Herpes Simplex Virus Clearance During Purification of a Recombinant Adeno-Associated Virus Serotype 1 Vector

Guo-jie Ye, Marina M. Scotti, Darby L. Thomas, Lijun Wang, David R. Knop, and Jeffrey D. Chulay

Abstract

Gene delivery vectors based on adeno-associated virus (AAV) have potential utility for treatment of many genetic disorders. Current AAV vector manufacturing methods employ helper viruses to deliver functions needed to produce replication-defective recombinant AAV (rAAV) vectors, and clearance of infectious helper virus from the drug substance is essential for ensuring the safety of rAAV-based therapies. We have developed a manufacturing method for the production of rAAV vectors using a recombinant herpes simplex virus type 1 (rHSV) complementation system in suspension baby hamster kidney cells. The manufacturing process includes three primary unit operations, detergent lysis of the cell harvest and two downstream column chromatography steps, which achieve viral clearance. These unit operations inactivate and remove HSV, including replication-competent HSV present at low levels in rHSV helper stocks. Here we report results quantifying the reduction in HSV achieved during rAAV vector purification. Clearance of HSV was at least 6.84 log\textsubscript{10} with 1% Triton X-100, 4.34 log\textsubscript{10} with CIM Q column chromatography, and 2.86 log\textsubscript{10} with AVB affinity chromatography. Combined, these three orthogonal methods achieved clearance of at least 14.04 log\textsubscript{10} of HSV. The total input quantity of rHSV in a 100-liter production batch is approximately 1.2 \cdot 10^{12} plaque-forming units (pfu), and after purification, the concentration of residual rHSV in the resulting drug substance of approximately 450 ml would be less than 2.42 \cdot 10^{-5} pfu/ml. A rAAV vector produced using this method was used in a clinical trial in which subjects receive up to 100 intramuscular injections of 1.35 ml each, which would contain a maximum of 3.27 \cdot 10^{-3} pfu of HSV. These results support the safety of rAAV vectors produced using our rHSV complementation method.

Introduction

Evaluation and characterization of virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. The objective of viral clearance studies is to assess process steps that can be considered effective in inactivating or removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. According to an International Conference on Harmonization guidance document, this should be achieved by the deliberate addition (“spiking”) of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps (ICH Expert Working Group, 1999).

Applied Genetic Technologies Corporation (AGTC, Alachua, FL) has developed a manufacturing method for production and purification of recombinant adeno-associated virus (rAAV) vectors using a recombinant herpes simplex virus (rHSV) complementation system in suspension baby hamster kidney (sBHK) cells (Thomas et al., 2009). The system employs two ICP27-deficient rHSV helper viruses, one containing the AAV2 \textit{rep} and AAVX \textit{cap} genes (where X is any AAV serotype, e.g., rHSV-rep2/cap1 contains the AAV1 \textit{cap} gene), and the second containing a gene expression cassette comprised of the AAV2 inverted terminal repeat sequences flanking the transgene of interest and appropriate regulatory elements (e.g., a human alpha-1 antitrypsin gene in rHSV-CB-hAAT). The rHSV helper viruses are ICP27 deficient, rendering them replication-incompetent in normal cells because of a deletion in the essential \textit{UL54} (ICP27-encoding) gene; however, they can be propagated in V27 cells (Vero cells stably transformed with the \textit{UL54} gene), as previously described (Kang et al., 2009; Thomas et al., 2009). This rHSV helper virus system efficiently provides all \textit{cis-} and \textit{trans-} acting AAV components as well as the requisite helper
functions for generation of rAAV. After production of rAAV in sBHK cells by co-infection with both rHSV helper viruses, downstream unit operations include lysis of the infected producer cells to liberate rAAV particles, incubation with Benzonase to digest unpackaged host and viral DNA, concentration and buffer exchange, column chromatography operations, and tangential flow filtration concentration and buffer exchange (Fig. 1).

