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Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A

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Sindbis virus can infect a broad range of insect and vertebrate cell types due to the widespread distribution of the cellular receptor for the virus. The development of Sindbis virus vectors that target specific cell types could have important implications for the design of gene therapy strategies. To achieve this goal we have designed and constructed Sindbis virus particles displaying the IgG-binding domain of protein A. The protein A-envelope chimeric Sindbis virus vector has minimal infectivities against baby hamster kidney and human cell lines. When used in conjunction with monoclonal antibodies that react with cell-surface antigens, however, the protein A-envelope chimeric virus was able to infect human cell lines with high efficiency. Infection rates were 90% or higher for human lymphoblastoid cells. A variety of cells could be targeted by changing the monoclonal antibody without generating a new recombinant virus.

Keywords: gene therapy vector, alphavirus, targeting, protein A

Sindbis virus, a member of the *Alphavirus* genus, has received considerable attention for use as virus-based expression vectors. Many properties 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 nondividing cells, and high levels of expression[1-4]. However, a major drawback to the use of Sindbis virus vectors is that these vectors lack target-cell specificity. For mammalian cells, at least one Sindbis virus receptor is the high-affinity laminin receptor, whose wide distribution and highly conserved nature may be in part responsible for the broad host range of the virus[1,5]. It is desirable to alter the tropism of the Sindbis virus vectors to permit gene delivery specifically to certain target cell types. This will require both the ablation of endogenous viral tropism and the introduction of novel tropism. In the mature Sindbis virion, a plus-stranded viral genome RNA is complexed with capsid protein C to form icosahedral nucleocapsid that is surrounded by lipid bilayer in which two integral membrane glycoproteins, E1 and E2, are embedded[1]. Although E1 and E2 form a heterodimer that functions as a unit, the E2 domain appears to be particularly important for binding to cells. Monoclonal antibodies (Mabs) capable of neutralizing virus infectivity are usually E2-specific[6,7], and mutations in E2, rather than E1, are more often associated with altered host range and virulence[8,9]. A Sindbis virus mutant was identified with an insertion in E2 resulting in defective binding to mammalian cells[10].

Several attempts to alter the host range of viruses have been reported to date. For retrovirus-based vectors, direct modifications of the envelope protein of murine leukemia virus (MLV) redirects viral tropism. A recombinant virus containing a fragment encoding a single Fv antibody chain at the N-terminal region of the MLV *env* gene has been shown to recognize the corresponding epitopes[11] and infect human cells[12,13]. Kasahara et al.[14] have made a chimeric ecotropic virus containing an erythropoietin-envelope fusion protein. This chimeric virus has been shown to infect human cells bearing the erythropoietin receptor. In these systems, however, each targetable vector much be constructed de novo, and only very low levels of infectivity were observed[13,14]. Furthermore, virions constructed to directly bind to specific targets in human cells are intrinsically unsafe, as wild-type recombinants could possibly go on to cause cancer in patients treated with such vectors.

We describe the construction of a recombinant Sindbis virus vector displaying protein A (PA)-envelope chimeric proteins to redirect the viral tropism. PA, derived from *Staphylococcus aureus*, has a strong affinity for the Fc region of various mammalian IgGs[15]. In contrast to the targeted retroviral vectors described above, the PA-envelope chimeric virus vector, once successfully generated, needs no further modification to target distinct cells. The targeting is achieved simply by changing the complementary Mab (Fig. 1A). More importantly, we demonstrate that this chimeric virus used in conjunction with Mabs can infect human cells and transfer a marker gene, bacterial β-galactosidase, with high efficiency.

