Genetic engineering of a modified herpes simplex virus 1 vaccine vector

XueQiao Liu, Eeva Broberg, Daisuke Watanabe, Timothy Dudek, Neal DeLuca, David M. Knipe

Abstract

The herpes simplex virus 1 (HSV-1) d106 mutant virus is a multiple immediate-early gene deletion mutant virus that has been effective as an AIDS vaccine vector in rhesus macaques (Kaur A, Sanford HB, Garry D, Lang S, Klumpp SA, Watanabe D, et al. Ability of herpes simplex virus vectors to boost immune responses to DNA vectors and to protect against challenge by simian immunodeficiency virus. Virology 2007;357:199–214). Further analysis of this vector is needed to advance development into clinical trials. In this study we have defined the precise nature of the multiple IE gene mutations in the d106 viral gene and have used this information to construct a new transfer plasmid for gene transfer into d106. We tested the effect of an additional mutation in the U1–41 gene on d106 immunogenicity and found that it did not improve the efficacy of the d106 vector, in contrast with results from other studies with U1–41 gene mutants. The safety profile of d106 was improved by generating a new vector strain, d106S, with increased sensitivity to acyclovir. Finally, we have constructed a d106S recombinant vector that expresses the HIV clade C envelope protein. The d106S–HIVenv vector has retained the sensitivity to acyclovir, indicating that this phenotype is a stable property of the d106S vector.

1. Introduction

Viral vaccines were historically either inactivated viruses or live, attenuated viruses [1], but these types of vaccines have not been feasible for certain viruses such as the herpes viruses or HIV. Therefore, new types of vaccines including plasmid DNA vectors and replication-defective mutant viruses have been investigated. Replication-defective mutant viruses are genetically engineered or spontaneous mutant viruses that are defective for a viral function essential for replication in normal cells, but that can replicate in cells that express the missing viral gene product [2]. Replication-defective mutant viruses have been considered for vaccines for smallpox [3,4], genital herpes [5] and AIDS [6]. Similarly, replication-defective mutant adenoviruses [7], poxviruses [8], and alphaviruses [9] have been studied as vaccine vectors. Adenoviruses have been extensively tested as AIDS vaccine vectors [10], but the failure of the recent STEP trial utilizing Ad vectors [11] raises the need for additional vector approaches.

We have constructed replication-competent and replication-defective HSV-1 recombinant viruses that express SIV gene products and have used these to immunize rhesus macaques. The initial recombinants expressed a number of HSV gene products in addition to SIV proteins and induced SIV-specific humoral and cellular immune responses that resulted in partial protection of macaques against mucosal SIVmac239 challenge infection [12]. A second-generation HSV vector, HSV-1–d106, has multiple IE gene mutations; thus, in normal cells it expresses only two viral gene products, ICP0 and ICP6 [13]. HSV-1–d106 causes minimal host protein shutoff, and minimal cytopathic effect, and shows prolonged expression of a transgene in infected cells [14]. HSV-1–d106 recombinants expressing SIV env, gag, and a rev–tat–nef fusion protein were constructed [14] and used to immunize rhesus macaques [15]. Immunized macaques showed reduced viral loads, which correlate with certain cellular and humoral immune responses [15]. Based on these results and those of further studies (Kaur et al., unpublished results), we are developing d106 recombinants as AIDS vaccine vectors for clinical trials. In this study we have defined the IE gene mutations in d106, constructed a new plasmid transfer vector, tested the effect of mutating the virion host shutoff (Vhs) function on immunogenicity, and improved the safety profile of the vector strain by making it more sensitive to a herpes antiviral drug, acyclovir.

2. Results

2.1. Sequencing of IE gene mutations in the d106 genome

The d106 mutant virus [13] was constructed to have mutations in the ICP4 and U1–54 ORF encoding ICP27 and in the intergenic regions.
Fig. 1. Organization of the HSV-1 genome. (A) Diagram of the structure of the HSV genome. The unique sequences are represented as a line, and the repeated sequences are represented as boxes. a = terminal repeats; b, b’ = L component inverted repeats; c, c’ = S component inverted repeats. (B) Expanded map of the right end of the genome showing the ORFs of the IE genes. (C) Expanded map of the right end of the d106 genome showing the deletions (open boxes) of ICP4 and ICP22/47 gene promoters, and the CMV-GFP cassette (open arrow) insertion in the ICP27 gene.