During expansion of rHSV helper viruses in cell culture, a potential undesirable byproduct is generation of replication-competent HSV (rcHSV) because of recombination between the partial ICP27 gene sequence in the rHSV helper virus and the ICP27 gene in the complementing V27 cells. This can result in low levels of ICP27-revertant rcHSV, which might then further propagate during rAAV production and carry through into the unprocessed bulk product.

In order to ensure the safety of rAAV gene therapy products, both rcHSV and replication-incompetent rHSV in the unprocessed bulk must be inactivated and/or removed during the harvest and purification processes that generate the rAAV drug substance. The cell harvest/detergent lysis and downstream column chromatography operations provide three orthogonal methods to inactivate and remove all HSV, including rcHSV and replication-incompetent rHSV. Specifically, during cell harvest operations a nonionic detergent (Triton X-100) is added to lyse infected cells and release the rAAV particles for subsequent processing. A consequence of this detergent lysis is inactivation of enveloped, infectious rHSV. In addition, efficient removal of HSV and other contaminants, including HSV protein and DNA, from the rAAV product is achieved by selective column chromatography steps using a CIM Q Monolith anion exchanger column coupled with an AVB Sepharose affinity column (Thomas et al., 2009). In the current study, we quantified the inactivation and removal of wild-type HSV KOS 1.1, as a worst-case surrogate for rcHSV.

Thaw and expand sBHK cells from WCB
↓
Infect with two rHSV helper viruses
↓
Harvest by detergent lysis, digest free DNA, increase NaCl concentration
↓
Clarification by depth filtration and filtration
↓
Concentration by TFF and buffer exchange to 0.5 M NaCl by diafiltration
↓
Filtration through a sterilizing grade filter, store at 2-8°C overnight
↓
CIM Q Monolith anion exchange chromatography, store at -80°C or proceed
↓
AVB affinity chromatography
↓
Eluate = pre-pool purified bulk
↓
Sample and store frozen at -80°C
↓
Thaw and pool batches of pre-pool purified bulk
↓
Concentrate and diafilter with formulation buffer = drug substance
↓
Sterile filter and dispense to vials = drug product

FIG. 1. Process flow diagram for production of the rAAV1-CB-hAAT drug product. A working cell bank (WCB) of suspension baby hamster kidney (sBHK) cells is expanded in cell culture and then infected with two recombinant herpes simplex virus (HSV) helper viruses as described in the text. After overnight culture, the cells are lysed with Triton X-100 detergent, DNA not contained within viral capsids is digested with Benzonase, and NaCl is added. After two filtration steps and tangential flow filtration (TFF) to the appropriate buffer, product is filtered and subjected to CIM Q anion exchange chromatography followed by AVB affinity chromatography. Eluates from multiple batches are pooled, concentrated by TFF, diafiltered, and then sterile filtered through a 0.2 μm filter to generate the drug product.
replication-incompetent rHSV, by the processes used to recover and purify the rAAV1-CB-hAAT vector that was evaluated in a recently completed clinical trial (Flotte et al., 2011; Mueller et al., 2013). Inactivation and removal of HSV was assessed at specific purification steps throughout the manufacturing processes, and the cumulative clearance of the process was calculated.

Materials and Methods

Supplementary Data are available online at www.liebertpub.com/humc

Results

Detection of low levels of rcHSV in rHSV stocks

V27 cells harbor a 2443 base pair (bp) BamHI-HpaI fragment of HSV DNA that contains the ICP27 gene and flanking sequences. ICP27-deficient rHSV helper viruses, which have a deletion of a 1629 bp BamHI-Stul fragment including most of the ICP27 gene sequence, contain a truncated ICP27 gene and flanking sequence that overlaps the sequence in V27 cells by 814 bp. This creates the potential for the rHSV to acquire the ICP27 gene sequence from the V27 cells by homologous recombination events during virus propagation. In preliminary experiments, the presence of rcHSV in rHSV helper virus stocks was evaluated by adding $3 \times 10^8$ plaque-forming units (pfu) of rHSV from each of 17 separate lots to monolayers of Vero cells at an MOI of 10 followed by two serial passages on Vero cells (see Supplementary Table S1). A single aliquot from six lots was tested for rcHSV, of which one was positive and five were negative. Duplicate aliquots from 11 lots were tested for rcHSV, of which both aliquots were positive for 4 lots, both aliquots were negative for 1 lot, and one aliquot was positive and the other aliquot was negative for 6 lots.