Results

Construction of PA-envelope Sindbis virus helper plasmid. To modify the Sindbis virus envelope protein, the DH-BB helper plasmid (Fig. 1B), constructed by deletion of the region between BspMII and BamHI sites of the full-length Sindbis virus cDNA clone[16], was utilized. When RNA from DH-BB is cotransfected with recombinant RNA from the Sindbis virus expression vector, the structural proteins expressed in trans, from the DH-BB RNA transcript allows packaging of the recombinant RNA into virions. As DH-BB does not contain a packaging signal, it will not form a defective interfering particle or be packaged with recombinant RNA. Two modified Sindbis virus helper plasmids were constructed: DH-BB-Bst into which a BstEII cloning site was inserted, and DH-BB-ZZ into which two IgG-binding domains of PA were inserted in the E2 region (Fig. 1B). Native PA has five homologous IgG-binding domains (E, D, A, B, and C). We used the synthetic Z domain, which is based on the B domain of protein PA (ref. 17). The insertion position, between codons 71 and 74 amino acids in E2, was

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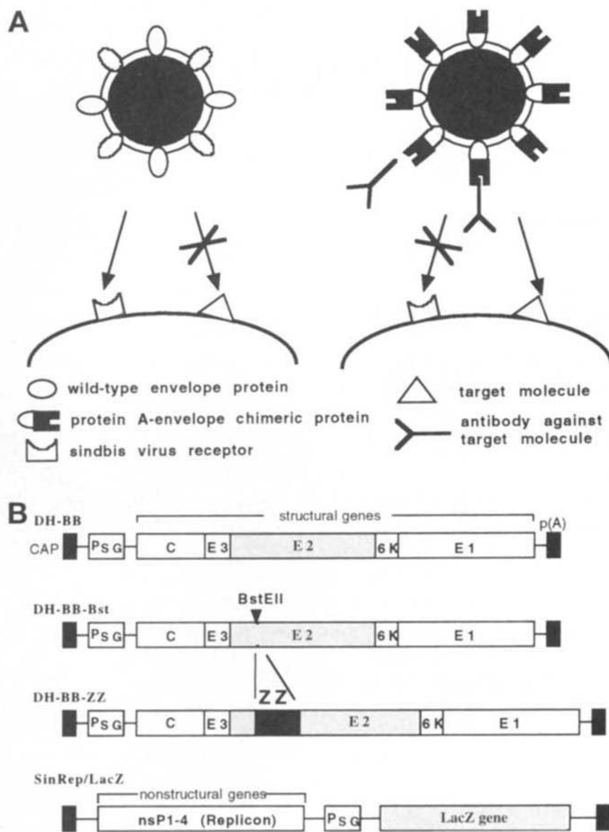

Figure 1. (A) Strategy for retargeting a Sindbis virus vector. A wild-type Sindbis virus (left) binds to mammalian cells via its conserved surface receptor. A recombinant Sindbis virus displaying the IgG-binding domain of protein A (right) permits binding to a novel target molecule on the cell surface when used with a corresponding monoclonal antibody. (B) Schematic representation of recombinant helper constructs and a SinRep/LacZ expression vector. 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-Bst contains a BstEII cloning site between amino acids 71 and 74 of the E2 glycoprotein. DHBB-ZZ contains the synthetic IgG-binding domain (ZZ) of PA at the BstEII site in DH-BB-Bst. SinRep/LacZ contains the packaging signal, nonstructural protein genes for replicating the RNA transcript and lacZ gene. P_{SG}: Sindbis viral subgenomic promoter; C: capsid; nsP1-4: nonstructural protein genes 1–4; ZZ: synthetic IgG-binding domain of protein A; p(A): polyadenylation signal.

chosen because mutations in this region allow normal particle assembly and release and block virus entry at the level of attachment[10].

Expression and incorporation of chimeric envelopes into virions. After linearization of helper and SinRep/LacZ plasmids, in vitro transcription was performed and the quality of RNA was checked on agarose gels (data not shown). To examine the expression of the recombinant envelope, recombinant helper RNA was cotransfected with RNA from SinRep/LacZ plasmid into baby hamster kidney (BHK) cells by electroporation. The transfection efficiency was usually nearly 100% (data not shown). Lysates from transfected cells were first analyzed for expression of structural protein by using anti-Sindbis virus immune ascitic fluid. DH-BB-Bst helper RNA expressed a 50 to 55 kDa envelope (E1 and E2) and a 33 kDa capsid protein, which is the same protein profile as the parental virus produced by DH-BB (Fig. 2A). A band of 60 kDa corresponding to the E2 precursor PE2 was also detected. In the protein profile expressed by DH-BB-ZZ RNA, a major band between 65 to 70 kDa, which is the estimated molecular weight of PA-E2 and PA-PE2 chimeric protein, was observed as well as the 33 kDa capsid protein. These results suggest that the mutants were correctly expressed and processed.

