

In Vivo Antitumor Activity of Sindbis Viral Vectors

Jen-Chieh Tseng, Brandi Levin, Tadamichi Hirano, Herman Yee, Christine Pampeno, Daniel Meruelo

Background: Sindbis virus, a blood-borne virus transmitted by mosquitoes, has been used as a vector to efficiently express exogenous genes *in vitro* and *in vivo* and to induce apoptosis. Because Sindbis virus infects mammalian cells by interacting with the high-affinity laminin receptors, which are expressed at higher levels in several human cancers than in normal cells, we determined whether a Sindbis viral vector could be used to target cancers *in vivo*. **Methods:** C.B-17-SCID mice with established xenografts were given daily intraperitoneal injections of the Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene. Control mice were untreated or received injections with phosphate-buffered saline. Tumor size was measured daily. Expression of β -galactosidase and Factor VIII (a marker for endothelial cells) was determined by immunohistochemical staining of tumor sections. Apoptosis was analyzed by TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) staining. C.B-17-SCID beige mice, which lack natural killer (NK) cells, were used to assess the importance of NK cells in antitumor efficacy of Sindbis viral vectors. **Results:** Tumors from mice treated with SinRep/LacZ were statistically significantly smaller than tumors from control mice. This effect was observed for tumor xenografts derived from BHK (kidney, hamster), LS174T (colon, human), HT29 (colon, human), and CFPAC (pancreas, human) cells. Expression of β -galactosidase co-localized with that of Factor VIII in tumor sections. Tumors from SinRep/LacZ-treated mice contained more apoptotic cells than tumors from control mice. Complete tumor regression was observed in three of five C.B-17-SCID mice but in none of five C.B-17-SCID beige mice treated with SinRep/LacZ. **Conclusion:** Sindbis viral vectors efficiently targeted tumors *in vivo*, were apparently delivered through the circulation, and were more effective in the presence of NK cells. [J Natl Cancer Inst 2002; 94:1790–1802]

Effective gene therapy vectors for cancer treatment must have target specificity and high gene expression efficiency. Viral vectors, such as retroviruses and adenoviruses, have been developed to deliver heterologous genes into tumors *in vivo*, but these vectors have several limitations. For example, although retroviral vectors mediate stable gene transfer by genomic integration, they are limited by their relatively low transfer efficiencies (1–4) and by possible modifications of the germ line (5). Furthermore, most retroviral vectors are susceptible to lysis by human serum components (6–8), greatly limiting their *in vivo* applications, especially for intravenous administrations. Although adenoviral vectors appear to be more efficient than retroviral vectors for gene delivery *in vivo*, they may be limited by the high immunogenicity of adenoviral proteins, which can be toxic to patients (9,10). Use of adenoviruses for gene therapy is also limited in that they cannot be delivered through the bloodstream but rather are commonly administered by intratumoral injection (11–13).

Another virus that has drawn interest as a gene transfer vector is the Sindbis virus, which belongs to the *alphavirus* genus in the *Togaviridae* family. Sindbis viral vectors for gene transfer have been well studied *in vitro* (14–19) but have not been widely used for *in vivo* gene therapy, except for some reports of *in vivo* Sindbis virus-mediated gene transfer into the central nervous system (15,16) and into antigen-presenting cells, which were used for vaccination against various pathogens (20–25) and for cancer immunotherapy (26).

Sindbis viral vectors have several advantages over other viral vectors. First, Sindbis viral vectors show extremely high gene transfer efficiency into mammalian and insect cells. Second, Sindbis viral vectors are amplified in the cytoplasm of infected cells, where they can transcribe 10^5 RNA molecules that can potentially express high levels of exogenous gene product within a few hours after infection. The level of RNA amplification would allow for prolonged gene expression were it not for the fact that the virus induces apoptosis in infected cells (27–30). Third, because Sindbis virus has an RNA genome, it does not have a DNA phase in its life cycle and therefore may avoid potential complications associated with chromosomal integration (23). Fourth, the Sindbis RNA genome is relatively small (the entire genome has fewer than 12 000 nucleotides) and easy to manipulate. New Sindbis viral vectors have been engineered that are capable of nonreplicative infection, increasing the safety aspects of the vector (14). Finally, because Sindbis virus is a blood-borne virus (31) that can cross the blood–brain barrier (15), Sindbis viral vectors should be able to reach most cells of the body.

Sindbis virus is also well known for its ability to induce apoptosis in infected mammalian cells (27–30). Furthermore, the high-affinity 67-kd laminin receptor, whose expression is increased in many types of human cancers (32–42), is known to mediate Sindbis viral infection of mammalian cells (43,44). We tested whether Sindbis viral vectors, unmodified with respect to a target tissue and carrying no cytotoxic gene, could be targeted to tumor cells and could induce cell death via apoptosis. Severe combined immunodeficient (SCID) mice bearing tumor xenografts were given intraperitoneal injections of the Sindbis viral vectors to determine the antitumor activity of the Sindbis viral vectors and to establish the route of delivery. The importance of natural killer (NK) cells in antitumor effects was also assessed by examining the effect of Sindbis viral vectors on the growth of tumor xenografts in SCID beige mice, which lack NK cells.

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MATERIALS AND METHODS

Cell Lines

Baby hamster kidney (BHK), human ovarian carcinoma (ES-2), colon carcinoma (HT29), pancreatic cancer (CFPAC), ovarian cancer (SKOV-3), epidermoid carcinoma of the vulva (A431), renal cancer (A498), bladder cancer (HT1197), and colon carcinoma (LS174T) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human hepatocellular carcinoma cell line HuH-7 was provided by Dr. H. Yamamoto (Hyogo College of Medicine, Japan) and was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Inc., Woodland, CA). BHK and ES-2 cells were maintained in minimum essential medium Eagle alpha modification (α MEM; JRH Biosciences, Lenexa, KS) supplemented with 5% FBS. HT29 cells were maintained in McCoy's 5A medium (Iwakata & Grace modification; Mediatech, Inc., Herndon, VA) supplemented with 10% FBS. CFPAC, SKOV-3, and A431 cells were maintained in DMEM (low glucose modified; JRH Biosciences) supplemented with 10% FBS. A498, HT1197, and LS174T cells were maintained in MEM with Earle's salts and L-glutamine (Mediatech, Inc.) supplemented with 10% FBS. All media were supplemented with 100 μ g/mL of penicillin-streptomycin (Mediatech, Inc.) and 0.5 μ g/mL of amphotericin B (Mediatech, Inc.).

Vectors

The Sindbis virus-based expression system SinRep/LacZ was originally obtained from Invitrogen (Carlsbad, CA). This vector encodes the packaging signal, a nonstructural polyprotein nsp1-4 for replicating the RNA transcript, the promoter for subgenomic transcription, and the bacterial β -galactosidase LacZ gene. DH-BB, a helper DNA template that contains the structural genes (capsid, E3, E2, 6K, and E1) required for packaging the virus was also obtained from Invitrogen. A DNA fragment containing the firefly luciferase gene (Luc) was excised from the *NheI* and *XbaI* sites of the plasmid pGL3 (Promega, Madison, WI) and subcloned into the *XbaI* site of the SinRep plasmid (Invitrogen) to construct the SinRep/Luc vector. To construct a Sindbis viral vector containing genes for interleukin 12 (IL-12), we first constructed the Sindbis viral vector SinRep/2P_{SG}, which contains a secondary subgenomic promoter that is responsive to the Sindbis replicase. Two DNA oligonucleotide primers (sequence 5'-CGCGTAAAGCATCTCTACGGTGGTCCTA-ATAGTGCATG-3' and its complementary strand 5'-CACTAT-TAGGACCACCGTTCGAGATGCTTTA-3') containing the subgenomic promoter sequence were annealed and ligated into the *MluI* and *SphI* sites of the SinRep plasmid. The murine IL-12 α subunit gene (mp35; ATCC 87596) and the IL-12 β subunit gene (mp40; ATCC 87595) were subcloned into the *MluI* and the *StuI* sites of SinRep/2P_{SG}, respectively, to produce the SinRep/IL12 plasmid.

In Vitro Transcription and Transfection for Sindbis Viral Vector Production

To generate the Sindbis viral vectors, the vector plasmids were first transcribed *in vitro* to generate Sindbis viral vector RNA. The RNA would then be transfected into cells, where it would be translated, replicated, and packaged into viral particles, which would be used to infect tumor cells. Plasmids for the

in vitro transcription of Sindbis viral RNAs (SinRep/LacZ, SinRep/IL12, and DH-BB) were prepared with the Qiagen plasmid kit (Valencia, CA). The helper DNA template DH-BB and a replicon plasmid (SinRep/LacZ or SinRep/IL12) were digested with *XhoI* to linearize the templates. SinRep/Luc plasmids were digested with *NotI* because the Luc gene contains an *XhoI* site. Digested plasmids were purified by phenol-chloroform extraction and ethanol precipitation. *In vitro* transcription reactions were carried out using the mMESSAGE mMACHINETM high-yield capped RNA transcription kit (SP6 version; Ambion, Inc., Austin, TX) to produce capped mRNA transcripts. The quality of the transcribed RNA was checked on 1% agarose gels.