The rcHSV from one batch (rc921) was isolated by plaque purification. Expression of the immediate-early HSV proteins ICP0 and ICP27 and the late HSV capsid protein ICP5 was evaluated in Vero cells infected with rc921 or with a control rHSV stock that had previously tested negative for rHSV. ICP27 protein expression and viral DNA replication are required for subsequent expression of the late HSV capsid protein ICP5, but not for expression of the immediate-early HSV protein ICP0. In Vero cells, only ICP27-revertant or wtHSV will express the ICP5 protein. As shown in Supplementary Fig. S1, Vero cells infected with rc921 expressed ICP0, ICP27, and ICP5, while cells infected with replication-incompetent rHSV expressed ICP0 but not ICP27 or ICP5. This indicated that rc921 is an ICP27-revertant rHSV and confirmed that the rcHSV present at low levels in rHSV helper virus stock acquired replication competence during passage in V27 cells because of a recombination event between the ICP27 gene and flanking sequences in the V27 cell and the residual truncated ICP27 gene and flanking sequences in the rHSV virus.

Viral inactivation by Triton X-100 detergent

The time course of the effect of 1% Triton X-100 detergent on inactivation of HSV at 37°C is summarized in Table 1. HSV titers were determined by culture on V27 cells, which support the growth of both rcHSV and replication-incompetent rHSV. In the unprocessed bulk generated as shown in Figure 2, the HSV titer before addition of wild-type HSV was $2.8 \times 10^6$ pfu/ml with very small plaques, typical of plaques formed by recombinant HSV and not revertant HSV that acquired a functional ICP27 gene. The HSV titer in the unprocessed bulk after spiking with wtHSV was $4.65 \times 10^6$ pfu/ml, with only a modest decrease in titer after 30 or 120 min at room temperature. After removal of 1 ml for HSV assay and addition of 1 ml of 10% Triton X-100, the theoretical HSV titer was $0.9 \times 4.65 \times 10^6 = 4.18 \times 10^5$ pfu/ml. However, complete inactivation of HSV in the Triton X-100-treated unprocessed bulk was observed at every time point after addition of Triton X-100. The sample in which a nominal 3 pfu of HSV was added to a 1:500 dilution of Triton X-100-treated unprocessed bulk gave an estimated titer of 2.3 pfu/ml, and each of two 25 ml aliquots of a 1:500 dilution of Triton X-100-treated unprocessed bulk tested 30 and 120 min after spiking with wild-type HSV had no pfu detected. Therefore, the titer in the Triton X-100-treated unprocessed bulk at 30 and 120 min after spiking with wtHSV was <3 pfu per 0.05 ml or <60 pfu/ml.

From these data we can calculate that HSV clearance achieved by treatment with 1% Triton X-100 was at least $\log_{10} (4.18 \times 10^6/60) = 6.84 \log_{10}$.

Viral clearance by CIM Q column chromatography

Using the mock DF pool generated as shown in Figure 2, HSV clearance during CIM Q column chromatography was

