Int. J. Cancer: **80,** 110–118 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

CELL-SPECIFIC TARGETING OF A THYMIDINE KINASE/GANCICLOVIR GENE THERAPY SYSTEM USING A RECOMBINANT SINDBIS VIRUS VECTOR

Yasushi IIJIMA, Kouichi OHNO, Hiroshi IKEDA, Keisuke SAWAI, Brandi LEVIN and Daniel MERUELO*

Department of Pathology and Kaplan Cancer Center, New York University Medical Center, New York, New York, USA

Transfer of the herpes simplex virus type 1 thymidine kinase (*HSV-TK*) gene into tumor cells using virus-based vectors in conjunction with ganciclovir (GCV) exposure provides a potential gene therapy strategy for the treatment of cancer. The possibility of using a novel targetable Sindbis virus expression vector containing the *HSV-TK* gene was examined. Baby hamster kidney (BHK) cells and several human tumor cells infected with a Sindbis virus containing the *HSV-TK* gene showed strong expression of HSV-TK protein. Cells transduced with the *HSV-TK* gene exhibited increased TK activity, ranging from 3- to 20-fold over an average baseline level. The human HeLa-CD4$^+$ cells infected with recombinant Sindbis virus containing the *HSV-TK* gene were sensitive to low concentrations of GCV (0.1–1 μg/ml) and the 50% growth inhibitory concentration (IC_{50}) was 0.6 μg/ml. We also demonstrated applications of cell type-specific Sindbis virus-mediated antigen-antibody targeting of the HSV-TK/GCV system *in vitro.* Sindbis virus containing the *HSV-TK* gene packaged in a helper virus displaying the IgG-binding domain of protein A on its envelope could infect various tumor cell lines in the presence of specific antibodies that recognize antigens on their surfaces. HSV-TK-transduced tumor cell lines exhibited sensitivity to GCV. Our data suggest the potential for targeted gene therapy of the HSV-TK/GCV system using a cell type-specific recombinant Sindbis virus vector-antibody system. *Int. J. Cancer* 80:110–118, 1999.

Various systems of somatic gene therapy for cancer have been developed, including transfer of toxic or ''suicide'' genes. Some suicide genes may express products that are directly toxic for the cell, such as diphtheria toxin or *Pseudomonas* exotoxin, both of which inhibit protein synthesis, while others act by transforming a prodrug into a toxic metabolite; *e.g.,* the bacterial cytosine deaminase converts 5-fluorocytosine into 5-fluorouracil (5-FU), an agent currently used in cancer therapy (Mullen *et al.,* 1992). The gene encoding herpes simplex virus type 1 thymidine kinase (*HSV-TK*) is the most widely investigated suicide gene. The expression of the *HSV-TK* gene in eukaryotic cells confers sensitivity to nucleoside analogs, such as acyclovir and ganciclovir (GCV), which possess strong activity against herpes virus infections. Unlike the mammalian thymidine kinase, HSV-TK efficiently phosphorylates these nucleotide analogs, which are subsequently converted into di- and triphosphate forms by cellular kinases. The GCV triphosphate forms then compete with normal nucleotides for DNA synthesis in mammalian cells, thus inhibiting cell growth and causing cell death (Matthews and Boehme, 1998).

Reports describing the use of the HSV-TK/GCV system for the treatment of cancer have used retroviral vectors for gene transfer (Moolten and Wells, 1990). Although retroviruses have the advantage of mediating stable gene transfer with a low potential for immunogenicity, this vector delivery system has some problems with respect to therapeutic use including difficulties in producing high titers of retrovirus, the fact that only actively dividing cells are capable of being infected and the possibility of insertional mutagenesis (Mulligan, 1993). The adenovirus vector system, while capable of delivering genes with high efficiency to a wide spectrum of non-dividing cells *in vivo* (Engelhart *et al.,* 1993*a*,*b*), unfortunately, has produced only transient expression with different gene products. Transient expression may result from a strong immune response of host cells against the adenovirus (Engelhart *et al.,* 1993*a*).

Sindbis virus, a member of the *Alphavirus* genus, has received considerable attention for use as a virus-based expression vector. Many characteristics of alphavirus vectors make them a desirable alternative to other virus-derived vector systems being developed, including rapid engineering of expression constructs, production of high titered stocks of infectious particles, infection of non-dividing cells and high levels of expression (Strauss and Strauss, 1994).

One of the most important problems of the HSV-TK/GCV system using virus-based expression vectors may be how to enhance the tumor specificity or viral infection. One strategy would be to employ a tumor-specific promoter, such as the α-fetoprotein promoter (Huber *et al.,* 1991). Another possible method uses procedures for selective delivery of the virus vector to the tumor. However, thus far such approaches have required *de novo* construction of each specific and targetable vector for each application and only very low levels of infectivity have been observed (Kasahara *et al.,* 1994).

Sindbis virus-based vectors suffer from the same problem for cell targeting use in that they naturally infect most cells. For mammalian cells, the high-affinity laminin receptor has been identified as one Sindbis virus receptor. The wide distribution and highly conserved nature of the laminin receptor may be, in part, responsible for the broad host range of the virus (Strauss and Strauss, 1994). Given the advantages discussed above for Sindbis virus vectors, we decided to examine whether it was possible to alter the tropism of the Sindbis virus vectors to permit gene delivery specifically to certain target cell types.

This requires both the ablation of endogenous viral tropism and the introduction of a novel tropism. In the mature Sindbis virus virion, a plus-stranded viral genome RNA is complexed with capsid protein C to form an icosahedral nucleocapsid that is surrounded by a lipid bilayer in which 2 integral membrane glycoproteins, E1 and E2, are embedded (Strauss and Strauss, 1994). Although E1 and E2 form a heterodimer that functions as a unit, the E2 domain appears to be particularly important for viral binding to cells. Monoclonal antibodies (MAbs) capable of neutralizing virus infectivity are usually E2 specific (Olmsted *et al.,* 1986), and mutations in *E2,* rather than *E1,* are more often associated with altered host range and virulence (Lustig *et al.,* 1988). A Sindbis virus mutant was identified which contained an insertion in *E2* and exhibited defective binding to mammalian cells (Dubuisson and Rice, 1993). We envisioned that this mutant would be useful for development of targetable Sindbis virus vectors.

We have demonstrated the successful construction Sindbis virus vectors that target specific cell types (Ohno *et al.,* 1997). We have designed and constructed Sindbis virus particles displaying the IgG-binding domain of protein A. We inserted the gene of synthetic protein A into the envelope gene (*E2*) of the Sindbis virus helper vector. The chimeric helper RNA was transfected and expressed in

Grant sponsor: National Institutes of Health; Grant numbers: CA68498 and CA22247.

*Correspondence to: Department of Pathology and Kaplan Cancer Center, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA. Fax: (212) 263-8211.
E-mail: merue01@mcrcr.med.nyu.edu

Received 15 May 1998; Revised 6 August 1998

baby hamster kidney (BHK) cells, and virus particles with IgG-binding activities were recovered. The protein A-envelope chimeric Sindbis virus vector has minimal infectivities against BHK and human cell lines. However, this chimeric virus, used in conjunction with MAbs, could infect human cells and transfer a test gene, β-galactosidase, with high efficiency.

The purpose of this report is to demonstrate that transfer of the *HSV-TK* gene to BHK cells and several human tumor cells also can be accomplished using a recombinant Sindbis virus vector. In addition, cellular killing occurs *in vitro* following GCV administration. Moreover, when we used a recombinant Sindbis virus vector displaying a protein A IgG-binding domain in the presence of MAbs, we demonstrated cell-specific expression of the *HSV-TK* gene and the tumor targeting of the HSV-TK/GCV gene therapy system.

MATERIAL AND METHODS

Cell lines

BHK cells were obtained from Invitrogen (San Diego, CA) and maintained in minimum essential medium alpha-modification (αMEM; JRH Bioscience, Lenexa, KS) supplemented with 5% fetal bovine serum (FBS; Gemini, Calabasas, CA). HeLa $CD4^+$ clone 1022 (NIH AIDS Research and Reference Reagent), which expresses CD4 on the cell surface, was grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS). A human Burkitt's lymphoma cell line Daudi (ATCC CCL213; Rockville, MD), (ATCC CRL1582) and a human small cell lung cancer (SCLC) cell line NCI-H526 (ATCC CRL5811) were grown as suspension cells in RPMI 1640 (JRH Bioscience) supplemented with 10% FCS.