Both DH-BB and SinRep/LacZ or SinRep/IL12 RNAs (20 μ L each of the *in vitro* transcription reaction mix) were electroporated into BHK cells, as described (45). Electroporated cells were transferred into 10 mL of α MEM containing 5% FBS and incubated at 37 °C for 12 hours. Adherent BHK cells were then washed with phosphate-buffered saline (PBS) and incubated in 10 mL of Opti-MEM I medium (Invitrogen) without FBS. After 24 hours, culture supernatants were collected and aliquots (3-4 mL) were stored at -80 °C.

Virus Quantification

To quantify the number of viral vector particles in the collected culture supernatants (above), serial dilutions (300 μ L each) of an aliquot containing SinRep/LacZ vectors were added to 2×10^5 BHK cells in 12-well plates. After incubating for 1 hour at room temperature, the cells were washed with PBS and incubated with 2 mL of α MEM at 37 °C for 24 hours. SinRep/LacZ infection was determined by fixing the cells in PBS containing 0.5% glutaraldehyde at room temperature for 20 minutes, washing them three times with PBS, and then staining them with PBS containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Fisher Scientific, Pittsburgh, PA), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM MgSO₄ at 37 °C for 3 hours. After staining with the X-Gal solution, cells that expressed LacZ stained blue. Blue-stained cells were counted and viral titers were estimated by determining the number of LacZ colony-forming units (CFU) per mL of aliquot.

To determine the viral titer of SinRep/IL12, we used an indirect method in which the Sindbis virus RNA levels in culture supernatants from SinRep/IL12-producing cells were compared with Sindbis virus RNA levels that corresponded to the known CFU/mL from culture supernatants of SinRep/LacZ-producing cells. Viral RNAs were isolated from serial dilutions of 300 μ L of Opti-MEM I medium containing either SinRep/LacZ or SinRep/IL12 vectors after the addition of 1 mL of TRIzol[®] reagent (Invitrogen). After ethanol precipitation, all RNA samples were resuspended in 50 μ L of RNase-free water. Vector RNA levels were determined by reverse transcription-polymerase chain reaction (RT-PCR), with a primer pair specific to Sindbis genomic RNA: 5'-AGCTTCCCAGCAATTTGAGGT-3', 5'-ACGCATG-GGGCAGACACAAT-3'. RT-PCR was performed with the Platinum Quantitative RT-PCR ThermoScriptTM One-Step System (GIBCO-BRL) for 35 cycles, with each cycle consisting of steps at 95 °C, 60 °C, and 72 °C. The PCR products were separated on 2% agarose gels and visualized after staining with ethidium bromide. Relative band intensities were visually compared, and relative SinRep/IL12 CFUs were obtained by comparing the RT-PCR band intensities of SinRep/LacZ RNA serial dilutions

with the RT-PCR band intensities of SinRep/IL12 RNA serial dilutions. The RT-PCR results of SinRep/IL12 were compared with the corresponding RT-PCR and β -galactosidase assay results of the SinRep/LacZ vector.

To determine the viral titers in supernatants from cells infected with SinRep/Luc vectors, 2×10^5 BHK cells were plated on 12-well plates and infected with serial dilutions of the vector in Opti-MEM I (300 μ L). After an overnight incubation, the luciferase activities in cell lysates were determined by aspirating the culture medium from the wells and adding 200 μ L per well of culture medium and 200 μ L per well of Steady-Glo[®] reagent (Promega Corp., Madison, WI). Plates were incubated with gentle rocking for 5 minutes until the cells detached from the plates. The cell lysates were then transferred to 12 \times 47 mm cuvettes (BD Pharmingen, San Diego, CA), and the luciferase activity of each lysate was determined by taking a 30-second reading with a LUMI-ONE portable luminometer (Bioscan, Inc., Washington, DC).

***In Vitro* Assay of Sindbis Infectivity of Different Cell Lines**

To determine the infectivity of the Sindbis viral vector of different cell lines, 300 μ L of Opti-MEM I medium containing approximately 10^7 CFU of SinRep/Luc viral vector particles was incubated at 37 °C for 1 hour with 2×10^5 each of BHK, LS174T, ES-2, HT29, CFPAC, PC-3, HuH-7, SKOV-3, A498, HT1197, or A431 cells that were previously cultured in 12-well plates. The multiplicity of infection (MOI) was determined by dividing the CFU by the number of cells infected. For all experiments, the MOI was about 50. After the incubation, the infection medium was replaced with normal culture medium and the cells were incubated overnight at 37 °C. The next day, cells were assayed by the Steady-Glo[®] Luciferase Assay System (Promega Corp.). For each cell line, two to four independent experiments were performed. With each experiment, control cells were mock-infected with 300 μ L of normal culture medium and incubated at 37 °C for 1 hour.

Cell Viability Assay After Sindbis Infection

To determine the viability of cells infected with Sindbis virus vectors, 2×10^5 BHK, CFPAC, ES-2, HT29, LS174T, or SKOV-3 cells were seeded in 12-well plates. The next day, on day 0, 300 μ L of Opti-MEM I medium containing $\approx 10^7$ CFU of SinRep/LacZ viral vector particles was added to each well. After 1 hour, the infection medium was removed, the cells were washed with PBS, and normal culture medium (1 mL per well) was added. On the designated day (day 0, 1, 2, 3, or 4), floating cells in the culture medium were collected and pooled with the harvested adherent cells. All cells were centrifuged for 5 minutes at 600g. Cell pellets were resuspended into 100 μ L of PBS and diluted 1:10 with a trypan blue solution (Mediatech, Inc.), of which 10 μ L was counted with the use of a hemacytometer. Viable cells exclude trypan blue, whereas dead cells do not. Cell viability was determined in duplicate with the following formula: (number of viable cells/number of total cells) \times 100%.

Animal Models and *In Vivo* Transfection

All animal experiments were performed in accordance with institutional guidelines. All experiments used 6- to 8-week-old SCID mice (C.B-17-SCID and C.B-17-SCID beige mice), which were obtained from Taconic (Germantown, NY). Male and female mice were used for the experiments.

BHK cells (5×10^6) were injected subcutaneously into the right flank of the abdomen of C.B-17-SCID or C.B-17-SCID beige mice. After 10 days, when the BHK tumors had reached a size of at least 1 cm², the mice were randomly assigned to one of three groups: control (n = 5), SinRep/LacZ (n = 5), or SinRep/IL12 (n = 5). Each mouse in the experimental groups received a single daily intraperitoneal injection of 0.5 mL of Opti-MEM I containing $\approx 10^7$ – 10^8 CFU of SinRep/LacZ or SinRep/IL12 viral vector particles. Three control mice received an injection of 0.5 mL of PBS, and two were left untreated. The day of first treatment was designated day 1. The size of BHK tumors was measured daily and calculated by the formula ($\pi/6$) \times (length, cm) \times (width, cm)². Control mice were followed for 12 days, and treated mice were followed for 5 weeks.

For human tumor models, LS174T, HT29, and CFPAC cells (initial injection of 4×10^6 cells) were grown as subcutaneous tumors in C.B-17-SCID mice for 4 weeks to allow the tumors to reach a substantial size before treatment was begun. Tumor-bearing C.B-17-SCID mice were randomly assigned to control or experimental groups, and they received daily intraperitoneal treatments of PBS or 0.5 mL SinRep/LacZ containing 10^7 – 10^8 CFU of viral vector particles. Experimental groups were treated for 6–7 weeks. Tumor sizes [(length, cm) \times (width, cm) \times (height, cm)] were recorded daily. There were four mice per group for experiments with LS174T and CFPAC tumors and five mice per group for experiments with HT29 tumors.

To establish the hepatic HuH-7 tumor model, C.B-17-SCID mice were anesthetized, and a transverse incision was made in the left flank through the skin and peritoneum, exposing the spleen. Mice were injected with 2×10^6 HuH-7 cells in 250 μ L of DMEM containing 10% FBS into the portal vein through the splenic hilus by using a 27.5-gauge needle. Eight weeks after tumor injection, when the tumors were palpable, mice received an intraperitoneal injection of 250 μ L of SinRep/LacZ ($\approx 10^7$ CFU) once or on three consecutive days. Control mice received an intraperitoneal injection of 250 μ L of Opti-MEM I medium. In this experiment, all mice were euthanized the day after the final injection.

Immunostaining

C.B-17-SCID mice were injected subcutaneously with 5×10^6 BHK cells. After 7 days, the mice were injected daily with SinRep/LacZ (treated) or left untreated (control). After the various treatments, the mice were euthanized and the tumors were removed. One portion of the tumor was fixed in 10% neutral-buffered formalin for at least 12 hours, embedded in paraffin, sectioned (5- μ m thick), and processed for immunohistochemistry. The other portion of the tumor was prepared for the β -galactosidase detection assay (*see* below).

For the hepatic tumors, sections were mounted onto electrostatically charged glass slides and then incubated at 60 °C overnight before being processed for immunohistochemistry. For the other tumors, slides with sections (5- μ m thick) of the paraffin-embedded tumors were prepared, deparaffinized by three washes in xylene, and then rehydrated through a graded ethanol series (100%, 90%, and 70%). Some slides were processed for immunohistochemical staining to detect bacterial β -galactosidase (mouse monoclonal antibody, clone 2E9, 1:20 dilution; BioDesign International; Saco, ME) or Factor VIII (rabbit polyclonal

antibody, pre-diluted read-to-use solution; Ventana Medical Systems; Tucson, AZ) on a NexES automated immunostainer (Ventana Medical Systems) by a standard streptavidin–biotin horseradish peroxidase complex detection method with 3,3-diaminobenzidine (DAB) as a chromagen (Ventana Medical Systems). Negative controls (human normal tissues, including tonsil, colon, skin, liver, and kidney, obtained from residual surgical pathology specimens) were included in all experiments.

