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The Oncolytic Effect of Recombinant Vesicular Stomatitis Virus Is Enhanced by Expression of the Fusion Cytosine Deaminase/Uracil Phosphoribosyltransferase Suicide Gene

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ABSTRACT

Vesicular stomatitis virus (VSV) has recently been demonstrated to exhibit significant oncolytic capabilities against a wide variety of tumor models *in vitro* and *in vivo*. To potentially enhance the oncolytic effect, we generated a novel recombinant VSV (rVSV) that expressed the fusion suicide gene *Escherichia coli* cytosine deaminase (CD)/uracil phosphoribosyltransferase (UPRT). rVSV encoding the CD/UPRT fusion gene (VSV-C:U) exhibited normal growth properties and generated high levels of biologically active CD/UPRT that could catalyze the modification of 5-fluorocytosine into chemotherapeutic 5-fluorouracil (5-FU), which exhibited considerable bystander effect. Intratumoral inoculation of VSV-C:U in the presence of the systemically administered prodrug 5-fluorocytosine produced statistically significant reductions in the malignant growth of syngeneic lymphoma (A20) or mammary carcinoma (TSA) in BALB/c mice compared with rVSV treatments or with control 5-FU alone. Aside from detecting prolonged therapeutic levels of 5-FU in VSV-C:U-treated animals harboring TSA tumors and enhancing bystander killing of tumor cells, we demonstrated marked activation of IFN-γ-secreting cytotoxic T cells by enzyme-linked immunospot analysis that may have also facilitated tumor killing. In conclusion, the insertion of the fusion CD/UPRT suicide gene potentiates the oncolytic efficiency of VSV by generating a strong bystander effect and by contributing to the activation of the immune system against the tumor without detrimentally altering the kinetics of virus-mediated oncolysis and may be useful in the treatment of malignant disease.

INTRODUCTION

The delivery of suicide genes into malignant cells affords the opportunity of increasing the efficacy of tumor eradication while limiting toxic side effects that are often found with systemic chemotherapy (1). Suicide gene therapy involves the transduction of cancer cells with a nonmammalian gene encoding an enzyme that converts a relatively innocuous, systemically delivered prodrug into a highly toxic chemotherapeutic (1). However, a major issue when considering enzyme-prodrug systems for the treatment of cancer remains the effective transport of the suicide gene into the appropriate tumor tissue. Although a number of strategies have been used to target tumor cells effectively, including the use of replication-defective virus vectors, problems associated with low levels of gene transfer and expression are encountered frequently (2).

Recently, we genetically modified a replication-competent oncolytic virus, VSV,[1] to carry foreign genes such as cytokines (3). We reasoned that this approach could improve tumor cell killing by combining the effect of direct viral oncolytic activity with the augmented antitumor immune responses or, in the case of suicide genes, bystander cytotoxic action. VSV is an enveloped, negative-stranded RNA virus with a simple genetic structure of five genes and well-characterized immunobiology (4). VSV is relatively innocuous in humans, inducing at the most a flu-like syndrome (5). However, the majority of the population lacks antiviral antibodies that could conceivably limit virus treatment, and in general, the incidence of exposure to the VSV is low (6). VSV is a nonintegrating virus that replicates in the cytoplasm, does not undergo genetic reassortment, and has no known transforming potential (4). Evidence indicates that the mechanism of VSV-induced oncolysis involves taking advantage of defects in the IFN host defense system prevalent in tumor cells. Lack of an effective IFN response allows propagation of virus, leading to rapid cytolysis (7, 8). Our attempts at improving the efficacy of VSV tumor therapy have indicated that rVSV is easy to generate and is amenable to high-titer production and purification (3). rVSV variants generated thus far have also demonstrated greater oncolytic activity than the WT virus in tumor therapy studies and appear greatly attenuated *in vivo* compared with the WT parent virus (3).

CD is a well-characterized enzyme-prodrug system that functions similar to the herpesvirus TK paradigm (9, 10). However, CD is an enzyme found in bacteria and fungi and not mammalian cells that can catalyze the hydrolytic deamination of the relatively nontoxic antifungal agent 5-FC to the commonly used chemotherapeutic 5-FU. 5-FU can be further modified by cellular enzymes into potent pyrimidine antimetabolites, which induce cell death by inhibiting thymidine synthetase and DNA and RNA synthesis (11). 5-FU has also been reported to diffuse freely through cellular membranes to generate a strong direct bystander effect (12). Reports using human colorectal xenograft murine models indicated that transfection of 4% of the tumor cells with *Escherichia coli* CD gene led to survival rates of 60% (13). However, limited success has been reported in several experiments that used selected breast and pancreatic tumor models because such cells were found to be relatively resistant to 5-FU, possibly attributable to defects in downstream cellular pathways that are responsible for the metabolism of this enzyme (14, 15). In support of this hypothesis, expression of bacterial or yeast UPRT in mammalian cells was found to greatly enhance the sensitivity of the cells to 5-FU (15, 16). UPRT is an enzyme that catalyzes the conversion of uracil and 5-FU directly into UMP and 5-fluoro-UMP, respectively, by bypassing rate-limiting reactions controlled by the cellular enzymes. Coexpression of CD and UPRT has been reported to increase the sensitivity to 5-FC 10–168 times when compared with CD alone, depending on the experimental model used (15–17).

To examine whether the CD/5-FC system could be used with rVSV as a vector delivery system, we generated a novel rVSV suicide gene model expressing the *E. coli* CD/UPRT fusion gene (rVSV-C:U). We show here that the expression of the foreign gene does not detrimentally affect VSV replication and results in the generation of high levels of CD and UPRT enzyme activity after infection of the cell. Importantly, rVSV-C:U retained selective oncolytic activity and displayed

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[1] The abbreviations used are: VSV, vesicular stomatitis virus; rVSV, recombinant VSV; CD, cytosine deaminase; UPRT, uracil phosphoribosyltransferase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; TK, thymidine kinase; HPLC, high-performance liquid chromatography; GFP, green fluorescent protein; hIFN, human IFN; TLCS, TLC sheet; BHK, baby hamster kidney; MOI, multiplicity of infection; WT, wild-type; HI-VSV, heat-inactivated VSV; GCV, ganciclovir; ELISPOT, enzyme-linked immunospot; CPE, cytopathic effect.

effective bystander effect *in vivo* and *in vitro* with concomitant tumor cell killing. Our data indicate that incorporating CD-based strategies into rVSV-mediated gene therapy protocols may improve the oncolytic potential of this tumor treatment approach.

MATERIALS AND METHODS

Chemicals. [^{3}H]Cytosine (9.5 Ci/mmol), [2-^{14}C]uracil (54 mCi/mmol), 5-phosphoribosyl-1-pyrophosphate, uracil, cytosine, UMP, 5-FC, low EEO type 1 agarose, and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO), and streptavidin-alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). hIFN-α-2a and gancyclovir were obtained from Roche Laboratories, Inc. (Nutley, NJ), and 5-FU was purchased from Pharmacia & Upjohn Co (Kalamazoo, MI). Antimouse IFN-γ antibodies and biotinylated anti-IFN-γ monoclonal antibodies were acquired from PharMingen (San Diego, CA). TLCSs and polyethyleneimine-impregnated TLCSs were acquired from EM Science (Gibbstown, NJ), and Nunc MaxiSorp plates were obtained from Nunc (Naperville, IL).

Cell Culture. Human multiple myeloma cell line 8226/Dox40 was a gift from Dr. L. H. Boise (University of Miami; Ref. 18), and the murine T-cell lymphoma EL4 and the murine B-cell lymphoma A20 were gifts from Dr. J. Rosenblatt (University of Miami; Refs. 19 and 20). The TSA mammary adenocarcinoma cell line was a gift from Dr. A. Rakmilevich (University of Wisconsin, Madison, WI; Ref. 2). B16(F10) melanoma cells, 293T embryonal kidney cells, and the BHK cell line BHK-21 were purchased from American Type Culture Collection (Manassas, VA). HMVEC, a human microvascular endothelial cell line, was acquired from Clonetics Corp. (San Diego, CA). Cells were grown in suspension or in monolayer in the recommended culture medium with fetal bovine serum.

Construction of Recombinant Virus. The recovery of the infectious recombinant viruses and the growth, purification, concentration, and titration of the WT and recombinant viruses were performed according to the methods described previously (21, 22) The C:U gene was PCR amplified from the pORF-codA::upp expression vector (InvivoGen). The primers were 5′-GAACTCGAGCCACTATGGTGTCGAATAACGCTT and 3′-GTCGAGCTAGCGAATTTCGACAAGCTTATTTCGTACC, introducing *Xho*I and *Nhe*I restriction sites, respectively. The C:U cDNA was then excised from the amplification product with *Xho*I and *Nhe*I and cloned into the *Xho*I/*Nhe*I site of pVSV-XN2 (23) using a Zero Blunt TOPO shuttle vector (Invitrogen, Carlsbad, CA). The plasmid pVSV-XN2 contains the entire VSV genome and has unique *Xho*I and *Nhe*I sites flanked by VSV transcription start and stop signals. After recovery of the infectious rVSV in BHK cells, monoclonal virus was obtained by plaque purification and further purification, and concentration was achieved by sucrose centrifugation. Virus stock titers were measured by standard plaque assay.

