplicate, using 250 ng of DNA in each reaction. Thermal cycling was initiated with 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

To quantify copy numbers, plasmid DNA containing the 4070A *env* target gene was used for determining a standard curve of plasmid that was assayed for each experiment. Murine fibroblast genomic DNA served as reference DNA, which was serially diluted and run in parallel to establish the calibration curve and to infer copy numbers from the cycle thresholds (Cts), assuming a conversion factor of 6.6 pg of DNA per diploid genome. The normalized gene dose, N , is given by the following ratio:

$$N = \frac{\text{Copy number of target gene (4070A env)}}{\text{Copy number of reference gene (ApoB)}} \\ = \frac{\text{Copy number of env gene}}{\text{Number of cells}}$$

Results and discussion

Design and in vitro propagation of replicative vectors

We firstly engineered two fully RRV by introducing the *EGFP* reporter gene, preceded either by a copy of the viral spliced acceptor (*SA*) region or an IRES, downstream of the *env* gene of a MoMLV proviral genome (Fig 1A). We transfected these constructs into cultured cells and monitored the level of transgene expression over time. Transfection of the RRV-*SA* led to heterogeneous interexperimental propagation kinetics. Transgene propagation usually started at a rather slow pace followed by a burst, leading to transduction efficiencies that reached up to 80%, as illustrated in a representative experiment (Fig 2A). In contrast, RRV-*IRES* propagation kinetics was always extremely rapid, with transduction efficiencies that could reach 100% (Fig 2A).

We also evaluated a second replicative vector system that relies on helper-dependent propagation of a DRV rescued by a wt MLV helper virus (Fig 1B). Such a system offers the advantage to allow propagation of multiple available

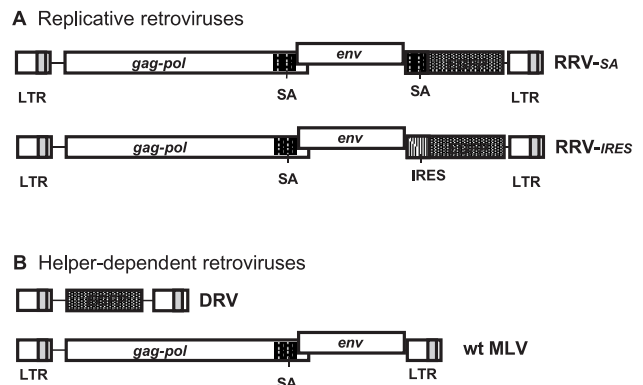


Figure 1 Schematic representation of replicative vector systems. Three replicative systems were used: **(A)** the two RRVs harbor the complete wt MoMLV genome with an additional splice acceptor site (RRV-*SA*) or an internal ribosomal entry site (RRV-*IRES*) and the *EGFP* reporter gene introduced downstream of the *env* gene; **(B)** the helper-dependent system consists of two vectors: a wt MLV complete genome and a conventional defective retrovirus (DRV) containing the *EGFP* reporter gene.