For each tumor sample, one slide was stained with hematoxylin and eosin and another was stained for β -galactosidase. A pathologist (H. Yee), who was blinded to the treatments, reviewed the hematoxylin and eosin-stained sections. For all tumors, the samples were interpreted as positive if they had any staining for bacterial β -galactosidase and negative if they had no staining for bacterial β -galactosidase.

TUNEL Assay to Detect Apoptotic Cells

TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) staining kit (Boehringer Mannheim, Indianapolis, IN) was used according to manufacturer's instructions. Briefly, sections (5- μ m thick) of formalin-fixed tumors were deparaffinized and rehydrated with sequential washes in xylene, 100% ethanol, 70% ethanol, and PBS. The slides then were microwaved for 1 minute in boiling 0.1M citrate buffer solution (pH 6.0) before being placed on an automated immunostainer (NexES). The slides were incubated with

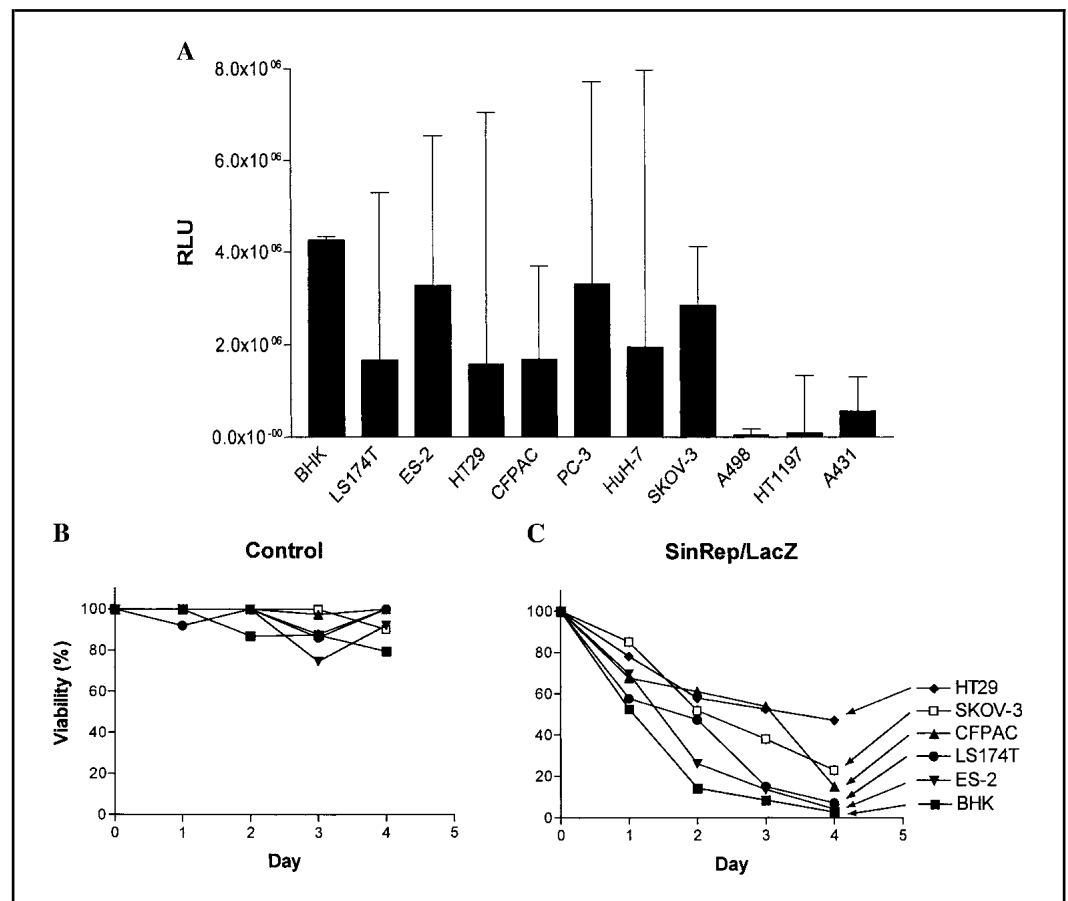
TUNEL reaction mixtures (100 μ L) for 60 minutes at room temperature and then with 100 μ L of Converter-POD, which is an anti-fluorescein peroxidase conjugate. After appropriate washes, 100 μ L of a DAB substrate solution was added to the slides and incubated for 2 minutes at room temperature. A pathologist (H. Yee), blinded to the treatments, reviewed the TUNEL-stained sections. TUNEL-positive staining was recorded only if the positive staining occurred in morphologically apparent apoptotic bodies.

β -Galactosidase Activity Assay

The activity of the bacterial β -galactosidase protein in tumors was detected by using the All-in-One™ β -Gal assay reagent kit (Pierce, Rockford, IL). The tumors were homogenized in 3 mL of PBS with a glass Pyrex homogenizer and a type B pestle (30 strokes). Homogenized samples were centrifuged at 1000g at 4°C for 10 minutes. The supernatants were harvested, and the pellets were resuspended with 2 mL of PBS. Fifty-microliter samples of each aliquot (supernatant and resuspended pellet) were mixed with an equivalent volume of the β -galactosidase assay reagent in one well of a 96-well plate and incubated at 37°C for 30 minutes; the absorbance at 405 nm was determined spectrophotometrically. The protein concentration of each sample was determined by the DC Protein Assay (BioRad Laboratories, Hercules, CA), and the results of the β -galactosidase assay were adjusted per 100 μ g of protein.

Fig. 1. A) Hamster (BHK) and human tumor (LS174T, ES-2, HT29, CFPAC, PC-3, HuH-7, SKOV-3, A498, HT1197, and A431) cell lines were infected *in vitro* with a Sindbis viral vector containing a firefly luciferase gene (SinRep/Luc). The cells (about 2×10^5 cells per well) were plated in 12-well plates and infected for 1 hour at a multiplicity of infection (MOI) of 100. After an overnight incubation, luciferase activity expressed in relative luciferase units (RLU) in cell lysates was quantified. **Bars** represent the mean values with upper 95% confidence intervals (CIs) from two to five replicates. (The number of replicates was BHK, four; LS174T, three; ES-2, four; HT29, three; CFPAC, five; PC-3, three; HuH-7, two; SKOV-3, five; A498, four; HT1197, two; and A431, five.)

B and C) Hamster and human tumor cell lines (2×10^5 cells per well) were mock-infected (control) or infected with the Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene at an MOI of about 50. Cell viability was determined daily by using the trypan blue exclusion assay. Data points represent the mean percentage of viable cells from two replicates. On day 4, the mean values of viability with 95% CIs for each cell line were as follows: BHK, mean = 2.7%, 95% CI = -1.1% to 6.5%; ES-2, mean = 4.2%, 95% CI = -19.4% to 27.7%; LS174T, mean = 7.0%, 95% CI = -31.1% to 45.1%; CFPAC, mean = 15.0%, 95% CI = -48.5% to 78.5%; SKOV-3, mean = 24.0%, 95% CI = 10.6% to 37.3%; HT29, mean = 47.0%, 95% CI = -54.7% to 148.6%.



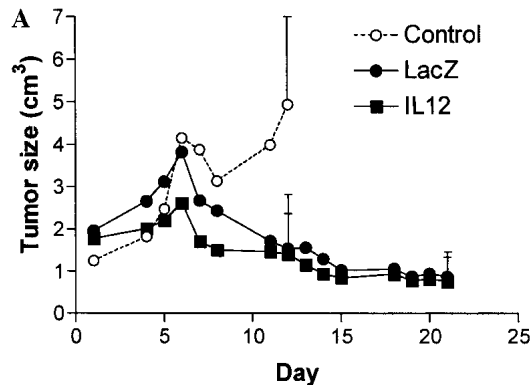
Statistical Analysis

The *in vitro* infectivity data were analyzed using a standard Student's *t* test. The tumor size data, obtained from different mouse models, were analyzed with repeated-measure two-way ANOVA using GraphPad Prism, version 3.0a for Macintosh (GraphPad Software, San Diego, CA). All *P* values presented in this study are two-tailed. The statistical significance of each factor of variation was determined by the *F* ratio, which is equal to the mean square of the specific factor divided by the residual mean square. The *F* ratio is presented in the format *F* (*df* of factor, *df* of residual) and determines the *F* distribution. The *P* value is the integration of the particular *F* distribution from the

F ratio calculated to positive infinity. In our analysis of animal tumor models, the SinRep/LacZ treatments are considered statistically significant relative to the control group if the *P* value of either SinRep/LacZ × time or SinRep/LacZ was less than .05. If the *P* value of SinRep/LacZ was less than .05 and the *P* value of SinRep/LacZ × time was greater than .05, the SinRep/LacZ treatment was still considered statistically significant, and the analysis indicates a parallel pair of tumor growth curves (treated and control groups). The *P* value for Subject (matching) determines whether the tumor size variation, if any, was caused by the different individuals (mice) within a group. If the *P* value was greater than .05, then the variation in tumor size was not caused by the difference within individual mice.