CD Enzyme Assay Measured Spectrophotometrically. CD activity was measured directly by using cell lysates or indirectly by measuring 5-FU released in the culture media. BHK cells (5×10^6) were infected with rVSV-GFP at MOI = 5.0, rVSV-C:U at MOI = 0.1, or rVSV-C:U at MOI = 5.0. To measure 5-FU in culture media, 5 mM 5-FC was added to the media 2 h postinfection. Samples of culture media (10 μl) were collected at different time points and quenched with 990 μl of 0.1 M HCl. To measure C:U activity using cell lysates, 6 h postinfection cells were washed twice with PBS, resuspended in PBS at a concentration of 5×10^6 cells/100 μl PBS, and exposed to five cycles of thawing/freezing. Fifty μl of each sample were mixed with 950 μl of 3 mM solution of 5-FC in PBS and incubated at 37°C. At different time points, 50-μl samples were collected and quenched with 950 ml of 0.1 mM HCl (24).

The concentration of 5-FU and 5-FC for each condition was determined spectrophotometrically using the following formulas: 5-FC (mM) = $0.119(A_{290}) - 0.025(A_{225})$; and 5-FU (mM) = $0.185(A_{225}) - 0.049(A_{290})$. Each experiment was performed independently three times.

CD and UPRT Chromatographic Assays. CD and UPRT enzymatic activities were measured as described previously (14, 15, 25). 293T cells were transfected with C:U gene using the LipofectAMINE system. Confluent BHK cells were infected with rVSV-C:U at MOI = 0.1 or MOI = 5.0. For CD activity, 6 h postinfection BHK-infected cells, 293T-transfected cells, and 293T and BHK control cells were harvested in a mixture of 100 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM DTT at a concentration of 1×10^6 cells/10 μl lysis buffer and frozen. At the time of enzymatic measurements, cells had gone through five freeze/thaw cycles, and cellular debris was removed by centrifugation (5 min at 14,000 rpm). For UPRT activity, BHK-infected cells (6 h postinfection) or control BHK cells were harvested in lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, and 1% Triton X-100] at a concentration of 1×10^6 cells/10 μl lysis buffer and incubated for 30 min at 4°C. Ten μl of cell lysate were mixed with 10 μl of [^{3}H]cytosine (0.5 μCi) for CD activity or with 10 μl of reaction buffer [100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 5-phosphoribosyl-1-pyrophosphate, and 1 μl of uracil-5,6-^{3}H (1 μCi)] for UPRT activity and incubated at 37°C for 2 h. Ten μl of each reaction and 10 μl of a marker solution (unlabeled cytosine and uracil or uracil and UMP) were loaded on TLCSs for CD activity or on propidium iodide-TLCSs for UPRT activity and chromatographed in butanol/water (86:14). The bands corresponding to cytosine and uracil or to uracil and UMP were cut out under short-wave UV illumination, and the radioactivity was assayed by liquid scintillation counter. Each experiment was performed independently three times.

Kinetics of Cytolytic VSV and One-Step Growth Curve. BHK-21 cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 10. After 45 min, infection media were removed; cells were washed five times with PBS and covered with fresh media. At the indicated time points, samples of culture media were collected, and viral titers were measured by standard plaque assay (5). For analysis of virus replication and cytolysis after infection with low-dose virus, BHK cells were plated at 10^6 cells/well in 6-well dishes and infected 18 h later with MOI = 0.01 of WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U for 45 min, washed once with PBS, and covered with fresh media. At 12,18, 24, and 28 h, cells were examined by microscopy. At 24 and 48 h, cell viability was assayed by trypan blue exclusion, culture media samples were collected, and viral titers were measured by standard plaque assay.

***In Vitro* Evaluation of the Effect of 5-FU Bioactivation and Effect of 5-FU and 5-FC on Virus Replication.** TSA or BHK cells were plated at a density of 5×10^5 cells in 6-well dishes and treated (in triplicate) with different concentrations of 5-FU or 5-FC (1, 3, 6, and 10 μM and mM, respectively). After 24 h, cells were infected with HI-VSV or rVSV-GFP at MOI = 0.1 or 10. The percentage of infected cells was evaluated at 4, 12, and 24 h by fluorescent microscopy. Samples of culture media collected at 24 h were used to measure viral titers by standard plaque assay. Cell survival was measured by trypan blue staining at 24 h. A20 cells were infected with HI-VSV, rVSV-GFP, or rVSV-C:U at MOI = 0.1. After 4 h, infected cells were mixed in different proportions with noninfected cells at a total concentration of 5×10^5 cells/well in 12-well dishes, and 1 mM 5-FC was added to the media. Cell survival was evaluated by trypan blue staining after 48 h. HMVEC, A20, EL4, 8226/Dox40, or TSA cells were infected with rVSV-C:U at MOI = 0.1 or mock infected (HI-VSV) in the presence or absence of 1 or 3 mM 5-FC (in the case of A20 cells). Samples of culture media from each condition were collected after 24 h and incubated at 60°C for 10 min to inactivate the virus and to preserve the 5-FU activity (26). Aliquots of treated culture media were mixed in different ratios with corresponding fresh media and added to cells freshly plated at a density of 2×10^5 cells/well in 6-well plates. Cell survival was assayed by trypan blue exclusion after 96 h. Complete inactivation of VSV by heating at 60°C was checked by standard plaque assay.

Virus/Suicide Gene System-Induced Cell Killing. HMVEC, TSA, B16(F10), 8226/Dox40, or EL4 cells were treated with rVSV-C:U at MOI = 0.1, in the presence or absence of 3 mM 5-FC. Cells treated with 5-FC alone, with HI-VSV, or with WT-VSV at MOI = 0.1 were used as control. Cell survival was evaluated by trypan blue exclusion after 18 h.

IFN Protection Assay. HMVEC or 8226/Dox40 cells were cultured in duplicate in 6-well dishes at a density of 5×10^5 cells/well, and 12 h later, hIFN-α (1000 UI/ml) was added to the culture media in half of the dishes. After 24 h, culture media were removed, and hIFN-α-treated cells and untreated cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 0.1. Postinfection samples of culture media (24 h) were collected for viral titers, and cell viability was determined by trypan blue exclusion. A20 or control EL4 cells were infected with rVSV-GFP at MOI = 0.1, 10, or 100 and then cultured in 25-cm flasks at a density of 10^6 cells/ml. Postinfection cells (4, 8, 24, and 48 h) were resuspended, and samples

were analyzed by fluorescent microscopy and flow cytometry for calculation of the percentage of GFP-positive cells. A20 cells were infected with rVSV-C:U at MOI = 0.1 in the presence or absence of 1 mM 5-FC. Control cells were treated with 5-FC alone, HI-VSV, or infected with rVSV-TK in the presence of 10 μg/ml GCV or with WT-VSV at MOI = 0.1. Treated cells were cultured at a density of 5×10^5 cells/ml in 25-cm flasks. At 24, 48, and 72 h postinfection, cells were resuspended, and samples were collected for cell survival evaluation by trypan blue staining.

Sensitivity to 5-FU. HMVEC or TSA cells were plated in 6-well dishes at a concentration of 2×10^5 cells/well. A20 or EL4 cells were cultured in 25-cm flasks (kept upright) at the same density. Twelve h later, increasing concentrations of 5-FU were added to the culture media. After 96 h, cell survival was assessed by trypan blue counting and spectrophotometric measurements at 490. IC_{50} level was defined as the concentration of 5-FU that induced 50% cell killing compared with control untreated cells (27).

Experimental Tumor Models. All *in vivo* experiments were performed in accordance with the University of Miami animal care guidelines. BALB/c mice (8-week-old) were purchased from The Jackson Laboratory. A20 (10^6) or TSA (10^5) cells (in 100 μl of PBS) were injected in the right flank of each mouse. Once tumors were established (4–5-mm diameter), mice were randomized in groups of five and six mice each. One group of mice received five daily i.p. injections of 30 mg/kg 5-FU. The other groups of mice received two injections, 3 days apart, of 2×10^7 of either HI-VSV, rVSV-GFP, rVSV-TK (two groups), or rVSV-C:U (two groups). Starting 24 h after the first virus injection, one group of mice that received an injection with rVSV-TK received 100 mg/kg GCV, daily, i.p. for 7 days, and one group of mice that received an injection with rVSV-C:U received 500 mg/kg 5-FC, daily, i.p. for 10 consecutive days. Tumors were measured every other day using a caliper, and tumor volumes were estimated using the following formula: volume = length $\times$ width^2/2. Mice were sacrificed when tumors measured >15 mm in any diameter. Results were reported as (*a*) mean tumor volume of each group at the time point when the first mouse had to be culled and (*b*) survival of each group of mice over the period of 45 days of follow-up. For statistical analysis, Student's *t* test was used (3). For rechallenge experiments, at the end of 45 days, surviving mice free of A20 cells, a group of five control mice, and the mice that previously received the injection with A20 cells but did not develop tumors were rechallenged with the same number of 10^6 A20 cells in the contralateral flank.