Virions produced by DH-BB and DH-BB-Bst RNA contain capsid and envelope (E1 and E2) proteins indicating that the mutation in DH-BB-Bst does not affect virus assembly (Fig. 2B). The PA-E2 chimeric protein was also incorporated into virions and exhibited IgG-binding activity, which is not detected in that of DH-BB and DH-BB-Bst (Fig. 2B and C). These results demonstrate that DH-BB-ZZ produces recombinant Sindbis pseudovirions displaying the IgG-binding domain in its envelope. The E1 protein, which was expressed in transfected cells (Fig. 2A), could not be detected in the virions produced by DH-BB-ZZ RNA. This may be because the antisera being used using reacts poorly—or not at all—with the E1 protein. The gels used may not adequately separate E1 and E2. While we have assumed that the band running around 50k PA is E1, this band may represent a small amount of E2 in which the PA insert has been deleted. E1 migrates slower than E2 and the presumptive E1 observed is migrating faster than expected. It is also possible that the antisera used does recognize E1. In this case, the effect could be due to loss of a sugar group in E1 through lack of chaperoning by E2, which could be one reason for misfolding of E1 and reduced incorporation into virions.

Infection with viruses carrying mutant envelopes. Recombinant virus infectivity of hamster and human cells was determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce bacterial β-galactosidase gene. Viruses derived from DH-BB and DH-BB-Bst helper showed very high infectious titer (10^8 LacZ CFU/ml) against BHK cells whereas viruses produced by DH-BB-ZZ showed very low infectivity (10^3 LacZ cfu/ml) suggesting that the protein A insertion into E2 blocked virus binding to host cells sup-

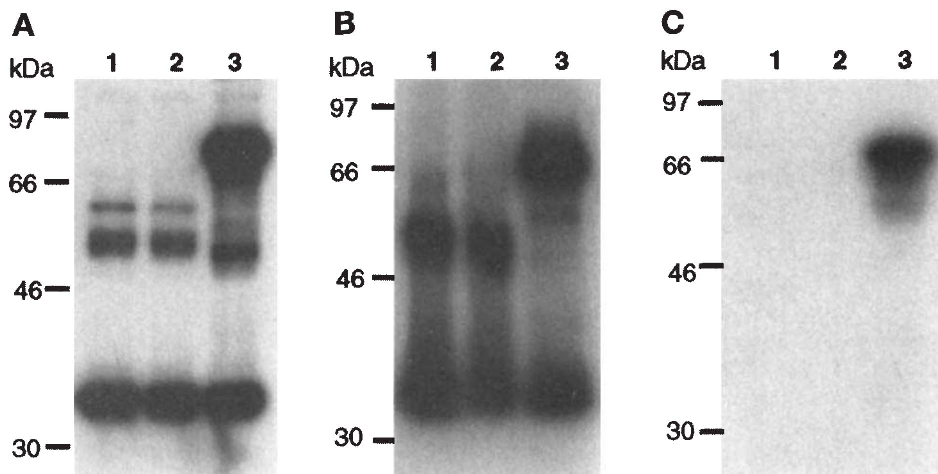

Figure 2. Western blot analysis of Sindbis viral structural protein components (A). Cell lysates from BHK cells transfected with helper RNA and (B and C) pellets of viral particles produced from these cells. Viral proteins were stained with diluted anti-Sindbis virus mouse immune ascitic fluid to detect all structural components (A and B) or with HRP-conjugated goat anti-mouse IgG to detect protein A-envelope chimeric protein (C). Lane 1: DH-BB; lane 2: DH-BB-Bst; lane 3: DH-BB-ZZ.