Fig. 2. Effect of Sindbis viral vectors on BHK tumor growth in severe combined immunodeficient (SCID) mice. **A**) BHK cells (5×10^6) were inoculated subcutaneously 10 days before treatment with Sindbis viral vectors containing the bacterial β -galactosidase gene (SinRep/LacZ) or murine interleukin 12 genes (SinRep/IL12) was started (on day 1). Intraperitoneal injections of SinRep/LacZ or SinRep/IL12 viral vectors (10^7 – 10^8 colony forming units [CFU]) were given daily. Control mice received daily intraperitoneal injections of phosphate-buffered saline or were left untreated. Tumor volumes were also recorded daily and calculated by the formula $(\pi/6) \times (\text{length, cm}) \times (\text{width, cm})^2$. On day 12, the control group was euthanized because of excessive tumor burden.

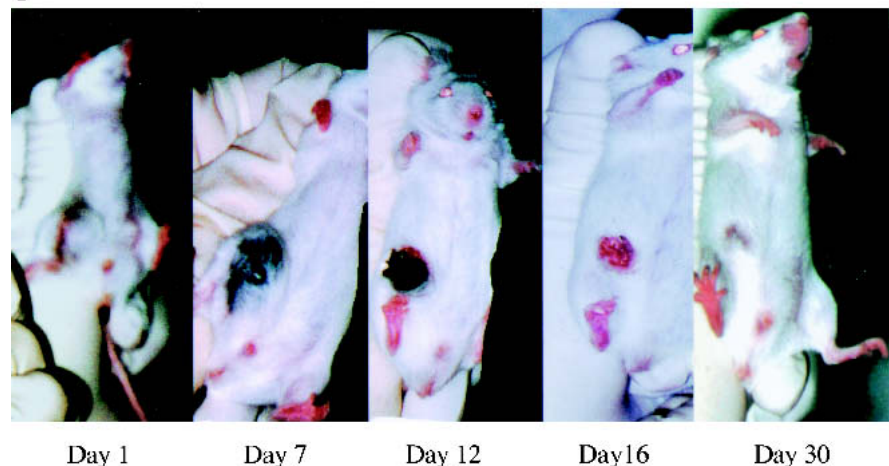
Data points represent the mean tumor size of five mice in each group. **B**) Statistical analysis of the effects of the Sindbis viral vector treatments were determined by a repeated measure two-way ANOVA. Tumor size was statistically significantly decreased by treatment with SinRep/LacZ or SinRep/IL12 compared with that in the control group after 12 days of treatments (Sinrep/LacZ × time, [$F(7, 32) = 5.14, P < .001$]; SinRep/IL12 × time, [$F(7, 32) = 3.43, P = .008$]). Compared with the tumor size in mice treated with Sinrep/LacZ vector, tumor size in mice treated with SinRep/IL12 was statistically significantly smaller (IL-12, [$F(1, 60) = 6.06, P = .017$]). There was no statistically significant interaction between vector type and treatment time, suggesting that both SinRep/LacZ and SinRep/IL12 have similar kinetics (IL-12 × time, [$F(14, 60) = 0.63, P = .829$]). **C**) Typical time course of BHK tumor regression during the SinRep/LacZ treatment on SCID mice. Treatment was started on day 1.



B

Source of variation	<i>df</i>	Sum of squares	Mean square	<i>F</i>	<i>P</i> value	% total variation
SinRep/LacZ						
SinRep/LacZ × time	7	40.8	5.83	5.14	.001	22.9
Time	7	35.62	5.09	4.49	.001	20.0
SinRep/LacZ	1	10.23	10.23	5.92	.021	5.7
Subjects (matching)	32	55.36	1.73	1.53	.119	31.0
Residual	32	36.3	1.13			
SinRep/IL12						
SinRep/IL12 × time	7	34.98	5.00	3.43	.008	21.4
Time	7	27.42	3.92	2.69	.026	16.7
SinRep/IL12	1	37.58	37.58	69.55	<.001	22.9
Subjects (matching)	32	17.29	0.54	0.37	.997	10.6
Residual	32	46.59	1.46			
IL12						
IL12 × time	14	5.22	0.37	0.63	.829	2.6
Time	14	77.19	5.51	9.32	<.001	39.0
IL12	1	7.35	7.35	6.06	.017	3.7
Subjects (matching)	60	72.8	1.21	2.05	.003	36.8
Residual	60	35.5	0.59			

C



RESULTS

Infectivity of Several Human Tumor Cell Lines *In Vitro*

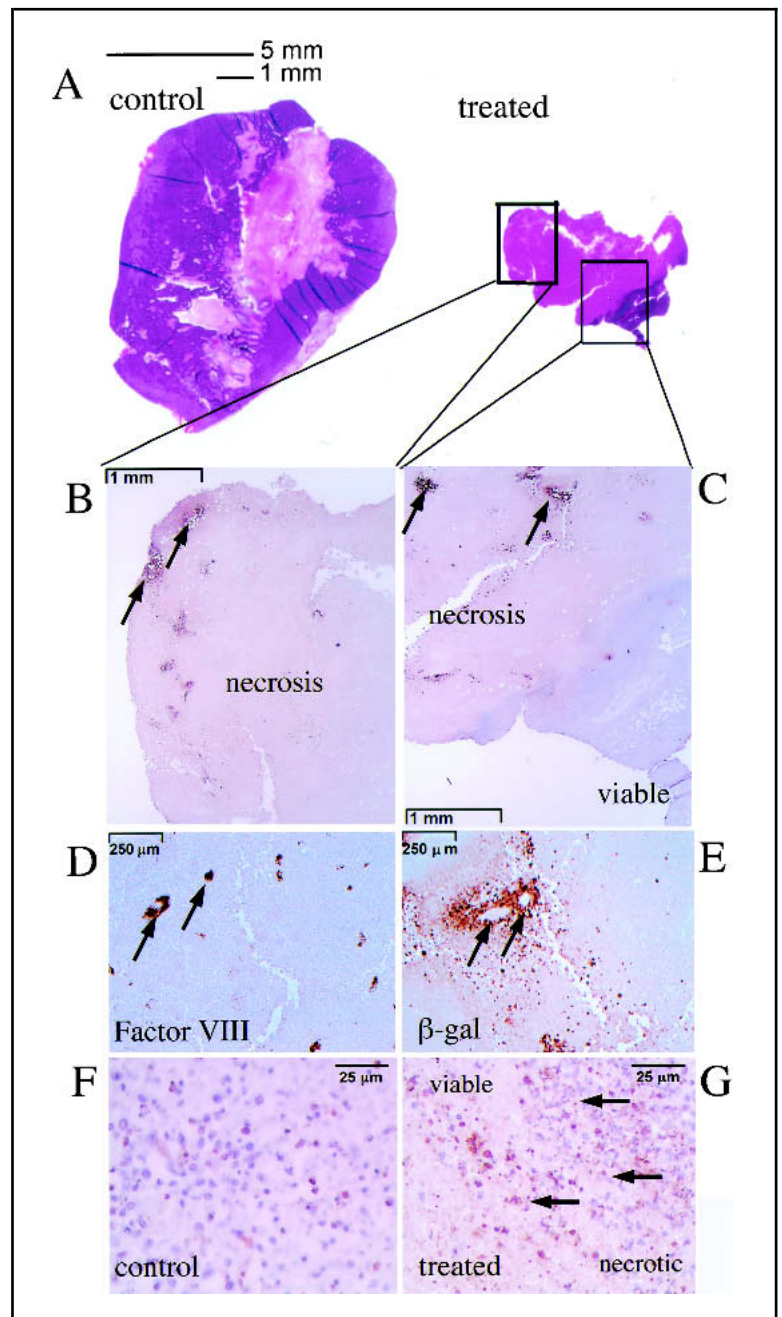
The alphaviruses have a broad host range that includes both invertebrates (mosquitoes or other hematophagous insects) and vertebrates (14). We first tested whether a Sindbis viral vector was capable of infecting several mammalian cell lines *in vitro*. The Sindbis viral vector SinRep/Luc, which carries the firefly luciferase gene, infected LS174T (colon), ES-2 (ovarian), HT29 (colon), CFPAC (pancreatic), PC-3 (prostate), HuH-7 (liver), and SKOV-3 (ovarian) cells, with high efficiency (Fig. 1, A). By contrast, the SinRep/Luc vector infected A498 (kidney), HT1197 (bladder), and A431 (epidermoid carcinoma of the vulva) cells with low efficiency relative to the efficiency with which it infects BHK cells. Similar levels of luciferase activity

were detected in all control mock-infected cells (approximately 150 relative luciferase units [RLU]) (data not shown).