MAbs

Anti-CD4 (mouse IgG_1), anti-HLA-DR (mouse IgG_{2a}) and anti-NCAM 16 (CD56) (mouse IgG_{2b}) were purchased from Becton Dickinson (San Jose, CA).

Plasmids

pSinRep5 (Bredenbeek *et al.,* 1993) (Invitrogen) is a Sindbis virus expression vector used to generate recombinant RNA molecules for transfection or infection of eukaryotic cell lines. It contains the Sindbis virus non-structural protein genes 1–4 (nsP1–4) for replicating RNA transcripts *in vitro,* the promoter for subgenomic transcription and a multiple cloning site. The multiple cloning site allows insertion of genes of interest behind the subgenomic promoter for expression in BHK cells or the cell line of choice. Three unique linearization restriction sites located 3′ to the multiple cloning site allow linearization of the plasmid prior to *in vitro* transcription. The recombinant RNA transcripts are synthesized using the SP6 promoter and transfected into BHK cells. A Sindbis virus-based expression vector SinRep/LacZ (Bredenbeek *et al.,* 1993) (Invitrogen; Fig. 1) contains the packaging signal, nsP1–4 for replicating the RNA transcript and the *lac*Z gene. A helper plasmid DH-BB (Bredenbeek *et al.,* 1993) (Invitrogen; Fig. 1), which contains the genes for the structural proteins (capsid, E3, E2, 6K and E1) required for packaging of the Sindbis viral genome, was used for construction of the recombinant envelope gene. A modified Sindbis virus helper plasmid has been constructed, DH-BB-ZZ, into which two IgG-binding domains of protein A were inserted in the *E2* region (Fig. 1). Native protein A has 5 homologous IgG-binding domains (E, D, A, B and C), and we have utilized the synthetic *Z* domain, which is based on the B domain of protein A (Ohno *et al.,* 1997).

Construction of the recombinant Sindbis virus expression vector SinRep/TK

The construction of the HSV-TK expressing Sindbis virus vector is shown in Figure 2. For the cloning of the *HSV-TK* gene, we utilized the unique *Mlu*I site at position 26 and the unique *Sma*I site at position 1106 of pHSV-106 (GIBCO-BRL) containing the *HSV-TK* gene. The first step in the cloning strategy was the introduction of a double-stranded oligonucleotide into a pBK-CMV (Stratagene, La Jolla, CA) plasmid at the *Bam*HI and *Eco*RI sites. The sequence encoded by the oligonucleotides is precisely that of the 5′ end of the open reading frame, upstream from the *Mul*I site and that of the 3′ end of the reading frame, downstream from the *Sma*I site, including these 2 restriction enzyme sites. The *Mul*I-*Sma*I fragment of the *HSV-TK* gene, cut from the pHSV-106, was inserted into the corresponding sites of this recombinant pBK-CMV plasmid, creating pBK-CMV-TK. Cleavage with *Spe*I and *Xba*I generated a fragment containing the whole open reading frame, which was ligated into *Xba*I-digested plasmid pSinRep5, resulting in the final Sindbis virus expression vector SinRep/TK. Clones containing inserts of proper size and orientation were sequenced to confirm that the correct reading frames were maintained (Fig. 1).

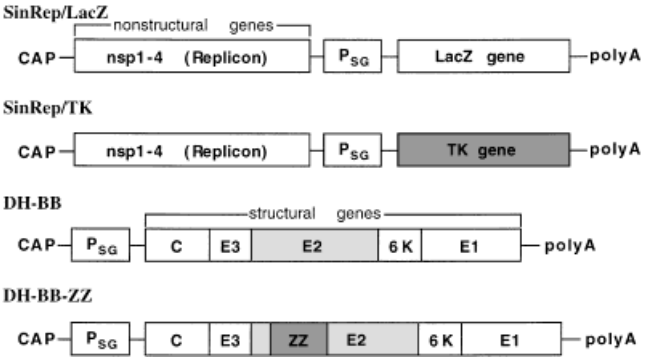

FIGURE 1 – Schematic representation of Sindbis virus expression vectors and helper vectors. SinRep/LacZ is a Sindbis virus-based expression vector that contains the packaging signal, non-structural genes for replicating the RNA transcript and *lac*Z gene. SinRep/TK is a Sindbis virus-based expression vector that contains *HSV-TK* gene at a cloning site (*Xba*I) in pSinRep5 plasmid. DH-BB is a parental helper plasmid that contains the genes for the structural proteins (capsid, E3, E2, 6K and E1) required for packaging of the Sindbis viral genome. DH-BB-ZZ is a modified Sindbis virus helper plasmid in which 2 IgG-binding domains of protein A (ZZ) were inserted in the *E2* region. P_{SG}, Sindbis virus subgenomic promoter; C, capsid; npP1–4, non-structural protein genes 1–4; ZZ, synthetic IgG-binding domain of protein A; poly A, polyadenylation signal.

In vitro *transcription and transfection for recombinant virus production*

Plasmids for *in vitro* transcription were prepared by use of Qiagen (Chatsworth, CA) columns. Two helper plasmids (DH-BB and DH-BB-ZZ) and 2 expression plasmids (SinRep/LacZ and SinRep/TK) were linearized by XhoI (DH-BB, DH-BB-ZZ and SinRep/LacZ) or NotI (SinRep/TK) restriction enzyme digestion and purified by phenol/chloroform extraction followed by ethanol precipitation. Transcription reactions were carried out by using InvitroScript Cap Kit (Invitrogen) to produce large quantities of capped mRNA transcript from the SP6 promoter. For cotransfections of helper and expression RNA into BHK cells, electroporations were performed. Electroporated cells were transferred to 10 ml of αMEM containing 5% FCS and incubated for 12 hr. Cells were then washed with phosphate-buffered saline (PBS) and incubated in 10 ml of Opti-MEM I medium (GIBCO-BRL) without FCS. After 24 hr, culture supernatants were harvested and aliquots were stored at −80°C. Four kinds of recombinant Sindbis virus particles—SinRep/LacZ/DH-BB (helper RNA from DH-BB cotransfected with expression RNA from SinRep/LacZ), SinRep/LacZ/DH-BB-ZZ (helper RNA from DH-BB-ZZ cotransfected with expression RNA from SinRep/LacZ), SinRep/TK/DH-BB (helper RNA from DH-BB cotransfected with expression RNA from SinRep/