<table>
<thead>
<tr>
<th>Sample</th>
<th>pfu</th>
<th>Initial dilution</th>
<th>Titering dilution</th>
<th>Volume tested (ml)</th>
<th>pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed bulk, $t = -10$ min</td>
<td>30, 26$^b$</td>
<td>1</td>
<td>$10^5$</td>
<td>1</td>
<td>$2.80 \times 10^4$</td>
</tr>
<tr>
<td>Unprocessed bulk, $t = 30$ min</td>
<td>194, 191$^b$</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>$1.93 \times 10^4$</td>
</tr>
<tr>
<td>Unprocessed bulk, $t = 120$ min</td>
<td>121, 124$^b$</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>$1.23 \times 10^4$</td>
</tr>
<tr>
<td>HSV-spiked unprocessed bulk</td>
<td>47, 46</td>
<td>1</td>
<td>$10^7$</td>
<td>1</td>
<td>$4.65 \times 10^6$</td>
</tr>
<tr>
<td>HSV-spiked TX100-treated unprocessed bulk, $t = 0.1$ min</td>
<td>0, 0</td>
<td>300</td>
<td>1</td>
<td>1</td>
<td>&lt;1500</td>
</tr>
<tr>
<td>HSV-spiked TX100-treated unprocessed bulk, $t = 30$ min</td>
<td>0, 0</td>
<td>500</td>
<td>1</td>
<td>$2 \times 25$</td>
<td>&lt;60</td>
</tr>
<tr>
<td>HSV-spiked TX100-treated unprocessed bulk, $t = 120$ min</td>
<td>0, 0</td>
<td>500</td>
<td>1</td>
<td>$2 \times 25$</td>
<td>&lt;60</td>
</tr>
<tr>
<td>3 pfu/well in 500-fold diluted TX100-treated unprocessed bulk</td>
<td>3, 2, 2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>5 pfu/well in 500-fold diluted TX100-treated unprocessed bulk</td>
<td>5, 12, 9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8.7</td>
</tr>
</tbody>
</table>

$^a$Replicate values of plaque-forming units (pfu) for each sample tested on V27 cells.

$^b$Plaques very small, indicative of rHSV, not replication-competent (revertant) HSV.

rHSV, recombinant herpes simplex virus.
Table 2. Clearance of HSV by CIM Q Column Chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial dilution</th>
<th>Titering dilution</th>
<th>Volume tested (ml)</th>
<th>pfu(^a)</th>
<th>pfu/ml</th>
<th>Volume (ml)</th>
<th>Total pfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-spiked Q column load</td>
<td>1</td>
<td>10(^6)</td>
<td>1</td>
<td>64, 50</td>
<td>5.70x10(^7)</td>
<td>1299</td>
<td>7.40x10(^{10})</td>
</tr>
<tr>
<td>Q peak</td>
<td>4</td>
<td>10(^2)</td>
<td>1</td>
<td>227, 206</td>
<td>8.66x10(^4)</td>
<td>39</td>
<td>3.38x10(^6)</td>
</tr>
</tbody>
</table>

\(^a\)Replicate values of plaque-forming units (pfu) for each sample tested on V27 cells.

evaluated using an 8 ml CIM Q column, with all other conditions maintained as closely as possible to those used in the cGMP process, in which 80 ml CIM Q columns are used. During the study, the CIM Q column load was controlled for volume, conductivity, and total protein. A mock DF pool matrix was generated as described in the Materials and Methods and spiked with wtHSV. An aliquot was removed and used for HSV assay, and the remaining ~1300 ml underwent CIM Q column chromatography. The Q peak was collected by step elution in a volume of 39 ml, a sample of which was used for HSV pfu titering starting at a 1:4 dilution with DMEM.

As shown in Table 2, the total amount of HSV in the CIM Q column load was 7.40x10\(^{10}\) pfu and the total amount of HSV in the Q peak was 3.38x10\(^6\) pfu. From these data we can calculate that the HSV clearance achieved by the CIM Q column chromatography was log\(_{10}\) (7.40x10\(^{10}\) / 3.38x10\(^6\)) = 4.34 log\(_{10}\).