The ICP4 open reading frame deletions in d106 virus (Fig. 1C) were isolated originally in the d120 mutant virus and estimated to be 4.12–4.15 kbp each [16], but the exact size of the deletions had not been defined. This deletion inactivates both copies of the essential ICP4 gene, making d106 virus replication dependent on ICP4 provided by the complementing cell line. Our sequencing results confirmed a 4222 bp deletion of nts 126,371–130,593 (Fig. 2B) and 147,641–151,862. These deletions represent 3361 bp deletion from each of the two ICP4 ORFs, leaving 535 bp or 178 codons in the 5′ end of the ICP4 ORF followed by a frame-shift.

In the original construction of the d106 mutant virus [13], the CMV-GFP cassette was inserted in the ICP27/UL54 gene between the BamHI (nts 113,322–113,327) and SalI (nts 114,517–114,522) sites (Fig. 1C). This deletion inactivates the essential ICP27/UL54 gene, making d106 replication dependent on ICP27/UL54 expressed from the gene in the complementing cell line. Our sequencing results confirmed that nts 113,328–114,516 were replaced by the CMV-GFP cassette, between the BamHI and SalI restriction endonuclease cleavage sites (Fig. 2A). In addition, the CMV-GFP expression cassette was inserted in the opposite orientation to the UL54 ORF and flanked on the left by one copy of the PacI linker (TTAAT- TAA) and on the right by three copies of the PacI linker, and this entire cassette was bounded by XbaI sites (Fig. 2A). Therefore, the deleted sequences, the orientation of CMV-GFP cassette insertion, and linker sequences are totally consistent with the original transfer plasmid and virus construction [13].

The ICP4 open reading frame deletions in d106 virus (Fig. 1C) were isolated originally in the d120 mutant virus and estimated to be 4.12–4.15 kbp each [16], but the exact size of the deletions had not been defined. This deletion inactivates both copies of the essential ICP4 gene, making d106 virus replication dependent on ICP4 provided by the complementing cell line. Our sequencing results confirmed a 4222 bp deletion of nts 126,371–130,593 (Fig. 2B) and 147,641–151,862. These deletions represent 3361 bp deletion from each of the two ICP4 ORFs, leaving 535 bp or 178 codons in the 5′ end of the ICP4 ORF followed by a frame-shift.

Fig. 2. Locations of deletions and GFP insertion in d106 genome. (A) DNA sequence of the HSV sequences flanking CMV-GFP cassette insertion between nts 113,327 and 114,516 of BamHI and SalI sites of ICP27 locus. (B) ICP4 gene deletions from nts 126,370 to 130,593. The second deletion from nts 147,641 to 151,862 is not shown. (C) Deletions within ICP22/47 promoters from nts 131,539 to 131,893 and 146,429 to 146,830.
146,695 was replaced by a 16-bp sequence, GCTCTAGATTAATTAA, used as a linker in the construction of the TGFΔ mutation (Fig. 2C). The 265 bp of deleted sequence contains three SP1 binding sites and a TTAATGARAT Oct-1 site within the promoter of the ICP22 and ICP47 genes from nts 131,536 to 131,543, nts 131,685 to 131,693, and nts 131,786 to 131,794. Deletion of the SP1 and Oct-1 sites is believed to result in the disruption of promoter activity and the loss of the transcription of the ICP4, ICP22, and ICP47 genes.

The mutations in the d106 viral genome were confirmed by the sequencing of another d106-derived recombinant, d106–27lacZ [14]. This virus was constructed by the replacement of the GFP cassette of IC27/U54 region of d106 with a lacZ cassette using homologous recombination by co-transfection of d106 genomic DNA and pd27–bgal plasmid DNA, which has HSV sequences from nts 112,700 to 113,326 (625 bp) and nts 114,591 to 115,957 (1366 bp) flanking the lacZ expression cassette. The β gene mutations in d106–27lacZ viral DNA were sequenced and found to contain the same sequence alterations as d106 described above.

