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13. ABSTRACT (Maximum 200 Words) The goal of the proposed work is to develop novel methods based on recombinant SV5 (rSV5) for targeting and killing predetermined populations of tumor cells. During the previous funding period, we have accomplished our proposed phases of the approved tasks. First, we have constructed new chimeric proteins composed of SV5 glycoproteins linked to a single chain antibody (sFv) specific for human HER-2. We have developed an assay to test functional interactions between these chimeras and HER-2, and have used flow cytometry to identify optimal forms of sFv for cell surface expression (Task 1). We are currently testing this chimera for expression in rSV5-infected cells. Second, we have generated human breast cancer cell lines which express the tet-repressor protein (Task 2). These stable cell lines are important as they will serve as the inducible target cells which will be induced to express varying levels of HER-2 to test the specificity of our rSV5 targeting model. Thus, our progress over the last year has resulted in identifying two important components for our research plan: a candidate anti-HER-2 sFv for inserting into rSV5 genome and cell lines which will be the targets for testing the specificity of targeted infection and killing.

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Introduction.

Viruses and virus-based vectors represent a powerful tool for the delivery of recombinant molecules to cells. However, a major drawback to current systems has been the inability to target and limit an infection by a recombinant virus to predetermined cell types or tissue. The goal of the proposed work is to develop a novel method based on recombinant SV5 (rSV5) for targeting and killing predetermined population of tumor cells. The hypothesis to be tested is that the cell-type specificity of an SV5 infection can be pre-determined by incorporating the appropriate foreign membrane protein into the viral envelope. In this work, recombinant SV5 (rSV5) will be engineered to incorporate a membrane-bound form of a single chain antibody (sFv) into its envelope. It is anticipated that the specificity of infection by this rSV5 will be limited to cells expressing the appropriate surface antigen that is recognized by this antibody. To test this hypothesis, virions will be isolated which contain in their envelope an sFv that is specific for HER2, a cell surface antigen that is overexpressed in a large number of carcinomas. Cell lines will be created which express varying levels of HER2. The cell-type specificity of infection by this recombinant virus will be tested to determine if the infection is limited to cancer cells expressing the cell surface HER2 antigen. This annual report describes the progress we have made in identifying two important components for our research plan: a candidate anti-HER-2 sFv for inserting into rSV5 genome and cell lines which will be the targets for testing the specificity of targeted infection and killing.

Body.

List of Approved Tasks.

Task 1. To determine the requirements for incorporation of the membrane-bound HER2-specific sFv into the SV5 envelope.

- a. Chimeric proteins containing portions of either the SV5 HN or the F protein link the sFv will be analyzed by flow cytometry and by indirect immunofluorescence to identify the construct which provides the most efficient cell surface expression.
- b. Incorporation of the chimeric sFv proteins into SV5 virions will be confirmed by expression in SV5-infected cells and western blotting of virions.

Task 2. To determine if the tropism of rSV5-sFv-F is restricted to cells which express cell-surface HER2.

- a. MCF cell lines which contain the HER2 gene under control of an inducible (tetracycline) promoter will be isolated by transfection of plasmids containing a drug resistant gene. Expression in the cell lines will be monitored by immunoblotting with anti-HER2 monoclonal antibodies
- b. Recombinant SV5 viruses (rSV5-sFv and rSV5-sFv-stop) that contain the above chimeric genes will be generated. Viruses will be characterized by biochemical and virological approaches.
- c. The specificity of infection by rSV5-sFv will be determined by flow cytometric analysis, western blotting of cell lysates and by virus neutralization assays.

Research Accomplished on Task 1.

The steady-state level of cell surface sFv-HN chimeric protein cannot be increased by the same mutations which increase cell surface expression of the WT HN protein. We have constructed a series of chimeric proteins consisting of a single chain antibody (sFv) specific for the human HER-2 protein linked to increasing amounts of either the SV5 F (F series) or HN protein (HN series). These chimeric proteins each contained an epitope tag that could be used for detection by immunoprecipitation or by western blotting as shown in the case of the HN series in Fig. 1B. Our immunofluorescence data have indicated that the level of cell surface expression of sFv-HN chimeras is very low (see below). Recently, the HN protein of SV5 has been shown to be efficiently internalized from the cell surface (Leser et al., 1999). The signal for internalization is composed in part by a glutamate residue at position 37 (E37) that abuts the HN transmembrane domain and this residue has been shown to be

important for high turnover from the cell surface (Leser et al., 1999). Changing E37 in the context of the full length HN molecule results in a higher steady state cell surface level of HN.

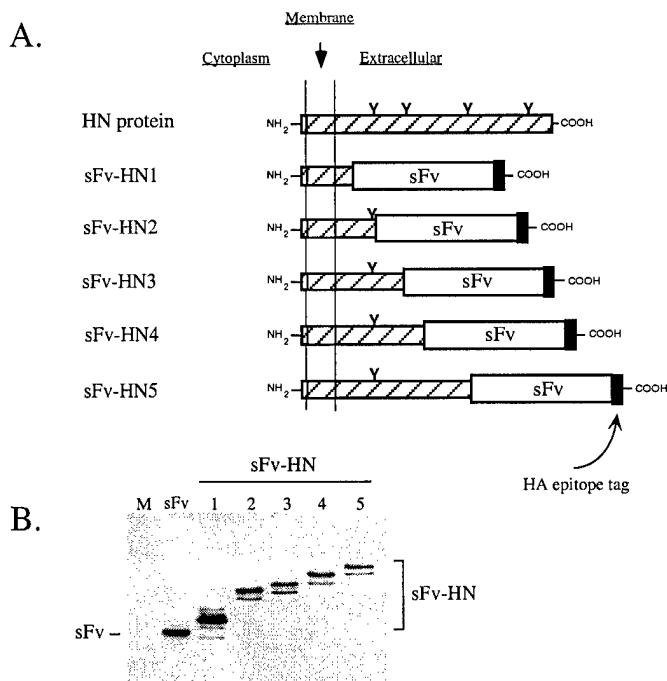


Fig. 1. A) Schematic of sFv-HN chimeric proteins. B) Western blotting with anti-HA antibody of extracts from cells expressing the WT sFv or chimeric proteins 1-5.

The high turnover of the HN protein from the cell surface would be an undesirable property of the sFv-HN chimeric molecule, since this could reduce steady-state levels needed for the incorporation into budding particles. As a means to increase the cell surface expression of sFv-HN, we were interested in determining if changing the E37 residue within the sFv-HN chimeric protein also leads to an increase in cell surface expression of the chimeric molecule as shown by Leser et al., 1999). A mutant protein was constructed using sFv-HN1 as the starting material. This mutant encoded the shortest HN fragment as shown in Fig. 1, linked to the anti-Her2 sFv and this was further modified to include the E37K mutation described by Leser et al (1999) as increasing cell surface expression of HN. Western blotting with the anti-HA antibody confirmed that the mutant protein was expressed to levels matching the wt protein (Fig. 2).

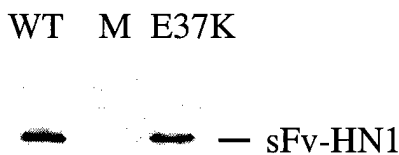


Fig. 2. Expression and western blotting of sFv-HN containing a mutation (E37K) in the internalization signal.

To determine the relative cell surface expression, A549 cells were infected with a vaccinia virus that expresses T7 RNA polymerase (rVV-T7) and then transfected with plasmids encoding the WT sFv-HN1 or E37K sFv-HN1 mutant each of which was under control of

the T7 promoter as described previously (Rassa and Parks, 2000). Cells were stained with the monoclonal antibody that recognizes the HA-tag on the chimeric protein and then analyzed by flow cytometry. Table 1 shows the mean cell surface fluorescence from an experiment in CV-1 monkey kidney cells. The results indicate that the cell surface expression of sFv-HN1 is not significantly enhanced over that of the WT chimeric protein. This experiment has been repeated with similar results in a number of cell lines.

Sample	Mean Fluorescence Intensity
Mock transfected	77
WT sFv-HN1	137
E37K	113

Cell surface immunofluorescence using the anti-HA antibody showed very few cells with bright staining, but those brightly stained cells were expressing very high levels of sFv-HN on their surface. This result indicates that there may be a block in the efficient transport of the chimeric protein to the surface. Alternatively, the transfection efficiency may be very low. We intend to do intracellular staining of permeabilized cells to distinguish between these possibilities.