For 5-FU measurements, TSA tumors were established by s.c. injection of 10^5 TSA cells in the right flank of each mouse. When the tumors reached approximately 10 mm in diameter, mice were divided into two groups. One group received 90 mg/kg 5-FU i.p., and animals were sacrificed at different time points after the injection. Blood samples and tumors were collected. The other group of mice received one intratumoral injection of 2×10^7 rVSV-C:U followed 24 h later by 500 mg/kg 5-FC i.p. Animals were sacrificed at different time points after having received the i.p. injection, and blood samples and tumors were collected. The 5-FU extraction procedures were performed as described previously (16). Plasma was separated by centrifugation from blood collected via the retroorbital sinus in heparinized tubes. Fifty μl of plasma were quenched with 500 μl of ethyl acetate/2-propanol/0.5 M acetic acid (84:15:1) and stored at −20°C. At the time of extraction, the samples were thawed, vortexed, and centrifuged. The organic supernatant was evaporated in a Speed Vacuum Concentrator (Savant) and resuspended in 50 μl of mobile phase, vortexed, sonicated for 10 s, centrifuged to remove particles, and analyzed by HPLC. 5-FU and 5-FC were separated isocratically on a Beckman Coulter Gold HPLC System equipped with 32 Karat software and photo diode array 168 detector. The eluates were monitored at 254 and 290 nm. A Whatman Partisil 5 SAX (4.6 $\times$ 250-mm) column with matching guard cartridge was used at a flow rate of 0.5 ml/min. The mobile phase was 10 mM $NH_4H_2PO_4$ buffer (pH 3.8) containing 7% methanol. 5-FU and 5-FC eluated at 6.5 and 7.1 min, respectively.

Viral Titer Measurement in the Tumors. TSA tumors were established in BALB/c mice by s.c. infliction of 10^5 TSA cells in the right flank. When tumors reached 10 mm in diameter, mice were randomized in three groups and treated intratumorally with two administrations, 3 days apart, of 2×10^7 HI-VSV (one group) or rVSV-C:U (two groups). One group of mice that received live virus intratumorally were also treated with 500 mg/kg 5-FC i.p. daily. Mice were sacrificed at days 2, 4, and 6, and the tumors were collected, weighed, and frozen. At the time of viral titer measurements, tumors were thawed and homogenized in 1 ml of PBS, and virus was measured by standard plaque assay.

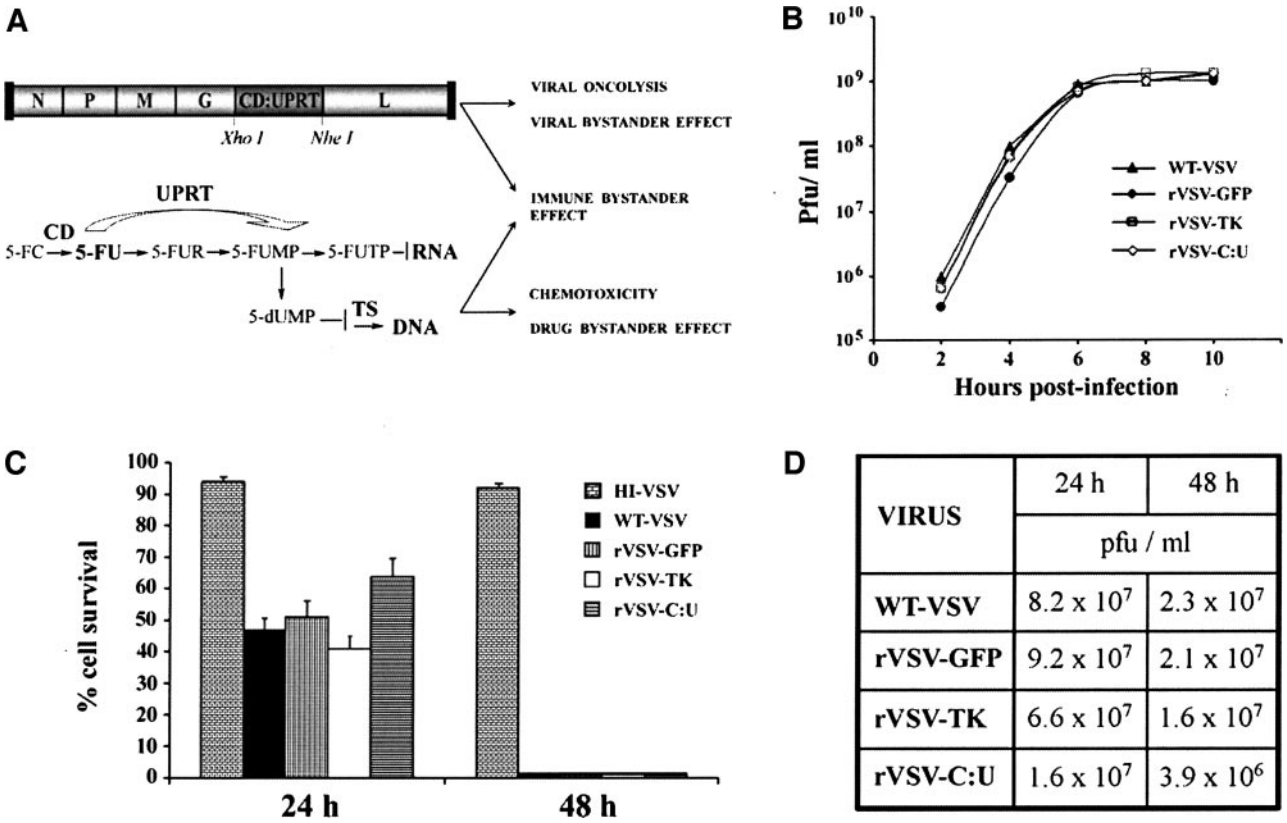
ELISPOT Assay. We followed the experimental protocol described previously in detail (28). Nunc MaxiSorp plates were coated with 100 μl/well of a 0.5 μg/ml solution of antimouse IFN-γ antibodies and incubated overnight at room temperature. Spleens were collected from the three groups of two mice each (treated with HI-VSV, rVSV-C:U, or rVSV-C:U + 5-FC) sacrificed on day 6 of the above-described experiment (see "Viral Titer Measurement in the Tumors"). Spleen cells were suspended in 10% RPMI 1640 and plated in the coated wells in triplicate using four different cell concentrations in 2-fold dilutions (from 10^6 cells/well of the highest concentration). Spleen cells were cultured in the presence or absence of 10^5 irradiated TSA cells/well. The plates were incubated for 24 h at 37°C in 5% CO_2. The plates were then washed and covered with biotinylated anti-IFN-γ monoclonal antibodies (100 μl/well) and incubated for 90 min at room temperature. After additional washes, 100 μl/well of 0.2 μg/ml of streptavidin-alkaline phosphatase were added, and the wells were incubated for 60 min at room temperature. After washing again, 100 μl of a 1:4 mixture of 3% melted low EEO type 1 agarose and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate in AMP buffer was added to each well. The developed spots were counted under the dissecting microscope. The triplicate wells with >50 and <100 spots were used to calculate the average of the two experiments ± SD.

RESULTS

Generation and Characterization of VSV-Expressing CD/UPRT. To evaluate whether VSV could be successfully generated to carry and express the *E. coli* CD/UPRT (C:U) fusion gene, cDNA-encoding C:U was cloned into the pVSV-XN2 plasmid (pVSV-XN2-C:U), which carries the full-length antigenomic VSV DNA (21, 22). The additional foreign transcription unit (C:U) was inserted between the VSV glycoprotein (G) and the polymerase gene (L) in a position flanked by unique *Xho*I and *Nhe*I restriction sites (Fig. 1*A*). After transfection with pVSV-XN2-C:U, recombinant virus was recovered in BHK cells 48 h later, and plaque purification of resultant recombinant viruses (rVSV-C:U) was carried out as described previously (29).

To investigate whether the insertion of the foreign suicide gene affected viral replication and infectivity, we analyzed rVSV-C:U production per cell after infection of BHK cells at a MOI of 10. Accordingly, one-step growth curve studies indicated that rVSV-C:U exhibited similar growth characteristics to WT-VSV or rVSV expressing the GFP (rVSV-GFP) or TK (rVSV-TK) and reached 10^9 viruses/ml within 10 h (Fig. 1*B*). Indeed, rVSV-C:U induced almost complete cytolysis of BHK cells (MOI = 0.01) within 48 h, similar to WT VSV (Indiana strain), rVSV expressing TK, or rVSV expressing GFP (Fig. 1*C*). Because of the rapid viral-induced killing, the addition of 5-FC had very little additional effect on the kinetics of BHK cell death (data not shown). The levels of viral production were also similar for rVSV-C:U and other control VSVs, even after infection of BHK cells with very low doses of virus (Fig. 1*D*). In conclusion, the C:U fusion gene does not appear to affect VSV replication, and rVSV-C:U exhibits *in vitro* growth characteristics comparable with WT VSV after infection of BHK cells.