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porting previous observations[10] (Table 1). The PA-envelope virus also showed minimal titer against human HeLa-CD4⁻ cells (10^2 LacZ cfu/ml). When virions were preincubated with anti-CD4 Mab, however, the PA-envelope chimeric virus could infect HeLa-CD4⁺ cells in an antibody dose-dependent manner (Table 1). When the viral supernatant was preincubated with 0.5 μg/ml Mab, the infectious titer was approximately 2.3×10^5 LacZ cfu/ml. The enhancement of infectivities by Mab was not observed with DH-BB- and DH-BB-Bst derived viruses. As determined by enzyme-linked immunosorbant assay (ELISA) using anti-Sindbis antibodies, the ratio of DH-BB to DH-BB-ZZ was slightly greater than to 1:1. When the ratio of virus particles, as determined ELISA assays using anti-Sindbis antibodies, is 1:1, the efficacies of infection of DH-BB and DH-BB-ZZ are comparable on HeLa-CD4⁺ cells (DH-BB is actually slightly more infectious), DH-BB-ZZ is more than 50% as infectious as DH-BB.

The PA-envelope chimeric virus with anti-CD4 Mab could not infect HeLa cells, which do not express CD4, indicating that the infection is dependent on both an antibody and a corresponding antigen (Fig. 3). These data demonstrate that the PA-E2 chimeric envelope derived from DH-BB-ZZ helper RNA can redirect Sindbis virus infection via a new receptor/antigen in the presence of recognizing antibody.

Viruses with PA-envelope could infect the adherent epidermoid carcinoma cell line A431 and glioblastoma cell line U87MG, which overexpress epidermal growth factor receptors (EGFR), only when virions were preincubated with anti-EGFR Mab (Fig. 4). Infectious titers of the recombinant virus with Mab (0.5 μg/ml) against A431 and U87MG cells were approximately 10^4 LacZ cfu/ml. Minimal infectivities (10^2 LacZ cfu/ml) were seen on these cells when infected without Mab. In contrast to adherent cells, the wild-type virus particles derived from DH-BB helper RNA have very low infectivities against the suspension cell lines and HL-60 (Fig. 5). The PA-envelope virus preincubated with corresponding Mabs (anti-HLA-DR for Daudi and anti-CD33 for HL-60), however, infects more than 90% of these cells. Infection by the PA-envelope virus of these cells was not observed in the absence of Mab.

The efficiency of infection, which varies between 20% to 90%, may depend on the receptor being targeted. Thus the efficiencies of infection for HeLa-CD4⁺, A431, and U87MG cells is somewhat lower than the efficiencies observed with Daudi or HL-60 cells.

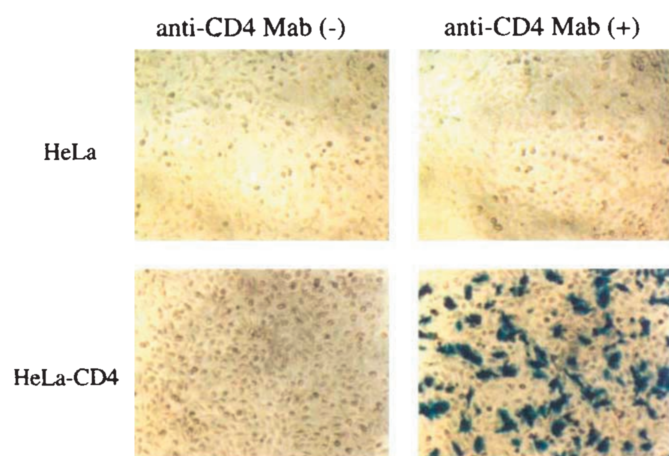

Figure 3. Infection of HeLa and HeLa-CD4⁺ cells with recombinant Sindbis virus derived from DH-BB-ZZ helper RNA. Viral supernatants (200 μl) were preincubated without or with anti-CD4 Mab (0.5 μg/ml) and added to cells (2×10^5) in 6-well plates. Viral infection was evaluated by X-Gal staining.

Discussion

In these experiments, the incorporation of E1 glycoprotein into virions could not be detected (Fig. 2C). While we believe we may be detecting E1 in transfected cells (Fig. 2A), the antisera we used may react poorly—or not at all—with the E1 protein. Further, the gels we used may not have separated E1 and E2, and the band running around 50k may represent a small amount of E2 in which the PA insert has been deleted. Further experiments will be conducted to determine whether E1 is indeed present in the virus or not, and whether it is modified in some manner (e.g., by loss of a sugar group). The interaction between E1 and E2 of alphaviruses is considered very important and E1 is deemed to play an important role in membrane fusion. A different result may provide new insights into the mechanism of Sindbis virus assembly.