The sFv-HN1 chimeric protein is functional, and forms complexes with human Her-2, its target receptor. The sFv which we have been using binds to the human Her-2 receptor. We have developed an assay to determine if the sFv-HN chimeric proteins have retained their ability to bind Her-2. A549 human lung cells were infected with rVV-T7 and then co-transfected with various amounts of a plasmid containing the Her-2 gene and a plasmid containing the sFv-HN1 protein, both of which were

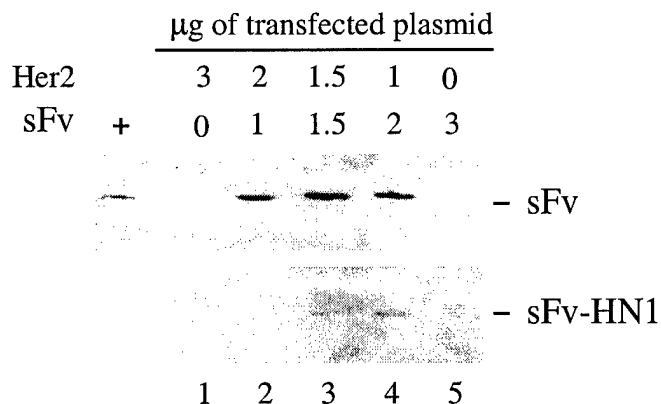


Fig. 3. Co-immunoprecipitation of sFv and sFv-HN1 along with Her-2 using antibodies to Her-2

under control of the T7 promoter. As a control for Her-2/sFv interactions, we used the soluble form of the sFv that was not linked to the membrane by the HN anchor. Cell extracts were prepared and immunoprecipitated with antibody to HER-2 before running on an SDS-PAGE gel. The immunoprecipitated proteins were analyzed by western blotting with the anti-HA epitope tag antibody. Thus, the only way we can detect the sFv-HN in this case, is if Her-2 and the sFv interact and they are co-immunoprecipitated with the anti-Her-2 antibody. As shown in Fig. 3, the soluble sFv specific for Her-2 was only detected in samples from cells co-transfected with both the plasmids encoding Her-2 and sFv (upper panel, lanes 2-4), but was absent in control samples lacking either plasmid (upper panel, lanes 1 and 5). This confirms the specificity of our assay for interactions. When this assay was applied to the membrane-bound chimera sFv-HN1, we detected a very low but reproducible interaction between sFv-HN1 and Her-2 (Fig. 3, lower panel, lanes 3 and 4). These data indicate that the membrane-bound sFv-HN1 chimeric protein is functional and can bind the Her-2 protein. This is an important result suggesting that the sFv-HN chimeras are candidate receptor binding protein to replace the HN gene in the rSV5 genome.

Very high level expression of the sFv-HN chimeric protein from a cellular promoter and studies to detect incorporation of sFv-HN into budding virions. We have chosen to focus on sFv-HN1 as the chimeric protein to insert into the rSV5 genome. We have initiated studies to incorporate sFv-HN1 into budding virions by transfecting cells with a plasmid encoding the sFv-HN1 and infecting the cells with rSV5 virus.

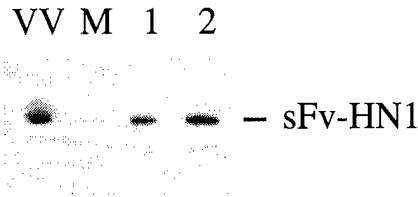


Fig. 4. Western blotting of expression levels from cells transfected with pCAGGS-sFV-HN1 or expressed from vaccinia virus.

The gene encoding sFv-HN1 was inserted into the pCAGGS vector which is driven by the cellular actin promoter. Fig. 4 shows that the expression level from the pCAGGS-sFv-HN1 vectors in two transfected cell samples (lanes 1 and 2) was very comparable to expression driven by vaccinia virus (lane VV). These results are important since they establish that cells transfected with pCAGG-sFv-HN can express high enough levels of the chimeric protein so that we should be able to detect incorporation of the molecule into budding virions. We have initiated these transfection/infection experiments.

Research Accomplished on Task 2.

Generation of human breast cancer cell lines expressing the tet-repressor protein. Our experimental plan includes the use of MCF cells as the target cell to test our hypothesis on targeting rSV5. We intend to isolate MCF cell lines that can be induced to express the Her-2 protein as the receptor for rSV5 containing an anti-Her2 sFv in the envelope. As a first step in this task, we have isolated cell lines that stably express the repressor of transcription from the Tet-inducible plasmids. These cells will then be transfected with the plasmid encoding the Her-2 gene under control of the Tet-inducible promoter. When Tet is added to the cells, transcription takes place due to inactivation of the Tet repressor.

Briefly, MCF human breast cancer cells were transfected with the pcDNA6/TR plasmid (Invitrogen) encoding the Tet repressor, and drug resistant (blasticidin resistant) cell colonies were isolated. To determine which stable cell line had the greatest induction from the Tet promoter, we screened cell lines for Tet-inducible expression of beta-galactosidase (Beta-gal) from a transfected pcDNA4TO-LacZ plasmid. Cell lines were transfected with plasmid in the presence or absence of Tet (10 µg/ml) in the media, and two days later cell lysates were analyzed for Beta-gal using OPTC substrate and OD at 429 nM.

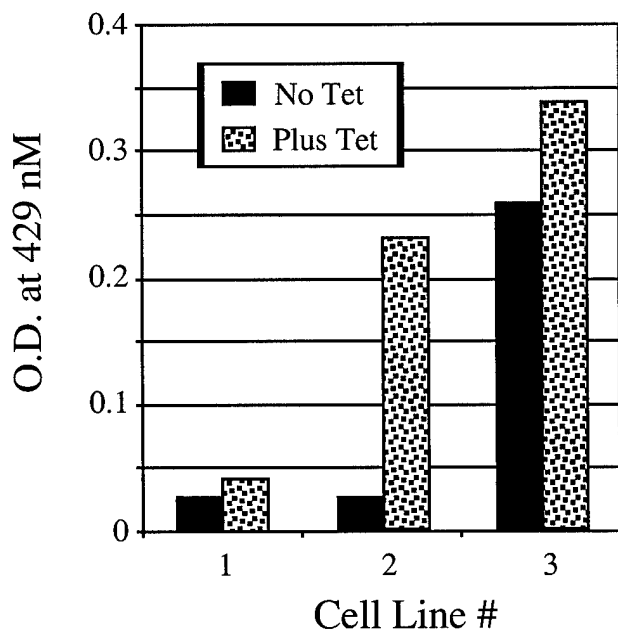


Fig. 5. Human breast cancer cell lines that show Tet-inducible expression of a transfected gene.

A typical result for three cell lines is shown in Fig. 5. Cell line #1 showed very low levels of Beta-gal and there was little induction of enzyme activity. Conversely, cell line #3 showed high Beta-gal activity in the absence of Tet, and there was little induction by adding Tet. Cell line #2 was the best candidate, since there was very low levels of gene expression without Tet and the addition of Tet led to a dramatic increase in Beta-gal activity. We are in the process of cloning the Her-2 gene into the Tet-inducible vector pcDNA6TO (Invitrogen). Once this is achieved, we will use cell line #2 as starting material to isolate stable MCF cell line that shows induction of Her-2 expression when exposed to Tet.

Additional work accomplished on designing rSV5 to selectively kill breast cancer cells.

As part of our efforts to develop rSV5 as a therapeutic vector for selective killing of cancer cells, we have constructed a rSV5 that expresses the herpes simplex thymidine kinase (rSV5-TK) gene. We have shown selective killing of a number of cancer cell lines by infection with rSV5-TK, but only when they are exposed to either ganciclovir (GCV) or acyclovir (ACV). This result has important implications for the use of rSV5 as a vector for cancer therapy. Our long term goal is to combine the selective killing with the TK/GCV system with our work on selective targeting described above to create a novel vector for cancer therapy. We are in the process of writing a manuscript describing this rSV5-TK virus and a preliminary copy is included in the appendix section of this report. The abstract from the paper is included below.

Abstract.

Engineering a Nonsegmented Negative Strand RNA Virus to Selectively Kill Infected Cells.

Griffith D. Parks, Virginia A. Young, Elizabeth K. Wansley, Jennifer L. Layer and Kelly M. Cooke*

In most tissue culture cell lines tested, infection with the paramyxovirus simian virus 5 (SV5) results in very little if any cell death. To determine if SV5 could be used as a vector for controlled killing of cells, a recombinant SV5 (rSV5-TK) was constructed to encode the herpes simplex virus thymidine kinase (TK) gene. MDBK cells infected with rSV5-TK showed a time-dependent loss of viability, but only in the presence of the prodrug acyclovir (ACV) or ganciclovir (GCV). In the presence of 5 µg/ml ACV, >90% of the rSV5-TK infected MDBK cells had lost viability within 48 hours, whereas cells infected with a control rSV5 expressing GFP showed only a slight reduction in growth rate with no detectable cell death. Time-lapse video microscopy of MDBK cells that were infected with rSV5-TK and cultured in the presence of ACV showed an accumulation of rounded cells displaying characteristics of apoptotic cell death. Titration experiments with GCV showed that rSV5-TK infection resulted in cell death for all mouse and non-human cell lines tested, although the kinetics and efficiency of cell death varied between cell types. An MDBK cell line persistently infected with rSV5-TK retained sensitivity to GCV-mediated cell killing after 15 passages in tissue culture. Our results showing controlled cell killing by an RNA virus has important implications for the therapeutic use of rSV5 in targeted infection of cancer cells.

Key Research Accomplishments.