VSV Expresses High Levels of Functional C:U. Confirmation of expression of the C:U gene by rVSV-C:U was next examined by measuring the enzymatic activity of CD using spectrophotometric assay and chromatographic enzyme assays as described previously (15). Essentially, for chromatographic identification, BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0, and cell lysates were retrieved at various times postinfection. This analysis indicated that labeled cytosine was effectively metabolized to uracil in lysates from rVSV-C:U-infected cells but not rVSV-GFP-infected cells (Fig. 2*A*). Spectrophotometric analysis confirmed expression and indicated that as much as 70 nM 5-FU/min was produced using cell lysates prepared



VIRUS	24 h	48 h
	pfu / ml	
WT-VSV	8.2×10^7	2.3×10^7
rVSV-GFP	9.2×10^7	2.1×10^7
rVSV-TK	6.6×10^7	1.6×10^7
rVSV-C:U	1.6×10^7	3.9×10^6

Fig. 1. Generation of rVSV-expressing C:U fusion gene and kinetics of virus cytolysis. *A*, C:U gene was inserted between the G and L genes of VSV cDNA, flanked by unique *Xho*I and *Nhe*I restriction sites. CD and UPRT enzymes expressed by the novel recombinant virus convert the 5-FC into 5-FU and its active metabolites. *5-FUR*, 5-fluorouridine; *5-FUMP*, 5-fluorouridine 5′-monophosphate; *5-FUTP*, 5-fluorouridine 5′-triphosphate; *5-dUMP*, 5-fluoro-2′-deoxiuridine-5′-monophosphate; *TS*, thymidylate synthase. *B*, one-step growth curve. BHK cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 10. Samples of the culture media were collected at the indicated time points, and viral titers were measured by the standard plaque assay. *C*, infectivity of rVSV-C:U. BHK cells were infected in double with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 0.01. Cell survival (24 and 48 h postinfection) was evaluated by trypan blue counting. *D*, samples of culture media were collected and used to measure viral titers. The results represent the average of at least two independent experiments, and the *error bars* represent the SE.

from 1 million cells infected with rVSV-C:U at a MOI of 0.1 or 5 (Fig. 2*B*). Likewise, high levels of UPRT activity were demonstrated by similar chromatographic analysis of rVSV-C:U-infected BHK cells (Fig. 2*C*). Collectively, our data indicate that rVSV-C:U expresses high levels of functional C:U.

Analysis of 5-FU Production and Bystander Effect. A major strength of any prodrug activation model is the potential to extend the cytotoxic therapeutic effect to untransfected target cells. In the case of C:U/5-FC, an efficient bystander effect has been reported because of the production of 5-FU, which can reach neighboring cells by simple diffusion. To evaluate the efficiency of CD catalytic activity, BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0 or with rVSV-GFP as a control, in the presence of 5 mM 5-FC. Samples of the infected culture media were collected at different times postinfection, and 5-FU production was measured spectrophotometrically. This analysis indicated that high levels of 5-FU production were detected in the culture media up to 72 h postinfection using rVSV-C:U but not rVSV-GFP (Fig. 3*A*). Thus, rVSV-C:U-infected cells produce large amounts of CD that are able to catalyze 5-FC into 5-FU beyond 24 h, after which almost all of the cells undergo virus-induced CPE.

Having confirmed the presence of 5-FU in the culture media, we next analyzed the extent of bystander effect. Cultured tumor cells (TSA, EL4, A20, and 8226/Dox40) or normal cells (HMVEC) were thus infected with rVSV-C:U or mock infected in the presence or absence of 5-FC. After 24 h, supernatants from these cells were recovered and incubated at 60°C to completely inactivate residual virus, a consequence that does not affect 5-FU activity (26). Heat-treated samples of culture medium from the infected or uninfected cells were mixed at different ratios with new media and added to freshly cultured cells. After 96 h, cell survival evaluation by trypan blue exclusion showed significant killing of tumor lines exposed to culture medium from rVSV-C:U + 5-FC-treated cells (Fig. 3*B*). Cell death (>50%) was observed even at 1:500 dilution of the media, demonstrating potent bystander activity independent of cell-to-cell contact. In contrast, no cell death was observed in cells treated with tissue-cultured medium from rVSV-C:U-infected cells in the absence of 5-FC or with 5-FC alone (Fig. 3*C*). HMVEC cells and normal murine BALB/c embryonic fibroblasts were significantly less affected by the rVSV suicide gene system, almost certainly because they were nonviable to infection and did not generate 5-FU (HMVEC IC_{50} to 5-FU was established at 3–4 μM and was similar to that for the following cancer cells tested: 3–4 μM TSA; 4–5 μM 8226/Dox40; 1 μM A20; and 10 μM EL4). Thus, 5-FU generated by rVSV-C:U exhibits the hallmarks of a functional bystander effect *in vitro*.

Because 5-FU is a chemotherapeutic that acts by inducing cell death, it was plausible that 5-FU could limit the replication and propagation of rVSV-C:U *in vitro* and *in vivo* (26). To address this concern *in vitro*, we evaluated the sensitivity of cancer cells pretreated with 5-FC or 5-FU to VSV replication and killing. Accordingly, TSA cells were treated with increasing concentrations of 5-FC and 5-FU for 24 h, followed by infection with rVSV-GFP at MOI = 0.1 or 10. The efficiency of VSV infectivity and oncolysis on cells pre-exposed to 5-FU and 5-FC was evaluated by measuring the percentage of infected cells using fluorescent microscopy and monitoring VSV-GFP replication. This was complemented by measuring virus titers and finally by the degree of cell killing by trypan blue exclusion analysis.

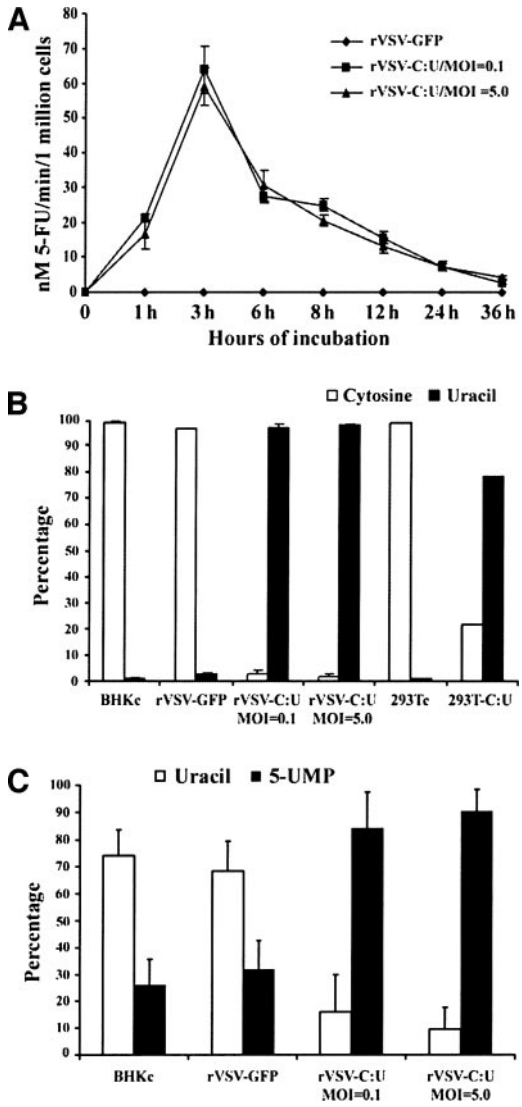


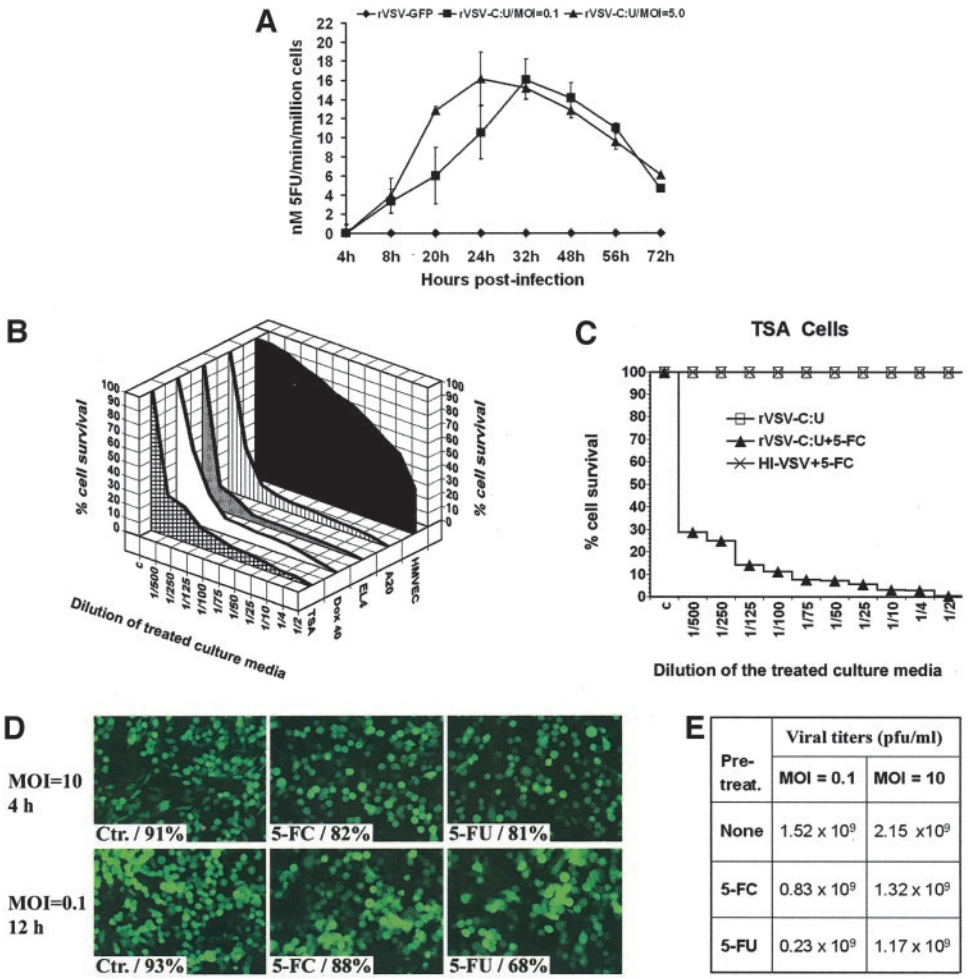
Fig. 2. rVSV expresses high levels of functional CD and UPRT. *A*, CD activity measured spectrophotometrically. BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0 or with rVSV-GFP. Six h postinfection, cells were harvested, and the cell lysates were incubated with 5-FC. At the specified time points, aliquots were collected, and CD activity, expressed as rate of 5-FU production, was calculated using spectrophotometric measurements at A_{290} and A_{255}. The data represent the average of three independent experiments, and the *error bars* represent the SD of the mean. CD activity (*B*) and UPRT activity (*C*) were measured chromatographically. Cell lysates were mixed with [^{3}H]cytosine and [^{3}H]uracil, respectively, and incubated for 2 h at 37°C. Samples were loaded on TLCSs. Bands corresponding to cytosine and uracil were evaluated by liquid scintillation counting, and results are presented as a percentage of total scintillation count for each sample. 293T cells transiently transfected with an empty vector or with the *E. coli* C:U fusion gene were used as control for the CD activity experiment. The data represent the average of two or three independent experiments, and the *error bars* represent the SE.

