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Infectivity of Herpes Simplex Virus Type-1 (HSV-1) amplicon vectors in dendritic cells is determined by the helper virus strain used for packaging

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Abstract

Herpes Simplex Virus Type-1 (HSV-1) amplicon vectors are being explored for a wide range of potential applications, including vaccine delivery and immunotherapy of cancer. While extensive effort has been directed towards the improvement of the amplicon “payload” in these vectors, relatively little attention has been paid to the effect of the packaging HSV-1 strains on the biological properties of co-packaged amplicon vectors. We therefore compared the biological properties of amplicon stocks prepared using a panel of primary HSV-1 isolates, a molecularly cloned strain used to package helper-free amplicons (designated here as F5), and two laboratory isolates (KOS and strain 17, which is the parent of the F5 clone). This analysis revealed considerable inter-strain variability in the ability of amplicon stocks packaged by different primary HSV-1 isolates to efficiently transduce established cell lines and primary human dendritic cells (DC). Amplicons packaged by both the F5 molecularly cloned virus and its lab-adapted parent (strain 17) were very inefficient at transducing DC, when compared to amplicons packaged by KOS or by several of the primary virus isolates. These findings have important implications for the future development of improved amplicon-based vaccine delivery systems and suggest that DC tropism may be an intrinsic property of some HSV-1 strains, independent of passage history or molecular cloning.

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Keywords

HSV-1 amplicon vectors; primary virus isolates; Herpes simplex virus type-1 (HSV-1); dendritic cells (DC)

1. Introduction

HSV-1 amplicon vectors represent a promising tool for multiple gene transfer applications including vaccine delivery. However, current methods for the generation of amplicon stocks rely on the use of highly passaged helper virus strains in order to produce infectious amplicon particles (Geller and Breakefield, 1988; Logvinoff and Epstein, 2001), or (in the case of helper-free amplicon stocks) on the use of molecularly cloned helper virus genomes that have been derived from laboratory-adapted strains (Fraefel et al., 1996; Saeki et al., 2001; Saeki et al., 1998; Stavropoulos and Strathdee, 1998).

Serial passage of herpesviruses in cultured cell lines is known to result in profound changes in the virus genome, including point mutations, alterations of splicing patterns and even deletions of large segments of viral DNA. This is exemplified by the genetic changes and associated loss of virulence that characterize the serially passaged Oka vaccine strain of varicella-zoster virus, when compared to the parental Oka strain (Argaw et al., 2000; Cohrs et al., 2006; Gomi et al., 2002). Similarly, laboratory-adapted strains of human cytomegalovirus (HCMV), such as the AD169 strain, possess an extensive genetic deletion (encompassing approximately 15 kb of the viral DNA genome) when compared to primary isolates (Cha et al., 1996; Dolan et al., 2004; Hahn et al., 2003; Murphy et al., 2003; Prichard et al., 2001). Since HCMV strains are typically propagated in fibroblasts, this presumably explains why laboratory adapted strains have lost the ability to infect endothelial cells, when compared to primary isolates (Hahn et al., 2004; MacCormac and Grundy, 1999; Sinzger et al., 1999; Wang and Shenk, 2005a; Wang and Shenk, 2005b).

Davison has noted that the genes which are most prone to mutation following prolonged passage of HCMV in cell culture often have roles in pathogenicity or tropism (Dolan et al., 2004). It is therefore reasonable to predict that changes in the genetic composition and biological properties of herpesviruses following adaptation to laboratory culture conditions are likely to result in loss of properties that may be desirable in the context of vaccine delivery and/or amplicon generation. For example, laboratory-adapted strains of HCMV not only lose the ability to infect endothelial cells, but also lose the ability to efficiently infect primary dendritic cells (Gerna et al., 2005; Hertel et al., 2003).