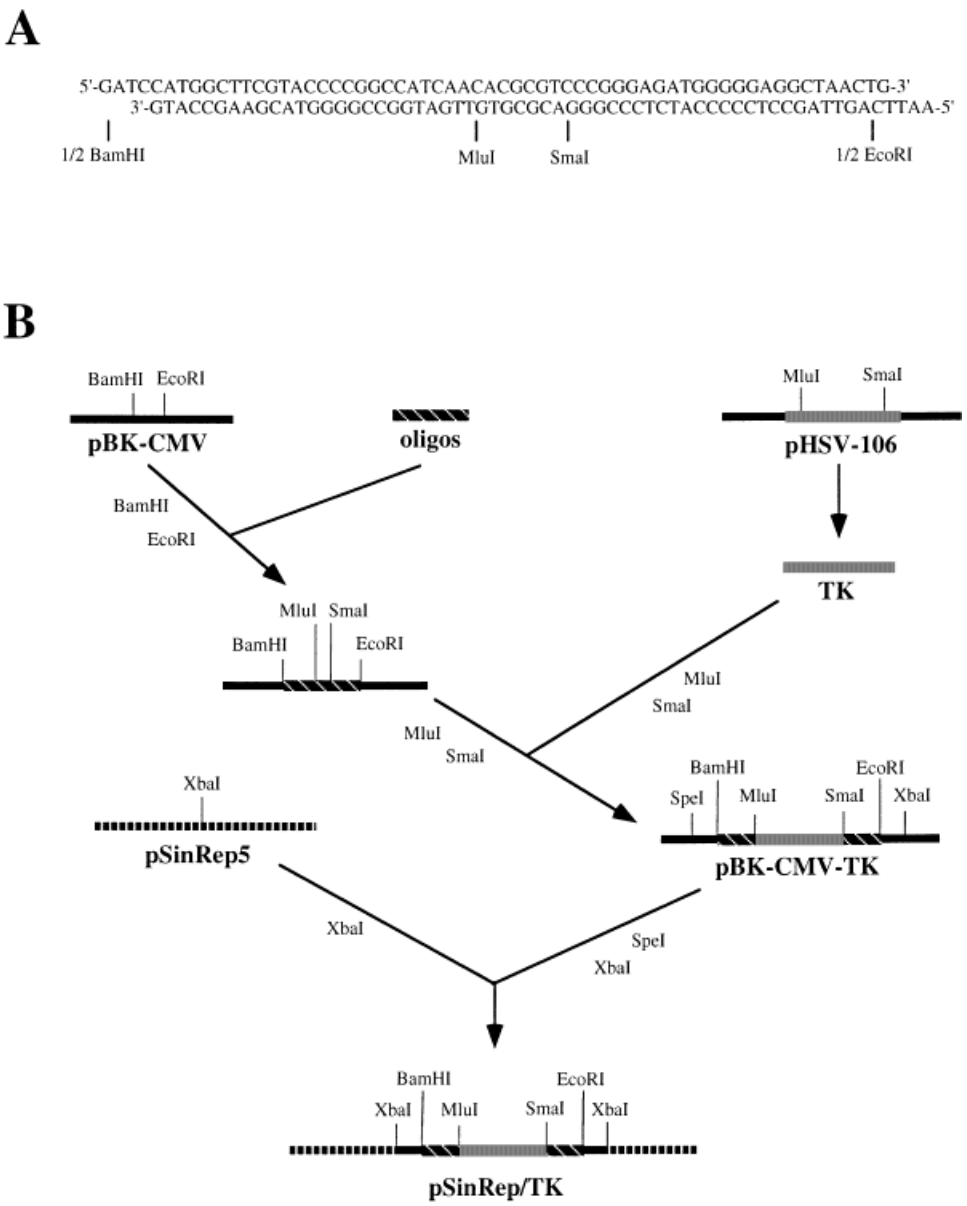

FIGURE 2 – Construction of the recombinant Sindbis virus expression vector SinRep/TK. *(a)* The 2 complementary oligonucleotides are designed in such a way as to complement the 3′ and 5′ ends of *HSV-TK* gene. *(b)* The annealed oligonucleotides were integrated first *via* the *Bam*HI and *Eco*RI sites into the plasmid pBK-CMV. The *Mlu*I-*SmaI* fragment of the *TK* was subsequently cloned into the corresponding sites of this recombinant plasmid. Cleavage with BamHI and EcoRI generated a fragment, containing the whole open reading frame of the *TK*, which was finally inserted in frame into the expression vector pSinRep5 to generate the plasmid SinRep/TK.

TK) and SinRep/TK/DH-BB-ZZ (helper RNA from DH-BB-ZZ cotransfected with expression RNA from SinRep/TK)—were produced.

Infection assays

Infectivity of recombinant chimeric viruses to BHK and human cell lines was determined by transfer of the Sindbis virus vector (SinRep/LacZ or SinRep/TK) that can transduce the bacterial β-galactosidase gene or *HSV-TK* gene. Viral supernatant dilutions (500 µl) were incubated with or without MAbs at room temperature for 1 hr, then added to adherent (2×10^5) and suspension (1×10^6) cells in 6-well plates. After 1 hr incubation at 37°C, cells were washed with PBS and incubated in growth medium at 37°C for 24 hr. Viral infection was evaluated by X-gal staining or immunohistochemistry using anti-HSV-TK antibody as described below and titers were estimated in LacZ colony forming units (cfu)/ml or TK cfu/ml.

X-gal staining

X-gal staining was performed as commercial protocol. Suspension cells were attached on slides using Cell Tak (Becton Dickinson). Briefly, cells were then fixed in PBS containing 0.5% glutaraldehyde for 15 min followed by washing with PBS 3 times. Then cells were stained with PBS containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mM $MgSO_4$ at 37°C for 2 hr.

Immunohistochemistry for HSV-TK

Suspension cells were attached on slides using Cell Tak. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1%

saponin and washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) 3 times. The cells were reacted with a polyclonal rabbit anti-HSV-TK antibody (courtesy of W.C. Summers, Yale University, New Haven, CT) at room temperature for 1 hr at 1:1,000 dilution. After washing with TBST for 1 hr, the cells were incubated with 1:500 diluted alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG antibodies (Southern Biotechnology Associate, Birmingham, AL) for 1 hr. Then the cells were washed with TBST for 1 hr and the bound primary antibody was detected with 1-Step NBT/BCIP kit (Pierce, Rockford, IL) as a substrate for AP.

Western blotting for HSV-TK

Adherent and suspension cells were infected with viral supernatants as described above, without or with MAbs; 24 hr after infection, cells were washed with PBS and resuspended into 250 mM Tris-HCl buffer, pH 8.0. The cells were then frozen in a dry ice/ethanol bath and thawed at 37°C 3 times. The cell debris was pelleted by centrifuging, and supernatants were used as cell lysates. The cell lysates were mixed with electrophoresis loading buffer (125 mM Tris-HCl, pH 6.8, 10 mM β-mercaptoethanol, 2% glycerol and 0.01% bromophenol blue). Proteins were solubilized by boiling for 5 min. Solubilized samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4.5% stacking gel and 10% separating gel. After electrophoresis, the gels were rinsed with transfer buffer containing 25 mM Tris, 192 mM glycine and 20% ethanol. Electroblotting of the proteins to nitrocellulose paper was performed at 250 mA for 2 hr at 4°C. Residual gels were stained with Coomassie blue (0.012% Coomassie blue, 40% methanol, 7% acetic acid) to evaluate equal blotting and transfer of proteins. After electroblotting, non-specific protein binding was blocked using 5% dry milk in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 24 hr. Polyclonal rabbit anti-HSV-TK antibody was diluted 1:2,000 in 5% dry milk in TBS and then incubated at room temperature for 1 hr followed by washing with TBST. Antibody-HSV-TK complexes were determined using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Pierce).

In vitro *TK activity assays*

The assay of thymidine kinase activity in cell lysates was performed by modification of the DEAE-cellulose paper method of Klemperer *et al.* (1967). Reaction mixtures contained 10 μM [methyl-^{3}H]thymidine (specific activity, 5.0 Ci/mmol; Amersham, Arlington Heights, IL). The cell lysate was added to the reaction mixture and incubated at 37°C for 5, 15, 30 and 60 min. For termination, reaction mixtures were heated at 95°C for 3 min. Denatured protein was removed by centrifugation for 5 min at 14,000*g* and supernatant was applied onto Whatman DE-81 paper. After washing the papers twice with 4 mM ammonium acetate and once with methanol, bound thymidine monophosphate was determined by scintillation counting. In these experiments, the enzymatic reaction was always linear with time for at least 30 min after addition of the enzyme. Thymidine kinase activity of cell lysates is defined as μmol of thymidine monophosphate/min/cell lysate from 10^{10} cells. The results are presented as the mean ± SD of triplicate samples.

In vitro *GCV Sensitivity of HeLa-CD4$^+$ cells infected with viral supernatant (SinRep/TK/DH-BB)*

HeLa-CD4$^+$ cells were cultured in 24-well plates at a density of 8×10^4 cells/well for 24 hr. Immediately before infection, the culture medium was removed from the wells and suspensions of viral supernatant derived from BHK cells transfected with SinRep/TK and DH-BB (SinRep/TK/DH-BB) or SinRep/LacZ and DH-BB (SinRep/LacZ/DH-BB) at a cfu of 6×10^3 were placed onto the cell monolayers. After incubation at 37°C for 1 hr, the medium containing virus was removed and the cells were washed with PBS 3 times. Then fresh medium containing various concentrations of GCV (0–10 μg/ml) was added to the cells. The cells were cultured at 37°C in 5% CO_2 for 2 hr; 18 hr before harvest, 0.5 μCi [methyl-^{3}H]thymidine (Amersham) was added to each well. Sensitivity of GCV was measured by [^{3}H]thymidine incorporation. Data are expressed as mean kcpm ± SD of triplicate wells.