### 2.2. Derivation of a new transfer plasmid

Co-infection of infectious HSV DNA with linear viral DNA sequences into cells to allow homologous recombination is one approach used to introduce new sequences into the viral genome [17], and this approach has been used to introduce expression cassettes into the HSV genome to generate vaccine vectors [12,14]. We had used the pPs27pd1 plasmid (Fig. 3) to introduce a lacZ expression cassette into the ICP27/U54 locus of d106 to construct d106–27lacZ, as described above. This transfer plasmid has proven useful, but the recombination efficiency was low, possibly due to its short HSV sequences flanking the expression cassette (only 625 bp on one side). Therefore, we constructed a new transfer plasmid by amplifying two 1.3 kbp flanking sequences from d106 viral DNA, one from nts 112,000 to 113,303 (1324 bp) and another from nts 114,541 to 115,860 (1320 bp). Two DNA fragments from nts 112,000 to 113,303 (F1) and nts 114,541 to 115,860 (F2) were PCR-amplified from d106 DNA using primer pairs A and B, respectively (Table 1). The PCR fragments were gel purified and ligated with TA plasmid DNA. The F1 insert was removed from TA–F1 by EcoRI and HindIII digestion and inserted into pUC19 plasmid between the EcoRI and HindIII sites to construct the pUC19–F1 plasmid. The F2 insert was removed from TA–F2 by EcoRI digestion and inserted into the EcoRI site of pUC19–F1 to construct pUC19–F2–F1. The CMV immediate-early promoter/enhancer-multi-cloning site-SV40 polyadenylation signal [CMV-MCS-poly(A)] cassette was removed from pCIΔAf III [12] by BgIII and BamHI digestion and ligated into the p54–53 plasmid cleaved with BgIII and BamHI and filled in with Klenow to generate the pd27B plasmid. The new pd27B plasmid (Fig. 3) and used as the transfer plasmid for the construction of a recombinant virus expressing HIV clade C envelope, as described below, and other microbial antigens (Colgrove and Knipe, in preparation).

### 2.3. Effect of vhs inactivation on immunogenicity of d106 vectors

Previous studies have reported that inactivation of the vhs function can increase immunogenicity of HSV strains in some situations [18–20], although not all situations [21]. We wanted to determine if a vhs mutation could increase immunogenicity of a recombinant d106 viral vector expressing HIV gag. We first established a quantitative ELISPOT assay for CD8+ T cells specific for HIV gag. We constructed a d106–HIV gag recombinant virus by the insertion of an HIV gag expression cassette into d106 as described in Section 4. This recombinant expressed gag protein for at least 36 hpi in Vero cells (Fig. 4A). We immunized groups of mice (n = 6) with 5 × 10^5, 5 × 10^4, or 5 × 10^3 PFU of d106–HIV gag at days 0, 21, and 42. Splenocytes were collected from each recombinant-infected mice (three mice per group per time point) at 7 days after each boost and were stimulated with an MHC-I gag peptide. The gag-specific CD8+ T cell responses were proportional to the dose of vector inoculated (Fig. 4B). Furthermore, the CD8+ T cell responses were higher after the priming immunization (4 weeks) than after the boosting immunization (7 weeks) (Fig. 4B). Therefore, this system provided a quantitative assay for comparing the T cell responses to different d106 constructs.
We then constructed a vector lacking vhs by inserting the HIV gag expression cassette into a vhs-deleted, d106-derived recombinant virus named RJ-1 [22], as described in Section 4. The RJ-1–HIV gag recombinant expressed slightly less gag protein than the d106–gag recombinant (Fig. 4A). We infected mice with the d106–HIV gag and RJ-1–HIV gag recombinants and measured CD8+ T cell responses by ELISPOT assays and antibody responses by ELISAs. In contrast with previous results with vhs mutant viruses, the RJ-1–HIV gag virus induced lower CD8+ gag-specific T cell responses (Fig. 5A) and lower p24-specific antibody responses than the d106 vector (Fig. 5B). HSV-specific antibody responses were similar for the two vectors (Fig. 5C). These results indicated that mutation of vhs yields no improvement in immunogenicity in the context of the d106 mutant virus.