Current methods for the generation of helper-free HSV-1 amplicon stocks involve the transient transfection of amplicon plasmid DNA into packaging-permissive cells [i.e., baby hamster kidney cells (BHK) or 2-2 cells], together with a bacmid construct that contains a non-packageable HSV-1 genome (Fraefel et al., 1996; Saeki et al., 2001; Saeki et al., 1998; Stavropoulos and Strathdee, 1998). For this purpose, our laboratory uses a bacmid (pBAC-V2; (Stavropoulos and Strathdee, 1998)) that was derived from a set of overlapping cosmid clones containing the intact genome of HSV-1 strain 17 (Brown et al., 1973; Cunningham and Davison, 1993). The biological properties of this molecularly cloned virus genome remain incompletely characterized, and may not be ideal for vaccine applications and/or efficient production of amplicon stocks. To this end, we conducted experiments to systemically compare the properties of a reconstituted infectious virus stock derived from the original HSV-1 cosmid panel (designated herein as F5, and generously provided by Dr. Andrew Davison; (Cunningham and Davison, 1993)) with those of a set of 19 clinical HSV-1 isolates that had been only minimally passaged, and two additional laboratory-adapted HSV-1 isolates (KOS

and strain 17, which is the parental virus from which the molecularly cloned F5 stock was derived). This analysis revealed considerable variability in the efficiency with which amplicon stocks packaged by these viruses were able to transduce established cell lines and primary human dendritic cells (DC). In most cases, the amplicon stocks generated using the minimally passaged primary isolates outperformed the F5-based stock. Moreover, amplicons packaged by both the molecularly cloned F5 virus and its lab-adapted parent (strain 17) were equally inefficient at transducing DC, suggesting that this property is intrinsic to strain 17 and not an artifact of the molecular cloning process. These findings have important implications for the current generation of HSV-1 amplicon vectors, and suggests ways in which amplicon-based vaccine platforms can be enhanced in the future.

2. Materials and Methods

2.1 Expansion of clinical HSV-1 isolates

Nineteen clinical isolates of HSV-1 were obtained from the UR Clinical Microbiology Laboratory (courtesy of Dr. Marilyn Menegus). Samples were selected from individuals with mild disease symptoms (i.e., with no evidence of encephalitis) and were provided without patient identifying information in an approximate volume of 1 ml each. One hundred microliters of each isolate was used to infect 1.1×10^6 VERO cells in 60-mm culture dishes and incubated at 34°C. Viral propagation was assessed by monitoring apparent cytopathic effect (CPE). The length of time required by the isolates to reach 100% CPE ranged between 3 and 5 days. Each dish was then incubated at 37°C for 2 h to enhance viral release from the host cells. The cells and supernatants were collected and frozen at -80°C (represented the P0 stock). Each isolate was then further expanded (P1 stock generation). The P0 stocks were thawed at 37°C, subjected to sonication to liberate any virus residing within host cells and centrifuged to remove the majority of cellular debris. The supernatants (approximately 4 ml each) were used to infect 4×10^6 Vero cells per T-75 flask. Propagation was monitored as previously mentioned; 100% CPE was observed between 4 and 5 days of incubation. The P1 viral stocks were generated, stored in 1-ml aliquots, and frozen at -80°C until titered by a previously described plaque-based method (Geschwind et al., 1994).

2.2 Analysis of virus growth kinetics

VERO cells were used to determine the growth kinetics of selected primary HSV-1 isolates, as well as the reconstituted F5 virus stock (generously provided by Dr. Andrew Davison; (Cunningham and Davison, 1993)) and the laboratory adapted isolates, KOS (gift of Dr. John Blaho) and strain 17 (a non-syncytium forming, *syn*⁺ (Ruyechan et al., 1979), stock of strain 17 kindly supplied by Dr. Nigel Fraser). Cells were plated at a density of 2×10^5 cells/well in 24-well tissue culture plates. Eight time points were selected for determination of viral propagation (0, 2, 6, 12, 18, 24, 36, 48 hours) and each well was infected with 4×10^4 pfu (MOI = 0.2). Viral medium was aspirated at the conclusion of each time point and cells were lysed in 100 µl 100 mM potassium phosphate, pH 7.8 and 0.2% Triton X-100 containing 1 mM DTT for 10 min. at 25°C. The resulting lysates were collected and frozen at -80°C. Total DNA was obtained as described (Bowers et al., 2000), and the resulting DNA concentration was determined by spectrophotometric analysis. Transduction analysis was performed using quantitative real-time DNA PCR (qRT-PCR) specific for the ICP0 gene of HSV-1.