In vitro *GCV sensitivity of the cells infected with viral supernatant (SinRep/TK/DH-BB-ZZ) with or without MAb*

HeLa-CD4$^+$, Daudi or H526 cells were cultured in 24-well plates (8×10^4 cells/well). Viral supernatant (200 μl for HeLa-CD4$^+$, 40 μl for Daudi and H526) derived from BHK cells transfected with SinRep/TK and DH-BB-ZZ (SinRep/TK/DH-BB-ZZ) was preincubated with or without MAb (0.5 μg/ml of anti-CD4 or anti-HLA-DR, 20 μg/ml of anti-NCAM) and added to each well. The protocol followed was as described above for GCV sensitivity of HeLa-CD4$^+$ cells.

RESULTS

Infection with recombinant viruses

Infectivities of recombinant viruses against hamster and human cells were determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce the bacterial β-galactosidase gene. As shown in Table I, viruses derived from DH-BB (SinRep/LacZ/DH-BB) helper had a very high infectious titer (8.2×10^7 LacZ cfu/ml) against BHK cells, whereas viruses produced by DH-BB-ZZ (SinRep/LacZ/DH-BB-ZZ) showed very low infectivity (2.7×10^3 LacZ cfu/ml), supporting previous observations which suggest that protein A insertion into E2 blocks virus binding to host cells. SinRep/LacZ-DH-BB wild-type virus showed high infectious titer against human HeLa-CD4$^+$ cells (4.0×10^5 LacZ cfu/ml) and human SCLC cells, H526 (1.2×10^6 LacZ cfu/ml), but showed very low infectivities against Burkitt's lymphoma cells, Daudi. SinRep/LacZ/DH-BB-ZZ virus also showed minimal titer (10^2 LacZ cfu/ml) against HeLa-CD4$^+$ cells and H526 cells and very low infectivities against Daudi cells. However, virions that were preincubated with MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi, anti-NCAM for H526), and the protein A-envelope chimeric virus (SinRep/LacZ/DH-BB-ZZ) could infect the cells (Table I, Fig. 3). When the viral supernatant was preincubated with 0.5 μg/ml MAb, the infectious titer was approximately 10^5–10^6 LacZ cfu/ml.

Next, we determined whether recombinant virus particles, capable of transducing the *HSV-TK* gene, could infect hamster and various human cells. Recombinant Sindbis virus particles packaged by DH-BB (SinRep/TK/DH-BB) have high infectious titer (4.0×10^7 TK cfu/ml) against BHK cells, whereas virus particles packaged by DH-BB-ZZ (SinRep/TK/DD-BB-ZZ) have very low infectivity (1.2×10^2 TK cfu/ml) (Table II). SinRep/TK/DH-BB virus showed high infectivities against HeLa-CD4$^+$ and H526 cells, but could not infect Daudi cells. On the other hand, virus particles packaged by DH-BB-ZZ (SinRep/TK/DH-BB-ZZ) could efficiently infect these cells only when virions were preincubated

TABLE I – INFECTION BY WILD-TYPE AND RECOMBINANT SINDBIS VIRUS PARTICLES (SinRep/LacZ)[1]

Cell line	Titer (LacZ cfu/ml)		
	DH-BB	DH-BB-ZZ MAb(−)	DH-BB-ZZ MAb(+)
BHK	8.2×10^7	2.7×10^3	N.D.[2]
HeLa-CD4$^+$	4.0×10^5	<500	3.4×10^5
Daudi	<10	<10	6.2×10^5
H526	1.2×10^6	<500	1.0×10^6

[1]Viral supernatants derived from BHK cells transfected with SinRep/LacZ and DH-BB or SinRep/LacZ and DH-BB-ZZ were preincubated with or without 0.5 μg/ml of MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi and anti-NCAM for H526) and used to infect BHK, HeLa-CD4$^+$, Daudi and H526 cells in serial dilutions to determine virus titer. See Material and Methods for details.–[2]N.D., not determined.

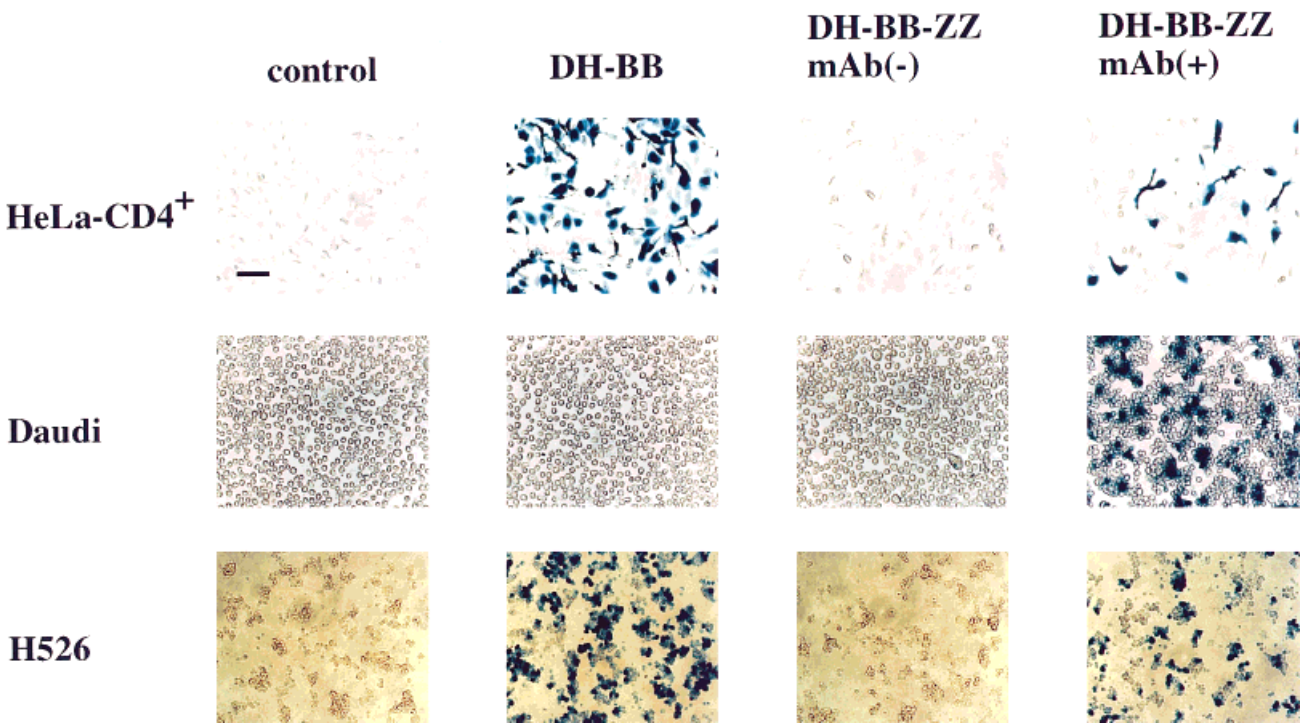

FIGURE 3 – Infectivities of recombinant Sindbis virus particles which are transducing the bacterial *lac*Z gene on HeLa-CD4$^+$, Daudi and H526 cells. Viral supernatants, SinRep/LacZ/DH-BB (500 µl) or SinRep/LacZ/DH-BB-ZZ (500 µl) preincubated with or without MAb (0.5 µg/ml) were added to adherent (2×10^5) and suspension (1×10^6) cells in 6-well plates. After 1 hr incubation at 37°C, cells were washed with PBS and incubated in growth medium for 24 hr. Viral infection was evaluated by X-gal staining. Control samples are uninfected cells. Scale bar: 50 µm.

