

Current Good Manufacturing Practice Production of an Oncolytic Recombinant Vesicular Stomatitis Viral Vector for Cancer Treatment

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Abstract

Vesicular stomatitis virus (VSV) is an oncolytic virus currently being investigated as a promising tool to treat cancer because of its ability to selectively replicate in cancer cells. To enhance the oncolytic property of the nonpathogenic laboratory strain of VSV, we generated a recombinant vector [rVSV(MΔ51)-M3] expressing murine gammaherpesvirus M3, a secreted viral chemokine-binding protein that binds to a broad range of mammalian chemokines with high affinity. As previously reported, when rVSV(MΔ51)-M3 was used in an orthotopic model of hepatocellular carcinoma (HCC) in rats, it suppressed inflammatory cell migration to the virus-infected tumor site, which allowed for enhanced intratumoral virus replication leading to increased tumor necrosis and substantially prolonged survival. These encouraging results led to the development of this vector for clinical translation in patients with HCC. However, a scalable current Good Manufacturing Practice (cGMP)-compliant manufacturing process has not been described for this vector. To produce the quantities of high-titer virus required for clinical trials, a process that is amenable to GMP manufacturing and scale-up was developed. We describe here a large-scale (50-liter) vector production process capable of achieving crude titers on the order of 10^9 plaque-forming units (PFU)/ml under cGMP. This process was used to generate a master virus seed stock and a clinical lot of the clinical trial agent under cGMP with an infectious viral titer of approximately 2×10^{10} PFU/ml (total yield, 1×10^{13} PFU). The lot has passed all U.S. Food and Drug Administration-mandated release testing and will be used in a phase 1 clinical translational trial in patients with advanced HCC.

Introduction

ONCOLYTIC VIROTHERAPY is emerging as a promising strategy to treat cancer because of the unique ability of these replication-competent viruses to selectively replicate only in cancer cells. Early oncolytic viruses shown to be effective against solid tumors in laboratory animals were derived from adenovirus or herpes simplex virus type 1, and hundreds of patients with various types of solid tumors have already been treated in various clinical translational trials (Barker and Berk, 1987; Martuza *et al.*, 1991; Whitley *et al.*, 1993; Mineta *et al.*, 1994, 1995; Bischoff *et al.*, 1996; Heise *et al.*, 1997; Wickham *et al.*, 1997; Hallenbeck *et al.*, 1999; Miyatake *et al.*, 1999; Alemany *et al.*, 2000; Fueyo *et al.*, 2000; Ganly *et al.*, 2000; Khuri *et al.*, 2000; Nemunaitis *et al.*, 2000; Rainov and Ren, 2003; Kasuya *et al.*, 2005). Subsequently, a number of

other animal and human viruses are being developed as potential oncolytic agents for cancer treatment, including reovirus, Newcastle disease virus, measles virus, vaccinia virus, influenza virus, and vesicular stomatitis virus (VSV), among others (Kirn *et al.*, 2001). Despite their efficacy in animal models, a major limitation to the successful use of oncolytic viruses to treat cancer in patients is their relatively poor replication efficiency and limitations in spreading within the solid tumor mass *in vivo*, which typically contains complex stromal structures interspersed with the tumor cells. To overcome these obstacles, more virulent oncolytic viruses that retain tumor specificity and/or oncolytic viruses molecularly engineered to express transgenes that exert bystander effects for enhanced tumor cell killing have been used (Hardcastle *et al.*, 2007; Singleton *et al.*, 2007; Thorne *et al.*, 2007; Gainey *et al.*, 2008; Kuhn *et al.*, 2008; Raykov *et al.*, 2008; Xie *et al.*, 2009;

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Ziauddin *et al.*, 2010). Another limitation is the neutralizing antiviral antibody responses that are elicited within several days in immune-competent hosts, which shortens the window of opportunity for intratumoral replication. This can be addressed through the use of viruses such as VSV with a short life cycle (8–10 hr), which permits robust intratumoral virus replication before the onset of high-titer neutralizing antibodies (Shinozaki *et al.*, 2005).

VSV is an enveloped, single-strand RNA virus belonging to the family Rhabdoviridae, genus *Vesiculovirus*, with 16 distinct serotypes, of which 6 can cause animal or human disease (Rose and Whitt, 2001). A high percentage of people living in endemic areas such as the central and southwestern United States and Canada may have been infected. Infections in humans are asymptomatic in most cases or result in a mild febrile illness, oral vesicular lesions, malaise, and pharyngitis (Fields and Hawkins, 1967; Letchworth *et al.*, 1999). Two cases of VSV meningoencephalitis have been reported in children (Quiroz *et al.*, 1988). The envelope G-protein of VSV binds to the surface of most insect and mammalian cell types, accounting for the wide tissue tropism of VSV. VSV replicates in the cytoplasm of infected cells, which die within hours as a result of robust viral mRNA and protein synthesis. Viral replication is inhibited in normal cells because of their responses to type I interferons (IFNs), thereby sparing the cell from cytopathic destruction. In tumor cells, however, viral replication is uninhibited because of cellular defects in the interferon response pathway, resulting in apoptotic cell death (Stojdl *et al.*, 2000a). This selective oncolytic property of VSV makes it a potentially effective agent for selective anti-tumor treatment (Giedlin *et al.*, 2003). Treatment of human melanoma xenografts with wild-type VSV in nude mice resulted in regression or growth inhibition of the established tumor (Stojdl *et al.*, 2000a). This finding has been postulated to be due to the fact that IFN-responsive antiviral pathways are defective in many tumors including those of human origin, and thus VSV can replicate within these cells regardless of IFN treatment (Stojdl *et al.*, 2000b). Viruses such as VSV and Newcastle disease virus (NDV) activate type I interferon responses in normal cells, which in turn induce protein kinase R (PKR) phosphorylation and inhibition of protein synthesis and viral replication (Kirn *et al.*, 2001; Kasuya *et al.*, 2005). Most tumor cells are defective in type I interferon response to various degrees and PKR remains underphosphorylated, permitting protein synthesis and viral replication to proceed.