Cytotoxic Effects of Sindbis Viral Vector Infection

Sindbis virus infection of mammalian cells is extremely cytotoxic because the virus induces apoptosis (27–30). We used the trypan blue exclusion assay to determine the viability of six different cancer cell lines after infection with the Sindbis viral vector SinRep/LacZ. Compared with the viability of mock-infected cells, the viability of all six cell lines rapidly declines over a 5-day period (day 0 to day 4) after infection with the SinRep/LacZ vector (Fig. 1, B and C). BHK cells were highly sensitive to the cytotoxic effects induced by SinRep/LacZ vector, with the majority of cells (mean viable percentage = 2.7%, 95% CI = -1.1% to 6.5%) dead 4 days after infection (Fig. 1,

Fig. 3. Severe combined immunodeficient (SCID) mice bearing BHK tumors were treated with the Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene for three consecutive days or were left untreated. Tumors were removed, fixed in formalin, sectioned, and processed for hematoxylin and eosin staining (A) or for immunohistochemistry by using a standard streptavidin–biotin horseradish peroxidase complex detection method with 3,3-diaminobenzidine (DAB) as chromogen (B–G). A) The darker purple area indicates the viable tumor tissue and the lighter pinkish area indicates the necrotic tissue. B and C) Immunohistochemical staining of the boxed areas in (A) with a mouse monoclonal antibody specific to bacterial β -galactosidase (encoded by LacZ) shows that the β -galactosidase protein (brownish dots, indicated by arrows) are mostly present in the peripheral necrotic area of the tumor. D and E) Consecutive tumor sections (5- μ m apart) were stained with a rabbit anti-Factor VIII polyclonal antibody, which detects endothelial cells (D) and anti- β -galactosidase (E). The same region is shown for (D) and (E). The tubal structure in the β -galactosidase stained section co-localizes with the Factor VIII signal, suggesting the presence of a blood vessel (indicated by black arrows). F and G) Tumor sections from control and Sindbis viral vector-treated mice were stained for apoptosis by using the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) assay. Intense and confined positive TUNEL signals were detected along the border between viable and necrotic areas of treated tumors (G), whereas the TUNEL signals on the border between viable and necrotic tissues were much less intense and more diffuse (F). A larger number of sharp and clear apoptotic bodies (indicated by arrows) were observed in the border regions of treated tumors (G).



C). Similar results were observed with ES-2 cells. Substantial cytotoxic effects were also observed in LS174T, CFPAC, HT29, and SKOV-3 cells infected with SinRep/LacZ vector. In control experiments, mock-infected cell lines showed no substantial cell death (Fig. 1, B).

In Vivo Antitumor Effects of Sindbis Viral Vector

Because BHK cells were the most sensitive to infection (Fig. 1, A) and cytotoxicity induced by the Sindbis viral vector (Fig. 1, C), we first established an SCID mouse model with BHK subcutaneous xenografts to test the *in vivo* antitumor efficacy of the SinRep/LacZ vector (Fig. 2). All mice in the control groups (those who received PBS or were untreated) were euthanized on day 12 of the experiment because of excessive tumor burden. By contrast, tumors in mice who received the SinRep/LacZ vector began to decrease in size by day 6 to 7 of treatment (Fig. 2, A). The repeated-measure two-way ANOVA (from day 0 to day 12) indicated that the SinRep/LacZ vector dramatically reduced the BHK tumor size (Fig. 2, B). After 30 days of treatment, com-

plete tumor regression was observed in four of five mice treated with the SinRep/LacZ vector (Fig. 2, C).

Tumors from mice treated with the SinRep/LacZ vector (mean size of treated tumors on day 12 = 1.54 cm³, 95% CI = 0.69 to 2.39 cm³) were statistically significantly smaller ($P = .003$) than tumors from control mice (mean size of control tumors on day 12 = 4.94 cm³, 95% CI = 2.90 to 6.99 cm³) (Fig. 3, A). Examination of tumor sections from mice treated with the SinRep/LacZ vector revealed that approximately 90%–95% of the tumors were necrotic, with only a rim of viable tumor cells (Fig. 3, A). Examination of tumor sections from control mice revealed that no more than 30% of those tumors were necrotic and that the necrotic areas were principally confined to the center of the tumor mass. Necrotic tumor cells were identified by their eosinophilic staining pattern and by a loss of cellular organization and membrane integrity.

We next assessed expression of the LacZ gene product, bacterial β -galactosidase (Fig. 3, B and C). Bacterial β -galactosidase immunoreactivity was observed only in necrotic areas of tumors from mice treated with the SinRep/LacZ vector. Bacte-

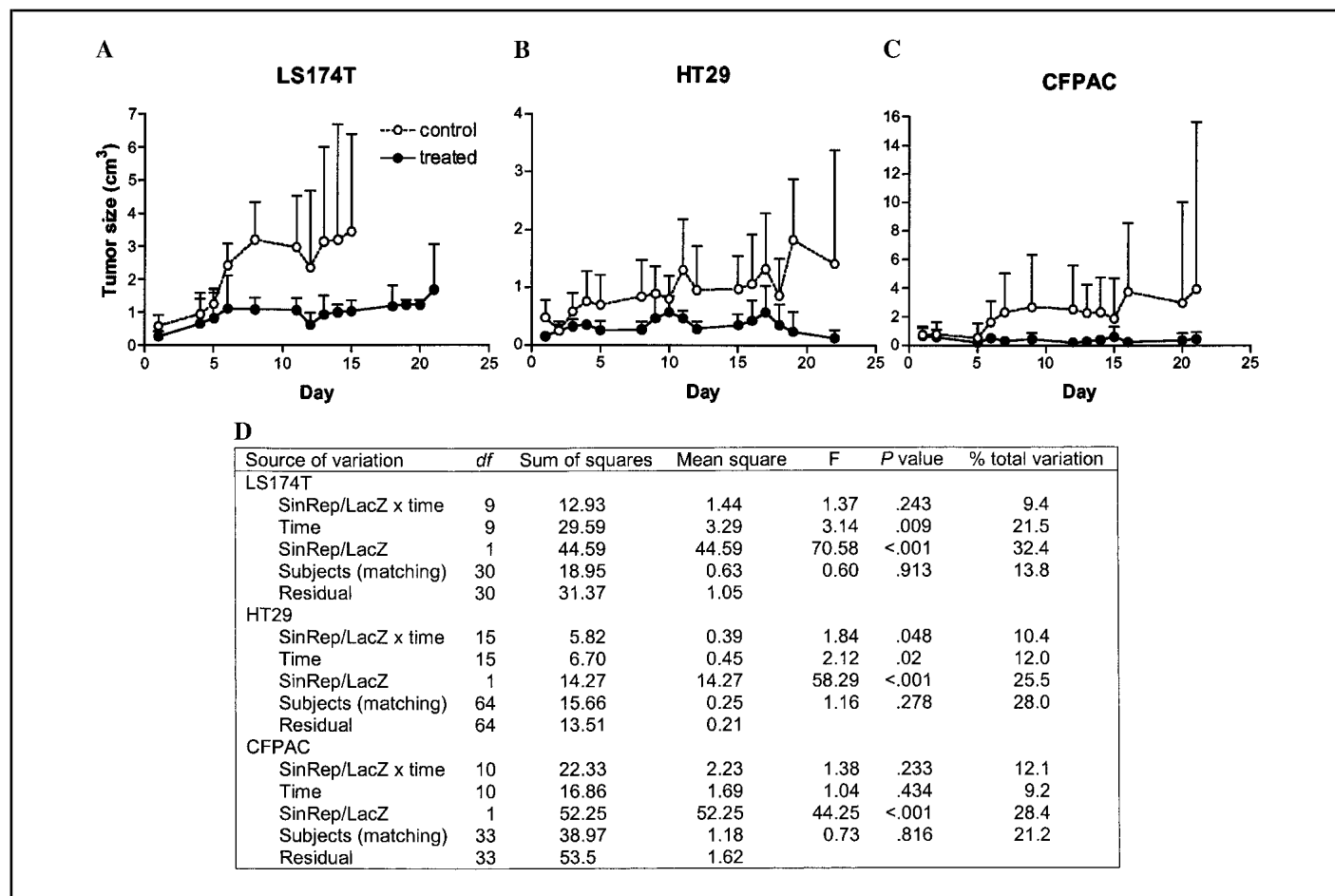


Fig. 4. Effect of Sindbis viral vectors on human tumors in severe combined immunodeficient (SCID) mice. Approximately 4×10^6 human colon LS174T (A), colon HT29 (B), or pancreatic CFPAC (C) cells were grown in SCID mice as subcutaneous tumors. After 4 weeks, daily intraperitoneal treatments (started on day 1) of Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene (10^7 – 10^8 colony forming units) were given to experimental groups. Control mice received intraperitoneal injections of phosphate-buffered saline. Tumor volume was measured daily and tumor size calculated as (length, cm) \times (width, cm) \times (height, cm). Control mice bearing LS174T tumors were euthanized on day 15 because of excessive tumor burden. **Data points** represent mean

tumor size of four mice (LS174T and CFPAC) or five mice (HT29) and the **error bars** represent the upper 95% confidence intervals. **D**) Statistical analysis of the effects of the Sindbis viral vector treatments were determined by a repeated measure two-way ANOVA. In mice treated with SinRep/LacZ, LS174T tumors ($F(1, 30) = 70.58; P < .001$), CFPAC tumors ($F(1, 33) = 44.25; P < .001$), and HT29 tumors ($F(1, 64) = 58.29; P < .001$) were all statistically significantly smaller compared with those in untreated control groups. Individual subjects within a group did not statistically significantly affect the tumor size (Subjects [matching]: LS174T, $P = .913$; HT29, $P = .278$; CFPAC, $P = .816$).