- The steady-state level of cell surface sFv-HN chimeric protein cannot be increased by the same mutations which increase cell surface expression of the WT HN protein.
- The sFv-HN1 chimeric protein is functional, and forms complexes with human Her-2, its target receptor.
- The sFv-HN1 chimeric protein has been identified as the candidate attachment protein for insertion into the rSV5 genome.
- Cell lines have been isolated that show inducible expression of a gene under control of the Tet-on operon.

Reportable Outcomes.

- 1) Development of human breast cancer cell lines expressing the tet repressor protein for use in inducible expression of genes.
- 2) Identification of candidate chimeric sFv-HN genes for interactions with Her-2 receptor.
- 3) Manuscript describing the construction of a novel recombinant SV5 that selectively kills cancer cell lines. This is the first example of an engineered RNA virus that kills cells in a controlled manner.

Conclusions.

The goal of the proposed work is to develop a novel method based on recombinant SV5 (rSV5) for targeting and killing predetermined population of tumor cells. This annual report describes progress we have made toward this goal by identifying two important components for our research plan: a candidate anti-HER-2 sFv for inserting into rSV5 genome and cell lines which will be the targets for testing the specificity of targeted infection and killing. These are important preliminary steps to know which sFv-HN chimera to use in the construction of rSV5 vectors. However, we will need to improve the efficiency of cell surface expression of the sFv-HN chimera. In the future, this could be improved by attaching the sFv to the full length HN protein and this may improve the folding of the hybrid molecule. Alternatively, we have initiated plans to test a sFv linked to the vesicular stomatitis virus (VSV) G protein as the attachment protein for rSV5 targeting. SV5 and VSV can form pseudotypes containing the viral glycoproteins and this could eliminate the possible formation of hetero-oligomers between the full length HN and the sFv-HN in our transfection/infection protocols.

Human breast cancer cell lines have been developed which express the Tet repressor protein. These cells show an induction of gene expression when cultured in the presence of Tet. These cell lines will be valuable for establishing the next generation cells that show inducible expression of a number of important genes such as the Her-2 protein for our experiments.

References.

Leser GP, Ector KJ, Ng DT, Shaughnessy MA, Lamb RA (1999) The signal for clathrin-mediated endocytosis of the paramyxovirus SV5 HN protein resides at the transmembrane domain-ectodomain boundary region. *Virology* **262**:79-92

Rassa, J. C., G. M. Wilson, G. A. Brewer, and G. D. Parks. (2000) Spacing constraints on reinitiation of paramyxovirus transcription: The gene end U tract acts as a spacer to separate gene end from gene start sites. *Virology* **274**:438-449.

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**Engineering a Nonsegmented Negative Strand RNA Virus to
Selectively Kill Infected Cells**

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Running title: Paramyxovirus expressing thymidine kinase

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ABSTRACT

In most tissue culture cell lines tested, infection with the paramyxovirus simian virus 5 (SV5) results in very little if any cell death. To determine if SV5 could be used as a vector for controlled killing of cells, a recombinant SV5 (rSV5-TK) was constructed to encode the herpes simplex virus thymidine kinase (TK) gene. MDBK cells infected with rSV5-TK showed a time-dependent loss of viability, but only in the presence of the prodrug acyclovir (ACV) or ganciclovir (GCV). In the presence of 5 µg/ml ACV, >90% of the rSV5-TK infected MDBK cells had lost viability within 48 hours, whereas cells infected with a control rSV5 expressing GFP showed only a slight reduction in growth rate with no detectable cell death. Time-lapse video microscopy of MDBK cells that were infected with rSV5-TK and cultured in the presence of ACV showed an accumulation of rounded cells displaying characteristics of apoptotic cell death. Titration experiments with GCV showed that rSV5-TK infection resulted in cell death for all mouse and non-human cell lines tested, although the kinetics and efficiency of cell death varied between cell types. An MDBK cell line persistently infected with rSV5-TK retained sensitivity to GCV-mediated cell killing after 15 passages in tissue culture. Our results showing controlled cell killing by an RNA virus has important implications for the therapeutic use of rSV5 in targeted infection of cancer cells.

INTRODUCTION

There has been remarkable progress in recent years on the development of reverse genetics systems for manipulating negative strand RNA viruses. As such, a number of non-segmented negative strand RNA viruses have been engineered to express a variety of foreign proteins (reviewed in Conzelmann, 1996; Garcia-Sastre, 1998; Pekosz et al., 1999; Palese, 1995). The ability to recover paramyxo- and rhabdoviruses from cDNA has raised the possibility of using these viruses as therapeutic vectors for the delivery of therapeutic genes or toxins for cancer therapy.

The paramyxovirus simian virus 5 is a prototype of the rubulaviruses, a group that includes mumps virus, human parainfluenza virus type 2 (HPIV2) and SV41. The ~15 kb negative sense RNA genome encodes a series of tandemly linked genes (Fig. 1A) that code for the nucleocapsid protein (NP) which binds the genomic RNA, the phospho- (P) and large (L) proteins which together constitute the viral polymerase, the matrix (M) protein, and the membrane proteins HN and F which are responsible for cell attachment and fusion, respectively. The small hydrophobic (SH) protein serves an unknown function in the viral lifecycle, and is reported to be dispensable for growth in tissue culture (He et al., 1998). A reverse genetics system has been developed for the recovery of recombinant SV5 from cDNA (He et al., 1997).

SV5 has inherent properties which could be exploited to generate novel therapeutic vectors. One such property is that infection with SV5 results in little if any cell death in most cell lines tested. We reasoned that a paramyxovirus which does not induce a dramatic cytopathic effect could serve as a vector for selective killing of infected cells, since the

noncytopathic vehicle could be engineered to express a foreign protein that would induce cell death in a controlled manner and by mechanisms which are understood.

A prototype system for controlled cell killing is based on the thymidine kinase (TK) protein from herpes simplex virus. TK has been used extensively as a "suicide gene" in a variety of cancer therapies (reviewed in Mesnil and Yamasaki, 2000; Moolten, 1994). Within a cell, TK selectively phosphorylates the nontoxic prodrug acyclovir (ACV) or ganciclovir (GCV), converting them into potent intracellular drugs which are incorporated into host DNA and ultimately induce cell death by apoptosis (Elion et al., 1977). Thus, cell death is controlled, since it occurs only when cells express TK and then are exposed to the prodrug. To test our hypothesis on the use of rSV5 as a vector, we have engineered a recombinant SV5 (rSV5-TK) to express the TK gene and tested cells for sensitivity to selective killing by rSV5-TK. We show that infection of a variety of cell lines with rSV5-TK results in very little if any virus-mediated cell death, but extensive cell killing can be induced when rSV5-TK infected cells are exposed to ACV or GCV. Our results have implications for the use of rSV5 as a therapeutic vector for controlled killing of targeted cells.

MATERIALS AND METHODS

Cells, viruses and plaque assays. Cultures of cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 containing 10% fetal calf serum (FCS). The 4T1 murine breast cancer cell line was kindly provided by Fred Miller (University of Michigan). To determine doubling times, 10^5 cells were plated in 3.5 cm dishes and viable cell numbers were determined at various timepoints using a Coulter Counter. Vaccinia virus MVA expressing T7 RNA polymerase was the kind gift of B. Moss and was grown and titered on BHK cells (Wyatt et al., 1995). Single step growth kinetics and SV5 plaque assays were carried out as described previously (Parks et al., 2001). An MDBK cell line persistently infected with rSV5-TK was established by infecting a cell monolayer ($\sim 10^6$ cells) at an moi of ~ 50 and passing the resulting cells every 4-5 days.

Recovery of rSV5 expressing TK. The full length SV5 infectious cDNA clone pBH276 (He et al., 1997) was kindly provided by B. He and R. A. Lamb (Northwestern University). A SphI-Sall DNA fragment encoding the HN-L junction was modified by a PCR using Pwo polymerase (Boehringer Mannheim) and subcloned into pGem3 to generate pG3-HNL-MCS, such that the HN-L junction contained a multiple cloning site with EcoRV and Sall restriction enzyme sequences. A plasmid (pPEP78) encoding the herpes simplex TK gene was kindly provided by Dr. Peter Pertel (Northwestern University). The TK gene was modified by a PCR to remove the internal SphI site and add a 3' terminal Asp718 site without changing the encoded amino acids. The resulting PCR product was inserted into pG3-HA such that the translation open reading frame was fused at the 3' end to a ten amino acid segment recognized by an anti-HA antibody (HA tag; YPYDVPDYA). The TK-HA gene was inserted into pG3-HNL-MCS such that the gene was flanked on the 3' and 5 sides by transcription

signals from the NP-P and HN-L junctions, respectively (Fig. 1A; Rassa and Parks, 1999), and the overall length was a multiple of six (Murphy and Parks, 1997). A SphI-SalI DNA fragment was excised and then inserted into the corresponding sites in the SV5 full length cDNA clone (He et al., 1997) to yield pRSV5-TK.

SV5 virus expressing the HA-tagged TK protein was recovered from pRSV5-TK as described previously (He et al., 1997) with minor modifications (Parks et al., 2001). Virus stocks were generated from single plaques by growth in MDBK cells. Wild type (WT) rSV5 and rSV5-GFP were similarly isolated from pBH276 and pBH311, respectively (He et al., 1997).