The PA-envelope chimeric Sindbis virus vector showed minimal infectivities against BHK and other human cell lines. However, when used in conjunction with Mabs that react with cell surface antigens, the PA-envelope chimeric virus was able to transfer the LacZ gene into human cell lines with high efficiency. The new tropism of the recombinant virus depends on antigen-antibody interaction because the PA-envelope virus could not infect targeted cells without Mab and corresponding antigen on cell surface (Fig. 3). Taken together, the PA-E2 chimeric envelope derived from DH-BB-ZZ helper RNA can effec-

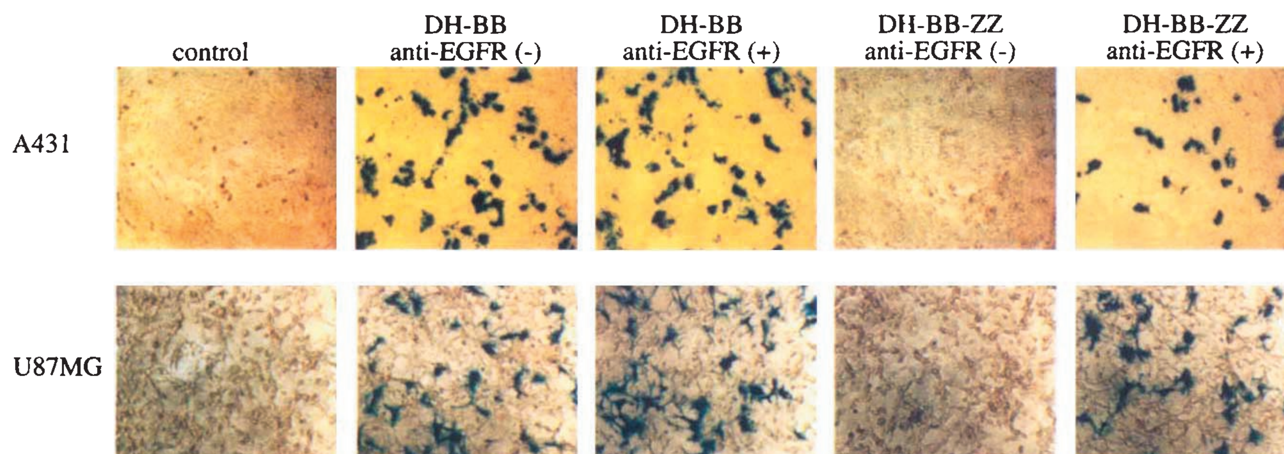

Figure 4. Antibody-dependent infectivities of recombinant Sindbis virus particles on A431 and U87MG cells. Viral supernatants (20 μl for DH-BB, 500 μl for DH-BB-ZZ) were preincubated without or with anti-EGFR Mab (0.5 μg/ml) and added to cells (2×10^5) in 6-well plates. Viral infection was evaluated by X-gal staining.

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Table 1. Infection by wild-type and recombinant Sindbis virus particles*.

Cell line	Preincubation*	Titer (LacZ cfu/ml) DH-BB	DH-BB-Bst	DH-BB-ZZ
BHK	(-)	1.23×10^8	1.33×10^8	3.23×10^3
HeLa-CD4⁺	anti-CD4 Mab (ng)			
	0	1.1×106	1.0×106	<500
	20	1.1×106	N.D.	3.8×104
	100	1.2×106	N.D.	1.7×105
	500	1.0×106	1.1×106	2.3×105

*Viral supernatants derived from BHK cells transfected with DH-BB, DH-BB-Bst, and DH-BB-ZZ were preincubated without or with Mab (0 to 0.5 μg/ml) and subjected to infection of BHK and HeLa-CD4⁺ cells in serial dilutions to determine virus titer. As determined by ELISA using anti-Sindbis antibodies, the ratio of DH-BB to DH-BB-ZZ was slightly greater than to 1:1. N.D. not determined.