2.3 Quantitative real-time PCR

Total DNA from cells infected with HSV-1 isolates was analyzed using qRT-PCR. Briefly, 12.5 ng of DNA was loaded into 25 µl PCR reactions and analyzed using the 7300 Real Time PCR System (Applied Biosystems). ICP0 gene copy number was determined using the pCI110 plasmid (kindly provided by Dr. Khaled Tolba, University of Miami) as a standard curve. Primers (Fwd: 5'-ATGTTTCCCGTCTGGTCCAC-3') (Rev: 5'-

CCCTGTCGCCTTACGTGAA-3') and probe (5'-CCCCGTCTCCATGTCCAGGATGG-3') were designed using the Primer Express 3.0 software (Applied Biosystems). Data were normalized using cellular genomic DNA (for the 18S rRNA gene) using primers (Fwd: 5'-CGGCTACCACATCCAAGGAA-3') (Rev: 5'-GCTGGAATTACCGCGGCT-3') (Probe: 5'-TGCTGGCACCAGACTTGCCCTC-3').

2.4 Packaging and propagation of an amplicon vector by HSV-1 isolates

Twenty T-150 flasks containing 8×10^6 Vero cells per flask were transfected with 56 μ g of pHSVlac plasmid DNA (Geller and Breakefield, 1988), using the Lipofectamine 2000 transfection reagent (Invitrogen). Each flask was incubated overnight at 37°C. The following day, the expanded HSV-1 clinical isolates and the reconstituted F5 clone were used to infect each flask at an MOI of 0.2. Four days later, each flask was incubated at 37°C for 2 hours to enhance viral release from the host cells. The cells and supernatants were collected and frozen at -80°C to create a P0 stock and further expanded to generate a P1 stock. Each P1 stock was subsequently subjected to sucrose-gradient concentration. The concentrated viral stocks were resuspended in 500 μ l of DPBS containing calcium and magnesium and frozen as 50 μ l aliquots at -80°C. Wild-type HSV-1 titers were determined by plaque assay on VERO cells and amplicon titers were determined using X-gal histochemistry on NIH3T3 cells as described (Bowers et al., 2000).

2.5 Human dendritic cells

Human dendritic cells (DC) were differentiated from CD14+ monocytes, as outlined in our previous studies (Maguire et al., 2006). Briefly, leukocyte concentrates received from the New York Blood Center (New York, NY) or whole blood samples were layered on a Lymphoprep™ (Axis-Shield, Oslo, Norway) cushion and human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. CD14+ monocytes from buffy coats obtained from the Lymphoprep interface were enriched by positive selection with anti-CD14 MACS beads (Miltenyi Biotec, Auburn, CA). Monocytes were cultured in Stemline Dendritic Cell Maturation Media (Sigma, St. Louis, MO) supplemented with 2 mM L-glutamine, and 50 ng/ml recombinant human GM-CSF (R&D Systems, Minneapolis, MN) and 25 ng/ml recombinant human IL-4 (R&D Systems). Media was replenished every 2 days. After 9 days in culture, the monocyte-derived human DC were used in HSV-1 amplicon transduction assays.

2.6 Amplicon transduction assays

Assessment of amplicon transduction efficiency was performed in differentiated human DC. Cells were incubated at 37°C/5% CO₂ under humidified conditions. For infections, 3×10^5 cells were seeded into 24-well plates and allowed to adhere overnight at 37°C/5% CO₂. Cells were then transduced at various multiplicities of infection (MOI) ranging from 0.001 to 0.1 (unless otherwise specified) with amplicons generated using the different isolates as helper virus. At 24 hours post transduction, cultures were harvested for analysis. Transduction efficiency was assessed either by enzymatic assay for β -galactosidase activity, or by a histochemical staining method. The enzymatic assay was performed using the GalactoLite Plus kit (Applied Biosystems) according to manufacturer's directions. Briefly, cells were lysed with 200 μ l lysis buffer supplemented with 1 mM dithiothreitol (DTT). Lysates were clarified by centrifugation at 13,000 rpm for 7 min at 4°C and protein concentration was measured using Bradford reagent (BioRad, Hercules, CA). Five microliters of cleared lysate (1 μ g total protein) were assayed for β -galactosidase activity. Light emission was measured in a white 96-well plate, using a luminometer (SpectraCount Version 3.0, Packard) and measurements of β -galactosidase activity were normalized to total protein content.