Western blotting. CV-1 cells plated in 24 well dishes were infected with rSV5 WT, rSV5-GFP or rSV5-TK at an moi of ~20 for one hour. Cells were washed and covered with DMEM/2% FCS. At each timepoint, cells were washed with PBS, lysed in gel loading buffer and equal aliquots of cell lysate were run on a 10% polyacrylamide gel (Lamb and Choppin, 1976). Proteins were transferred to nitrocellulose using a semi-dry electrophoretic transfer cell (15V, 15 min). For dot-blot analysis, the protein concentration of infected cell lysates was determined using the BCA assay (Pierce Chemicals). Equivalent amounts of protein were serially diluted in PBS and applied to nitrocellulose using a vacuum manifold. Nitrocellulose membranes were blocked (5% milk in PBS), and probed using a rat monoclonal antibody specific for an HA epitope (anti-HA clone 3F10, Roche Molecular Biochemicals) or rabbit antisera to the SV5 NP and P proteins (Parks et al., 2001). Proteins were visualized using HRP-conjugated goat anti-rabbit and ECL (Pierce Chemicals).

Cell killing assays and time-lapse video microscopy. Between $1-5 \times 10^5$ cells were mock infected or infected at an moi of ~20 with rSV5-GFP or rSV5-TK for one h. Cells were

covered in media containing 2% FCS and various concentrations of ACV or GCV (Sigma Chemicals) as described in the figure legends. At the indicated times, cell monolayers were washed, trypsinized (for adherent cells), and viable cell numbers were determined by trypan-blue exclusion.

Time-lapse video microscopy was carried out on monolayers of MDBK cells infected with rSV5-GFP or rSV5-TK and cultured in the presence of 5 $\mu\text{g/ml}$ ACV. Pictures were recorded at 10 s intervals on standard VHS tape using a nisisi. The accumulation of rounded cells that did not undergo cell division was determined as a percentage of the total number of cells in the field.

RESULTS

Growth properties of rSV5 expressing the herpes simplex TK protein. The TK gene from herpes simplex virus was modified by a PCR to be flanked by gene start and gene end signals from the SV5 NP-P/V and HN-L junctions, respectively (Rassa and Parks, 1999). To facilitate detection of the TK gene product, the 3' end of the TK open reading frame was extended to include an eleven amino acid epitope from the influenza HA protein. Previous work has indicated that extensions to the COOH terminus of TK does not affect enzyme activity (Loimas et al., 1998). The resulting TK-HA gene was inserted at the HN-L junction encoded in the SV5 infectious cDNA clone (Fig. 1A). Virus was recovered from the resulting plasmid as described previously (He et al., 1997) and was designated rSV5-TK.

For some negative strand RNA viruses, the insertion of a foreign gene can influence the viral growth characteristics (e.g., Sakai et al., 1999; Skiadopoulos et al., 2000). To determine if inserting the TK gene into the SV5 genome affected the rate of virus growth, a single step growth analysis was carried out on cells infected at a high moi with WT rSV5, rSV5-TK or a control virus containing the GFP gene at the HN-L junction (rSV5-GFP; He et al., 1997). As shown in Fig. 1B, the insertion of TK did not appear to affect growth, since each of the rSV5 viruses showed nearly identical growth rates as well as final virus titers when measured in MDBK cells.

Western blotting with HA antibody was carried out to determine a timecourse of TK expression in cells infected with rSV5-TK. As shown in Fig. 1C, cells infected with rSV5-TK synthesized a ~45 kDa polypeptide that was not detected in mock infected cells (lane M). The kinetics of TK appearance in cells infected with rSV5-TK was slightly delayed relative to NP and P, consistent with the position of the TK gene in a 3' promoter-distal site. A faster

migrating form of TK was also detected at late times in infection and may represent a degradation product.

ACV-mediated killing of MDBK cells infected with rSV5-TK. A titration experiment was carried out to determine if cells infected with rSV5-TK were sensitive to killing by the prodrug ACV. MDBK cells were mock infected or infected with rSV5-TK or rSV5-GFP at an moi of ~20 and the cells were cultured in the presence of various concentrations of ACV. After 48 h, the fold change in the number of viable cells was determined by counting cells that excluded trypan blue staining.

In the absence of ACV, infection of MDBK cells with either rSV5-GFP or rSV5-TK did not affect the fold change in cell number or cell viability (Fig. 2A). These data are consistent with the proposal that SV5 infection is largely non-cytopathic in MDBK cells. Likewise, the growth of mock infected or rSV5-GFP infected MDBK cells was not significantly affected by the presence of ACV, with only a ~2-3 fold change in cell number seen at ACV concentrations as high as 20 $\mu\text{g/ml}$. By contrast, including as little as 1 $\mu\text{g/ml}$ ACV in the growth media of cells infected with rSV5-TK resulted in no increase in the number of viable cells over the two day assay period. At 20 $\mu\text{g/ml}$ the number of viable cells had decreased from the original number of rSV5-TK infected cells by 2-3 fold.

A timecourse of cell viability in the presence of ACV was carried out using MDBK cells infected with rSV5-GFP, rSV5-TK or mock infected. During the first 24 h p.i., the number of mock infected or rSV5-GFP infected cells increased ~2-3 fold and by 48 h p.i. cell numbers were ~3-4 fold higher. These changes in growth were not significantly affected by including ACV in the media (dashed lines, Fig. 2B). Likewise, cells infected with rSV5-TK and cultured without added ACV showed growth kinetics that closely matched that of uninfected cells.

However, in the presence of 10 $\mu\text{g/ml}$ ACV, rSV5-TK infected cell numbers did not significantly increase after the first day p.i., and viable cell numbers decreased 2-3 fold by 48 h p.i. These data indicate that treatment of rSV5-TK infected MDBK cells with ACV results in an initial slowing of cell growth, followed by a significant loss of cell viability.

Timelapse video microscopy was used to more closely monitor the changes in individual rSV5-TK infected MDBK cells after exposure to ACV. Fig. 3 shows selected pictures from a representative experiment where MDBK cells were infected with rSV5-TK (panels A-C) or rSV5-GFP (panels D-F) and cultured in the presence of 10 $\mu\text{g/ml}$ ACV. By 24 h p.i. with rSV5-TK, there was an accumulation of cells rounded up without undergoing cell division (panel B). By 48 h p.i., many of the cells had acquired characteristics of apoptotic cells including cell rounding, membrane blebbing and accumulation of cellular debris. Although some rounded cells are seen in panel E with rSV5-GFP infected cells, these rounded cells were undergoing cell division. The number of rounded cells that did not undergo cell division was calculated as a percentage of the total number of cells in the field. As shown in the graph in Fig. 3 for rSV5-TK infected cells, there was an initial lag in accumulation of rounded cells but by 48 h p.i. nearly 70% of the cells had rounded up and many of the cells had disintegrated into apoptotic bodies (panel C). By contrast, for rSV5-GFP infected cells exposed to ACV, only ~2% and ~5% of the infected cells had rounded up without undergoing cell division by 24 and 48 h p.i., respectively (Fig. 3, panels E and F). These data suggest that the combination of rSV5-TK infection and ACV treatment induces a loss of MDBK cell viability that has characteristics of apoptosis.

Differential susceptibility of various cell types to GCV-mediated killing by rSV5-TK.

Various cell lines were tested for their sensitivity to selective cell killing by rSV5-TK. GCV was used for these assays, due to the lower non-specific effect on mock infected cells. Fig. 4 shows the results obtained using six representative cell lines: bovine kidney MDBK cells, murine breast cancer 4T1 cells, human monocytic-like U937 cells, human lung alveolar A549 cells, human HeLa cells, and human breast cancer SKBR-3 cells.

In the absence of added GCV, the growth of cells infected with either rSV5-GFP or rSV5-TK was slower compared to uninfected cells (Fig 4, 0 GCV bars). Culturing uninfected control cells with 200 μ M GCV also slowed the growth of some cell lines (Fig. 4, white bars). Likewise, the combination of infection with the control virus rSV5-GFP and GCV treatment resulted in slower growth, but the effect was not a dramatic. By contrast, the combination of infection with rSV5-TK and GCV treatment showed a significant reduction of cell numbers in each cell line tested, although the effect varied between cell lines. MDBK cells were the most sensitive to GCV-mediated rSV5-TK killing, with a loss of viability seen with as little as 2 μ M GCV. For the 4T1 murine breast cancer cell line, there was a lower overall GCV-mediated cell killing and significant losses were only seen at higher GCV concentrations.

Each of the human cell lines tested showed a different profile of GCV-mediated rSV5-TK killing. The U937 human monocytic-like cell line was similar to MDBK cells in susceptible to GCV-mediated killing by rSV5-TK, while HeLa cells showed loss of viability only at the highest concentration of GCV tested. Human lung 5 were the least sensitive to GCV-mediated killing. Although the profile for A549 cells is similar to that of U937 cells, A549 cell death was delayed until 4 days p.i. as opposed to the 2 days needed for killing of the other cell lines.