2.4. Acyclovir resistance of and engineering of a sensitive d106S strain

In our studies of the properties of the HSV-1–d106 virus, we observed that d106 virus was partially resistant to a herpes antiviral drug, acyclovir, in having a 50% inhibitory concentration (IC50) of 146 μM, as compared with the parental virus, HSV-1 KOS, which showed an IC50 of 13 μM (Fig. 6). For an optimal safety profile, an HSV vector should be sensitive to acyclovir, so we backcrossed d106 with KOS WT virus to generate a new version of d106 that was acyclovir-sensitive. For the backcross, we co-infected E-11 complementing cells with KOS and d106 viruses at an MOI of 3 and harvested the progeny virus. The resulting progeny virus stock was

Table 1

| Primer pair A | Forward 5′-CCC AAGCTT HindIII GATATC EcoRV ACTAGT SpeI ATTTAAAT SwaI AACGTT AclI nt 1 12000GCCTGTCGTGTCTGCGCTTTAAGCnt 1 12023-3  |
| Reverse 5′-CGGGATCC BamHI nt 1 13324TCCAGGCTACACGTGGCCTCGGnt 1 13303-3 |
| Primer pair B | Forward : GAAGATCT BglII nt 1 14541GGCCGCAGCGCACAGGTCATCGnt 1 14562-3  |
| Reverse : CGGAATTC EcoRI GATATC EcoRV ACTAGT SpeI ATTTAAAT SwaI AACGTT AclI nt 1 15838GAGACGTCCTTAATCGTCCCGACnt 1 15860-3 |

Fig. 4. HIV gag expression and CD8+ T cell response induction by HSV recombinants. (A) HIV Gag protein expression in Vero cells infected with d106–gag and RJ-1–gag recombinant viruses at various times post-infection (hours), as detected by Western blots. M =mock-infected. (B) Effect of varying the dose of immunizing virus on HIV Gag-specific CD8+ T cell responses. Groups of mice (n=6) were immunized with 5 × 104, 5 × 105, or 5 × 106 PFU d106–gag, at days 0, 21, and 42. Splenocytes were collected from each recombinant-infected mice (three mice per group per time point) at 7 days after each boost and were stimulated with an MHC-I Gag peptide. Results are shown as the mean number of interferon-γ spot-forming cells (SFC)/106 splenocytes ± standard deviation.

Fig. 5. Effect of a vhs mutation on d106 immunogenicity. Mice were inoculated with 2 × 106 PFU of d106–gag or RJ-1–gag virus, followed by two booster inoculations at week 3 and week 6. Splenocytes were collected at week 4 and week 7 for ELISPOT as described in Fig. 4. Serum samples were collected at week 0, week 3, and week 6 prior to booster inoculation, and week 9 after booster. (A) HIV gag-specific CD8+ T cell responses as measured by ELISPOT. (B) HIV Gag antibody responses, and (C) anti-HSV antibody responses induced by d106–gag and d106Δvhs-gag (RJ-1–gag) recombinant viruses determined by ELISA. The results are shown as mean of OD405 ± standard deviation.
used to infect E-11 cells at high dilutions so that well-isolated viral plaques were formed. Green fluorescent plaques were picked and screened for sensitivity to 30 μM acyclovir and 60 μM acyclovir. Four of 140 plaque isolates showed sensitivity to 30 μM acyclovir and were further plaque-purified. These 4 isolates were screened for growth on E-11 cells containing the ICP4 and ICP27/UL54 genes, V827 cells containing the ICP27/UL54 and ICP8 genes, and Vero cells, the parental cell line containing no HSV genes. One plaque isolate with high sensitivity to acyclovir formed green plaques on E-11 but not on Vero and V827 cells, and this virus was designated d106S. The acyclovir sensitivity of d106S was assayed in comparison with KOS and d106 (Fig. 6). The results showed that the IC50 for d106S was 6.6 μM, about 2-fold lower than KOS and 20-fold lower than d106, indicating that we had generated an acyclovir-sensitive vector strain, d106S.