**Viral clearance by AVB affinity chromatography**

Using the peak from the CIM Q anion exchange chromatography generated as shown in Figure 2, HSV clearance during AVB affinity chromatography was evaluated in two replicate experiments using a 5 ml AVB column, with all other conditions maintained as closely as possible to those used in the GMP process, in which a 150 ml AVB column is used (Table 3). In the first experiment, 35 ml of the thawed Q peak matrix was mixed with 3.9 ml of wtHSV, an aliquot was removed for HSV assay, and the remaining ~38 ml underwent AVB affinity chromatography. The AVB peak was collected by step elution in a volume of 13.8 ml, a sample of which was used for the HSV pfu titering assay starting at a 1:3 dilution with DMEM. The second experiment was identical except that 3.3 ml of wild-type HSV was added to a 30 ml aliquot of Q peak matrix and the AVB peak was collected by step elution in a volume of 10.5 ml, a sample of which was used for the HSV pfu assay starting at a 1:4 dilution with DMEM.

In the first experiment the total amount of HSV in the AVB column load was 1.76x10\(^{10}\) pfu and the total amount of HSV in the AVB peak eluate was 2.43x10\(^{7}\) pfu. From these data we can calculate that the HSV clearance achieved by AVB column chromatography was log\(_{10}\) (1.76x10\(^{10}\) / 2.43x10\(^{7}\)) = 2.86 log\(_{10}\).

In the second experiment the total amount of HSV in the AVB column load was 1.29x10\(^{10}\) pfu and the total amount of HSV in the AVB peak eluate was 4.51x10\(^{6}\) pfu. From these data we can calculate that the HSV clearance achieved by AVB column chromatography was log\(_{10}\) (1.29x10\(^{10}\) / 4.51x10\(^{6}\)) = 3.46 log\(_{10}\).

The HSV clearance achieved by the AVB column chromatography step is therefore at least 2.86 log\(_{10}\) (the smaller value from the two experiments).

**Discussion**

rAAV vectors have previously demonstrated their utility in the development of gene-based therapies (Mueller and Flotte, 2008; Ortolano et al., 2012) and have been evaluated in 117 gene therapy clinical trials as of June 2014 (Gene Therapy Clinical Trials Worldwide, 2014). These trials address a broad array of clinical indications, including ocular diseases, cancer, muscular dystrophy, Parkinson’s disease, cardiovascular disorders, immune disorders, and a variety of diseases resulting from single-gene defects. Despite their potential promise, the development of rAAV-based gene therapies has been hampered in part because of limitations in manufacturing these vectors at a scale sufficient to support preclinical and clinical development (Clement et al., 2009; Kotin, 2011).

Previous reports have shown that our rHSV complementation system utilizing suspension-adapted mammalian cells provides a scalable method for producing rAAV vectors capable of supporting clinically relevant expression levels of wild-type human alpha-1 antitrypsin (hAAT). We have previously reported that the methods used to purify rAAV1-CB-hAAT are highly efficient in removing HSV DNA (Ye et al., 2011). Although the absence of rHSV is a release criterion for each clinical batch of rAAV vector and we have never detected rHSV in any process intermediate after the initial detergent lysis step used to recover the

Table 3. Clearance of HSV by AVB Affinity Chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial dilution</th>
<th>Titering dilution</th>
<th>Volume tested (ml)</th>
<th>pfu(^a)</th>
<th>pfu/ml</th>
<th>Volume (ml)</th>
<th>Total pfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-spiked AVB column load</td>
<td>10</td>
<td>10(^6)</td>
<td>1</td>
<td>52, 39, 48</td>
<td>4.63x10(^8)</td>
<td>37.9</td>
<td>1.76x10(^{10})</td>
</tr>
<tr>
<td>AVB peak</td>
<td>3</td>
<td>10(^3)</td>
<td>1</td>
<td>567, 602, 593</td>
<td>1.76x10(^6)</td>
<td>13.8</td>
<td>2.43x10(^7)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-spiked AVB column load</td>
<td>10</td>
<td>10(^6)</td>
<td>1</td>
<td>37, 44, 35</td>
<td>3.87x10(^8)</td>
<td>33.3</td>
<td>1.29x10(^{10})</td>
</tr>
<tr>
<td>AVB peak</td>
<td>4</td>
<td>10(^3)</td>
<td>1</td>
<td>96, 113, 113</td>
<td>4.29x10(^5)</td>
<td>10.5</td>
<td>4.51x10(^6)</td>
</tr>
</tbody>
</table>

\(^a\)Replicate values of plaque-forming units (pfu) for each sample tested on V27 cells.
vector, it was important to demonstrate that any residual rHSV or rcHSV could be removed or inactivated during vector purification.