Our data demonstrated that infection of TSA cells with high titers of rVSV-GFP (MOI = 10) was not influenced by pretreatment with 10 μM 5-FU or 10 mM 5-FC used in the assay (the cells' IC_{50} for 5-FU was determined at 3 μM). The percentage of infected cells was 80–90% by 4 h (Fig. 3*D*). At lower dose infection (MOI = 0.1), the percentage of infected cells at 12 h was slightly lower after 5-FU pretreatment (Fig. 3*D*), although by 24 h almost 100% of cells were infected (data not shown). Viral production in TSA cells pretreated with 5-FU was <1 log lower than in control cells, remaining at high levels of 2×10^8. Cell death evaluation by trypan blue exclusion showed almost complete cell death within 24 h, both in control and in 5-FC- and 5-FU-pretreated conditions (data not shown). Taken together, these *in vitro* assays demonstrate that exposure to relatively high levels of 5-FC and 5-FU did not significantly influence VSV replication and oncolytic activity.

Evaluation of the *in Vitro* Oncolytic Efficiency of rVSV-C:U/5-FC. We next evaluated the combined oncolytic efficiency of rVSV-C:U/5-FC on different cancer cell lines or normal cells. Accordingly, rVSV-C:U, rVSV-GFP, HI-VSV, or WT-VSV was used to infect TSA, K562, 8226/Dox40, and EL4 cancer cells or normal HMVEC cells at a MOI of 0.1. Results indicated that all recombinant viruses killed the cancer cell lines (>75% cell death) within 24 h (Fig. 4*A*). In contrast, rVSV-C:U + 5-FC or VSV-GFP was found to induce a low fraction of HMVEC killing (20–25%) when treated similarly (Fig. 4*A*). Pretreatment with type I IFN completely prevented virus replication in the normal HMVEC cells but not in the cancer cell lines. For example, rVSV-C:U titers were 2000 times lower in infected HMVEC cells than in 8226/Dox40 cells by 24 h postinfection and decreased to an undetectable level in HMVEC cells, but not 8226/Dox40 cells, when pretreated with IFN-α (Fig. 4, *B* and *C*). These data confirm earlier findings that at least one potential mechanism of VSV-mediated oncolysis exploits defects in the IFN pathway present in tumor cells (30). In addition, these data demonstrate the selective oncolytic efficiency mediated by rVSV-C:U/5-FC.

Because rVSV-C:U infection in the absence of 5-FC clearly exhibits a viral-induced oncolytic effect alone, we next examined the contribution of 5-FU in cancer cell killing. For this experiment, mouse A20 lymphoma cells were used because they were found to exhibit different levels of virus-induced CPE when infected with rVSV-GFP and observed by fluorescent microscopy. For reasons that remain to be clarified, a small population of A20 cells was found to remain uninfected, possibly because some cells differentiated into a subpopulation that did not interact efficiently with the virus (Fig. 4*D*). However, in general, a high level of viral replication was achieved in the infected population, with viral titers reaching 10^7 by 24 h postinfection (data not shown). Nevertheless, when 5-FC (1 mM) was added to the culture medium harboring A20 cells infected with rVSV-C:U, almost complete cell death could be demonstrated by trypan blue staining after 48 h (Fig. 4*E*). In contrast, the dose of 5-FC used (1 mM) did not affect A20 cell growth when used alone. To further distinguish between the contribution of the virus alone and 5-FU bioactivation in A20 cell killing, cells were infected with rVSV-C:U, rVSV-GFP, or HI-VSV, and after 6 h, they were mixed at different ratios with uninfected cells and cultured in the presence of 1 mM 5-FC. Cell viability was measured after 48 h. The difference observed between HI-VSV + 5-FC and rVSV-GFP + 5-FC essentially represents the effects of the virus alone, whereas the difference between rVSV-GFP + 5-FC and rVSV-C:U + 5-FC accounts for the additional oncolytic effect generated by 5-FU (Fig. 4*F*). Collectively, these data indicate increased killing of A20 cells using rVSV-C:U + 5-FU, emphasizing that bystander effects can contribute toward the efficacy of oncolysis.

***In Vivo* Activity of the rVSV-C:U/5-FC System.** To evaluate the oncolytic effect of the rVSV-C:U system *in vivo*, we chose to investigate two aggressive s.c. tumor models, A20 and TSA, that differed slightly in their response to 5-FU. For example, the A20 lymphoma model showed a higher sensitivity to 5-FU (IC_{50} = 1 μM) compared with the TSA mammary prototype (IC_{50} = 3–4 μM). Our goals were to evaluate the efficiency of the VSV-directed C:U/5-FC model and to compare it with the previously published rVSV-TK/GCV model. Accordingly, s.c. tumors were grown in the left flank of BALB/c immunocompetent mice. When tumors became palpable, HI-VSV, rVSV-C:U, or rVSV-TK was injected intratumorally (2×10^7 plaque-forming units), followed or not followed by the administration of the respective prodrug, 5-FC or GCV. As an additional control, 5-FU was administered daily for 5 days i.p. Virus treatments were repeated once



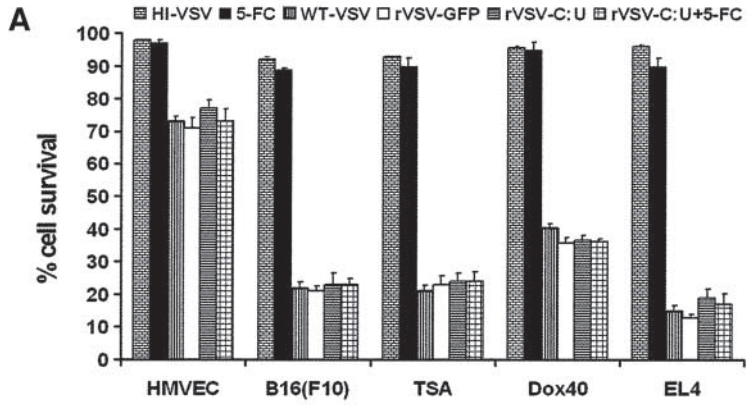
Pre-treat.	Viral titers (pfu/ml)	
	MOI = 0.1	MOI = 10
None	1.52 x 10^9	2.15 x10^9
5-FC	0.83 x 10^9	1.32 x 10^9
5-FU	0.23 x 10^9	1.17 x 10^9