The differential sensitivity of various cell lines to GCV-mediated cell killing was not strictly related to cell growth rate. As shown in Fig. 5, MDBK cells had the shortest doubling time (~11.5 h) and were the most sensitive to GCV-mediated killing by rSV5-TK. However, 4T1 cells had a similar doubling time (DT ~12 h), and were significantly less sensitive to GCV-mediated killing. U937 and A549 cells had similar doubling times (~20 and 17.5 h, respectively), but A549 cells took 4 days to show a significant loss of viability. Sensitivity to GCV-mediated killing was also not strictly correlated with rSV5-TK expression levels (not shown).

GCV-mediated killing of MDBK cells persistently infected with rSV5-TK. The above results indicate that cells infected for short periods of time with rSV5-TK are susceptible to GCV-mediated killing. We established an MDBK cell line that is persistently infected (PI) with rSV5-TK, to determine if these cells had retained their sensitivity to GCV-mediated killing. Immunofluorescence microscopy with anti-P antibodies demonstrated the presence of SV5 antigens in >95% of the PI cells for over 15 passages spanning two months (not shown). The PI cells showed no obvious difference in growth characteristics or morphology relative to uninfected or acutely infected MDBK cells. As shown in Fig. 5A, exposure of the PI cell line to 2 μ M GCV resulted in a dramatic loss of cell viability, with greater cell killing at higher GCV concentrations. Thus, the PI cell line appears to be at least as sensitive to GCV-mediated cell killing as the acutely rSV5-TK infected cells.

Dot blot hybridization of cell extracts was carried out to determine if the relative sensitivity of the PI cells and cells acutely infected with SV5-TK to GCV-mediated cell killing correlated with the level of TK protein expression. Cell lysates were prepared from mock infected MDBK cells, the PI cell line, or MDBK cells at 48 h p.i. with rSV5-TK. Serial dilutions

of lysates were analyzed using antibodies for the SV5 NP and TK proteins. As shown in Fig. 5B, the acute infection produced slightly more NP and TK protein at 48 h p.i, than was found for the PI cell line. Thus, the PI cell line retains sensitivity to GCV-mediated killing despite having lower levels of TK protein.

DISCUSSION

In this report we have tested the hypothesis that the nonsegmented negative strand RNA virus SV5 could be engineered to selectively kill infected cells. We reasoned that SV5 could serve as a vector for selective cell killing, since the noncytopathic vector could be engineered to express a foreign protein that would induce cell death in a controlled manner and by mechanisms which are understood. Our proposal was based on the observation that in most cell lines tested, rSV5 infection results in very little if any cell death. Likewise, in all cell types that we have tested to date, SV5 infection does not result in a shut-off of host protein synthesis or mRNA transcription (e.g., Paterson et al., 1984; Peluso et al., 1977). While SV5 infection of a few cell types, such as CV-1 and BHK, leads to extensive cell-cell fusion (e.g. Horvath and Lamb, 1992), this is not the most common outcome of infection and none of the cell lines tested here showed SV5-induced syncytia. Using time lapse video microscopy, we have found that only ~5% of rSV5-infected MDBK cells in a population have rounded up without undergoing cell division. In the case of A549 cells, it is difficult to find cells in the population that show any effects from rSV5 infection (not shown). Work is in progress to determine if these same properties are evident during an rSV5 infection of primary cells.

Our results indicate that SV5 infection has a minor effect on cell growth. When assayed over a two day period in the absence of ACV or GCV, rSV5-TK and rSV5-GFP infection resulted in a 2-3 fold decrease in cell number compared to mock infected cells (see Fig. 4). This observation is consistent with a previous careful analysis showing that SV5-infected HeLa cells proliferated more slowly than mock infected cells. Lin and Lamb (2000) have reported that SV5 infected Hela cells display both a small delay at the beginning of S phase and an extended length of the S phase of cellular growth. The SV5 V protein is

responsible for altering the HeLa cell cycle during infection (Lin and Lamb, 2000), and this may be due to the interaction of V protein with the cellular damage-specific DNA binding protein (DDB; Lin et al., 1998). For the purposes of using rSV5-TK in targeted cell killing, this small delay in entering S phase could be an advantage, since the cell would build up higher levels of phosphorylated GCV for incorporation into host DNA.

The mechanism of cell killing by the combination of TK plus ACV/GCV is thought to be by apoptosis (Hamel et al., 1996; Wallace et al., 1996). A number of viruses have been shown to encode components that act to block apoptotic pathways (Griffin and Hardwick, 1997; Roulston et al. 1999 and references therein). We initially anticipated that SV5 might have a mechanism(s) that inhibits apoptosis induced by an infection, and this mechanism might interfere with the ACV/GCV induction of cell death. Our results indicate that any SV5-mediated block in an apoptotic pathway can be overcome by the combination of ACV/GCV and TK. The variable sensitivities of cell lines to GCV-mediated killing by rSV5-TK could reflect either the status pathway in a particular cell line or the ability of SV5 to block the pathway.

While all cell types tested lost viability using the rSV5-TK plus GCV combination, the efficiency and degree of cell killing was not always the same. The most efficient and rapid cell killing occurred with MDBK cells, while HeLa cells were killed only at the highest GCV concentration tested and murine 4T1 cells showed the least efficient killing. A number of factors can contribute to the sensitivity of a cell line to GCV-mediated killing, including cell growth rate, level of TK expression, uptake of GCV, and the status of cellular apoptotic pathways. No consistent and direct correlation was evident between the efficiency of rSV5-TK killing and any one of these parameters. The efficiency of rSV5-TK killing of human A549

lung cells was unique among the cell lines tested, since significant cell killing was not evident until four days p.i.

Paramyxoviruses can frequently establish persistent infections both in cell culture and in vivo (reviewed by Randall and Russell, 1991). This property could be a potential disadvantage to the use of paramyxoviruses as safe therapeutic vectors for humans. The rSV5-TK infected MDBK PI cell line established here retained sensitivity to GCV-mediated cell killing, despite being passages for two months and having a lower level of TK expression than that found in an acute infection. This result supports the idea that including the TK gene in recombinant negative strand viruses could function to control or eliminate a persistent viral infection.

Selective cell killing and selective cell targeting are two critical features for a virus to be useful in targeted therapy of cancer cells (reviewed in Verma and Somia, 1997; Anderson, 1998). The results reported here with rSV5-TK provide proof-of-concept addressing the first requirement. Our approach has been to exploit the non-cytopathic nature of an SV5 infection, with the intent to engineer rSV5 vectors that kill cells by a mechanism that is controlled and that is understood. SV5 has inherent properties that can be also exploited to address the second requirement of selective cell targeting. The SV5 F protein is capable of promoting membrane fusion without the SV5 HN attachment protein (e.g., Horvath and Lamb, 1992; Lamb, 1993). This raises the possibility that the SV5 HN protein with a binding specificity for sialic acid, could be replaced with an attachment protein with specificity for a targeted cellular receptor. This approach has recently been demonstrated for another negative strand RNA virus, where selective cell targeting has been achieved using a recombinant measles virus that contains a chimeric attachment protein consisting of portions of the H attachment protein

linked to epidermal growth factor (Schneider et al., 2000). Work is in progress to engineer rSV5 to encode both the TK gene for controlled cell killing, as well as a chimeric attachment protein for targeting of the viral infection to selected cell types.

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FIGURE LEGENDS

Figure 1. Genome structure, protein expression and growth of rSV5-TK. Panel A. Structure of rSV5-TK. The SV5 genome is depicted as a rectangle with vertical bars denoting the intergenic regions. The 3' leader (le) and 5' trailer (tr) are shown as dark boxes. The herpes simplex TK open reading frame was modified to include a 3' end HA epitope tag, and was flanked on the 3' and 5' sides by the gene end - intergenic - gene start signals from the NP-P\ and the HN-L gene junctions, respectively. Panel B. Growth of rSV5-TK. MDBK cells were infected at a moi of ~20 with rSV5 WT, rSV5-GFP or rSV5-TK and media was harvested at the indicated times p.i. Virus titers were determined by plaque assay on CV-1 monolayers. Panel C. TK expression from rSV5-TK. MDBK cells were mock infected (M lane) or infected with rSV5-TK. At the indicated h p.i., cells were lysed and samples analyzed by western blotting with polyclonal antisera specific for the SV5 NP and P proteins (top panel) or an HA-specific monoclonal antibody (bottom panel).

Figure 2. Selective killing of MDBK cells infected with rSV5-TK in the presence of ACV. Panel A. Titration of ACV effect. Monolayers of MDBK cells were mock infected (white bars) or infected with rSV5-TK (black bars) or rSV5-GFP (hatched bars). Growth media were supplemented with ACV to the indicated concentrations. Two days later, viable cell numbers were determined by trypan exclusion, and are expressed as the log of the fold change from the initial number of infected cells. Data are a representative of four independent experiments. Panel B. Timecourse of ACV effect. MDBK cell monolayers were mock infected or infected with rSV5-TK or rSV5-GFP. The growth media were supplemented with either 0

(solid lines) or 10 $\mu\text{g/ml}$ ACV (dotted lines). At one and two days p.i., viable cell numbers were determined as described for panel A above.