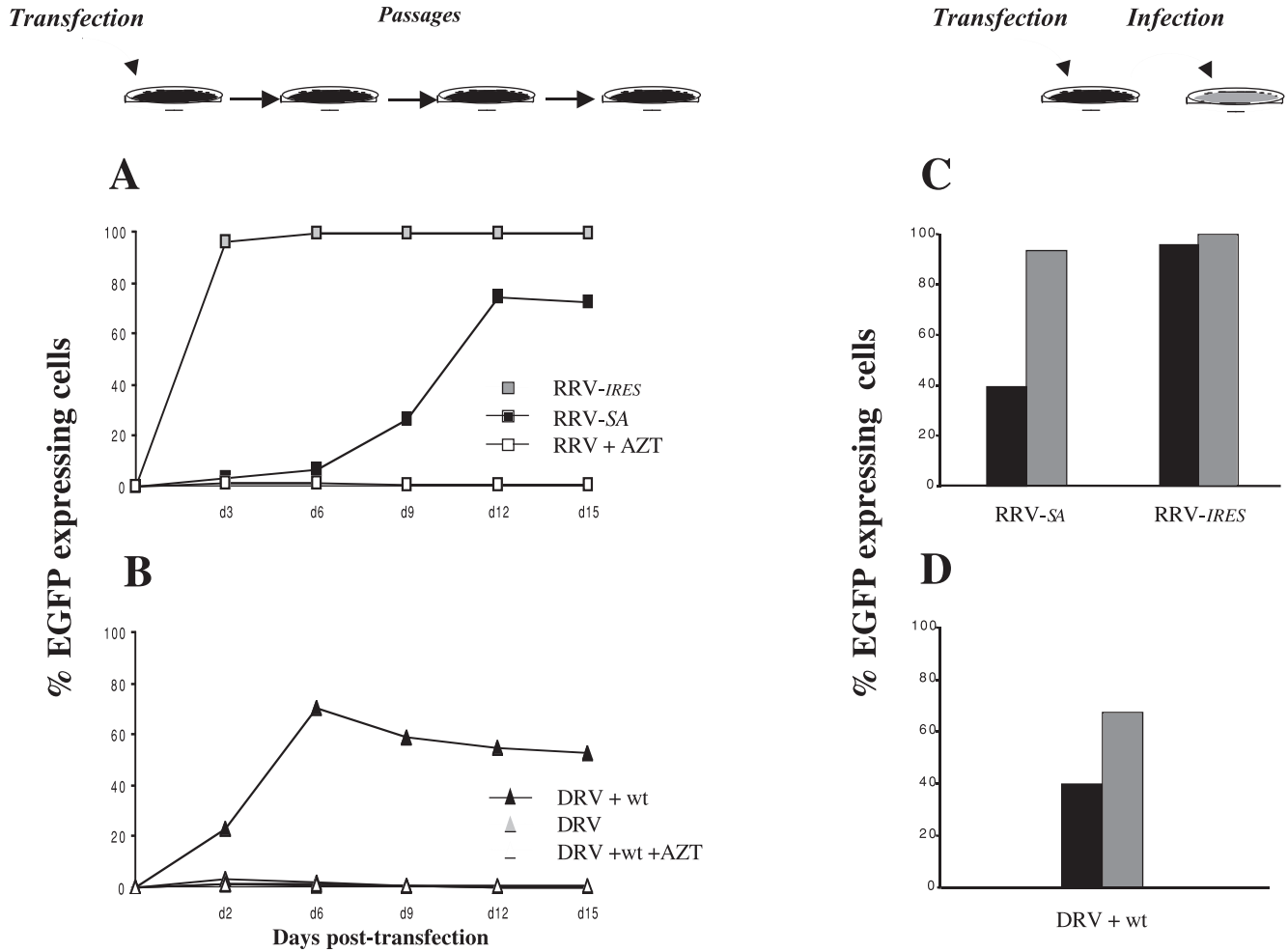


Figure 2 *In vitro* EGFP gene propagation by replicative and helper-dependent vector systems. *In vitro* transfection of the plasmid generating replicative retroviruses (RRV-*SA* or RRV-*IRES*) (A) or helper-dependent (DRV+wt) and control defective (DRV) retroviruses (B). Percentages of EGFP-expressing cells were evaluated by FACS at various passages after transfection of cells with plasmids. AZT (10 μ M) was added to the culture medium to inhibit retroviral replication. Propagation of viral particles released by transfection after infection of naive cell cultures. Percentages of EGFP-expressing cells were evaluated by FACS 3 days after transfection and infection (C, D). The graphs represent data from one experiment representative of at least three independent experiments for each vector system.

DRVs.²⁶ The propagation achieved with this helper-dependent vector system was reproducible and extremely rapid. Up to 30% of cells was already transduced after 2 days and the overall transduction efficacy reached 80–100% within 6–8 days (Fig 2B). Noteworthy, such transgene transduction levels imply that the vector genome is propagated more rapidly than the wt virus genome. Indeed, occupancy of the retrovirus receptors by endogenously produced env proteins on a cell infected with the wt virus should prevent its reinfection, a phenomenon called interference.³⁹ As infectious particles carrying the transgene can only be generated in cells coexpressing the defective vector and the wt virus genomes, it implies that the vector genome is more efficiently packaged than the wt virus genome. Presumably, this unexpected property renders the helper-dependent system remarkably efficient.