tively redirect Sindbis virus by antigen-antibody interaction. Using this vector we have been able to successfully infect every cell type we have tested. Generally speaking, greater than 20% of the target cell population is infected, but results of more than 90% infection are not uncommon. For some cells that are not infected by the unmodified Sindbis virus, like Daudi and HL-60, the unmodified virus infects 0% to 1.1% of the cells, whereas the PA-enveloped virus infects more than 90% of the cells. For other cell types, like HeLa-CD4⁺ cells, which can be infected by unmodified and modified Sindbis virus, the efficiency of DH-BB-ZZ is greater than 50% of DH-BB.

Several retrovirus and adenovirus-based cell-targeting vectors have been developed[11,13,14,18]. Compared with these retroviral and adenoviral retargeting vectors, in this approach it is not necessary to construct each targetable vector de novo. It is unlikely that the incorporation of different targeting elements in the envelope of the virus can always be achieved with equal success and without reducing the virus titers that could be obtained. Since the PA portion of the chimeric envelope binds to the Fc domain of the antibody[15], it allows flexibility with regards to the targeting elements, as any of a variety of Mabs can be selected. In addition, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule, without a DNA intermediate[1]. This is in contrast to retrovirus vectors, which must enter the nucleus and integrate into the host genome for initiation of vector activity. Thus, retrovirus-derived vectors have applications for long-term expression of foreign proteins, while alphavirus vectors are useful primarily for transient high-level expression. Furthermore, although adenovirus vectors can express high levels of foreign proteins, these systems are far more complex than alphaviruses and express many highly antigenic virus-specific gene products including structural proteins[19]. In contrast, current alphavirus vectors express only the four viral replicase proteins (nonstructural proteins nsP1 through nsP4) required for RNA amplification in the transduced cells.

There are several problems that we have to consider in this study. First, Sindbis virus infection of vertebrate cells usually results in cell death by apoptosis[20], with the notable exception of neuronal cells in which a persistent infection may be established[21]. Although this cytotoxicity may be suitable for gene therapy for cancer, long-term or inducible expression vectors would have broader application. Several possibilities exist for controlling this potential disadvantage, and they need to be explored. Second, the recombinant Sindbis virus vector developed in this study still has low infectivities even in the absence of antibody. There might be other sites than E2 or E1 that are involved in receptor binding[1]. Furthermore, different receptors have been identified on chicken embryo fibroblast[22] and mouse neuronal cells[23], suggesting that the virus can use more than one receptor. For safety reasons, it is necessary to develop recombinant Sindbis virus vectors that do not infect mammalian cells when not used with Mabs. Finally, the system we have described requires clean antibodies in sufficient amounts; however, we do not feel this is an obstacle to development of this system. Antibodies used for clinical imaging are produced abundantly and in very clean form, and have been shown to possess a high degree of clinical specificity. There is no reason why the system we have described cannot take advantage of the availability of these reagents.