To enhance the oncolytic property of the laboratory strain of VSV (Indiana) that is nonpathogenic to humans (Sudia *et al.*, 1967), we generated a recombinant vector [rVSV(MΔ51)-M3] expressing the secreted form of the murine gammaherpesvirus M3 protein, a viral chemokine-binding protein that binds to a broad range of chemokines (C, CC, CXC, and CX3C) with high affinity (Parry *et al.*, 2000; van Berkel *et al.*, 2000). When rVSV(MΔ51)-M3 was used in our hepatocellular carcinoma (HCC) animal model, it was shown to block inflammatory cell migration to the tumor site, which allowed for enhanced intratumoral virus replication leading to increased tumor necrosis and substantially prolonged survival (Wu *et al.*, 2008). These encouraging results led us to develop this vector for potential use in treating patients with HCC.

The current manufacturing process has been used under Good Manufacturing Practice (GMP) conditions to produce a

clinical lot of rVSV(MΔ51)-M3 with an infectious titer of approximately 2×10^{10} plaque-forming units (PFU)/ml (total yield, 1×10^{13} PFU). The lot is intended for use in phase 1 human clinical trials and has been characterized using the panel of testing described in this paper. The manufacturing methods used for vector production are amenable to further scale-up to provide larger quantities of the vector for future expanded clinical trials.

Materials and Methods

Growth of 293 cells

A vial of 293 cells from a fully characterized 293 master cell bank was thawed and expanded in culture. Cells were seeded at $2.5\text{--}4.0 \times 10^4$ cells/cm² in Nunc flasks of increasing size (T-75 and T-500) or 10-layer Cell Factories (Nunc/Thermo Fisher Scientific, Rochester, NY). Cultures were expanded in growth medium consisting of 10% fetal bovine serum (FBS; HyClone, Logan, UT) in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, East Rutherford, NJ) and passaged every 3–4 days by the addition of trypsin (Irvine Scientific, Santa Ana, CA).

Production of crude rVSV vector

293 cells were seeded in a 10-layer Nunc Cell Factory (Nunc/Thermo Fisher Scientific) at a density of 7.5×10^4 cells/cm² in DMEM (Biowhittaker) with 10% FBS (HyClone). Approximately 24 hr after seeding, the cell density was determined in one of the culture vessels to calculate the amount of seed virus required for infection at a multiplicity of infection (MOI) of 0.01. Before infection, the DMEM–10% FBS medium was removed and replaced with fresh virus production–serum-free medium (VP-SFM; Invitrogen, Carlsbad, CA) containing the appropriate amount of seed virus. The supernatant containing the rVSV vector was harvested approximately 24 hr after infection.

Clarification of crude virus supernatant

The harvested crude viral supernatant was clarified with a filter train, which consisted of a Milligard LPB (low protein-binding) Opticap XL-4 dual membrane (1.2- μm /0.5- μm) filter in-line with a Milligard LPB Opticap XL-4 single-layer (0.2- μm) filter (Millipore, Billerica, MA), for up to 10 liters of crude viral supernatant. Benzonase (EMD Chemicals, Gibbstown, NJ) was added to the clarified supernatant at a concentration of 50 U/ml to digest host cell nucleic acids. Digestion was carried out at 37°C for 4 hr. After digestion, the supernatant was held at 4°C overnight before purification by column chromatography.

Chromatographic purification

Benzonase-treated supernatant was purified by anion-exchange chromatography, using a Poros HQ-50 (Applied Biosystems/Invitrogen, Foster City, CA) packed column. A 5×20 cm column was used to process 10 liters of culture at a linear flow rate of 1.5 cm/min. The column was equilibrated with 150 mM NaCl in 20 mM Tris, pH 7.5, before loading. After loading, the column was washed with 4 column volumes of equilibration buffer until the absorbance at 260 nm returned to baseline. A second wash step was performed

with 4 column volumes of 300 mM NaCl in 20 mM Tris, pH 7.5. The elution step was performed with 1.2 M NaCl in 20 mM Tris, pH 7.5. Ultraviolet (UV) absorbance at 260 nm was used to monitor and collect the elution peak, typically between 300 and 370 ml.