Preliminary experiments confirmed that propagation of ICP27-deleted rHSV in V27 cells can generate very low levels of rcHSV when rHSV acquires the ICP27 gene through homologous sequence recombination events during propagation. The ratio of rHSV to replication-incompetent rHSV is extremely low, since testing $3 \times 10^5$ pfu of rHSV by serial passage on Vero cells detected rHSV in less than half of the lots of rHSV tested, and testing duplicate aliquots from individual lots often detected rcHSV in only one of two aliquots. Each assay contained a positive control flask to which 3 pfu of wtHSV was added and the positive control was always positive. Based on the Poisson distribution, an aliquot of a suspension containing an average of 3 pfu per aliquot would have a 95% probability of containing at least 1 pfu per aliquot and an aliquot of a suspension containing an average of 0.7 pfu per aliquot would have a 50% probability of containing at least 1 pfu per aliquot. This suggests that the average level of rcHSV in rHSV stocks is less than 1 pfu per $3 \times 10^5$ pfu.

Although the level of rcHSV in rHSV stocks is very low, it is important to ensure complete removal or inactivation of any rcHSV that might be present. Results of this viral clearance study demonstrate that the three orthogonal methods employed during harvest and purification of the drug substance achieved a cumulative clearance of at least $6.84 + 4.34 + 2.86 = 14.04 \log_{10}$ of rHSV. From this total clearance value, a simple deterministic risk assessment of the theoretical residual level of infectious rHSV in a single dose of rAAV1-CB-hAAT can be made. The total input quantity of rHSV in a 100-liter production batch is about $1.2 \times 10^7$ pfu/ml $\times 100$ liters $= 1.2 \times 10^{12}$ pfu. A $14.04 \log_{10}$ reduction of this value is $1.2 \times 10^{12} \div 10^{14.04} = 1.09 \times 10^{-2}$ pfu remaining in the final formulated bulk product. This is a maximum value, assuming that no other titer reduction is contributed by other steps during the purification process, such as the final 0.2 μm filtration of drug substance to generate the drug product.

The volume of the drug substance derived from a 100-liter production batch is about 450 ml, and the maximum concentration of residual rHSV is therefore $1.09 \times 10^{-2} \div 450 = 2.42 \times 10^{-5}$ pfu/ml. The volume of the highest single dose administered to a patient in the clinical trial of rAAV1-CB-hAAT was 100$\times$1.35 ml (Flotte et al., 2011), which therefore contained a maximum of $135 \times 2.42 \times 10^{-5} = 3.27 \times 10^{-3}$ pfu of residual rHSV.

However, this is a worst-case estimation based on the maximum theoretical level of residual rHSV, and it does not account for loss of input rHSV during culture of rHSV-infected cells before harvest of the unprocessed bulk. Measurement of infectious rHSV in development batches of unprocessed bulk has shown that the titer of residual rHSV is $<10^5$ pfu/ml (>$100$-fold lower than the input rHSV), or $<1 \times 10^{10}$ pfu per 100 liters (data not shown). A $14.04 \log_{10}$ reduction of this value is $<1 \times 10^{10} \div 10^{14.04} = <9.12 \times 10^{-5}$ pfu in 450 ml = $<2.03 \times 10^{-7}$ pfu/ml, which is likely more representative of the actual manufacturing scenario. This would imply that the highest single dose of 100$\times$1.35 ml administered to a patient would contain $<135 \times 2.03 \times 10^{-7} = <2.74 \times 10^{-5}$ pfu of rHSV.