X gal histochemistry was performed by staining with X-gal substrate. Briefly, transduced cells were pelleted by low speed centrifugation, washed with PBS, and fixed for 5 min at 25°C in a

2% formaldehyde/0.2% glutaraldehyde solution. The cells were then washed with PBS and stained with X-gal (20 mg/ml X-gal; Sigma) in dimethyl sulfoxide (DMSO), in KFe(CN)/PBS solution. Cells were then incubated at 37°C for 45 min, and observed using phase-contrast light microscopy using an Olympus IX81 inverted fluorescent microscope. Images were acquired using a CCD digital photo camera, and then evaluated using Image Pro Plus software (version 4.5.1). Statistical analyses were performed using GraphPad Prism software.

3. Results

3.1 Primary HSV-1 isolates vary in their ability to propagate amplicon vectors

The major goal of this study was to compare the biological properties of HSV-1 amplicon stocks generated using a panel of primary HSV-1 isolates with those of an amplicon stock generated using a reconstituted, molecularly cloned virus stock that is widely used in the production of helper-free amplicon particles (designated here as F5) (Cunningham and Davison, 1993; Stavropoulos and Strathee, 1998). Underlying this comparative analysis is the hypothesis that minimally passaged clinical HSV-1 isolates will permit the generation of amplicon stocks with more desirable properties (e.g., expanded host range) than is possible using the current helper virus genome.

We generated virus stocks that contained a co-propagated amplicon vector encoding a β -galactosidase transcription unit (so as to allow us to conveniently assess virally-mediated gene transfer into cultured target cells of interest). The ability to efficiently package and propagate amplicon stocks is posited to be an important criterion with respect to identifying new HSV-1 strains that may have promise for use as helper viruses in the generation of amplicon stocks. We therefore compared the ability of our panel of HSV-1 isolates to propagate amplicon stocks. To do this, we generated amplicon-containing stocks using each of the various isolates, and then used functional assays to separately measure the titer of the helper virus and the amplicon vector. Helper virus was quantitated by measuring virus plaque forming units (PFU) in VERO cells, while amplicon was titered by measuring β -galactosidase expressing, blue-forming units (BFU) in 3T3 cells. The ratio of amplicon:helper virus was then determined, and the results are presented in Table 1. This analysis revealed that the primary HSV-1 isolates varied in their ability to propagate amplicon stocks, but that several of them outperformed the F5 virus stock in this regard.

3.2 Primary HSV-1 isolates vary in their ability to infect established cell lines

To examine the biological properties of amplicon stocks packaged by the panel of clinical isolates, or the F5 control strain, we exposed two established cell lines (VERO and 293 cells) to helper-containing amplicon stocks at a MOI of 0.1 (in this experiment, and subsequent experiments, the infecting MOI was defined in terms of the titer of the lacZ-encoding amplicon vector, as measured in VERO cells; see Methods). Cultures were then harvested at 24 hours post infection and β -galactosidase activity was assayed from cell lysates. The results showed that amplicon stocks packaged by most of the clinical isolates were able to elicit higher levels of gene expression in both VERO (Figure 1A) and 293 cells (Figure 1B), when compared to the F5 virus stock.

3.3 Primary HSV-1 isolates vary in their ability to infect monocyte-derived human DC

One of the goals of our studies was to determine if, by using different HSV-1 isolates strains to package amplicon particles, we could generate amplicon stocks able to transduce biologically important cell types like dendritic cells. To address this hypothesis, human monocyte-derived cultured DC were exposed to amplicon-containing virus stocks derived from each of the 19 primary isolates and from the F5 strain, at an MOI of 0.1. Twenty-four hours later, cells were harvested and analyzed. Quantitation of β -galactosidase activity in cell lysates

(Figure 2A-C), showed that amplicon vectors packaged by the primary HSV-1 isolates varied considerably in their ability to transduce DC, but that the great majority of the primary isolates were able to significantly outperform the F5 virus stock, in terms of their ability to generate amplicon particles that could efficiently transduce DC. To confirm that differences in DC transduction efficiency were reproducible, and not a reflection of a specific donor, we repeated this analysis using DC that were isolated from multiple donors. This analysis revealed very similar findings, irrespective of the source of the DC (Figure 2).

Finally, we assayed β -galactosidase expression using a histochemical staining method (Figure 3). This allowed us to visualize individual β -galactosidase positive cells. As a result, we were able to show that the increased levels of β -galactosidase activity measured in the GalactoLight assay (Figure 2) were also associated with an increase in the number of β -galactosidase-positive cells. This strongly suggests that increased levels of β -galactosidase expression measured in the GalactoLight assay can be attributed, at least in part, to an increase in the percentage of the dendritic cell population that became transduced by the amplicon vector (and not simply because of an increase in the per-cell level of reporter gene expression). Consistent with this, there was a statistically significant correlation between the level of β -galactosidase expression, as measured by the GalactoLight assay versus the histochemical staining method; Pearson $r = 0.506$, $p < 0.05$ (data not shown).