TABLE II – INFECTION BY RECOMBINANT SINDBIS VIRUS PARTICLES (SinRep/TK)[1]

Cell line	Titer (TK cfu/ml)		
	DH-BB	DH-BB-ZZ MAb(−)	DH-BB-ZZ MAb(+)
BHK	4.0×10^7	1.2×10^2	N.D.[2]
HeLa-CD4$^+$	2.4×10^5	<500	2.0×10^5
Daudi	<10	<10	5.3×10^5
H526	8.0×10^5	<500	7.5×10^5

[1]Viral supernatants derived from BHK cells transfected with SinRep/TK and DH-BB or SinRep/TK and DH-BB-ZZ were preincubated with or without 0.5 µg/ml of MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi and anti-NCAM for H526) and used to infect BHK, HeLa-CD4$^+$, Daudi and H526 cells in serial dilutions to determine virus titer. See Material and Methods for details.–[2]N.D., not determined.

with MAbs (Table II, Fig. 4). Infectious titers of the recombinant virus (SinRep/TK/DH-BB-ZZ) against HeLa-CD4$^+$, Daudi and H526 cells were approximately 10^5–10^6 TK cfu/ml in the presence of MAb. Again, minimal infectivities (10^2 TK cfu/ml) were observed for HeLa-CD4$^+$ and H526 cells in the absence of antibody. The infectious titers were determined for replicate experiments by measuring the expression of the *lacZ* gene or the *HSV-TK* gene (see Material and Methods for details).

Western blot analysis

Western blotting was performed to compare the level of HSV-TK protein expression, after infection with recombinant Sindbis virus particles, produced in BHK cells transfected with SinRep/TK RNA transcribed *in vitro*. HSV-TK proteins were detected by using polyclonal anti-HSV-TK antibodies. All protein samples were prepared from the same number of cells for each cell line. A Coomassie blue stain confirmed equal loading and protein transfer among all cell lines. SinRep/TK/DH-BB virus infected BHK cells show high expression of HSV-TK by Western blot assay between 12 and 48 hr after infection; expression decreases 72 hr after infection (data not shown). Next, we investigated expression of the *HSV-TK* gene in HeLa-CD4$^+$, Daudi and H526 cells infected with recombinant Sindbis virus particles (Fig. 5). Cell lysates from uninfected cells (lane 1), cells infected with SinRep/LacZ/DH-BB (lane 2), cells infected with SinRep/TK/DH-BB (lane 3) and cells infected with SinRep/TK/DH-BB-ZZ preincubated with (lane 5) or without (lane 4) 0.5 µg/ml of MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi and anti-NCAM for H526) at 24 hr postinfection were analyzed for expression of the HSV-TK protein with a polyclonal rabbit anti-HSV-TK antibody. The proteins were expressed in HeLa-CD4$^+$ and H526 cells infected with SinRep/TK/DH-BB and SinRep/TK/DH-BB-ZZ which were preincubated with MAbs. HSV-TK protein was also expressed in Daudi cells infected with SinRep/TK/DH-BB-ZZ preincubated with MAb but could not be detected in cell lysates from these cells infected with SinRep/TK/DH-BB, in agreement with results observed in cell infection experiments (Fig. 4). All uninfected cells and cells infected with control Sindbis virus particles (SinRep/LacZ/DH-BB) did not express proteins detected by anti-HSV-TK antibody.

TK activity

In vitro enzyme activity assays using radiolabeled substrates were carried out to determine the efficacy of recombinant Sindbis virus vector to express protein in target cells. Activities of TK were measured by phosphorylation of [methyl-^{3}H]thymidine and SinRep/TK/DH-BB infected BHK cells show high activities of HSV-TK between 12 and 48 hr after infection with activity decrease 72 hr after infection (data not shown). Figure 6 shows the results of TK activity assays of cell lysates from HeLa-CD4$^+$, Daudi and H526 cells infected with recombinant Sindbis virus particles. In HeLa-

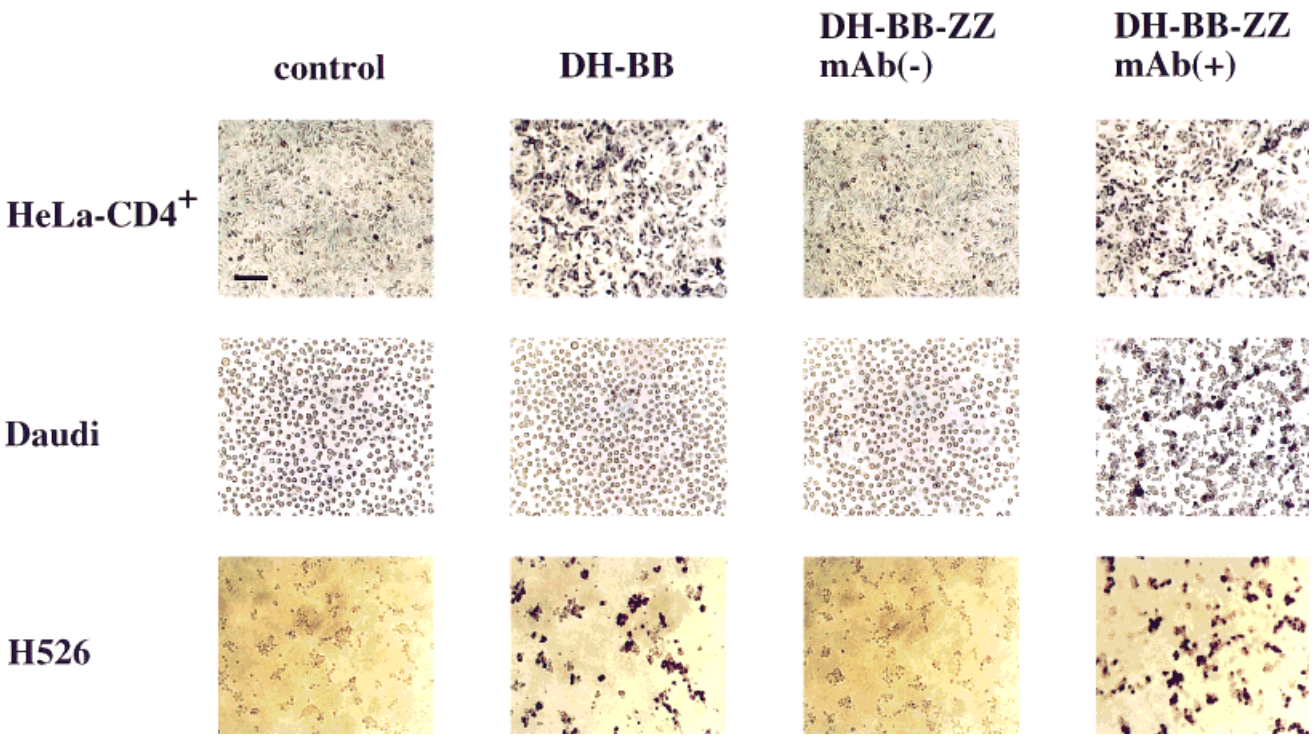

FIGURE 4 – Infectivities of recombinant Sindbis virus particles which are transducing *HSV-TK* gene on HeLa-CD4$^+$, Daudi and H526 cells. Viral supernatants, SinRep/TK/DH-BB (500 µl) or SinRep/TK/DH-BB-ZZ (500 µl) preincubated with or without MAb (0.5 µg/ml) were added to adherent (2×10^5) and suspension (1×10^6) cells in 6-well plates. After 1 hr incubation at 37°C, cells were washed with PBS and incubated in growth medium for 24 hr. Viral infection was evaluated by immunohistochemistry using anti-HSV-TK antibody. Control samples are uninfected cells. Scale bar: 50 µm.

CD4$^+$ and H526 cells, basal expression was detected, averaging 0.06 µmol/min 10^{-10} cells for uninfected and SinRep/LacZ/DH-BB infected cells, and 0.16 µmol/min 10^{-10} cells for cells infected with SinRep/TK/DH-BB-ZZ preincubated without MAb. Cells infected with SinRep/TK/DH-BB or SinRep/TK/DH-BB-ZZ preincubated with MAb (anti-CD4 for HeLa-CD4$^+$ and anti-NCAM for H526) exhibited increased TK activity, ranging from 3- to 20-fold over an average baseline level. In Daudi cells, basal expression levels were low, ranging from 0.05 to 0.06 µmol/min 10^{-10} cells. Levels of SinRep/TK/DH-BB infected cells also could not be increased. However, SinRep/TK/DH-BB-ZZ virus particles preincubated with anti-HLA-DR antibodies in Daudi cells generated 0.28 µmol/min 10^{-10} cells, an increase of more than 5-fold over that of controls. These levels of TK activities correlated with the amount of protein expression determined by Western blot analysis.