Importantly, when AZT, a reverse transcriptase inhibitor commonly used to treat HIV infection²⁴ but also known to be efficient against murine retroviruses,⁴⁰ was added to

these cultures, transgene propagation was blocked (Fig 2, A and B).

Passage of replicative vectors

We next verified that the increasing proportion of EGFP⁺ cells observed after DNA transfection depended on proper release of infectious viral particles. We infected naive cells with SNs collected from transfected cell cultures, and analyzed EGFP expression 3 days thereafter. With SN from cultures transfected with a DRV, no transgene expression was detected. In contrast, with SN from cultures transfected with the RRV (Fig 2C) or helper-dependent (Fig 2D) vector systems, the transgene was readily transferred. When AZT was added during one such passage, it fully blocked infection and further propagation (data not shown). Altogether, these results indicate that propagation is indeed the result of production of infectious retroviral particles carrying a transgene.

In vivo propagation of replicative vectors in nude mice

To evaluate transgene propagation *in vivo*, we first generated tumors by injecting mixtures made of 98% DHDK12 tumor cells and 2% vector-producing cells (VPC) into nude mice. The VPCs were either cloned conventional packaging cells producing defective retrovirus DRV as control, or VPCs obtained from cells transduced with the replicative vectors generated in the previous *in vitro* experiments. Fourteen days

after injection, tumors were removed, and tumor cell suspensions were analyzed by FACS. This experimental scheme, by establishing the initial percentage of transduced cells, allowed quantification of subsequent transgene propagation. With the defective vectors, the proportion of cells expressing EGFP remained near 2%. In marked contrast, with the replicative vectors, up to 85% (RRV) and 70% (helper-dependent) of tumor cells expressed EGFP (Fig 3A).

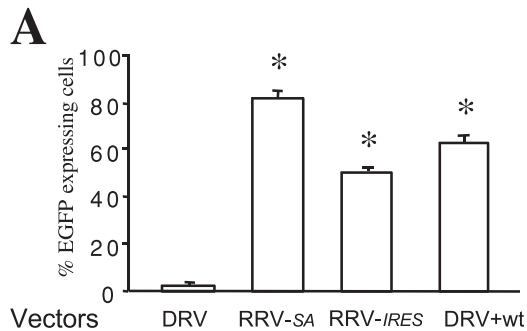
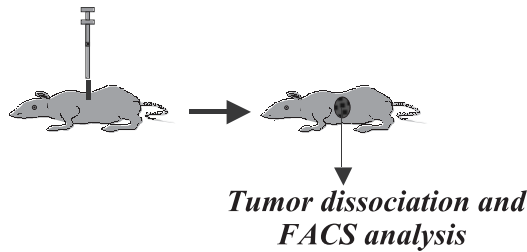
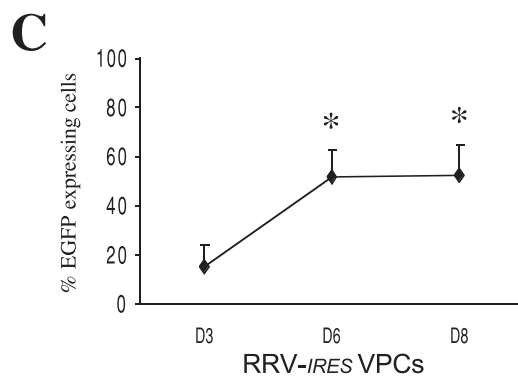
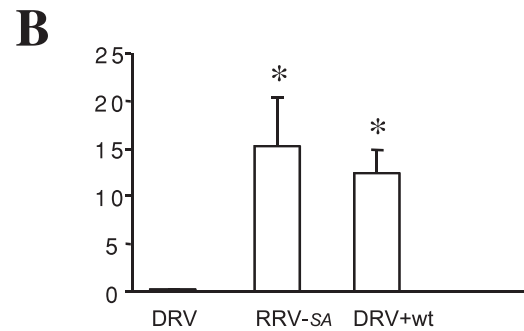
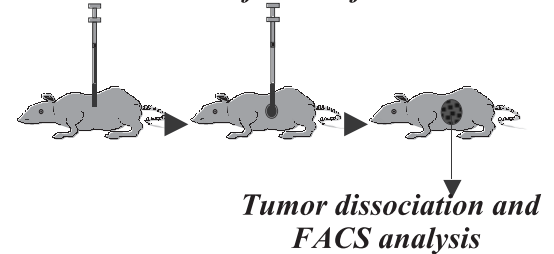
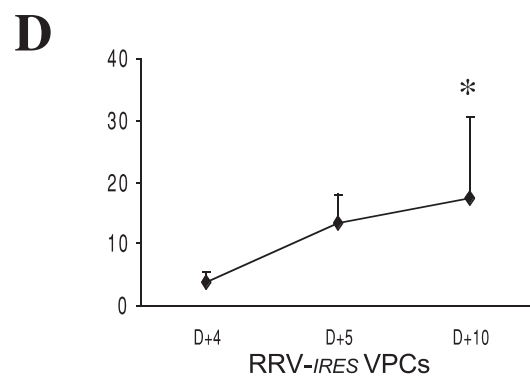
D0 : Co-injection of tumoral cells 98%+VPCs 2%**DHDK12 cells/ SWISS (nu/nu) mice****D0 : Injection of tumoral cells** **D8-10 : Intratumoral injection of VPCs****B16-F10 cells/ C57BL6 mice**