3.3 Amplicon-mediated gene expression in VERO cells correlates strongly with expression in 293 cells, but more weakly with expression in DC

One possible outcome of using different HSV-1 strains to package amplicon stocks is that there may be variation in the ability of the resulting amplicon particles to transduce different cell types, as a result (for example) of differences in virus envelope glycoprotein composition or tegument proteins. We therefore conducted linear regression analysis of cell transduction data for the VERO and 293 cell lines, and the primary dendritic cells. The associations between gene expression levels (averaged over three replicates) were examined in a pairwise fashion for the three different cell types using linear regression and correlation analysis. Figure 4 shows the graphical results of this analysis, while Table 2 provides a statistical summary of the results. As noted in Table 2, there was a very strong, highly significant correlation between the magnitude of amplicon-mediated gene expression in the two cultured cell lines (VERO cells and 293 cells); Pearson $r = 0.934$, 95% confidence interval 0.838 to 0.974, $p < 0.0001$. In contrast, the association between lacZ gene expression levels in VERO cells and primary DC was somewhat weaker and failed to achieve statistical significance (Pearson $r = 0.423$, 95% confidence interval -0.024 to 0.729 , $p = 0.063$). Similarly, the association between lacZ gene expression levels in 293 cells and primary DC was also relatively modest, although in this case it achieved statistical significance (Pearson $r = 0.525$, 95% confidence interval 0.107 to 0.785 , $p = 0.018$).

We performed formal tests for equality of the correlation coefficients for the VERO/293 cell comparison, and the VERO/DC and 293/DC comparisons. Because the correlations are statistically dependent, we used the T_2 statistic originally due to Williams (Williams, 1959) and described by Steiger (Steiger, 1980) for these comparisons. The results revealed that the correlation between the magnitude of amplicon-mediated gene expression in the two cultured cell lines (VERO cells and 293 cells, $r = 0.934$) was significantly different from the other two correlations (VERO cells and DC, 293 cells and DC, $p < 0.0001$ in each case).

3.4 Comparison of growth kinetics and biological properties of primary HSV-1 isolates versus laboratory-passaged viruses

The growth kinetics and biological properties of a representative subset of our primary HSV-1 isolate panel were compared directly to those of both a reconstituted, molecularly cloned virus

stock (designated here as F5) (Cunningham and Davison, 1993; Stavropoulos and Strathdee, 1998) and also to laboratory-passaged HSV-1 isolates, including both HSV-1 KOS and strain 17 (the isolate that was molecularly cloned in *E. coli*, and then used to produce the F5 virus stock).

We therefore first characterized the replication kinetics of two representative clinical HSV-1 isolates (1, 10) as well as the F5 stock and the laboratory isolates HSV-1 KOS and strain 17, in VERO cells. Cells were infected with virus stocks at a MOI of 0.2 (defined in terms of the infectious virus titer in VERO cells). Cultures were then harvested at predetermined time points and total DNA from infected cells was collected, and analyzed using a quantitative DNA PCR assay to measure ICP0 gene copy number. As shown in Figure 5, there were no significant differences in the replication kinetics of the primary and laboratory-adapted virus isolates. However, the molecularly cloned F5 strain replicated with delayed kinetics and to relatively low titers when compared to the other strains; Figure 5).

Figure 5 also shows that there is variation in the amount of viral DNA bound to the host cells at time zero (immediately after addition of virus and washing of the cells). Since we added a fixed number of infectious particles (PFU) to the VERO cells, this difference can be attributed to a difference in the genome (particle) to infectivity ratio for the various strains (KOS > 8,10,19, 17+ > F5).

We next compared the biological properties of amplicon stocks packaged by this same panel of primary and laboratory isolates in 293 cells. To do this, cells were exposed to helper-containing amplicon stocks at a MOI of 0.1. Cultures were then harvested at 24 hours post infection and β -galactosidase activity was assayed from cell lysates. The results showed that amplicon stocks packaged by primary isolate 19 efficiently transduced 293 cells, while stocks packaged by primary isolate 10 or the F5 strain were inefficient at transducing 293 cells (Figure 6; these results are consistent with data shown in Fig. 2). Amplicon stocks packaged by the two laboratory-passaged isolates (KOS, strain 17) were also efficient at transducing 293 cells (Fig. 6). This is consistent with the adaptation of these isolates to growth in continuous cell lines.