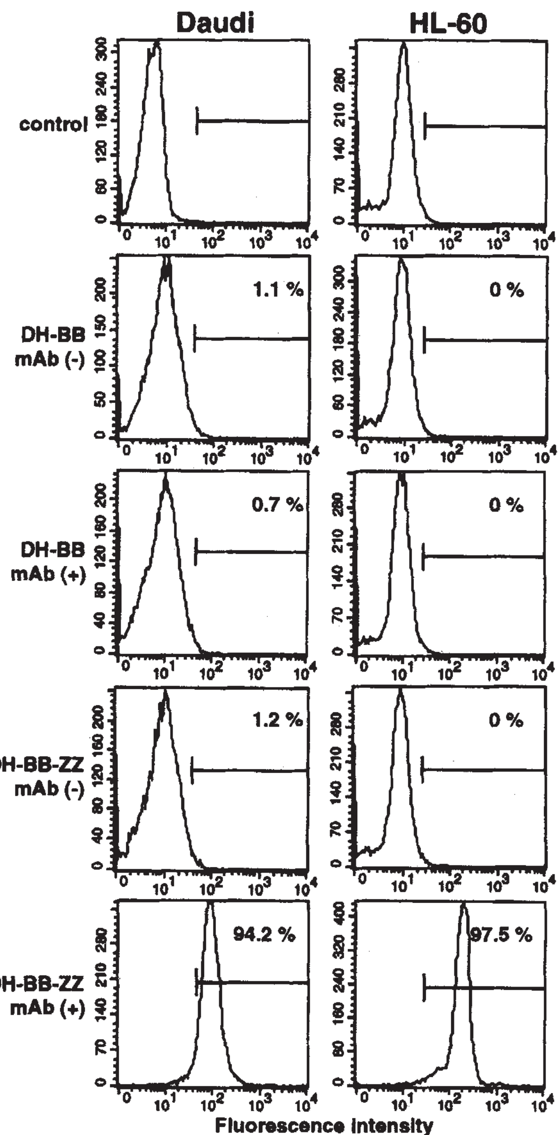

Figure 5. Antibody-dependent infectivities of recombinant Sindbis virus particles on Daudi and HL-60 suspension cell lines. Viral supernatants (500 μl) derived from DH-BB and DH-BB-ZZ transfected BHK cells were preincubated without or with 0.5 μg/ml of Mabs (anti-HLA-DR for Daudi and anti-CD33 for HL-60) and added to cells (1×10^6) in 6-well plates. Control shows uninfected cells. Viral infection was evaluated by FACS-gal analysis. Positive percent of infected cells are shown in each panel.

We have retargeted a Sindbis virus vector by using the PA-antibody interaction. A similar approach may be used with other viral vectors, such as retrovirus and adenovirus vectors by inserting the synthetic IgG binding domain (ZZ) of PA. The virus-based vectors displaying PA-envelope could be very useful and have a broad applicability for gene transfer and for the gene-therapy field.

Experimental protocol

Cell lines. BHK cells were obtained from Invitrogen (San Diego, CA) and maintained in minimum essential medium alpha-modification (αMEM, JRH Biosciences, Lenexa, KS) supplemented with 5% fetal bovine serum (FBS, Gemini Bio-Products, Calabasas, CA). A human epidermoid carcinoma cell line

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A431 (ATCC CRL1555), a human epitheloid carcinoma cell line HeLa (ATCC CRL2), and a human glioblastoma cell line U87MG (ATCC HTB14) were grown as monolayers in Dulbecco s modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% FBS. HeLa $CD4^+$ Clone 1022 (NIH AIDS Research and Reference Reagent Program), which expresses CD4 on its surface and a human Burkitt's lymphoma cell line Daudi (ATCC CCL213) (ATCC CRL1582) were maintained in RPMI 1640 (JRH Bioscience) supplemented with 10% FBS. HL-60, promyelocytic leukemia cell line (ATCC CCL240), was maintained in RPMI 1640 supplemented with 20% FBS.

Monoclonal antibodies (Mabs). A murine Mab of IgG2a type against the human EGFR was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-HLA-DR (mouse IgG2a), anti-CD4 (mouse IgG1), and anti-CD33 (mouse IgG1) were purchased from Becton Dickinson (San Jose, CA).

Plasmids. A helper plasmid DH-BB (Invitrogen, Fig. 1B)[16] 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 Sindbis virus-based expression vector SinRep/LacZ (Invitrogen, Fig. 1B)[16] contains the packaging signal, nonstructural protein genes 1-4 (nsP1-4) for replicating the RNA transcript and the lacZ gene. Plasmid pEZZ 18, which contains two synthetic Z domains based on the B domain of PA (ref. 24), was purchased from Pharmacia Biotech (Uppsala, Sweden). The phagemid pALTER-1 vector (Promega, Madison, WI) was used to introduce the BstEII site in the E2 region of DH-BB plasmid by oligo-directed site-specific mutagenesis.