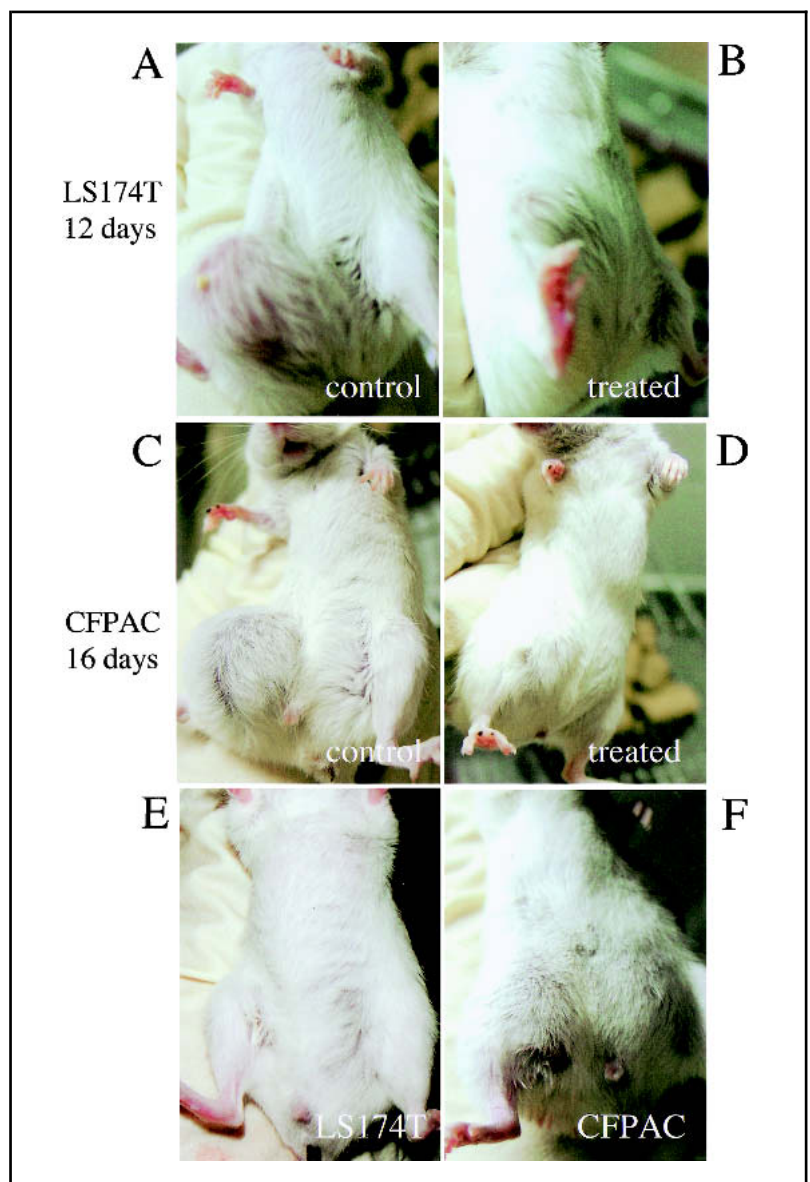
rial β -galactosidase was not detected in viable areas of the tumors. To determine whether expression of bacterial β -galactosidase was associated with the vasculature, we stained serial tumor sections with an antibody specific for Factor VIII, a marker for endothelial cells, and bacterial β -galactosidase (Fig. 3, D and E). Detection of Factor VIII expression corresponded to areas of bacterial β -galactosidase expression, suggesting that the Sindbis viral vectors were delivered to the tumor by the vasculature. Furthermore, the bacterial β -galactosidase was still detectable at the periphery of the treated tumors after three treatments. Because no bacterial β -galactosidase was detected in the central necrotic zone, we speculate either that this zone completely degraded the β -galactosidase antigen or that this zone contained fewer blood vessels and therefore did not receive the Sindbis viral vector.

We used TUNEL staining to determine whether BHK tumor cells were dying by apoptosis in mice treated with Sindbis viral vectors. In the control tumors, sparse TUNEL signals were detected along the border between viable and necrotic tissue (Fig. 3, F). By contrast, in the tumors from mice treated with the SinRep/LacZ vector, intense TUNEL signals were observed

along the border between viable and necrotic areas (Fig. 3, G). Compared with the control tumors, more sharp and clear apoptotic bodies were observed in the border regions separating viable and necrotic areas in tumors from treated mice. No TUNEL signals were observed in the central necrotic area of control or treated tumors (data not shown). This observation suggests that the SinRep/LacZ vector may suppress BHK tumor growth by inducing apoptosis.

We examined the *in vivo* antitumor activity of the SinRep/LacZ vector on different human tumor cells. Human cell lines LS174T, HT29, and CFPAC were injected subcutaneously into C.B-17-SCID mice, and tumors were grown to a defined size (approximately 0.5 cm³) before treatment. SinRep/LacZ vector treatments were administered to experimental groups five times a week, and PBS was administered to the control group. Tumor size was monitored over time (Fig. 4, A–C). Tumors from mice treated with the SinRep/LacZ vector were statistically significantly smaller than those from untreated mice after 2 weeks of treatment, regardless of the tumor type (Fig. 4, A–C) ($P < .001$). In each human tumor model, there were no statistically significant effects resulting from variation among individual subjects

Fig. 5. Effect of Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene on the growth of human colon LS174T and pancreatic CFPAC tumors in severe combined immunodeficient (SCID) mice. Control mice received mock treatments (phosphate-buffered saline). Treated mice received daily intraperitoneal injections of 10^7 – 10^8 colony forming units of SinRep/LacZ. **A** and **B**) Representative results for LS174T tumors after 12 days of mock treatments (**A**) or SinRep/LacZ treatments (**B**). **C** and **D**) Representative results for CFPAC tumors after 16 days of mock treatments (**C**) or SinRep/LacZ treatments (**D**). **E** and **F**) Complete regression of LS174T (**E**) or CFPAC (**F**) tumors was observed in some mice after 42 and 46 SinRep/LacZ treatments, respectively.



within the same group (Fig. 4, D; all *P* values for Subject (matching) were greater than .05). After about 2 weeks, LS174T and CFPAC tumors in mice treated with the SinRep/LacZ vector were dramatically smaller than tumors in control mice (Fig. 5, A–D). In mice treated with the SinRep/LacZ vector, complete tumor regression was observed in two of four inoculated with LS174T tumor cells and in one of four inoculated with CFPAC tumor cells (Fig. 5, E and F).

***In Vivo* Targeting of SinRep/LacZ Vector to HuH-7 Tumors to the Liver**

To determine whether intraperitoneal administration could result in delivery of the Sindbis viral vector to tumors in well vascularized organs such as liver, we established an HuH-7 liver tumor model in SCID mice. After 8 weeks, when tumor growth was evident by palpation, mice were injected intraperitoneally with the SinRep/LacZ vector. Tumor-bearing mice treated with SinRep/LacZ vector one time only or on three consecutive days were euthanized 24 hours after the final treatment. We determined whether the tumors expressed bacterial β -galactosidase by immunohistochemistry (Fig. 6). Bacterial β -galactosidase expression was not detected in normal liver tissue (Fig. 6, A) or in tumors from untreated control mice (data not shown). Bacterial β -galactosidase expression was not clearly detectable in tumors from mice that received only a single treatment with the SinRep/LacZ vector (data not shown) but could be detected in tumors from mice that received treatments on three consecutive days (Fig. 6, B). We observed necrotic areas in tumors from mice that received three consecutive SinRep/LacZ vector treatments (Fig. 6, C).

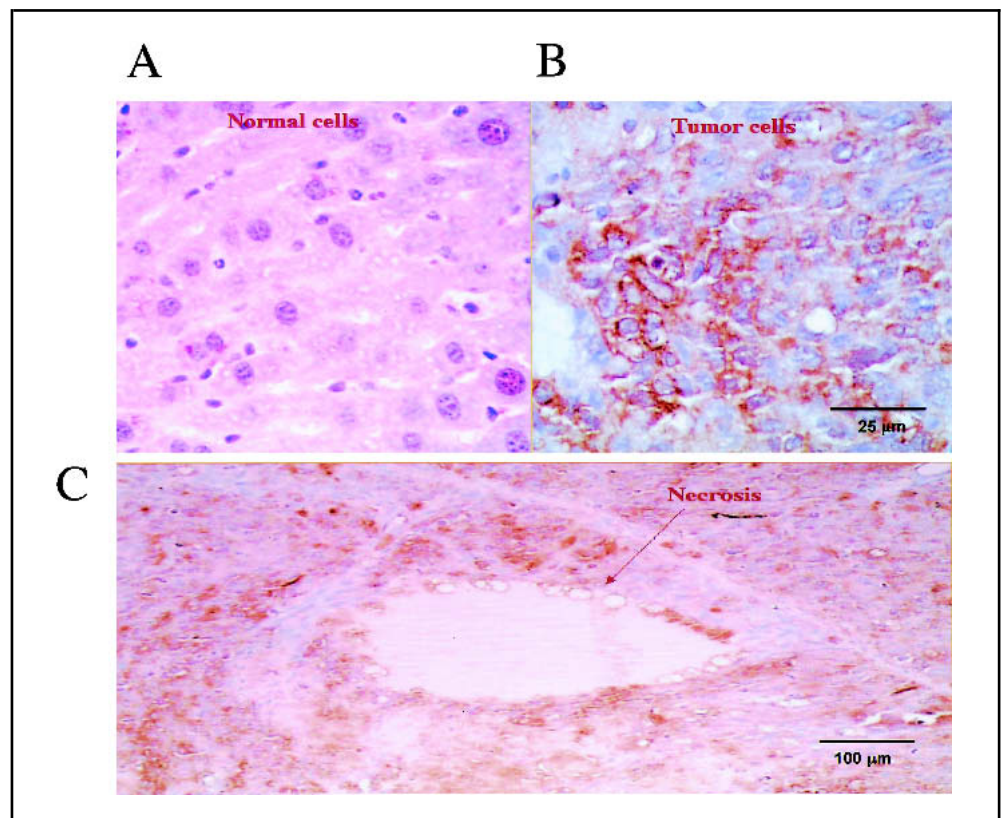
To determine whether the Sindbis viral vector infected normal tissues, we quantified the β -galactosidase protein activity in different tissues after one or three treatments (Fig. 7). Compar-