In order to confirm that observed differences in the levels of β -galactosidase expression at the 24 hour time point were not affected by differences in helper virus replication kinetics (and accompanying replication of amplicon genomes), an additional control was included in this analysis. Specifically, the experiment was performed in the presence and absence of acyclovir (ACV), at a dose of 1 μ g/ml (approx. 4.4 μ M); this exceeds the IC₉₉ for most primary HSV-1 isolates (Elion et al., 1977).

As shown in Figure 6, the levels of β -galactosidase expression were similar, either in the presence or absence of ACV. We infer that, at the early time point used in our experiments (24 hours), β -galactosidase is being produced exclusively off transcripts that derive from the original incoming amplicon genomes – and that newly synthesized amplicon genome template makes no significant contribution to β -galactosidase protein production at this time point.

Finally, we compared the ability of amplicon stocks packaged by primary and laboratory isolates of HSV-1 to transduce primary dendritic cells (see Fig. 2 for Methods). The results showed that amplicon stocks packaged by primary isolate 19 were the most efficient at transducing DC, followed by stocks packaged by the lab-adapted isolate KOS (for both donors, there was a statistically significant difference in results for primary isolate 19 versus the KOS strain; Figure 7). Other amplicon stocks, including those packaged by primary isolate 10 as well as the molecularly cloned F5 virus and the parental lab-adapted strain 17 were uniformly inefficient at transducing DC (Fig. 7).

4. Discussion

The generation of HSV-1 amplicon vectors depends on the use of helper virus strains that are, without exception, laboratory-adapted and highly passaged. This raises significant concerns with regard to the biological properties of the resulting amplicon stocks, particularly because serial passage of herpesviruses is known to result in changes in virus tropism. A reduction in the efficiency with which amplicon vectors transduce biologically important cell types may lead to inferior outcomes in gene transfer applications. Since our laboratory is investigating the use of HSV-1 amplicon vectors for vaccine delivery applications, we were especially interested in the question of dendritic cell tropism. We also wished to know whether primary HSV-1 isolates might differ in their ability to propagate amplicon vectors, since this could profoundly influence the generation of high-titer vector stocks.

The present experiments revealed that clinical HSV-1 isolates vary in their ability to propagate amplicon stocks, and also in their ability to generate amplicon particles that are capable of efficiently transducing cultured cell lines and primary human dendritic cells. Statistical analysis showed that there was a strong correlation between the strength of amplicon-mediated gene expression in VERO cells and in 293 cells. This suggests that amplicon-mediated gene expression in 293 cells and in VERO cells may be governed by a common set of helper virus-intrinsic biological factors (such as a common virion glycoprotein composition).

In contrast, our statistical analysis revealed that the correlation between the strength of amplicon-mediated gene expression in VERO cells and in primary dendritic cells was relatively modest (and did not achieve statistical significance). This, combined with our additional statistical analyses showing that this correlation was significantly lower than that between the gene expressions in the two cultured cell lines (VERO cells and 293 cells), suggests that the efficiency of amplicon-mediated gene expression in dendritic cells and in VERO cells may be controlled by two different sets of helper virus-intrinsic properties. Thus, there may be distinct HSV-1 genetic determinants for efficient gene expression in dendritic cells versus VERO cells or 293 cells.

Helper virus-intrinsic properties that could influence amplicon-mediated gene expression in dendritic cells might include variations in virion glycoproteins or other genes involved in virus attachment and entry, as well as differences in uncoating or intracellular transport, or gene expression (including effects at the level of gene transcription, mRNA stability or translation). Helper virus-intrinsic properties can also be expected to result in inter-strain variation in the ability to propagate amplicon stocks - due to such factors as variations in genes that control virus DNA replication and/or packaging. Future studies will be needed in order to explore these issues.