In vitro *GCV sensitivity of HeLa-CD4$^+$ cells infected with Sindbis virus particles (SinRep/TK/DH-BB)*

To test the ability of SinRep/TK/DH-BB virus particles to confer sensitivity to GCV, HeLa-CD4$^+$ cells were infected at a virus titer of 6×10^3 cfu with SinRep/TK/DH-BB or control virus particles (SinRep/LacZ/DH-BB) and exposed to various doses of GCV (0–10 µg/ml) for 2 hr. Sensitivity of GCV was measured by [^{3}H]thymidine incorporation. The cells infected with virus transducing the *HSV-TK* gene (SinRep/TK/DH-BB) were sensitive to low concentrations of GCV (0.1–1 µg/ml) and cytotoxicity increased with GCV concentration. There was 50% relative proliferation in HSV-TK transduced cells at 0.6 µg/ml GCV (IC_{50}) compared to untransduced cells, which showed only 26% inhibition at 10 µg/ml GCV. SinRep/LacZ/DH-BB infected cells also showed no increase in sensitivity to GCV (Fig. 7).

In vitro *GCV sensitivity of cells infected with Sindbis virus particles (SinRep/TK/DH-BB-ZZ) preincubated with or without MAbs*

To determine the sensitivity of HeLa-CD4$^+$, Daudi and H526 cells infected with SinRep/TK/DH-BB-ZZ targeting virus particles, viral supernatants (200 µl for HeLa-CD4$^+$, 40 µl for Daudi and H526) were preincubated with or without MAbs (0.5 µg/ml of anti-CD4 or anti-HLA-DR, 20 µg/ml of anti-NCAM) and added to the cells. After 1 hr incubation, the cells were exposed to various doses of GCV (0–10 µg/ml) for 2 hr and inhibition of cell growth was measured by [^{3}H]thymidine incorporation. As shown in Figure 8, cells of all 3 lines infected with SinRep/TK/DH-BB-ZZ and treated with MAb were rendered sensitive to doses of GCV that were lower than cells infected with virus particles only. Fifty percent of HSV-TK transduced cells were inhibited in proliferation at 10 µg/ml (HeLa-CD4$^+$), 7.5 µg/ml (Daudi) and 1.5 µg/ml (H526) (IC_{50}) compared to cells infected with virus particles only which showed 14% (Hela-CD4$^+$), 22% (Daudi) and 28% (H526) inhibition at the same GCV concentration.

DISCUSSION

We describe here the transfer of a therapeutic gene construct and the successful *in vitro* sensitization to drug of several human malignant tumor cell lines using recombinant Sindbis virus vectors. Moreover, we demonstrate the possibility of targeting gene therapy for human tumor cells using a recombinant Sindbis virus-based helper vector displaying protein A-envelope chimeric proteins on the virus surface complexed with cell type-specific MAbs. Our results suggest that recombinant Sindbis virus transfer of a suicide gene offers an alternative to recombinant retrovirus or adenovirus gene therapy systems.

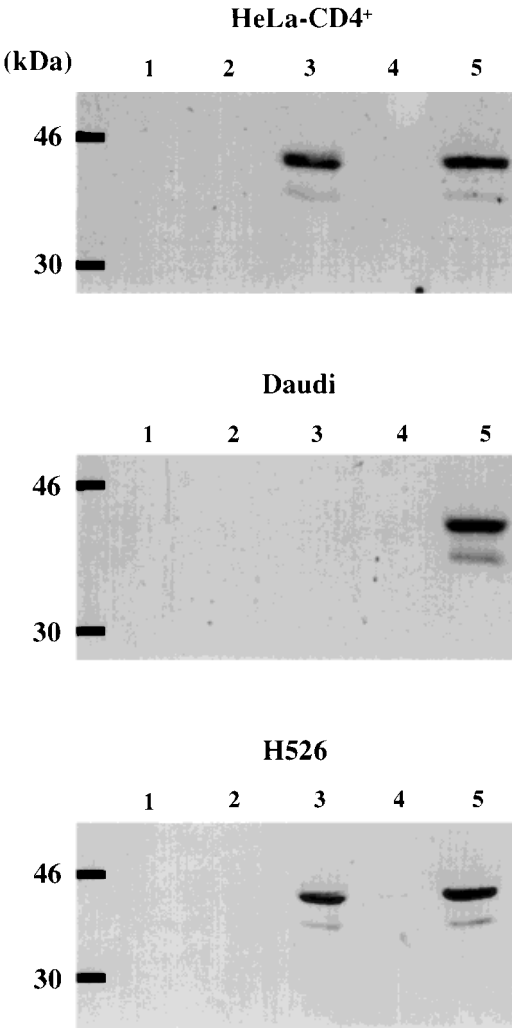

FIGURE 5 – Western blot analysis of HSV-TK protein expression in HeLa-CD4$^+$, Daudi and H526 cells infected with recombinant Sindbis virus particles. Cell lysates from uninfected cells (lane 1), cells infected with SinRep/LacZ/DH-BB (lane 2), cells infected with SinRep/TK/DH-BB (lane 3) and cells infected with SinRep/TK/DH-BB-ZZ preincubated with (lane 5) or without (lane 4) 0.5 µg/ml of MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi and anti-NCAM for H526) 24 hr postinfection were analyzed for expression of the HSV-TK protein with a polyclonal rabbit anti-HSV-TK antibody.

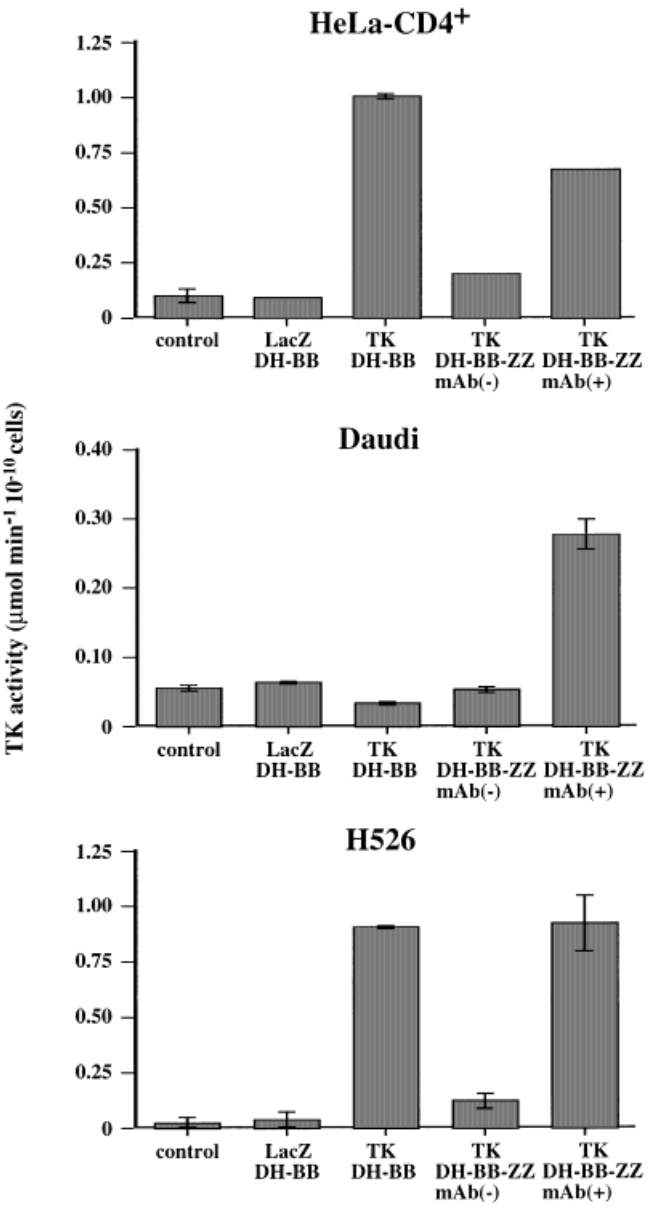

FIGURE 6 – TK activity of cell lysates from HeLa-CD4$^+$, Daudi and H526 cells infected with recombinant Sindbis virus particles. At 24 hr postinfection, the TK activity of cell lysates from uninfected cells, cells infected with SinRep/LacZ/DH-BB, cells infected with SinRep/TK/DH-BB and cells infected with SinRep/TK/DH-BB-ZZ preincubated with or without 0.5 µg/ml of MAb (anti-CD4 for HeLa-CD4$^+$, anti-HLA-DR for Daudi and anti-NCAM for H526) was assayed by metabolic formation of phosphorylated [methyl-^{3}H]thymidine. Results are given in mean mol/min 10^{-10} cells ± SD of triplicates.