Construction of the recombinant Sindbis virus structural gene. Altered Sites in vitro Mutagenesis System (Promega) was used to introduce a specific restriction site into the E2 region of Sindbis virus structural gene. First, a BssHII site was introduced between XbaI and HindIII sites of the pALTER-1 vector by using two compatible oligonucleotides, 5'-CTAGAGCGCGCAAA-3' and 5'-AGCTTTTGCGCGCT-3'. A fragment between SacI and BssHII of the DH-BB plasmid containing the E2 region of structural gene was cloned into the pALTER-1 vector. A single-stranded template of the recombinant pALTER-1 vector was prepared by infection of helper phage M13KO7. A mutagenic oligonucleotide (5'-ATGTCGCTTAAGCAGGTAACCACCGTTAAA-GAAGGC-3'), which introduces a BstEII cloning site between codons 71 and 74 amino acids in E2 polypeptides, and an ampicillin repair oligonucleotide (5'-GTTGCCATTGCTGCAGGCATCGTGGTG-3') were annealed to the single-stranded template, followed by synthesis of the mutant strand with T4 DNA polymerase. After transformation into *Escherichia coli*, mutants were selected in the presence of ampicillin and screened by direct sequencing of the plasmid DNA. The SacI-BssHII region of original DH-BB plasmid was replaced with the mutated fragment and the DH-BB-Bst plasmid was obtained (Fig. 1B). A region of protein A (ZZ) containing two synthetic IgG-binding domains was amplified by polymerase chain reaction (PCR) using pEZZ 18 as a template. Primers used for PCR amplification are ZZ-5 (5'-CACGATGAG-GTAACCGACAACAAATTCAAC-3') and ZZ-3 (5'-GGTCGAGGTTACCG-GATCCCCGGGTACCGA-3') both encoding unique BstEII sites. The resulting PCR products were digested with BstEII and inserted into predigested DH-BB-Bst plasmid at the BstEII site. Clones containing inserts of proper size and orientation were sequenced to confirm that the correct reading frames were maintained and the DH-BB-ZZ plasmid was obtained (Fig. 1B).

In vitro transcription and transfection for recombinant virus production. Plasmids for in vitro transcription were prepared by use of Qiagen (Chatsworth, CA) columns. All helper plasmids (DH-BB, DH-BB-Bst, and DH-BB-ZZ) and SinRep/LacZ plasmid were linearized by XhoI 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 SinRep/LacZ RNA into BHK cells, electroporations were performed as described before[2]. Electroporated cells were transferred to 10 ml of αMEM containing 5% FCS and incubated for 12 h. Cells were then washed with PBS and incubated in 10 ml of Opti-MEM I medium (Gibco-BRL) without FCS. After 24 h, culture supernatants were harvested and aliquots were stored at -80°C.

Immunoblot assay. Cells were lysed in 20 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 10% glycerol 24 h after transfection. Cell extracts were then sonicated and mixed with electrophoresis loading buffer (125 mM Tris-HCl, pH 6.8, 10 mM β-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue). Virus samples were pelleted by ultracentrifugation of the supernatants (10 ml) in an SW41 Beckmann Rotor (35,000 rpm, 2 h, 4°C) and resuspended in electrophoresis loading buffer. Cell extracts and viral samples were subjected to immunoblot analysis as described before[13] by using anti-Sindbis virus mouse immune ascitic fluid (ATCC VR-1248) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibodies (Pierce, Rockford, IL). Blot was visualized be enhanced chemiluminesence (DuPont NEN, Boston, MA).

Infection assays. Infectivity of recombinant chimeric viruses to BHK and human cell lines was determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce the bacterial β-galactosidase gene[16]. Viral supernatant dilutions were incubated with or without Mabs at room temperature for 1 h, then added to adherent (2×10^5) and suspension (1×10^6) cells in six-well plates. After 1 hour incubation at room temperature, cells were washed with PBS and incubated in growth medium for 24 h. Viral infection was evaluated by X-gal Staining and FACS-Gal as described below and titers were estimated in LacZ cfu per milliliter. CFU is defined in terms of cells staining blue by X-gal.

X-Gal staining and FACS-gal assay. For X-gal staining, commercial protocol was followed. Briefly, cells were fixed in PBS containing 0.5% glutaraldehyde for 15 min followed by washing with PBS three 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 h. The FACS-gal assays were performed as described[25].

Acknowledgment

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