Figure 3 *In vivo* propagation of replicative vector systems. *In vivo* propagation of replicative vector systems in athymic nu/nu Swiss mice. On day 0 (D0), mice were injected subcutaneously in the flank with a mixture of 1.6×10^7 DHDK12 tumor cells and 2% of the respective VPCs (**A**) or DHDK12 tumor cells alone (**B**). On D8–10, these mice received three intratumoral injections of 1.6×10^6 or 3×10^6 RRV-SA or DRV+wt and DRV VPCs, respectively (**B**). In (**A**) and (**B**), tumors were removed after 2 weeks of propagation, and the percentage of EGFP-expressing cells was evaluated by FACS. Data are means (\pm SE) from one experiment in which six tumors were analyzed for each condition. *Significant differences at $P < .01$ according to Mann-Whitney *U* test. *In vivo* kinetics of propagation after RRV-IRES VPC injections in syngeneic C57BL6 mice. A total of 1×10^6 B16-F10 tumor cells were coinjected subcutaneously in flanks of mice with 2% of RRV-IRES VPCs. Tumors on days 3, 6, and 8 (six tumors at each time point) were removed and the percentage of EGFP-expressing cells was evaluated by FACS (* $P < .001$ compared to D3 value) (**C**). Mice with subcutaneously established B16-F10 tumor cells were treated with three injections of 5×10^6 RRV-IRES VPCs (D8–10). On days 4, 5, and 10 after the first injection, tumors were removed and the percentage of EGFP-expressing cells was evaluated by FACS (* $P < .05$ compared to D4 value) (**D**).

We next tested vector propagation after injections of VPCs into established DHDK12 tumors, a setting more relevant to the therapeutic use of these vectors. Under our experimental conditions, these intratumoral injections lead to a very low multiplicity of infection (MOI); injected VPCs represented less than 0.5% of tumor cell numbers. Despite this, we observed significant transgene propagation (Fig 3B), with up to 20% or 15% transduced tumor cells with replicative or helper-dependent vectors, respectively.

Detection by fluorescence stereomicroscopy of transgene expression after intratumoral injection of *EGFP/RRV-IREs* VPCs revealed efficient propagation of the replicative vector throughout the excised tumor tissue. As early as 3 days after injection, part of the tumor tissue expressed a few punctuate green fluorescent dots (Fig 4C). On day 5, we observed

retroviral propagation, with clusters of transduced tumor cells (Fig 4D). Seven days after injection, we detected highly efficient transduction with remarkably homogenous *EGFP* fluorescence throughout the tumor tissue (Fig 4E). Higher magnification showed numerous bright *EGFP*-expressing tumor cells (Fig 4H). In contrast, under these conditions in control tumors that received injections of DRV VPCs, we could not detect any fluorescence signal (Fig 4B). The very rapid spread of RRV-*IREs* after intratumoral injection in mice demonstrates the potential of this RRV in the treatment of solid tumors.