Application of drug sensitization with the HSV-TK/GCV system in cancer was pioneered by Moolten (1986) and Moolten and Wells (1990), and has been tested in several clinical trials. In this study, we constructed a novel recombinant Sindbis virus vector containing the *HSV-TK* gene. Sindbis virus is being developed as a vector for expression of heterologous genes (Bredenbeek *et al.,* 1993) and has many advantages for expression vector systems. In Sindbis virus systems, because helper RNA (DH-BB, DH-BB-ZZ) does not contain a packaging signal, it will not form a defective interfering particle or be packaged with recombinant RNA. Furthermore, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule, without a DNA intermediate (Strauss and Strauss, 1994). This is in contrast with retroviruses, which must enter the nucleus and integrate into the host genome for initiation of vector activity. Thus, retrovirus vectors have applications for long-term expression of foreign genes, while alphavirus vectors are useful primarily for transient high level expression. Furthermore, although adenovirus vectors can express high levels of foreign genes, these systems are far more complex than alphaviruses and express many highly antigenic virus-specific gene products including structural proteins (Rosenfeld *et al.,* 1991). In contrast, current alphavirus vectors express only the 4 viral replicase proteins (nsP1–4) required for RNA amplification in the transduced cells.

BHK cells and several human tumor cells infected with the recombinant virus particles containing the *HSV-TK* gene (SinRep/TK/DH-BB) showed strong expression of HSV-TK protein (Fig. 5, Table II) and high activities of thymidine kinase (Fig. 6). The *in vitro* sensitivity of Hela-CD4$^+$ cells infected with recombinant Sindbis virus (SinRep/TK/DH-BB) to GCV reported here is similar to that in reports using the retrovirus or adenovirus vectors. The IC_{50} of GCV was 0.6 µg/ml. This dose is certainly acceptable with regard to considering eventual *in vivo* efficacy.

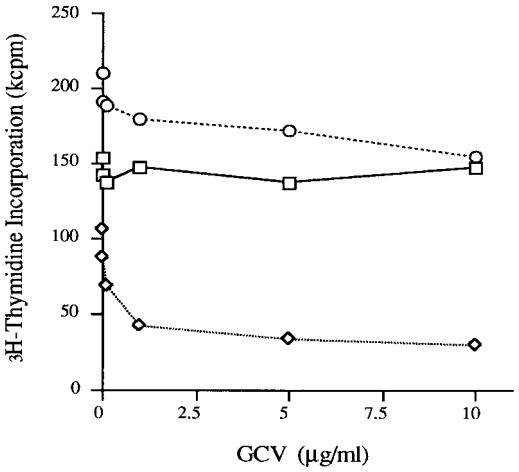

FIGURE 7 – Sensitivity of HeLa-CD4$^+$ cells infected with recombinant Sindbis virus particles (measured by [^{3}H]thymidine incorporation) to various concentrations of GCV (0–10 μg/ml). Comparison of GCV dose-response between uninfected cells (○), cells infected with control virus particles (SinRep/LacZ/DH-BB, □) and cells infected with SinRep/TK/DH-BB (◇) is shown. Results are given in mean kcpm ± SD of triplicates.

A major drawback to the use of virus-based vectors for a gene delivery system is the fact that these vectors lack target-cell specificity. To overcome this limitation, the specific expression of transfected foreign genes in tumor cells may be induced using the promoter element of genes usually transcribed only by tumor cells. In retrovirus and adenovirus expression vectors, several kinds of tumor-specific promoters ligated with *HSV-TK* genes have been examined to enhance the specificity (Huber *et al.,* 1991; Smythe *et al.,* 1994). Another strategy uses procedures for selective delivery of the virus vector to targeted tumor cells. Several retrovirus and adenovirus-based cell targeting vectors have been developed to date (Douglas *et al.,* 1996; Kasahara *et al.,* 1994). However, with these approaches, each specific and targetable vector must be constructed *de novo* and only very low levels of infectivity have been observed.

We have described the retargeting of a Sindbis virus vector by a novel utilization of the protein A-antibody complex (Ohno *et al.,* 1997). We constructed the recombinant Sindbis virus helper vector displaying protein A (PA)-envelope chimeric proteins on the viral surface. Recombinant Sindbis virus particles derived from this helper vector (DH-BB-ZZ) RNA have minimal infectivities against BHK and human cell lines. However, when used in conjunction with MAbs which react with cell surface antigens, the PA-envelope chimeric virus is able to transfer the *lac*Z gene into human cell lines with high efficiency. The new tropism of the recombinant virus is dependent upon the antigen-antibody interaction since the PA-envelope virus could not infect targeted cells without MAb and corresponding antigen on the cell surface. Taken together, the PA-chimeric envelope, derived from DH-BB-ZZ helper RNA, can redirect Sindbis virus infection with high efficiency by antigen-antibody interaction. As shown in Table I and Figure 4, PA-envelope chimeric virus has as good efficiency of infectivity as the Sindbis virus derived from DH-BB helper RNA, and at one of the suspesion cells, Daudi, it has much better infectivity than wild-type virus. In this report, we also demonstrate successful application of this cell type-specific recombinant Sindbis virus vector to the HSV-TK/GCV susceptibility system *in vitro.* Recombinant SinRep/TK particles were packaged with the PA-envelope chimeric helper virus (DH-BB-ZZ). Several human tumor cells that have specific antigen on their cell surface could be infected with the recombinant Sindbis virus displaying PA-envelope chimeric proteins (SinRep/TK/DH-BB-ZZ) preincubated with MAbs (Table II, Fig. 4) as well as infected with wild-type virus. Cells transduced with the *HSV-TK* gene have high activity of thymidine kinase