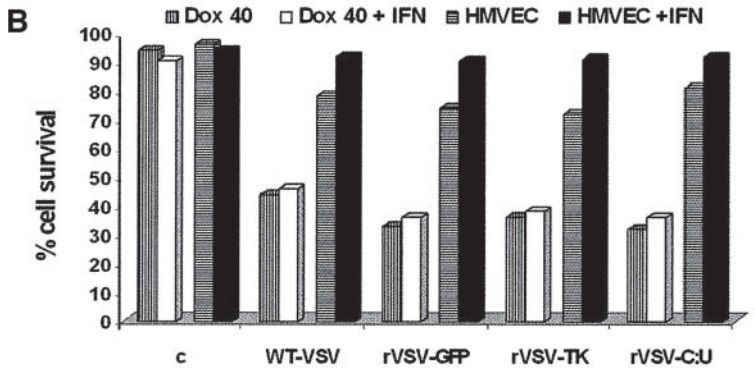
Fig. 3. 5-FU generated by the rVSV-C:U-prodrug system produces an efficient bystander effect and does not interfere significantly with the viral replication and oncolysis. *A*, BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0 or with rVSV-GFP and treated with 5-FC. At the specified time points, aliquots of the conditioning media were collected, and the rate of 5-FU generated in the media was determined using spectrophotometric measurements at A_{290} and A_{255}. *B* and *C*, evaluation of the bystander effect. HMVEC, A20, EL4, 8226/Dox40, or TSA cells were infected with rVSV-C:U at MOI = 0.1 or mock infected (HI-VSV) and treated or not treated with 5-FC. Culture media aliquots collected at 24 h were treated at 60°C for 10 min. to inactivate the virus and mixed in different ratios with fresh correspondent media before being added to freshly cultured cells. Cell survival was evaluated by trypan blue exclusion after 96 h. *D* and *E*, 5-FC and 5-FU do not interfere significantly with viral replication and cytolysis. TSA cells were pretreated with 10 mM 5-FC or 10 μM 5-FU for 24 h. Pretreated as well as untreated control cells were infected with rVSV-GFP at MOI = 0.1 or 10 and covered with fresh media. At 4 and 12 h, cells were analyzed and photographed by fluorescent microscope. Culture media samples were collected at 24 h, and viral titers were measured by standard plaque assay.

more after 3 days, and tumor volumes were monitored every other day. For these experiments, two end points were established. First, survival was monitored with the specification that mice were sacrificed when the largest tumor diameter reached 15 mm. Second, evaluation of the mean tumor volumes was determined at a time when the first animal in the experiment was sacrificed.

This study indicated that there was a significant reduction in A20 or TSA tumor growth using either rVSV-TK or rVSV-C:U in the absence of prodrug, compared with HI-VSV ($P < 0.001$; Fig. 5*A* for A20 and Fig. 6*A* for TSA). However, even more potent tumor inhibition was found in the virus suicide gene/prodrug groups (rVSV-TK/GCV and rVSV-C:U/5-FC; Figs. 5*A* and 6*A*). Student's *t* test analysis indicated that animals harboring A20 or TSA and receiving rVSV-C:U/5-FC treatment faired better than those receiving rVSV-C:U alone ($P < 0.05$). The same analysis for rVSV-TK/GCV did not reach statistical significance, indicating that generation of 5-FU may be a better prodrug system in these tumor models. Statistical significance was seen between the rVSV-C:U + 5-FC group and 5-FU group [$P < 0.001$ in the TSA experiments (Figs. 5*A* and 6*A*) and $P < 0.05$ in the A20 experiment]. There was no difference between the HI-VSV and the HI-VSV + 5-FC groups in either of the experiments (data not shown). Indeed, although many of the tumors appeared of similar size in a number of differently treated groups after 14 days, tumors in all groups except for the rVSV-C:U + 5-FC-treated group grew rapidly. Indeed, animals with A20 tumors survived for the duration of the experiment (45 days) when treated with rVSV-C:U + 5-FC (Fig. 5*B*). In the control groups HI-VSV or HI-VSV + 5-FC, all animals had to be sacrificed after 20 days. By 90 days, four of six animals in the rVSV-C:U + 5-FC group, two of five animals in the rVSV-TK +GCV group, one of five animals in the rVSV-C:U group, and one of five animals in the 5-FU group remained alive and free of tumors. In the TSA model, the same statistically significant difference in survival was observed between the rVSV suicide gene + prodrug groups and the control groups ($P < 0.05$; data not shown). The combined intratumoral administration of both rVSV-TK and rVSV-C:U (in conjunction with GCV and 5-FC) did not significantly achieve better results than rVSV-C:U + 5-FC in the A20 model alone (data not shown). Collectively, our data indicate that because of increased bystander effect, rVSV-C:U may be a better oncolytic agent than rVSV-TK.

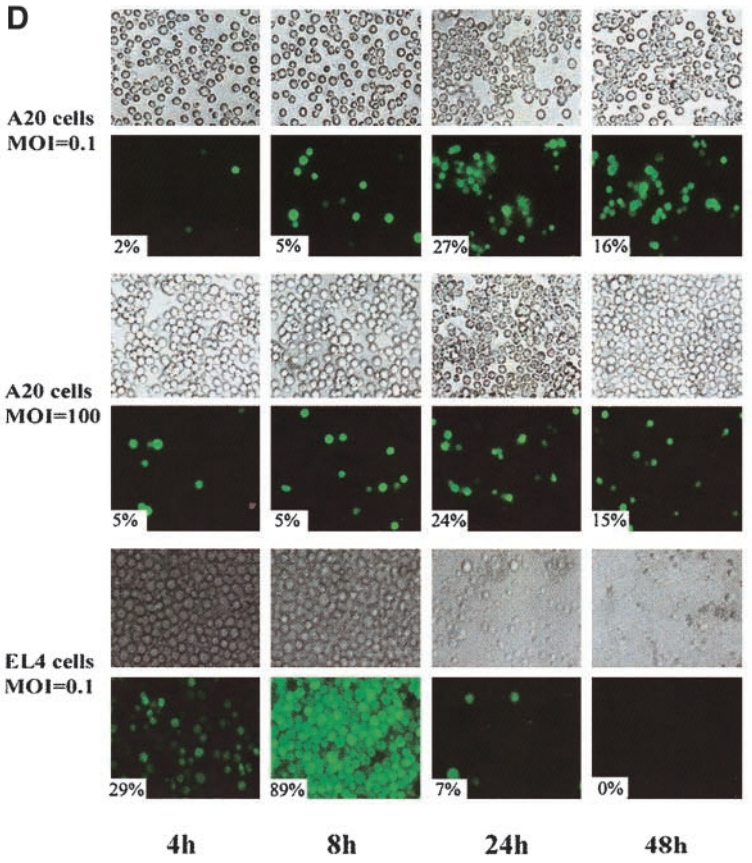
The Induction of Antitumor Immune Responses by rVSV-C:U. Previous data have demonstrated that lysis of cells, *e.g.*, through virus replication (CPE), can result in the enhanced generation of CTL activity, arguably by facilitating tumor antigen uptake by professional antigen-presenting cells (31, 32). To evaluate this, animals exhibiting no tumor formation after 45 days in the previously presented A20 model were rechallenged with the same number of A20 cells injected in the opposite flank of the animal (six of six animals in the rVSV-C:U + 5-FC group, two of five animals in the rVSV-C:U group, three of five animals in the rVSV-TK +GCV group, one of five animals in the 5-FU group, and zero of five animals in HI-VSV, HI-VSV + 5-FC, and rVSV-TK groups). Significantly, none of the rechallenged animals developed tumors at the site of cancer cell readministration, in contrast with a control group of new animals not treated previously, 4 of 5 of which developed tumors within 15 days postimplantation. These data indicate activation of the immune system against A20

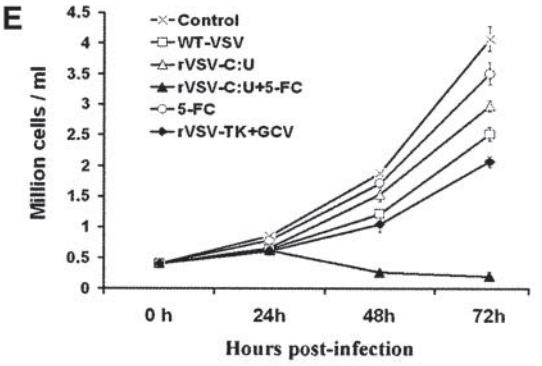




C

Cell Line	hINFα	Titer (pfu/ml)
Dox40	-	6.5×10^7
Dox40	+	4.8×10^7
HMVEC	–	3.3×10^4
HMVEC	+	< 100





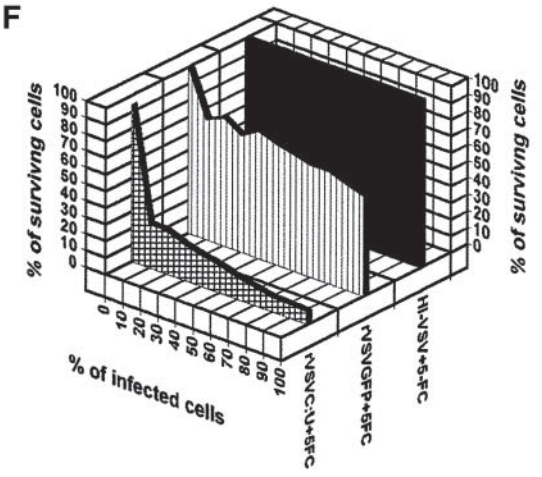
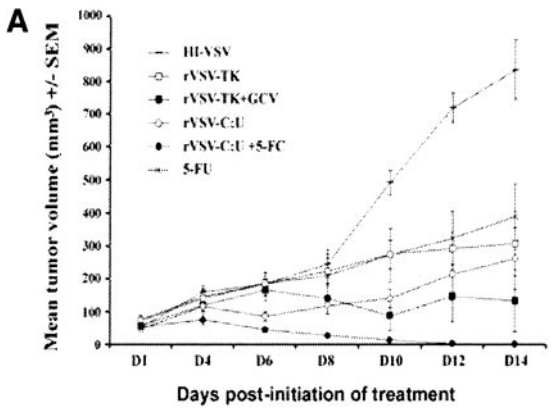
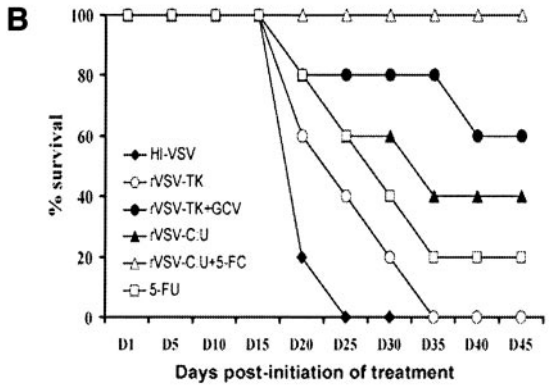