ing tumor cells from control-treated mice with those from mice who received a single SinRep/LacZ vector treatment revealed no substantial difference in β -galactosidase activity. However, mice that received three SinRep/LacZ vector treatments had noticeably higher β -galactosidase activity in tumor cell supernatants and in tissue pellet samples than control mice. No substantial increases in β -galactosidase activity were observed in liver, heart, lung, kidney, and testis tissues, regardless of whether the mice received one or three injections of the Sindbis viral vector. Compared with β -galactosidase activity in tumors, noticeably lower β -galactosidase activity was observed in the brain of one treated mouse. Despite the infectivity to brain cells, all mice who received one or three SinRep/LacZ vector injections were healthy and showed no abnormal behavior within the time frame of the experiment.

Role of NK Cells on Antitumor Effects of Sindbis Vectors

In the absence of an adaptive immune system, SCID mice rely on their innate immune system as a defense against pathogens and tumors. Among cells in the innate immune system, NK cells play an important role in antitumor activity. To determine the importance of NK cells in Sindbis viral vector-mediated antitumor activity, we induced subcutaneous BHK tumors in C.B-17-SCID mice and C.B-17-SCID beige mice and measured tumor growth over time (Fig. 8). C.B-17-SCID beige mice have an autosomal recessive mutation that makes them deficient in NK cells and impairs the chemotaxis and motility of macrophages. The growth of BHK tumors in C.B-17-SCID and C.B-17-SCID beige mice was similar (data not shown). Treatment with the SinRep/LacZ vector was more effective in C.B-17-SCID mice than in C.B-17-SCID beige mice because changes in tumor growth occurred faster in the C.B-17-SCID

Fig. 6. Effect of Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene on the growth of human hepatocellular HuH-7 carcinomas in severe combined immunodeficient (SCID) mice. SCID mice were injected with 2×10^6 human hepatocellular carcinoma HuH-7 cells via the portal vein through the splenic hilus. After 8 weeks, mice received daily intraperitoneal treatments of SinRep/LacZ (10^7 – 10^8 colony forming units) for 3 days. The expression of bacterial β -galactosidase (LacZ) protein in the liver was examined histologically by immunohistochemistry with a mouse monoclonal antibody specific to bacterial β -galactosidase by using a standard streptavidin-biotin horseradish peroxidase complex detection method with 3,3-diaminobenzidine (DAB) as chromogen, the day after the final treatment. **A**) Bacterial β -galactosidase was not detected in normal liver tissue (**A**) but was detected in the liver tumors of mice treated with SinRep/LacZ (**B**). A necrotic area was observed in tumors that were injected with SinRep/LacZ (**C**).



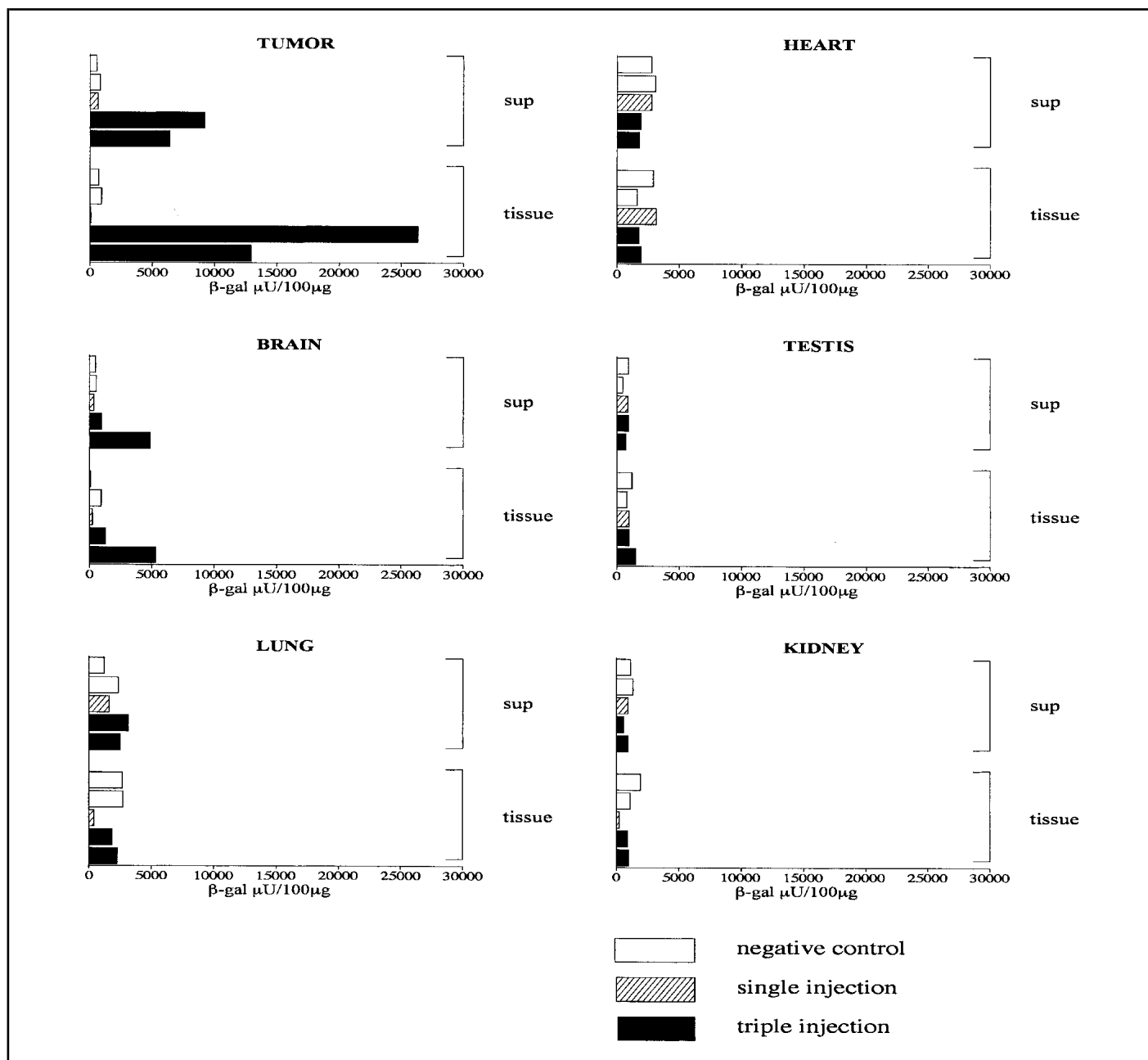


Fig. 7. β -Galactosidase activity in tissues from severe combined immunodeficient (SCID) mice that had human hepatocellular HuH-7 carcinomas and were treated with Sindbis viral vector SinRep/LacZ containing the bacterial β -galactosidase gene. SCID mice were injected with 2×10^6 human hepatocellular carcinoma HuH-7 cells via the portal vein through the splenic hilus. After 8 weeks, mice received daily intraperitoneal treatments of SinRep/LacZ (10^7 – 10^8 colony forming units) for 1 or 3 days. Within 24 hours of the last (or only) SinRep/LacZ treatment, β -galactosidase activity in different tissues was assayed by using a β -Gal assay reagent kit (Pierce, Rockford, IL), according to the