It is important to note that the unprocessed bulk product had low titers of HSV with a small plaque size (Table 1), which is characteristic of replication-incompetent rHSV and not rcHSV. HSV is an enveloped virus and, consequently, the initial treatment with Triton X-100, Benzonase, and additional NaCl followed by lysis used during vector purification. Any rcHSV that was present in the unprocessed bulk but below the limit of detection by plaque assay would be inactivated by the initial treatment with 1% Triton X-100. Any rcHSV that was present in the unprocessed bulk but below the limit of detection by plaque assay would be inactivated by the initial treatment with 1% Triton X-100, and would be further removed during CIM Q anion exchange chromatography and AVB affinity chromatography, providing a very robust safety margin to ensure that no rcHSV is present in the final product.

Regulatory agencies have not issued guidance on the maximum acceptable amount of replication-competent helper virus that can be present in rAAV vector preparations, but a limit of <1 pfu of replication-competent adenovirus in $3 \times 10^{10}$ vector particles has been proposed for adenovirus vectors (U.S. Food and Drug Administration, 2001). The maximum amount of residual HSV expected in a single rAAV dose of 100$\times$1.35 ml containing $5.4 \times 10^{14}$ AAV vector particles is $3.27 \times 10^{-3}$, or <1 rcHSV in $3 \times 10^{12}$ vector particles, and any residual HSV would likely be noninfectious as a result of the Triton X-100 detergent lysis used during vector purification.

**FIG. 2.** Process flow diagram for matrix generation. Suspension baby hamster kidney (sBHK) cells were expanded in cell culture; infected with two recombinant herpes simplex virus (HSV) helpers, rHSV-rep2/cap1 and rHSV-CB-hAAT; and cultured overnight. A 50 ml aliquot was used for a study of the time course of HSV inactivation by detergent, and 2 liters was used to generate a mock diafiltration (DF) pool that was used to evaluate HSV clearance by the CIM Q anion exchange chromatography step as described in the text. The remainder underwent subsequent processing using the methods shown in Fig. 1, including harvesting by treatment with Triton X-100, Benzonase, and additional NaCl followed by tangential flow filtration (TFF), DF, and CIM Q anion exchange chromatography. The peak from the CIM Q anion exchange chromatography (Q peak) was used to evaluate HSV clearance by AVB column chromatography. Additional details are provided in the Supplementary data available online at www.liebertpub.com/humc.
A limitation of this study is our inability to precisely quantify the level of clearance achieved in the Triton X-100 detergent lysis step, because no residual HSV was detected after this treatment. The concentration of our HSV stocks was $3.7 \times 10^9$ pfu/ml, and clearance in excess of the 6.84 log$_{10}$ reported here might have been achieved had we used a more concentrated HSV stock.

In summary, the use of Triton X-100 detergent lysis, CIM Q column chromatography, and AVB affinity chromatography effectively inactivates and removes HSV particles from rAAV vectors manufactured using an rHSV complementation system in sBHK cells. This viral clearance protocol resulted in greater than a 14.04 log$_{10}$ reduction in HSV, corresponding to a maximum of $3.27 \times 10^{-3}$ pfu of rcHSV or replication-incompetent HSV in a single patient dose of $>5 \times 10^{14}$ vg under the worst-case estimation, and a maximum of $<3.69 \times 10^{-5}$ pfu of replication-incompetent HSV when loss of input rHSV during culture of rHSV-infected cells is taken into consideration. These findings suggest that patients receiving rAAV vectors purified in this manner have a very low risk of HSV-related adverse events, and support the continued development of these vectors in the treatment of human disease.

Author Disclosure Statement

All authors have previously been employed by, or are currently employed by, Applied Genetic Technologies Corporation and own shares or share options in the company, and have a conflict of interest to the extent that this work potentially increases their personal financial interests.

References


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