These results are in agreement with those obtained using a RRV similar to RRV-*IREs*.²⁷

Of note, some solid tumors are not rapidly proliferating but have a low proliferative index, and ultimately, the

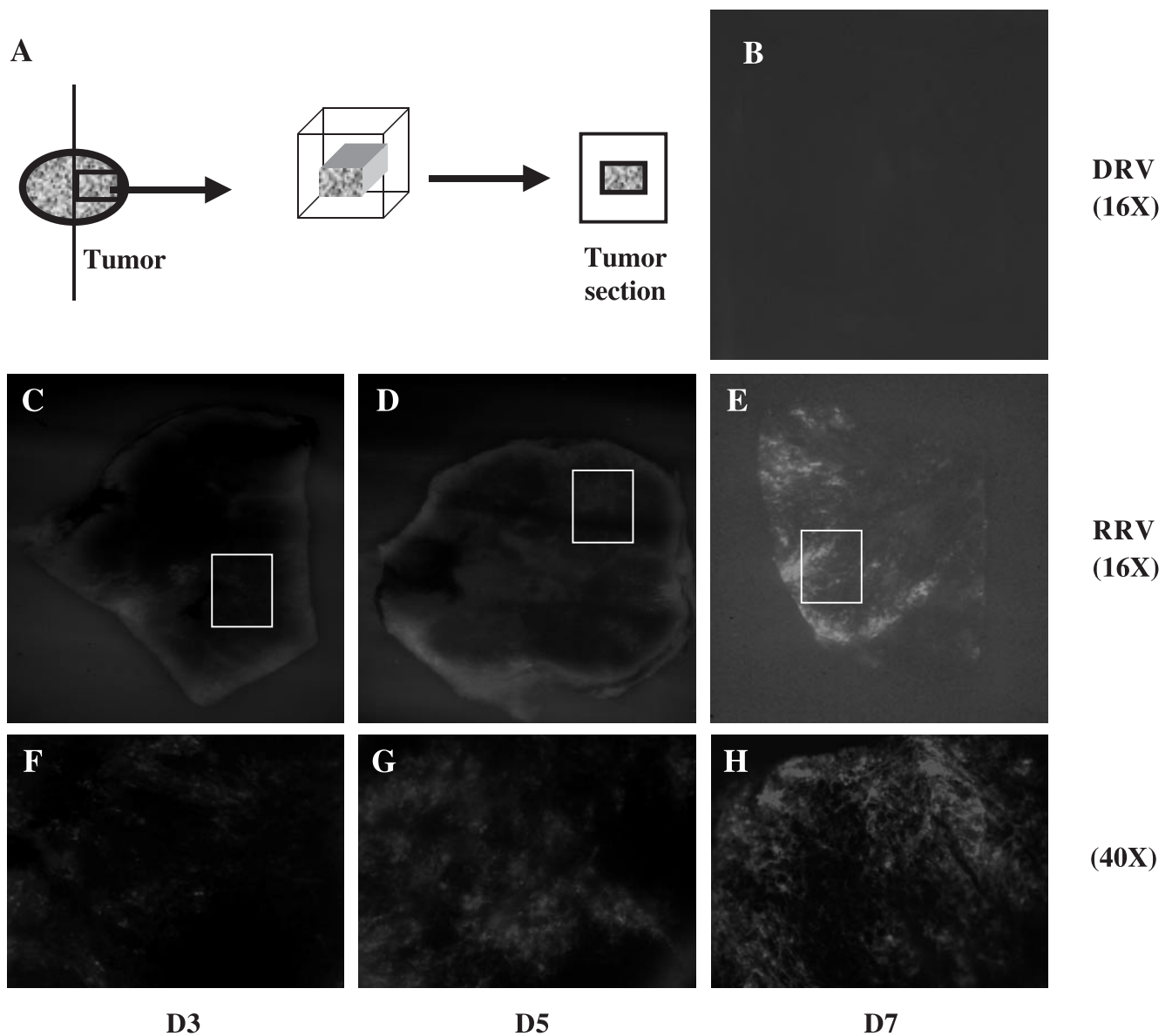


Figure 4 *In vivo* propagation of retroviral replicative vector. Schematic representation of pieces of tumor tissue sections analyzed by fluorescence stereomicroscopy (A). *EGFP* fluorescence images in mice tumors after one intratumoral injection of DRV (B) or RRV-*IREs* VPCs at low magnification ($\times 16$) (C–E) or at higher magnification ($\times 40$) (F–H). Each fluorescence image was captured and processed under the same conditions.