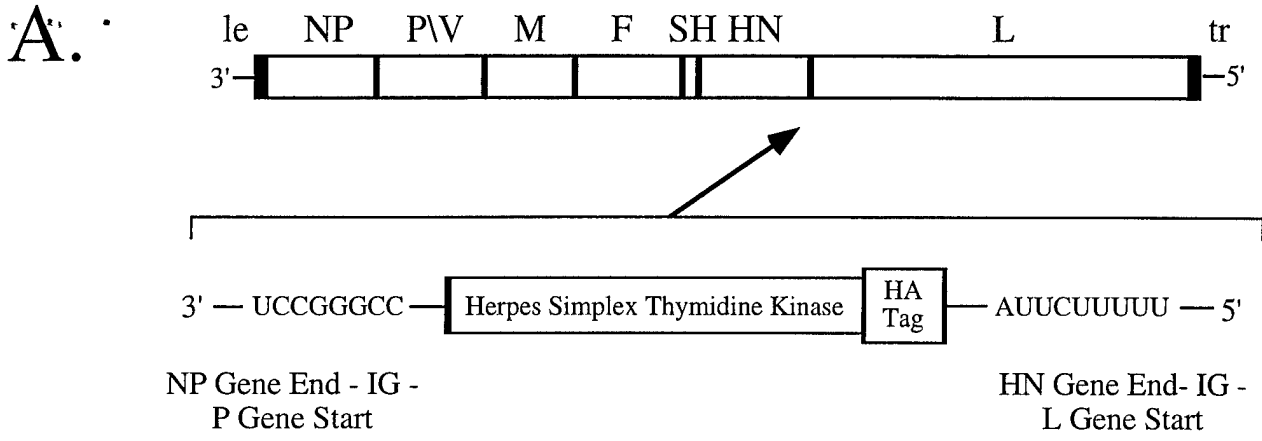
Figure 3. Timelapse video microscopy of MDBK cells infected with rSV5-TK or rSV5-GFP in the presence of ACV. Flasks of MDBK cells were infected with rSV5-TK (top row, A-C) or rSV5-GFP (bottom row, D-F) and incubated in medium containing 10 $\mu\text{g/ml}$ ACV. Timelapse video microscopy of a field of infected cells was carried out as described in Materials and Methods. Selected frames are shown representing time 0 (panels A and D), 24 h p.i. (panels B and E), and 48 h p.i. (panels C and F). The number of rounded cells that did not undergo cell division was determined at the indicated times p.i. and is expressed as a percentage of the total number of cells in the field. Note that most of the rounded cells photographed in panel E were not dead, as evidenced by their subsequently division. Data are representative of three independent experiments.

Figure 4. Differential GCV-mediated killing of rSV5-TK infected cells. MDBK, U937, 4T1, A549, HeLa or SKBR-3 cells were infected with rSV5-TK (black bars), rSV5-GFP (hatched bars), or mock infected (white bars), and the media was supplemented with the indicated concentrations of GCV. After two days, viable cell numbers were determined by trypan exclusion, and are expressed as the log of the fold change from the initial number of infected cells. For A549 cells, viable cell numbers were determined at 4 days p.i. Data are representative of at least two independent experiments. The doubling time (DT) of each cell line was determined as described in Materials and Methods.

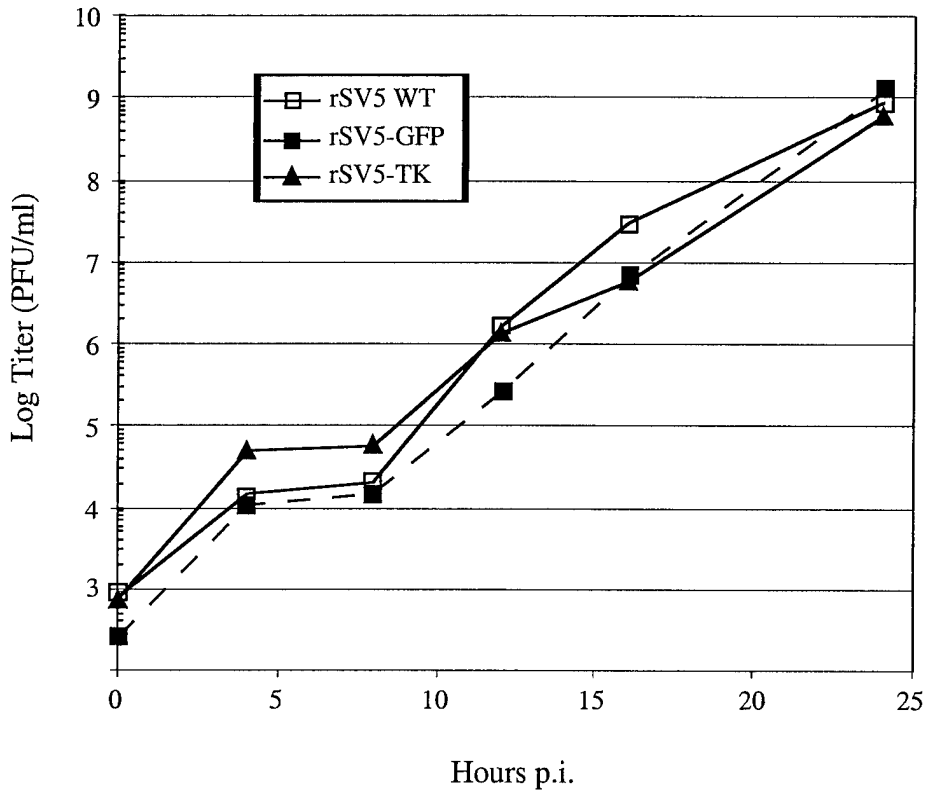
Figure 5. Cells persistently infected with rSV5-TK retain sensitivity to GCV-mediated killing. Panel A. Titration of the effect of GCV on MDBK cells persistently infected with rSV5-TK. A persistently infected cell line was established by infecting MDBK cells with

rSV5-TK and maintaining the cells through 15 passages. The growth media on monolayers of mock infected, rSV5-TK infected and the MDBK PI cell line were supplemented with GCV to the indicated concentrations. After 2 days, viable cell numbers were determined as described in the legend to Fig. 2. Panel B. Expression of TK protein in acute and persistently infected MDBK cells. Lysates were prepared from rSV5-TK persistently infected MDBK cells (P rows), MDBK cells infected for 48 hours with rSV5-TK (S rows) or from mock infected control cells (M rows). After normalizing protein concentrations (starting with 0.2 μ g of protein for NP samples and 2 μ g of protein for TK samples), serial dilutions of lysate were analyzed by dot-blot hybridization using antisera specific for the SV5 NP protein or the HA-tagged TK protein.

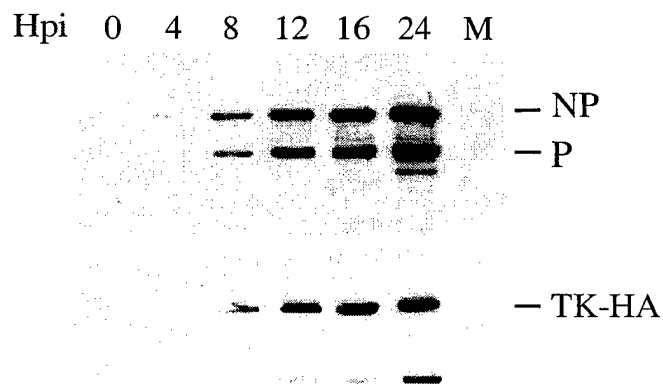
Figure 1



B.

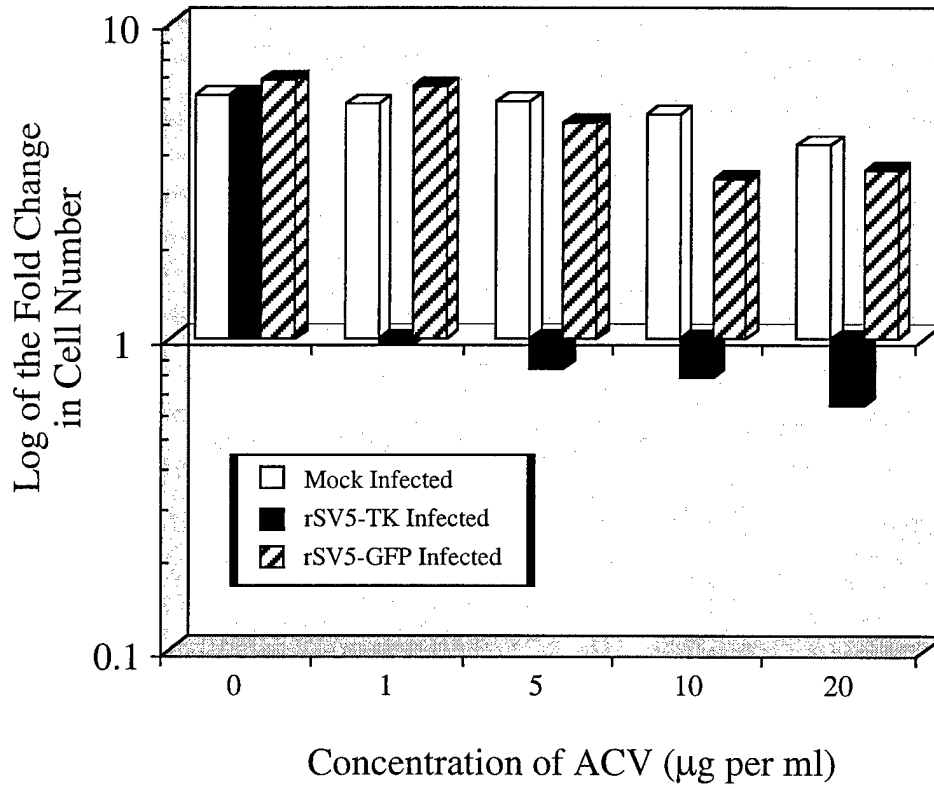


C.