Regardless of the underlying mechanism, the experiments reported here clearly show that the majority of minimally passaged clinical HSV-1 isolates significantly outperform a molecularly cloned HSV-1 strain that is presently used to produce amplicon stocks (F5), in terms of generating amplicon stocks that efficiently transduce primary dendritic cells. Interestingly, the unfavorable properties of the F5-based amplicon stocks appear to reflect the intrinsic properties of the parental, laboratory-adapted virus (strain 17) that was molecularly cloned and then used to generate F5. Amplicon stocks based on both strain 17 and the F5 virus were extremely inefficient in terms of their ability to transduce primary DC. In contrast, amplicons packaged by another laboratory-adapted isolate (KOS) were more efficient at transducing primary DC - although they were still inferior to stocks packaged by primary isolate 19. This suggests that it might be desirable to use minimally passaged, primary HSV-1 isolates to establish a new system for the production of both helper-containing and helper-free amplicon stocks. Efforts

are currently underway to molecularly clone the genomes of several of the clinical HSV-1 isolates described here, in order to test this prediction.

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Figure 1. Amplicon stocks propagated by most primary HSV-1 isolates are more efficient at transducing continuous cells than stocks generated using the packaging strain F5

(A) VERO and (B) HEK 293 A cells were transduced at an MOI of 0.1 with HSV-1 amplicon particles packaged in the presence of the various isolates. Transduced cells were assayed for total β-galactosidase activity 24 hours post infection. Cleared lysates (1 μg of total protein) were assayed for activity. Data represent mean values, calculated from three replicate measurements; measurements of β-galactosidase activity were normalized to total protein. Bars denote the standard deviation of the three individual values. The data show that amplicon stocks packaged by a majority of the primary isolates were more efficient at transducing both VERO and 293 cells than stocks that were generated using the packaging strain F5 (notable exceptions included virus isolates 1 and 10).

Figure 2. Amplicon stocks propagated by most primary HSV-1 isolates are more efficient at transducing dendritic cells than stocks generated using the packaging strain F5

Human monocyte-derived DC from three different donors were transduced at an MOI of 0.1 with HSV-1 amplicon particles packaged in the presence of the various isolates. (A-C) Amplicon-transduced human DC were assayed for total β -galactosidase activity 24 hours post transduction. Cleared lysates (1 μ g total protein) were assayed for β -galactosidase activity. Data represent mean values calculated from three replicate measurements; measurements of β -galactosidase activity were normalized to total protein. Bars denote the standard deviation of the three individual values.

Figure 3. Analysis of amplicon-mediated transduction of dendritic cells using X-gal histochemistry Human monocyte-derived DC were transduced at an MOI of 0.1 with lacZ-encoding HSV-1 amplicon particles packaged in the presence of the various isolates, and then assayed by X-gal histochemistry 24 hours post transduction. Staining was performed as described in Materials and Methods and stained cells were observed by phase contrast light microscopy.

Representative results for one donor are shown. The data show that amplicon stocks packaged by the primary isolates were more efficient at transducing primary human dendritic cells than stocks that were generated using the packaging strain F5. Numbers indicate the percentage of lacZ-positive cells within each culture.

Figure 4. Linear regression analyses for cell transduction data

Linear regression analysis of cell transduction data, for the VERO and 293 cell lines, and the primary dendritic cells. The associations between gene expression levels were examined in a pairwise fashion for the three different cell types using linear regression and correlation analysis. The figure shows the scatterplots and the computed least-squares regression line (GraphPad Prism). The data that were used in these analyses correspond to the datasets shown in Figure 2 and Figure 3 (DC Batch 1). It can be readily appreciated that there is a very strong correlation between the magnitude of amplicon-mediated transduction in the two cultured cell lines (293, VERO), but a weaker correlation between transduction efficiency in these cell lines

and amplicon-mediated gene expression in primary dendritic cells. A summary of the statistical analyses corresponding to these plots is provided in Table 2.

Figure 5. Representative primary HSV-1 strains and lab-adapted virus stocks replicate with approximately equivalent kinetics in VERO cells

VERO cells were infected with 4×10^4 pfu (MOI = 0.2) of two representative HSV-1 clinical isolates (10, 19), as well as the molecularly cloned F5 viral stock, and two laboratory-adapted strains (17 and KOS). The cultures were then sampled at selected time points (0, 2, 6, 12, 18, 24, 36, 48 hours), and viral genomic titers in cell lysates were measured by quantitative real-time PCR amplification, using oligonucleotide primers specific for the ICP0 gene. Viral genome titers were normalized in terms of the virus DNA load per 12.5 ng of total input cellular DNA. The results show that the virus strains replicated with essentially indistinguishable kinetics, with the exception of the.