transduction of spontaneously arising tumors will have to be assessed.

In vivo propagation of replicative vectors in immunocompetent mice

The immune response against retroviral or transgene antigens could limit the propagation of the RRVs. Actually, propagation of such vectors in immunocompetent mice has never been assessed. We generated tumors by injecting mixtures made of 98% B16F10 tumor cells and 2% RRV-*IRE5* VPC into immunocompetent mice, as described above. Kinetic experiments revealed a quite rapid and early propagation of RRV-*IRE5* viruses, the percent of EGFP⁺ cells rising from 2% to 17% in 3 days and reaching >50% on day 6 (Fig 3C). When RRV-*IRE5* VPCs were injected in established tumors, propagation could be observed all along the 10 days before the mice had to be sacrificed due to the size of the growing tumor. The proportion of transduced cells reached about 20% (Fig 3D). Altogether, these results show for the first time that viral propagation can be achieved in an immunocompetent host.

In vivo therapeutic effects of replicative vectors

We then assessed the therapeutic potential of replicative vectors with the suicide gene *EHV4-TK* fused to *EGFP* (*TK-EGFP*) to allow monitoring of transgene propagation. TK, or TK-EGFP, permits phosphorylation of the nucleoside analog GCV into the toxic triphosphated GCV, which induces cell death by blocking DNA elongation.⁴¹ TK-expressing cells are killed at GCV concentrations that are usually at least two logs lower than those toxic for parental cells. Experimental studies have demonstrated the efficacy of cancer suicide gene therapy *in vivo*.⁴² They have also revealed that its potential relies essentially on a bystander effect by which neighboring untransduced tumor cells are eliminated together with the transduced tumor cells during GCV administration.^{43–46} This effect is mainly immune-mediated^{35,47,48} and, consequently, suicide gene therapy is much less efficient in immunodeficient animals. We first tested the therapeutic efficacy of these vectors in nude mice injected with mixtures of 98% of tumor cells and 2% VPCs releasing helper-dependent replicative vectors carrying *TK-EGFP*. Significant transduction and corresponding therapeutic effects were obtained with replicative but not defective vectors in nude mice (not shown), in agreement with similar previous experiments.²⁶ Thus, in the absence of an immune response, the replicative vector system already provides a very significant therapeutic efficacy under conditions where defective vectors cannot.

We then wanted to assess the therapeutic potential of these vectors using a syngeneic glioblastoma model in immunocompetent rats. This model is relevant because: (a) the efficacy of recombinant TK-expressing adenovirus has already been proven; however, it reached vector doses that lead to sustained local brain inflammation and toxicity,³³ (b) brain tumors, notably inoperable glioblastoma, could be excellent targets for RRVs. Indeed, they have a very poor prognosis, no therapeutic alternative, and they offer a setting

where only the tumoral cells or endothelial cells from the tumor vasculature are dividing.

We implanted CNS-1 glioblastoma cells into the striata of Lewis rats, and then treated them with defective adenoviral vector or RRVs (DRV+wt). Rats treated with saline or defective vectors (DRV) died within 16 days (Fig 5, A and B). In contrast, rats treated with 10³ replicative retroviral particles had a significantly prolonged survival ($P < .001$, compared to saline) with 20% being cured (Fig 5A). A similar efficacy could only be achieved by injecting 10⁶ defective adenoviral particles (Fig 5B). These results emphasize the therapeutic potential of RRVs that appear 1000-fold more efficient than defective adenoviral vectors in this setting.