Fig. 4. Virus/suicide gene system retains selective oncolytic efficiency. *A*, tumor cells are sensitive to rVSV-C:U/5-FC. HMVEC, TSA, B16(F10), 8226/Dox40, or EL4 cells were infected with rVSV-C:U at MOI = 0.1, in the presence or absence of 5-FC added to the culture media. Cells treated with 5-FC alone, HI-VSV, or WT-VSV at MOI = 0.1 were used as control. Cell survival was evaluated by trypan blue exclusion after 18 h. *B* and *C*, Dox40 cells are resistant to IFN antiviral action. HMVEC or 8226/Dox40 cells were pretreated with 1000 IU/ml hIFN-α. After 24 h, pretreated as well as untreated control HMVEC and 8226/Dox40 cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 0.1. Twenty-four h postinfection, samples of culture media were collected for viral titer measurement, and cell viability was determined by trypan blue exclusion. *D*, A20 cells are less sensitive to infection with VSV. A20 cells or control EL4 cells were infected with rVSV-GFP at MOI = 0.1 or 100. At 4, 8, 24, and 48 h postinfection, cells were resuspended,





C

Initial Treatment	Re-challenged mice/ Treated mice	Mice growing tumors/ Re-challenged mice
None (new controls)	5	4/5
HI-VSV	0/5	0/0
rVSV-TK	0/5	0/0
rVSV-TK+GCV	3/5	0/3
rVSV-C:U	2/5	0/2
rVSV-C:U+5-FC	6/6	0/6
5-FU	1/5	0/1

Fig. 5. RVSV expressing C:U inhibits the tumor growth of the syngeneic A20 B-cell lymphoma tumor model in immunocompetent BALB/c mice. *A*, 10^6 A20 cells were injected s.c. in the flank of immunocompetent BALB/c mice. When tumors became palpable, mice were treated with 5-FU i.p., 2×10^7 plaque-forming units of HI-VSV, rVSV-TK, or rVSV-C:U injected intratumorally twice a day at 3-day intervals. Mice that received the active virus were randomized to receive or not receive GCV i.p. (for rVSV-TK) or 5-FC i.p. (for rVSV-C:U). Tumor size was measured every other day. Tumor volumes were calculated and are presented as average ± SE. Mice were sacrificed when the tumors reached 15 mm in the largest diameter. *B*, survival of the different therapeutic groups is presented over a period of follow-up of 45 days. *C*, table showing survival of animals from original experiments that were rechallenged with A20 cells.

cells, which may have contributed toward the antitumor activity of rVSV-C:U/5-FC.

To further characterize the mechanisms of rVSV-C:U/5-FC action, we measured the activation of T cells in the spleens of mice receiving tumor therapy by performing ELISPOT assay. Mice with s.c. TSA tumors were treated with HI-VSV, rVSV-C:U, or rVSV-C:U + 5-FC. Six days after the initiation of treatment, mice were sacrificed, and spleens were collected. Mononuclear cells were isolated, and IFN-γ release by single cells was measured in the presence or absence of radiated TSA cells as a source of *in vitro* stimulating antigen. We found that there were 30 and 44 times more T cells activated and secreting IFN-γ in the spleens of the mice that were treated with rVSV-C:U and rVSV-C:U + 5-FC, respectively, than in the spleens of mice bearing TSA tumors that received control HI-VSV (Fig. 6*B*). The number of secretory cells doubled in both viral treatment conditions when they were exposed to TSA cells as a source of antigen. Interestingly, the number of activated T cells was found to be almost double when rVSV-C:U was combined with generated 5-FU. These data indicate a strong immune component in the antitumor efficiency of rVSV-C:U/5-FC therapy.

We next evaluated, by HPLC, 5-FU levels in the blood after one single maximally tolerated dose of 5-FU or after intratumoral administration of rVSV-C:U in the presence of systemic 5-FC. Blood level measurement in samples collected at different time points revealed that systemic 5-FU administration produced high early peaks (up to 4.4 times higher than the rVSV-C:U + 5-FC by 15 min; Fig. 6*C*). However, exogenously added levels drop immediately, *in vivo*, so that after 25 min, 5-FU produced by the viral/prodrug treatment was detected at higher concentrations and maintained therapeutic levels over the next hour, thus indicating longevity of the system. We could not find any difference in the viral titers achieved by the rVSV-C:U virus in the presence or absence of prodrug administration when measured on day 4 (already 2 days through 5-FC treatment) or day 6 (after four administrations of 5-FC), suggesting that 5-FC and 5-FU did not affect VSV propagation in the tumor (Fig. 6*D*).

Thus, we demonstrate the presence of potent viral, chemotherapeutic, and antitumor immune components after treatment with rVSV-C:U/5-FC, indicating that this system may have promise for development as a therapeutic against malignant disease.

DISCUSSION

VSV has been recently characterized as a selective oncolytic virus showing significant *in vitro* and *in vivo* potential as a novel therapy for cancer (30). Previous studies have demonstrated that VSV is capable of inhibiting the growth of tumors harboring common genetic abnormalities such as p53, *myc*, or *ras* aberrations, both after local intratumoral administration and after systemic i.v. treatment (33). Furthermore, VSV has been shown to eradicate tumors in immunocompetent hosts without any substantial virus-associated CPE occurring in normal tissue (33). Recombinant adaptations of the virus that contain antitumor genes afford the opportunity to further increase the oncolytic efficiency of VSV, with concomitant specificity and attenuation (3). In this regard, previous studies have shown that the expression of cytokines such as interleukin-4 and suicide genes such as herpes simplex virus-TK increased the ability of VSV to suppress tumor growth, compared with WT-VSV, in melanoma and mammary cell carcinoma models (3).

A potential problem concerning viral tumor therapy in immunocompetent hosts remains the response of the immune system to virus infection, which may suppress a required oncolytic effect through inhibition of virus spread (35). One strategy to help avoid this di-

and samples were analyzed by fluorescent microscopy and flow cytometry to calculate the percentage of GFP-expressing cells. *E*, rVSV-C:U/5-FC displays potent oncolytic activity against A20 cells. A20 cells were infected with rVSV-C:U at MOI = 0.1, in the presence or absence of treatment with the prodrug 5-FC. A20 cells treated with 5-FC alone, infected with rVSV-TK in the presence of GCV, or infected with WT-VSV at MOI = 0.1 served as controls. Number of living cells was counted after trypan blue staining at 24, 48, and 72 h. *F*, measurement of 5-FU bystander effect in A20 lymphoma cells. A20 cells were infected with HI-VSV, rVSV-GFP, or rVSV-C:U at MOI = 0.1. After 4 h, infected cells were mixed in different proportions with noninfected cells, and 5-FC was added to the conditioning media. Cell survival was evaluated by trypan blue staining after 48 h. The data presented correspond to the average of three independent experiments.