To ensure that the d106S virus contained all of the mutations engineered into d106, we sequenced the regions of mutations in d106S. Our sequencing results confirmed that d106S contained the GFP insertion in the ICP27/UL54 gene, the deletions in the ICP4 gene, and the deletions within promoter regions of the ICP22 and ICP47 genes (data not shown). Therefore, we concluded that d106S contains the five IE gene deletions in the original d106 virus but has high sensitivity to acyclovir, suggesting that d106S has WT Pol and TK genes, which define acyclovir sensitivity.

2.5. Construction of d106S recombinant viruses expressing HIV envelope protein

We wanted to use the HSV-1–d106S virus as a vector for the expression of HIV gene products to serve as a candidate for a clinical trial vector. As the first application of this vector, we constructed a d106S recombinant virus that expresses the HIV clade C envelope (env) protein, d106S–HIVenvC, using homologous recombination.

The transfer plasmid was constructed as follows. Plasmid pVRC5309, which contains an expression cassette with a codon-optimized HIV (Clade C) envelope protein (envC) ORF, was digested with BamHI and NotI, and the resulting 1880 bp fragment containing the envC ORF was inserted into the pd27B plasmid digested with XbaI and NheI and filled with Klenow. The resulting plasmid, designated pd27B-5309, was linearized by SwaI digestion and co-transfected with d106S viral DNA into E-11 cells. Viral progeny was harvested, and recombinants were identified in a screen for non-fluorescent plaques, because homologous recombination leads to the incorporation of the CMV–env cassette into the ICP27/UL54 gene locus replacing the GFP expression cassette. Three isolates were plaque-purified, and expression of env protein was assessed (Fig. 7A). One of the recombinant viruses was chosen as the prototype strain and was shown to express env protein for at least 24 h (Fig. 7B). Furthermore, the recombinant virus was highly sensitive to acyclovir, as shown in Fig. 7C.
to acyclovir (Fig. 7C) with an IC50 of 6.1 μM. The d106–HIVenvC recombinant viruses expressed the env protein, and retained the acyclovir-sensitive property of d106S. Therefore, the acyclovir sensitivity of the modified vector, d106S, is stable through construction of new recombinant strains.

3. Discussion

The HSV-1–d106 recombinant virus has shown good immunogenicity and protective capacity in a rhesus macaque model of SIV infection [15], and we are developing this virus as an AIDS vaccine vector for clinical trials. In this study we have determined the precise nature of the multiple IE gene mutations in the d106 viral genome and have used this information to generate a transfer plasmid containing larger flanking sequences for improved efficiency of gene transfer into recombinant virus vectors. Previous studies with HSV mutants have shown increased immunogenicity with viral strains that have the U1 gene mutated and virion host shutoff function inactivated [18,19], but we observed slightly lower expression of the HIV gag protein from a vector lacking U1 and reduced cellular and humoral immunogenicity. Finally, we improved the safety profile of d106 for clinical use by generating a modified virus, d106S, that shows increased sensitivity to acyclovir.

3.1. Improved transfer plasmid

Previous studies have shown that the efficiency of gene transfer is proportional to the length of the sequences in the donor DNA [17]. We therefore used our data on the sequences surrounding the ICP27/U1.54 gene deletion insertion in d106 to make a new transfer plasmid, pd27B, with longer flanking sequences around the transgene site than the plasmid pd27pd1, which we had previously used to insert transgenes in the ICP27/U1.54 gene [14]. The plasmid pd27B contains flanking sequences of 1324 bp and 1320 bp as compared with 626 bp and 1366 bp for pd27pd1. The increased flanking sequences in pd27B have resulted in a higher frequency of recombination and greater ease of making recombinants (results not shown).