Altogether, these results outline important advantages of the RRVs compared to defective retroviral and first-generation adenoviral vectors in immunocompetent animals. Despite very low transduction efficacy, we and others have occasionally observed some therapeutic effect in clinical trials with *HSV1-TK* transduced into tumors by DRVs.^{49–52} We believe that the remarkable gain in transduction efficacy that could be obtained with the RRVs should translate into a significantly increased therapeutic benefit as suggested by our data. Whereas the paradigm for using retroviral vectors in humans has, thus far, been to avoid replication-competent retroviruses (RCRs), the results presented here should prompt careful reconsideration of using replicative viruses for treating cancer.

The major concern of using replicative vectors is the control of their putative dissemination, inside as well as outside the patient. Regarding patient safety, dissemination of replicative vectors outside the tumor could be an important issue, notably when using oncoviruses that can cause leukemia in animals. However, it should be kept in mind that drugs that occasionally induce leukemia are continuously used in the clinic if their risk/benefit ratio is nevertheless favorable. Also, the host immune response as well as the use of antiretroviral drugs should help limit dissemination. We investigated this by determining the biodistribution of replicative vectors after intravenous injection in immunodeficient as well as immunocompetent mice, with and without AZT treatment. Two weeks after RRV injections, the existence of proviral genomes was assessed in various tissues by quantitative PCR analysis (Table 1). In nude mice, proviral genomes could be detected in bone marrow and spleen, but only in the absence of AZT. In contrast, no proviral genome could be detected in any tissue analyzed from immunocompetent mice. These observations emphasize the potency of anti-MLV immune responses. They are consistent with previous experiments showing that even in the natural MLV host, establishment of a chronic infection and resulting pathogenic effects are mostly observed in mice that are immunodeficient or infected during the first days of their lives.⁵³ Similarly, the only report of leukemia arising in nonhuman primate after gene therapy with retroviral vectors relates to two severely immunodepressed macaques which received bone marrow transplants that had been cocultured *ex vivo* with RCR.⁵⁴ Thus, theoretically, viral dissemination in the patient should be at least partly controlled by efficient immune responses to MLV, and/or if needed by antiretroviral