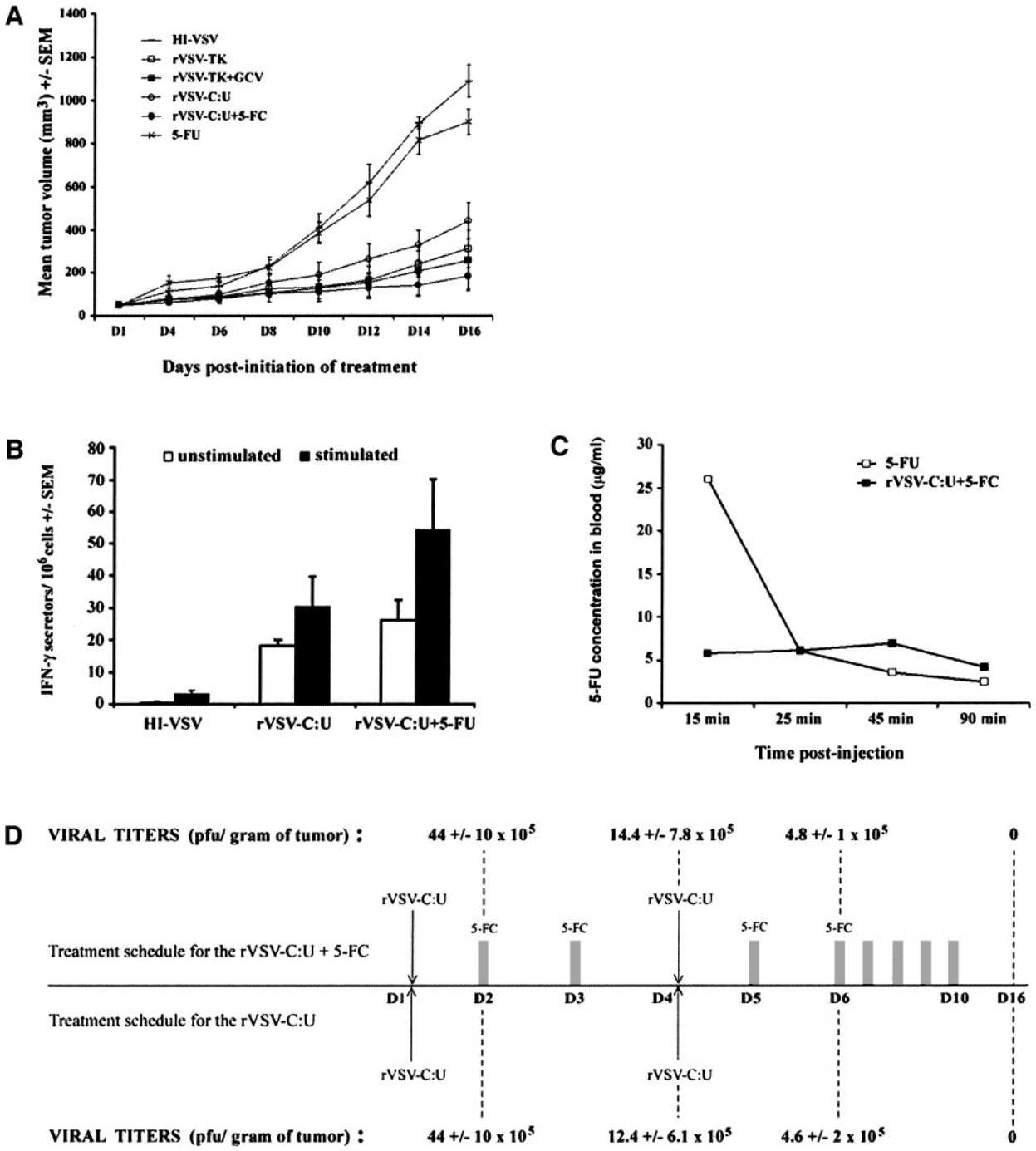


Fig. 6. Effect of intratumoral treatment with rVSV expressing C:U on syngeneic BALB/c TSA mammary tumors. *A*, rVSV-C:U/5-FC inhibits TSA tumor growth. TSA cells (10^5) were injected s.c. in BALB/c immunocompetent mice. When tumors became palpable, mice were treated with 5-FU i.p. or received 2×10^7 HI-VSV, rVSV-TK, or rVSV-C:U intratumorally, two administrations at 3-day intervals. Mice treated with the active virus received or did not receive GCV (for rVSV-TK) or 5-FU (for rVSV-C:U). Mean tumor volume ± SE is presented for all treatment groups. *B*, CTL activation after rVSV-C:U/5-FC treatment. BALB/c mice with TSA tumors grown s.c. received HI-VSV or rVSV-C:U as two intratumoral administrations, 3 days apart, with or without concomitant systemic administration of 5-FC. At day 6 postinitiation of treatment, mice were sacrificed, and the spleens were used in the ELISPOT assay. The number of activated spleen cells that secrete IFN-γ, counted in triplicate in two independent experiments, is presented as the mean ± SE. *C*, *in vivo* 5-FU levels in treated animals. In animals carrying s.c. TSA tumors, blood samples were drawn at different time points after systemic administration of a single maximally tolerated dose of 5-FU or after one systemic administration of 5-FC after intratumoral administration of rVSV-C:U. 5-FU level analysis was performed by HPLC. *D*, intratumoral viral replication is not affected by the suicide gene/prodrug system. Viral titers were measured by standard plaque assay at different time points in the TSA tumors grown s.c. and treated with rVSV-C:U intratumorally, in the presence or absence of the systemic 5-FC administration.

lemma may be the addition of a system capable of exerting a strong bystander effect, which may enhance the oncolytic efficacy of the virus therapy by eliminating uninfected tumor cells. In contrast to the TK/GCV system, the CD/UPRT/5-FC system may confer a stronger bystander activity that is independent of intercellular junctions (36–38). This may be important in the treatment of tumors with down-regulated intercellular gap junctions (36). Previous attempts to use viral agents in the therapy of cancer have been limited by lack of selectivity, inefficient replication of the virus in the tumor, or apprehension associated with the transforming potential of the virus itself (2, 39). Given this, we therefore decided to evaluate the antitumor/bystander potential of the recently characterized *E. coli* CD/UPRT

fusion suicide gene with the actively replicative, selectively oncolytic rVSV as a viral vector (15, 17).

The generation of rVSV-C:U was achieved, and impressively high levels of CD and UPRT activity were apparent. Spectrophotometric measurements of CD activity up to 64 ± 7 nM 5-FU/min were produced by approximately 1 million infected BHK cells. This is in comparison with a recently published value of 10 ± 2 nM 5-FU/min produced by 1 million BNL1 hepatocellular carcinoma cells that were retrovirally transduced with the *E. coli* CD gene (24). The CD gene from *E. coli* also compares favorably with results reported previously using the yeast CD/UPRT fusion gene (15). In addition, our results indicate that rVSV-C:U + 5-FC generates a very strong bystander killing effect. The amount of 5-FU produced by recombinant viruses in the media essentially eradicated >90% of a number of tumor cell types tested. This observation indicates that 5-FU effectively diffuses in and of cells and, unlike TK/GCV system, does not require cell-to-cell contact for bystander activity (36).

Other potential issues regarding virus gene therapy include taking into consideration the possibility that the rapid oncolytic effect of the virus itself could limit the amount of chemotherapeutic agent produced in the cell. Conversely, the chemotherapeutic drug could conceivably limit viral replication and subsequent propagation in the tumor (40, 41). However, we demonstrated high levels of 5-FU production in cancer cells *in vitro* and intratumorally *in vivo*. *In vitro* we showed that CD continues to transform the provided prodrug up to 72 h postinfection. In addition, our *in vitro* and *in vivo* data indicate that the generated 5-FU and the administered 5-FC do not significantly inhibit VSV replication, and the viral titers achieved in the tumors by the rVSV-C:U were similar in the presence or absence of prodrug administration. A probable explanation is that VSV is a RNA virus, and its cytoplasmic replication is less affected by the multiple mechanisms by which 5-FU inhibits DNA and RNA synthesis, altering cell growth and survival. This demonstration makes the rVSV a specifically suitable viral vector for the CD/UPRT/5-FC suicide gene therapy.

We have recently indicated that VSV selectively replicates in tumor cells because of such cells harboring a flawed IFN system. In this regard, IFN pretreatment completely protected the normal cells against rVSV-C:U replication and cytolysis, as demonstrated previously for the VSV oncolytic agents (30). In contrast, VSV oncolysis of tumor cells was extremely prompt and efficient, masking the contribution of the suicide gene system in all cancer cell lines except the mouse A20 B-cell lymphoma cells. In this particular cancer cell model, rVSV-C:U induced almost complete cytolysis only in the presence of the prodrug 5-FC, demonstrating a synergistic effect of the rVSV and the CD/UPRT/5-FC components in oncolysis of the lymphoma cells. This may reflect that some lymphoma cell types, such as chronic lymphocytic leukemia, exhibit selective resistance to VSV infection, perhaps because of tropism issues. Nevertheless, this finding allowed the opportunity to evaluate the bystander effect of the rVSV-C:U system. Accordingly, rVSV-C:U exhibited greater therapeutic oncolytic activity in the presence of systemically administered 5-FC compared with rVSV-C:U virus alone and with the previously published rVSV-TK/GCV system. Similar results were observed in the TSA mammary tumor model. The contribution of the viral oncolysis to suppression of the tumor can be compared with the measured effect of rVSV-C:U alone, in the context of demonstrating similar viral titers in tumors injected with the virus and exposed or not exposed to systemic 5-FC. The role of the generated 5-FU is revealed by the statistically greater therapeutic efficiency of rVSV-C:U + 5-FC compared with rVSV-C:U alone in both tumor models.

Another potential antitumor action invoked by rVSV-C:U/5-FC is the stimulation of the immune system. Robust immune responses have been documented previously in experiments using CD-based therapies, as demonstrated by the presence of intense inflammatory infiltrates found within the treated tumors (42), production of cytokines (43, 44), eradication of distant tumors (45), reduction of the bystander effect in nude mice (24), or after sublethal irradiation (46, 47). Although virus replication itself induces strong CTL responses against viral antigens presented on the surface of infected cells, it is plausible that CTL responses may be invoked against tumor antigens by this treatment also (48, 49). In our analysis, the generation of CTLs after VSV suicide gene treatment was indicated by the lack of any tumor growth in A20 cancer model rechallenge experiments. In addition, ELISPOT analysis confirmed the presence of IFN-γ-producing T cells specific to the tumor only in animals receiving rVSV-C:U treatment.

Collectively, our data indicate that VSV is able to generate high levels of CD and UPRT. In addition, 5-FU was found to participate in the killing of infected tumor cells and to exhibit bystander effect. Future studies could conceivably enhance VSV-based oncolytic activities by combining suicide genes with immunomodulatory cytokines or with radiotherapy, thus exploiting the radiosensitizing potential of 5-FU (50).

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