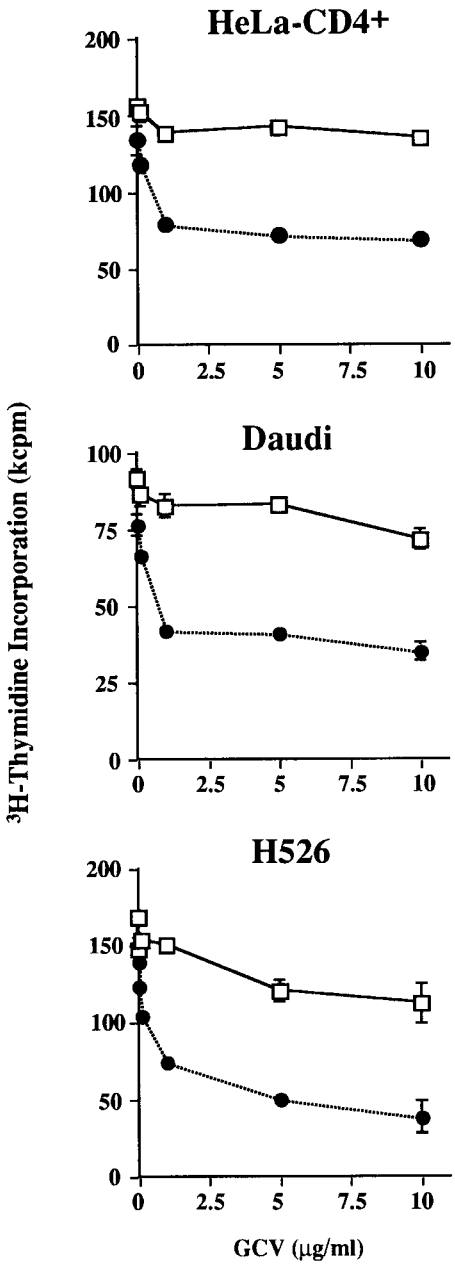

FIGURE 8 – Sensitivity of HeLa-CD4$^+$, Daudi and H526 cells infected with SinRep/TK/DH-BB-ZZ recombinant Sindbis virus particles preincubated with or without MAb to various concentrations of GCV. Comparison of GCV dose-response between cells infected with virus preincubated without MAb (□) and cells infected with virus preincubated with MAb (●) is shown. Viral supernatants (200 μl for HeLa-CD4$^+$, 40 μl for Daudi and H526) derived from BHK cells transfected with SinRep/TK and DH-BB-ZZ (SinRep/TK/DH-BB-ZZ) *in vitro* transcribed RNA were preincubated with or without MAbs (0.5 μg/ml of anti-CD4$^+$ or anti-HLA-DR, 20 μg/ml of anti-NCAM) and added to cells. After incubation at 37°C for 1 hr, the cells were washed with PBS 3 times. Fresh medium containing various concentrations of GCV (0–10 μg/ml) was then added to the cells. The cells were cultured at 37°C in 5% CO_2 for 2 hr; 18 hr before harvest, 0.5 μCi [methyl-^{3}H]thymidine was added to each cell sample. Sensitivity of GCV was measured by [^{3}H]thymidine incorporation. Results are given in mean kcpm ± SD of triplicates.

corresponding to levels of HSV-TK proteins observed by Western blot analysis (Figs. 5, 6). Moreover, cells infected with cell-specific virus particles preincubated with MAbs showed about 50% relative proliferation when challenged with GCV compared to cells in-

fected with virus particles only (Fig. 8). These data suggest the possibility of cell-specific HSV-TK/GCV gene therapy using recombinant Sindbis virus vectors complexed with targeting MAbs.

From the safe point of view, ideally targeting MAbs should be expressed on only targetable cancer cells. For example, viruses derived from DH-BB-ZZ helper RNA complexed with anti-Trop-2 Ab (Lipinski *et al.*, 1981) will infect choriocarcinoma and viruses with K:1-6F Ab (Andreasen and Olsson, 1987) will infect erythroleukemia cells but they may not infect normal cells, because the anti-Trop-2 Ab defines an antigen that is expressed on trophoblasts and choriocarcinoma and K:1-6F Ab mainly combines with erythroleukemia cells. Moreover, glioblastoma and squamous cell carcinoma are well known to have about 100 times as many epidermal growth factor (EGF) receptors as normal cells, so viruses derived from DH-BB-ZZ RNA with EGF receptor Ab can infect glioblastoma and squamous cell carcinoma efficiently. Although Sindbis virus infection of vertebrate cells is well known to result in cell death by apoptosis (Levine *et al.*, 1993), this cytotoxicity may be suitable for gene therapy of cancer cells. As shown in Figures 7 and 8, moreover, HSV-TK/GCV gene therapy using recombinant Sindbis virus vectors can suppress the cancer cell proliferation better than infection of Sindbis virus itself.

In conclusion, we have shown that the *HSV-TK* gene can be efficiently transferred to human tumor cells using a recombinant Sindbis virus vector resulting in cytotoxicity upon GCV exposure. Our results also show the potential for targeted gene therapy using the cell type-specific recombinant Sindbis virus vector-mediated antigen-antibody interaction.

ACKNOWLEDGEMENTS

The authors thank Dr. C. Pampeno for her frequent and helpful advice.

REFERENCES

ANDREASEN, R.B. and OLSSON, L., Identification and characterization of an antigen specific for normal erythroid precursor cells and its application in diagnosis of erythroleukemia. *Leukemia Res.,* **11,** 1045–1053 (1987).

BREDENBEEK, P.J., FROLOV, I., RICE, C.M. and SCHLESINGER, S., Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J. Virol.,* **67,** 6439–6446 (1993).

DOUGLAS, J.T., ROGERS, B.E., ROSENFELD, M.E., MICHAEL, S.I. and CURIEL, M.F.D.T., Targeted gene delivery by tropism-modified adenoviral vectors. *Nature (Biotechnol.),* **14,** 1574–1578 (1996).

DUBUISSON, J. and RICE, C.M., Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J. Virol.,* **67,** 3363–3374 (1993).

ENGELHART, J.F., SIMON, R.H., YANG, Y., ZEPEDA, M., WEBER-PENDLETON, S., DORANZ, B., GROSSMAN, M. and WILSON, J.M., Adenovirus-mediated transfer of the *CFTR* gene to lung of nonhuman primates: biological efficacy study. *Hum Gene Ther.,* **4,** 759–769 (1993*a*).

ENGELHART, J.F., YANG, Y., STRATFORD-PERRICAUDET, L.D., ALLEN, E.D., KOZARSKY, K., PERICAUDET, M., YANKASKAS, J.R. and WILSON, J.M., Direct gene transfer of human CFRT into human bronchial epithelia of xenografts with E1-deleted adenoviruses. *Nature (Genet.),* **4,** 27–34 (1993*b*).

HUBER, B.E., RICHARDS, C.A. and KRENITSKY, T.A., Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc. nat. Acad. Sci. (Wash.),* **88,** 8039–8043 (1991).

KASAHARA, N., DOZY, A.M. and KAN, Y.W., Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science,* **266,** 1373–1376 (1994).

KLEMPERER, H.G., HAYNES, G.R., SHEDDEN, W.I.H. and WATSON, D.H., A virus-specific thymidine kinase in BHK21 cells infected with herpes simplex virus. *Virology,* **31,** 120–128 (1967).

LEVINE, B., HUANG, Q., ISAACS, J.T., REED, J.C., GRIFFIN, D.E. and HARDWICK, J.M., Conversion of lytic to persistent alphavirus infection by the *bcl*-2 cellular oncogene. *Nature (Lond.),* **361,** 739–742 (1993).

LIPINSKI, M., PARKS, D.R., ROUSE, R.V. and HERZENBERG, L.A., Human trophoblast cell-surface antigens defined by monoclonal antibodies. *Proc. nat. Acad. Sci. (Wash.),* **78,** 5147–5150 (1981).

LUSTIG, S., JACKSON, A.C., HAHN, C.S., GRIFFIN, D.E., STRAUSS, E.G. and STRAUSS, J.H., Molecular basis of Sindbis virus neurovirulence in mice. *J. Virol.,* **62,** 2329–2336 (1988).

MATTHEWS, T. and BOEHME, R., Antiviral activity and mechanism of action of ganciclovir. *Rev. infect. Dis.,* **10,** S490–S494 (1998).

MOOLTEN, F.L., Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.,* **46,** 5276–5281 (1986).

MOOLTEN, F.L. and WELLS, J.M., Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vector. *J. nat. Cancer Inst.,* **82,** 297–300 (1990).

MULLEN, C.A., KILSTRUP, M. and BLAESE, R.M., Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. nat. Acad. Sci. (Wash.),* **89,** 33–37 (1992).

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

OHNO, K., SAWAI, K., IIJIMA, Y., LEVIN, B. and MERUELO, D., Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domain of protein A. *Nature (Biotechnol.),* **15,** 763–767 (1997).

OLMSTED, R.A., MEYER, W.J. and JOHNSTON, R.E., Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. *Virology,* **148,** 245–254 (1986).

ROSENFELD, M.A. and 13 OTHERS, Adenovirus-mediated transfer of a recombinant anti-antitrypsin gene to the lung epithelium *in vivo. Science,* **252,** 431–434 (1991).

SMYTHE, W.R., HWANG, H.C., AMIN, K.M., ECK, S.L., DAVIDSON, B.L., WILSON, J.M., KAISER, L.R. and ALBELDA, S.M., Use of recombinant adenovirus to transfer the herpes simplex virus thymidine kinase (*HSVtk*) gene to thoracic neoplasms: an effective *in vitro* drug sensitization system. *Cancer Res.,* **54,** 2055–2059 (1994).

STRAUSS, J.H. and STRAUSS, E.G., The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.,* **58,** 491–562 (1994).