A.

Figure 2



B.

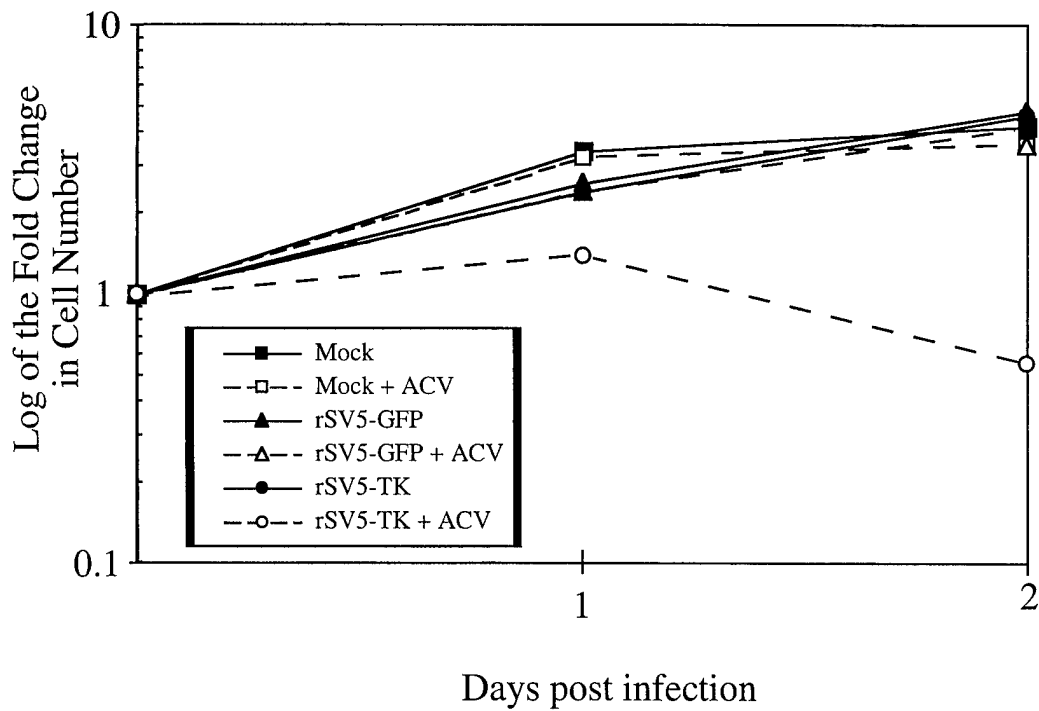


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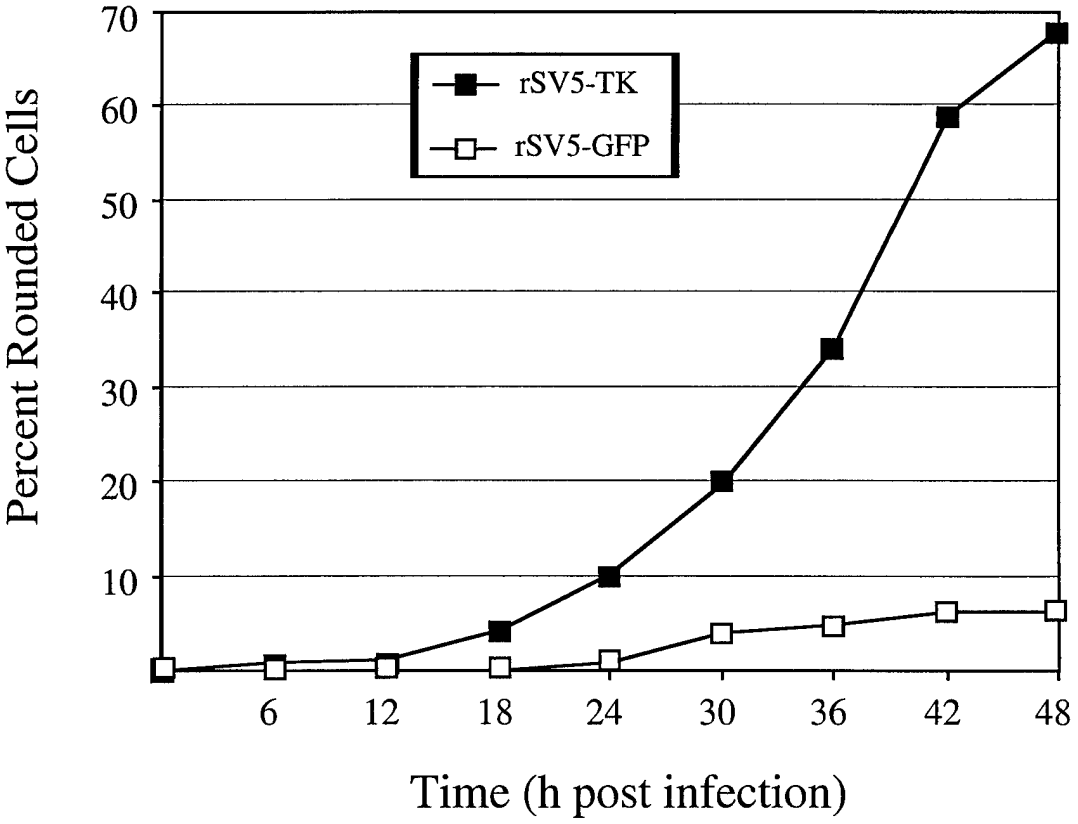
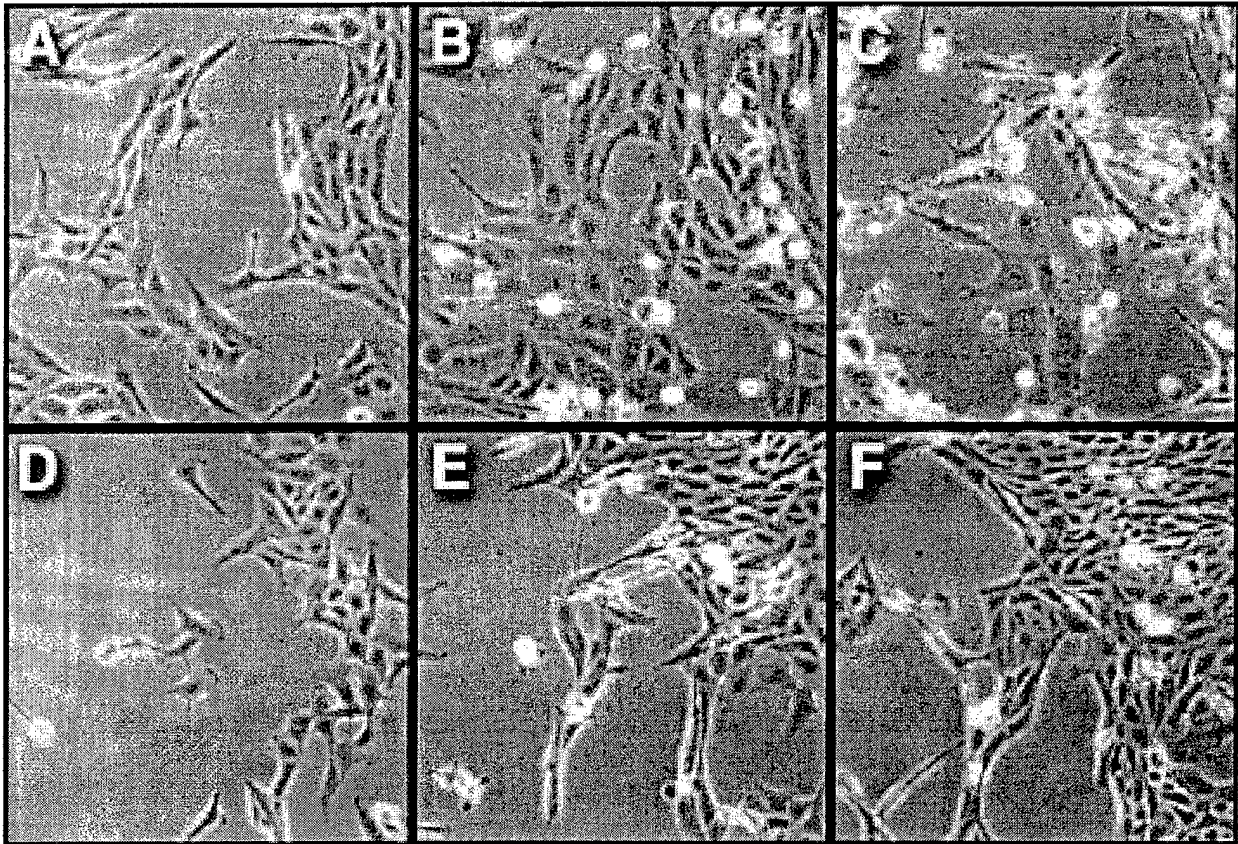
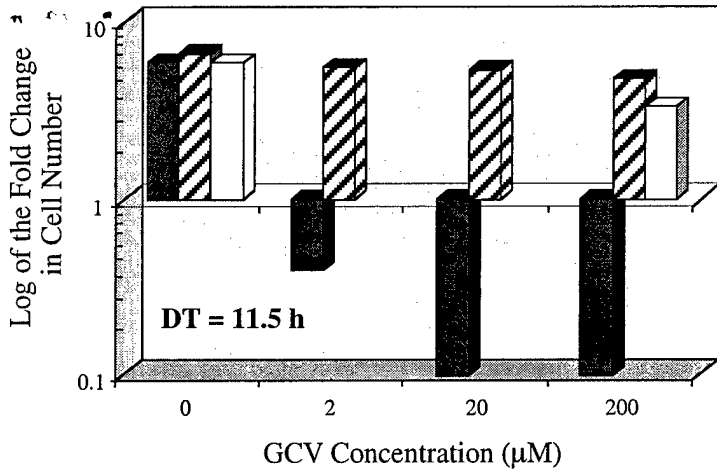
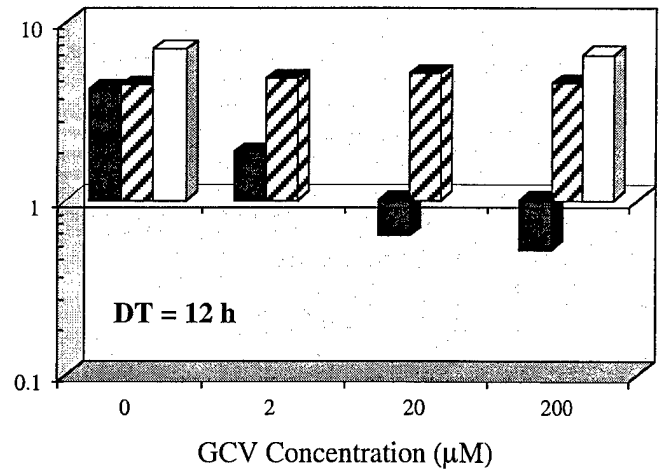