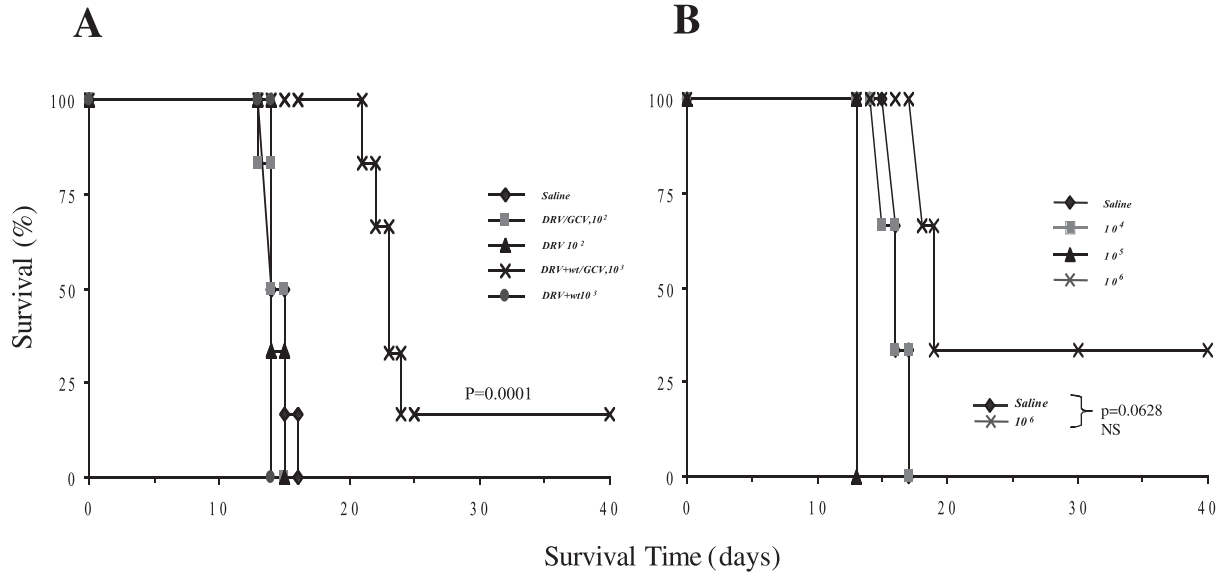


Figure 5 Comparison of the efficacy of retrovirus- versus adenovirus-mediated thymidine kinase expression (with GCV treatment) on the survival of Lewis rats intracranially injected with CNS-1 cells. **A:** Survival of CNS-1 tumor-bearing Lewis rats treated with intracranial injection of either replicative DRV+wt at 1×10^3 or DRV at 1×10^2 VPC supernatant particles or saline. One group of rats received intraperitoneal injection of GCV 24 hours postviral injection; the controls received only saline. Survival of rats treated with DRV+wt/GCV was significantly greater than for those treated with saline ($P < .0001$). **B:** Effect of increasing dose of Rad128, a replication-defective recombinant adenovirus encoding HSV-1-TK under control of the short immediate early human cytomegalovirus (sMIEhCMV) promoter, on survival of CNS-injected rats. Groups of rats bearing CNS-1 intracranial tumor, inoculated 3 days before intracranial injection with different doses of Rad128 or saline, received intraperitoneal injection of GCV 24 hours postviral injection. NS: nonsignificant.

drugs such as AZT. Altogether, as for any drug, treating patients with potentially toxic vectors will depend on the risk/benefit ratio they provide.

We thus believe that the major concern for using replicative vectors is with regards to environmental dissemination. In this respect, using replicative retroviruses should be comparatively safe. Indeed, retrovirus transmission occurs mainly through sexual or blood exposure, which can be readily prevented.²² Importantly, transmission through aerosols, which can occur with other viral vectors and easily leads to epidemic outbreak, does not occur with retroviral vectors.²² Although endemic retroviral dissemination with the human retroviruses HTLV-1 and-2 exists, retroviral epidemics have only been seen in humans with the more sophisticated HIV lentiviruses. Furthermore, blood or sexual

transmission of retroviruses depends on the viral load of the infected contact.⁵⁵ The patient's immune responses, optionally aided with antiretroviral drugs, should limit viral load and effectively prevent viral dissemination.⁵⁶

Finally, it must be emphasized that these first-generation replicative vectors may be improved further. Safety of these vectors can be increased by the addition of propagation constraints and such efforts are already in progress in our laboratory. It is possible to manipulate the retroviral long-term repeat (LTR) to incorporate tissue-specific or inducible regulatory sequences.⁵⁷⁻⁶¹ Retroviral genomes can also be incorporated in adenoviruses to benefit from the efficient adenovirus titers. Furthermore, strategies for redirecting the tropism of retroviruses or adenoretroviruses, toward target cells by modifying the viral envelope are currently being

Table 1 *In vivo* biodistribution of helper-dependent and defective vector systems using real-time PCR for 4070A *env* detection

	Immunocompetent Balb/c mice			Immunodeficient nude mice		
	DRV	DRV+wt	DRV+wt+AZT	DRV	DRV+wt	DRV+wt+AZT
Bone marrow	-	-	-	-	0.21	-
Spleen	-	-	-	-	0.0073	-
Liver	-	-	-	-	-	-
Lung	-	-	-	-	-	-
Gonads	-	-	-	-	-	-

Nude mice were injected intravenously with 2×10^4 particles of DRV or 2×10^5 particles of DRV+wt vectors. Balb/c mice were injected intravenously with 1×10^4 particles of DRV or DRV+wt vectors. Two weeks later, the indicated organs were harvested from injected and control mice. Estimation of 4070A envelope DNA copies per cell. Values were normalized to values obtained from DNA preparations of control organs. Data represent a number (*N*) calculated from at least three samples and each sample was performed in triplicate. Correlation coefficient in each experiment was between 0.99 and 1. *N* was normalized to control genomic DNA corresponding to organs isolated from noninjected mice.

developed.^{62,63} Primarily, such enhancements should be employed before applying these vectors in patients. Altogether, we believe that our data strongly support further use and development of replicative retroviruses for cancer gene therapy.

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