3.2. ACV sensitivity

HSV recombinants delivered for clinical purposes need to be susceptible to standard anti-HSV drugs so that the drugs, in the unlikely event that any revertant or recombinant that might arise, could control this virus. It is highly unlikely that a revertant could arise because the d106 strain has multiple deletion mutations in genes encoding functions that cannot be complemented by cellular gene products. It is also unlikely that the mutant virus could re-gain the missing viral genes from the complementing cell line due to the lack of homologous sequences for recombination. The missing functions cannot be provided by another herpesvirus, except for HSV-1 and HSV-2. However, a drug resistance mutation could be transferred from the vaccine strain to a naturally occurring HSV-1 or HSV-2 strain by homologous recombination. Therefore, it is essential that HSV strains used for clinical applications be sensitive to antiviral drugs. We observed that the d106 virus was moderately resistant to acyclovir so we backcrossed it with WT KOS virus to obtain a d106-derived strain, d106S, with acyclovir sensitivity similar to the WT KOS parental strain. The acyclovir sensitivity phenotype was maintained in d106S after construction of an HIV gag-expressing recombinant, indicating that this is a stable property of d106S.

3.3. Lack of an effect of vhs on d106 immunogenicity

The HSV U1.41 gene encodes the virion host shutoff function, a function which reduces host mRNA translation in HSV-infected cells [23]. The U1.41 protein is incorporated into the tegument layer of the virion and when delivered into the cytoplasm is activated to degrade mRNAs on polyribosome. Inactivation of the U1.41 gene and loss of the vhs function increases immunogenicity of certain virus strains [19,20,24] so it was surprising that this additional mutation did not increase immunogenicity of d106. This is likely explained by the fact that d106 already shows a defect in shutoff of host translation because of the mutation in the ICP27/U1.54 gene. Shutoff of host protein synthesis by HSV requires both vhs (U1.41) and ICP27 (U1.54) [25]. ICP27/UL54 inhibits splicing of host mRNA [26] and cell gene transcription [27], so it is also required for host shutoff. The vhs function is required for the ability of HSV to block dendritic cell activation [28]. Because either ICP27/U1.54 or U1.41 gene inactivation seems to enhance immunogenicity of HSV vaccine vectors and both are required for shutoff of host protein synthesis, the increased immunogenicity is likely due to a lack of host shutoff, and not other effects of vhs on dendritic cell function or innate responses [29].

3.4. Comparisons of different HSV vectors

In addition to replication-defective mutant HSV strains, HSV amplicons have been constructed that express the HIV gag protein [30]. These amplicons contain no known HSV ORF and express no HSV proteins. Immunogenicity studies using amplicons in mice have been published [30,31], but thus far no studies of immunogenicity in monkeys have been published. Recent studies have shown that the immediate-early ICP0 protein is required for chromatin modification and remodeling to allow efficient expression of genes on the viral genome [32,33]. Therefore, expression of ICP0 by a HSV vaccine vector is likely to prevent host chromatin silencing of the viral genome and enhance transgene expression from the viral genome and the immune responses elicited. This hypothesis is consistent with decreased transgene expression by the d109 virus, which does not express any IE genes [13].

In summary, we have modified the HSV-1–d106 vector system by generating the d106S virus strain with increased acyclovir sensitivity. Furthermore, the d106S virus strain shows the properties of limited cytopathic effect and prolonged expression of transgenes. We are now constructing and characterizing a set of d106S vectors expressing HIV proteins for future clinical trials.

4. Materials and methods

4.1. Plasmids

The pVRC3509 plasmid [34] containing a codon-optimized HIV clade C envelope protein (envC) ORF and pVRC4302 plasmid [35] containing the HIV gag–pol–nef expression cassette were provided by Dr. Gary Nabel, NIH. The pdl27CIA plasmid was described previously [12]. The TA (PCR 2.1) plasmid was obtained from Invitrogen (Carlsbad, CA).

4.2. Cells and viruses

Vero cells, V827 cells containing the ICP8 and ICP27 genes [36] and E-11 cells containing the ICP4 and ICP27 genes [13] were cultured as described. The HSV-1–d106–LacZ virus [14], the HSV–1–d106 virus [13], and the R1–virus (d106Δvhs) [22] were described previously. Low passage HSV-1 strain KOS virus was obtained from Dr. Priscilla Schaffer.

The HSV-1–d106–HIV gag and R1–HIV gag viruses were constructed as follows. The gag–pol ORF from the plasmid VRC4302 was removed by Hpal and EcoRI digestion followed by Klenow treatment to fill in the ends. This fragment was inserted into plasmid pdl27.CIA [12] at a filled-in AFLII site. A partial digest was performed
References