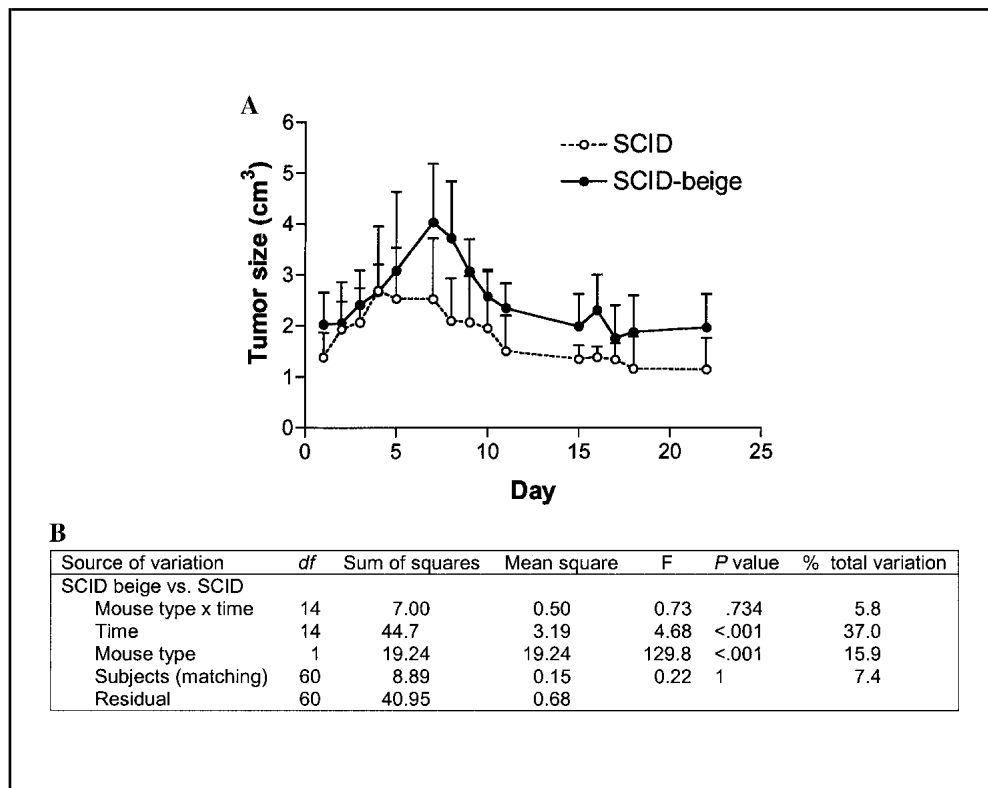
manufacturer's recommended protocol. Tissues from different mice were homogenized separately and centrifuged. β -Galactosidase activity was measured in both the supernatant (sup) and tissue pellet (tissue) fractions of each sample. The total protein concentration of each fraction was determined and used to normalize the β -galactosidase activity (expressed as β -gal μ U per 100 μ g total protein). **Bars** represent the activity of each individual mouse tissue. **Open bars** represent the activity of each individual control mice. **Hatched bars** represent mice who received a single treatment with SinRep/LacZ. **Solid bars** represent mice who received three treatments with SinRep/LacZ.

mice than in the C.B-17-SCID beige mice ($P < .001$) (Fig. 8, A). However, the overall tumor response was similar. Complete regression of tumors was obtained in three of five C.B-17-SCID mice but in none of five C.B-17-SCID beige mice.

To further examine the role of NK cells in the tumor response to treatments with the Sindbis viral vector, we constructed a Sindbis viral vector carrying the genes for the two murine IL-12 subunits, mp35 and mp40. IL-12 is a potent NK cell stimulatory factor (46) and has strong antitumor activity (47–50). BHK tu-

mors in mice treated with the Sindbis viral vector SinRep/IL12 were statistically significantly smaller than tumors in mice treated with the SinRep/LacZ vector (Fig. 2, B; IL12 gene, $P = .017$), suggesting that the SinRep/IL12 vector had enhanced antitumor activity. The kinetics of tumor reduction were similar in mice treated with either the SinRep/IL12 vector or the SinRep/LacZ vector (Fig. 2, A and B). Complete tumor regression was also observed in three of five mice treated with the SinRep/IL12 vector (data not shown).

Fig. 8. Effect of natural killer cells on the *in vivo* activity of the Sindbis viral vector SinRep/LacZ containing the β -galactosidase gene. **A)** Subcutaneous hamster BHK tumors were induced in C.B-17-SCID mice and C.B-17-SCID beige mice, which lack motile macrophages and natural killer cells. After 10 days, mice were mock treated (phosphate-buffered saline) or treated with daily intraperitoneal injections of SinRep/LacZ (10^7 – 10^8 colony forming units). Tumor volumes were recorded daily and calculated by formula: $(\pi/6) \times (\text{length, cm}) \times (\text{width, cm})^2$. Mock-treated C.B-17-SCID and C.B-17-SCID beige mice showed no differences in tumor growth (data not shown). **Data points** represent the mean tumor size of five mice and **error bars** represent the upper 95% confidence intervals. **B)** Statistical analysis of the effects of the Sindbis viral vector treatments were determined by a repeated measure two-way ANOVA. Treatments with Sindbis vectors resulted in similar kinetics of tumor regression because there was no statistically significant interaction between mouse type and time (mouse type \times time; $F(14, 60) = 0.73, P = .734$). However, treatments were more effective in C.B-17-SCID mice than in C.B-17-SCID beige mice (mouse type; $F(1, 60) = 129.8, P < .001$).



DISCUSSION

Our observations indicate that Sindbis viral vectors induce extensive necrosis in BHK tumors, especially at the tumor periphery. This pattern of necrosis differs from that seen in BHK tumors of untreated mice (Fig. 3, A), which is centrally located and typically associated with hypoxia and poor nutrition. The co-localization of the Sindbis viral vector and blood vessels within tumors suggests that the vector was delivered through the bloodstream (Fig. 3, D and E), which reflects the advantage of Sindbis viral vector as an antitumor agent. The clearance rate from circulation is an important factor for successful gene therapy viral vectors. Serum components such as complement have been shown to mediate lysis of retroviral vectors in human blood (6–8). Adenoviruses are unstable in the bloodstream (12), precluding their delivery to tumors via this route. By contrast, because they are naturally evolved alphaviruses, Sindbis virus and Venezuelan equine encephalitis virus are stable in the bloodstream (51–53), which is important for systematic administration to target distal or metastasized tumors.

A second advantage of Sindbis viral vectors is their ability to induce apoptosis. TUNEL staining of treated BHK tumor sections (Fig. 3, G) suggests that the apoptosis-inducing activity of the Sindbis viral vector plays a role in its antitumor activity. Sindbis viral vectors induce apoptosis in mammalian cells (27–30). We have shown in this article that Sindbis viral vectors also induce apoptosis (Fig. 3, G) and necrosis *in vivo* in BHK tumors, which was accompanied by complete tumor regression in some mice (Fig. 2).

A third advantage of Sindbis viral vectors is that the treatments, which comprised multiple injections of Sindbis viral vectors, did not appear to be toxic to the mice. This apparent lack of toxicity might explain why intraperitoneal injections of the Sindbis viral vectors almost selectively infected tumor cells and

some brain cells, with no substantial infection of the host's heart, lung, liver, and kidney (Fig. 6, A, and Fig. 7). The infection of brain cells resulted in no observable clinical central nervous system disorders in adult (6- to 8-week-old) mice, which are resistant to the neurovirulent effects of Sindbis virus. By contrast, in neonatal mice, Sindbis virus has neurovirulent effects (54).

In our mouse models, tumor regression is not likely the result of preferential Sindbis virus infection of human cells relative to murine cells. Successful vaccination with Sindbis viral vectors in adult mice (20–26) demonstrates that Sindbis viral vector infects mouse cells, although the level of infection may be lower than that in neonatal mouse brain cells. In humans, the mild symptoms (fever, rash, and arthralgia) resulting from Sindbis virus infection also suggests low infectivity and viral replication. Such mild symptoms contrast with the strong antitumor effects we observed in this study and imply that there is an inherent difference in the susceptibility of tumor cells and normal cells to Sindbis vector infection.

Sindbis viral vectors target cells via the high-affinity laminin receptor (43,44). An association has already been established between the increased expression of the 67 000 kd high-affinity laminin receptor and tumor progression for a wide variety of human cancers (32–42). Laminin receptors appear to be altered in number and degree of ligand occupancy in human carcinomas. It has been shown that many tumors (e.g., breast and colon carcinoma tissues) contain a higher number of exposed (i.e., unoccupied) receptors than do benign lesions (55–57). This may be the indirect result of defective basement membrane organization in carcinomas. By contrast, the laminin receptors on invading carcinoma cells may be distributed over the entire surface of the cell. These receptors may be unoccupied because the invading cells have lost basement membrane structure. Because unoccupied receptors are found at much higher levels in cancer

cells than in normal cells, an agent such as Sindbis virus, which is able to induce apoptosis after binding to the high-affinity laminin receptors, would be expected to preferentially limit tumor cell survival or expansion.

In addition to direct killing of tumor cells induced by Sindbis virus infection, the host immune system may contribute to tumor elimination in our tumor models. NK cells, which are important for innate immunity, possibly aid the antitumor effects of Sindbis viral vectors (Fig. 8). NK cells can be cytotoxic to tumor (58,59) and virus-infected cells (46). When the host antiviral mechanisms become activated, several signals, such as interferons, are released that can activate NK cells (46). NK cell activation may also result from the inflammation caused by tumor cell necrosis and the release of cell contents. IL-12 has been shown to be a potent NK cell stimulatory factor (46), and administration of IL-12 produces potent antitumor and antimetastatic activity against certain solid tumors (47–50). However, complete tumor eradication is usually limited to very small tumors. In our study, an IL-12-encoding recombinant Sindbis viral vector (SinRep/IL12) appeared to enhance the antitumor effects compared with the SinRep/LacZ vector (Fig. 2, A). Because Sindbis viral vectors express exogenous genes at very high levels, other antitumor therapeutic genes, such as tumor suppressor, cytotoxic, and cytokine genes, might be ideal candidates to boost the antitumor efficacy of Sindbis vectors.

Because tumor cells express more high-affinity laminin receptors than normal cells, Sindbis viral vectors apparently target and infect tumors. We have also developed Sindbis viral vectors that recognize other specific cell surface molecules, while retaining high infectivity and titers (45), and those vectors might, therefore, allow refinement of *in vivo* targeting. Our results suggest that Sindbis viral vectors, alone or in combination with other existing treatment modalities, might be useful new tools for cancer gene therapy.

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NOTES

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