Figure 4

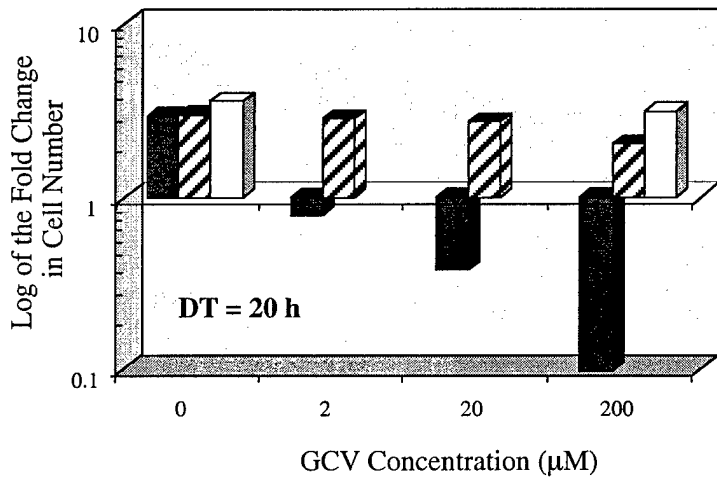
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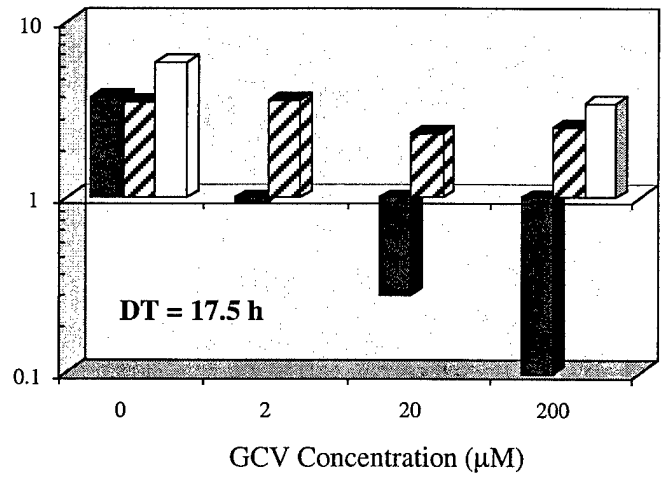
4T1



U937



A549



HeLa

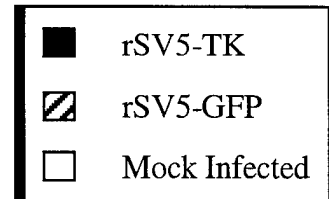
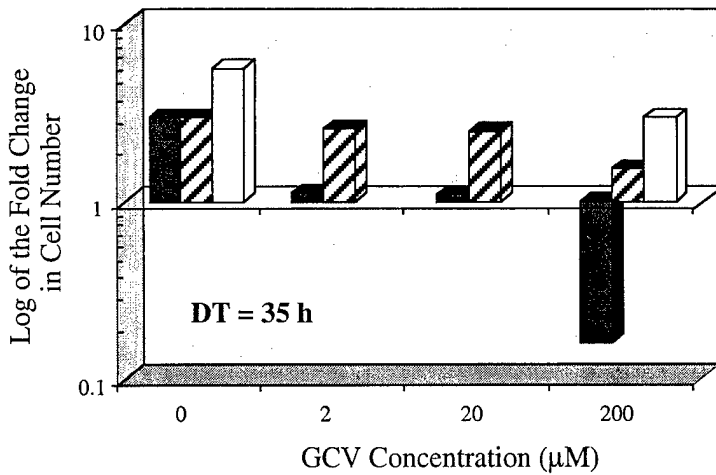
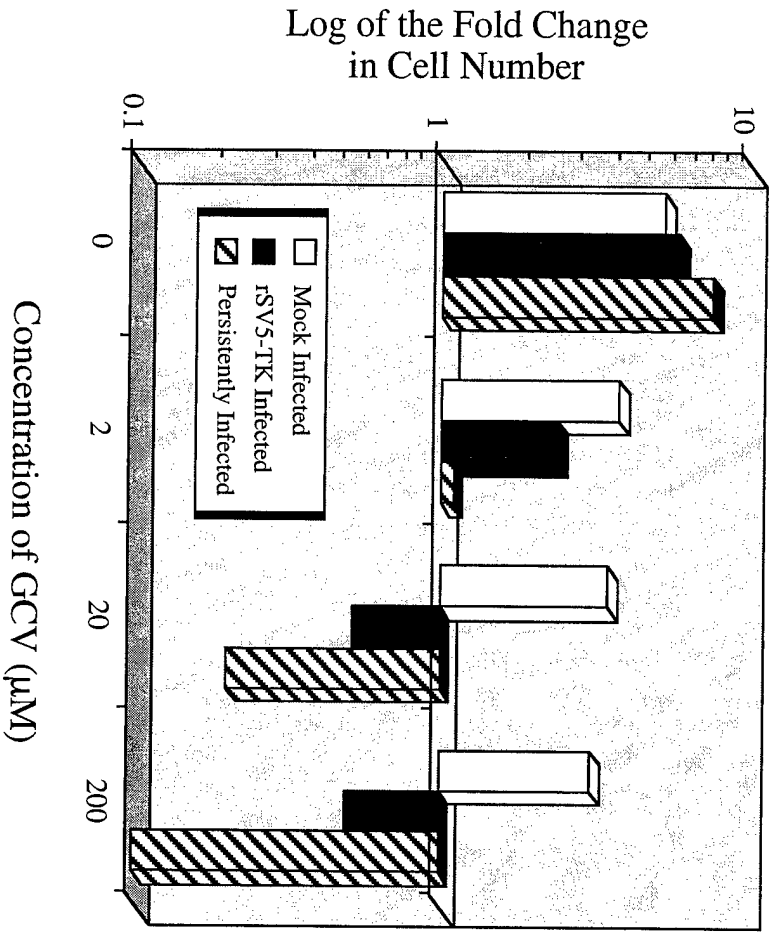


Figure 5

A.



B.