Figure 6. Amplicon stocks packaged by representative primary HSV-1 strains and lab-adapted virus stocks differ in their ability to transduce 293 cells

HEK 293 A cells were transduced at an MOI of 0.1 with HSV-1 amplicon particles packaged by the various isolates, and then cultured in the presence or absence of acyclovir (1 μ M).

Transduced cells were assayed for total β -galactosidase activity 24 hours post infection.

Cleared lysates (1 μ g of total protein) were assayed for activity. Data represent mean values, calculated from three replicate measurements; measurements of β -galactosidase activity were normalized to total protein. Bars denote the standard deviation of the three individual values.

The data show that acyclovir had no significant effect on amplicon-mediated gene expression, when measured at this early time point. the data also show that amplicons packaged by lab-adapted virus strains (KOS, 17) and primary isolate 19 were efficient at transducing 293 cells, whereas amplicons packaged by the molecularly cloned F5 virus and primary isolate 10 were inefficient at transducing this cell line.

Figure 7. Amplicon stocks packaged by representative primary HSV-1 strains and lab-adapted virus stocks differ in their ability to transduce primary dendritic cells

DC were transduced at an MOI of 0.1 with HSV-1 amplicon particles packaged by the various isolate. Transduced cells were assayed for total β -galactosidase activity 24 hours post infection. Cleared lysates (1 μ g of total protein) were assayed for activity. Data represent mean values, calculated from three replicate measurements; measurements of β -galactosidase activity were normalized to total protein. Bars denote the standard deviation of the three individual values. The data show results for DC prepared from two different donors (A, B). It can be readily appreciated that amplicons packaged by the parental, lab-adapted strain 17 and its molecularly cloned counterpart F5 were both poor at transducing DC. In contrast, amplicons packaged by the KOS strain and primary isolate 19 were much more efficient at transducing DC. Note that, for both donors, results for primary isolate 19 are significantly different (better) than those for strain KOS ($p < 0.01$ in both cases; one-way ANOVA with Tukey's post-test).

TABLE 1**Ability of primary HSV-1 isolates to package and propagate amplicon stocks**

Amplicon-containing stocks were generated using each of the various isolates. Wild-type (helper virus) titers were determined by plaque assay on VERO cells (pfu/ml) and amplicon titers were determined using X-gal histochemistry on NIH 3T3 cells (bfu/ml). The ratio of amplicon:helper virus was then determined. The results show that most of the primary isolates were able to efficiently propagate the amplicon plasmid, except for isolates 1 and 10.

ISOLATE	Amplicon : Helper Ratio	Amplicon Titer ($\times 10^7$ bfu/ml)	Helper Titer ($\times 10^7$ pfu/ml)
1	0.086	3	34.8
2	0.363	27	74.4
3	0.704	79.5	113
4	0.560	48	85.8
5	0.680	51	75
6	0.233	24	103
7	0.319	34.5	108
8	0.447	25.5	57
9	0.464	42	90.6
10	0.074	8.4	114
11	0.450	30	66.6
12	0.526	30	57
13	0.443	12.5	28.2
14	0.495	33	66.6
15	0.417	6.75	16.2
16	0.472	30	63.6
17	0.386	28.5	73.8
18	0.490	7.05	14.4
19	1.01	52.5	52.2
F5	0.470	28.5	60.6

TABLE 2**Pair-wise correlation analysis of cell transduction data**

A pairwise correlation analysis of cell transduction data is presented, for the VERO and 293 cell lines, and the primary dendritic cells. The associations between gene expression levels were examined in a pairwise fashion for the three different cell types (as noted in the column headings) using Pearson correlation coefficients (r). The data that were used in these analyses correspond to the datasets shown in Figure 2 and Figure 3 (DC Batch 1). The results show a very strong correlation between amplicon transduction efficiency in the two cultured cell lines (VERO, 293), but weaker correlations between cell line transduction efficiency and the efficiency of amplicon-mediated gene expression in primary dendritic cells (DC).

	293 v VERO	293 v DC	DC v VERO
Correlation coefficient (<i>Pearson r</i>)	0.934	0.525	0.4232
95% confidence interval (<i>for Pearson r</i>)	0.838 to 0.974	0.107 to 0.785	-0.024 to 0.729
P value	< 0.0001	0.018	0.063