Tangential flow ultrafiltration, formulation, and sterile filtration

The column eluate was diluted to 2 liters, using exchange buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA; pH 7.8), and then concentrated to 400 ml by tangential flow filtration (TFF) (GE Healthcare Life Sciences, Little Chalfont, UK). Next, the eluate was diafiltered in continuous mode, using 2.4 liters of exchange buffer for a 6-fold buffer exchange. The diafiltered material was further concentrated from 400 ml to approximately 100 ml, representing an approximately 90-fold volume reduction from the original supernatant. The concentrated bulk was prepared by adding glycerol to the diafiltered material to obtain a final concentration of 10% glycerol. The concentrated bulk was stored at -80°C until pooling of concentrated bulk subbatches for final sterile filtration.

Plaque-forming assay

Six-well plates were seeded with BHK-21 cells in DMEM–10% FBS at $2\text{--}4 \times 10^5$ cells per well and incubated overnight at $37 \pm 2^{\circ}\text{C}$ with $5 \pm 2\%$ CO_2 . Serial dilutions of the test article were performed in Dulbecco's phosphate-buffered saline with calcium and magnesium. The culture medium was aspirated and 1 ml of diluted test article was added to each well in triplicate. The plates were incubated for 30 min at $37 \pm 2^{\circ}\text{C}$ with $5 \pm 2\%$ CO_2 . At the end of the incubation period, inoculum was aspirated and the wells were overlaid with a mixture of equal volumes of 1% agarose and 2×minimal essential medium (MEM)–15% FBS and incubated overnight at $37 \pm 2^{\circ}\text{C}$ with $5 \pm 2\%$ CO_2 . Plaques were counted after overnight incubation. The virus concentration was determined by taking the average number of plaques per well, at the dilution that gave rise to 5–50 plaques per well, and then multiplying by the corresponding dilution factor. The assay was performed three times to obtain the final titer.

End-labeling assay

Forty microliters of rVSV(Δ M51)-M3 were heat inactivated at 65°C for 30 min. A reaction mixture containing equal volumes of each radiolabeled nucleotide (dCTP, dGTP, dATP, and dTTP) (MP Biomedicals, Solon, OH), Klenow enzyme (New England BioLabs, Ipswich, MA), and 10×buffer was added to each reaction tube. The original specific activity for each nucleotide was 3000 Ci/mmol and 10 mCi/ml. The contents were mixed by vortexing and the liquid was brought to the bottom of the tube by brief centrifugation at 5000 rpm. The reaction tubes were incubated at 25°C for 30 min. The end-labeling reaction was stopped by adding 10 μl of 0.5 M EDTA and then 0.5 μl of 10% sodium dodecyl sulfate (SDS) and 5 μl of 6×loading dye were added. The unincorporated radionucleotides were removed with a MicroSpin (G-50) spin column (GE Healthcare Life Sciences). The flowthrough was loaded onto a 3% agarose gel and run

for approximately 1.5 hr at 100 V. The dried gel was exposed to a storage phosphor screen (GE Healthcare Life Sciences) overnight in a Kodak film cassette (Carestream Health, Rochester, NY). The exposed storage phosphor screen was scanned with a Storm 860 PhosphorImager (Molecular Dynamics/GE Healthcare Life Sciences) and software.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

A 10% polyacrylamide gel (1.5 mm thick) was prepared and overlaid with a 3.5% stacking gel. A 10- μl aliquot of the rVSV(M Δ 51)-M3 clinical lot was mixed with 10 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA) before being loaded onto the gel. The gel was run at 180 V for approximately 1 hr and then stained with Coomassie Brilliant Blue R250 (Bio-Rad) to visualize the protein bands.

Sequencing of viral genome

Viral RNA was amplified by the strategy of reverse transcription (RT) and polymerase chain reaction (PCR). The rVSV(M Δ 51)-M3 genome is 12417 bp long and several RT-PCRs using different primer pairs were performed. The RT-PCR products were then purified by agarose gel separation. Sequencing of the internal fragments was performed on these PCR products, using primers in both directions. Sequencing of the two ends of the viral genome (~ 80 bp from the 3' end and ~ 100 bp from the 5' end) was done in one direction only. All samples were submitted to the DNA Core Sequencing Facility of Mount Sinai School of Medicine (New York, NY). The raw sequence data were analyzed with MacVector software (MacVector, Cary, NC) to align the obtained sequence data with the theoretically expected sequence (based on the VSV-Indiana strain, GenBank accession number J02428.1, and the murine herpesvirus M3, GenBank accession number AF127083).

Neutralization of rVSV

To perform the lot release assays to look for viral or mycoplasma contamination, the rVSV sample needed to be neutralized with antibody against rVSV. The neutralization procedure was performed by incubating the rVSV sample with antiserum to VSV (immune ascites fluid, cat. no. VR-1238AF; American Type Culture Collection [ATCC], Manassas, VA) at a 1:1 ratio for 30 min at room temperature.

Results

Production of master virus seed stock

A vial of the 293 master cell bank (MCB) was expanded until sufficient cells existed for initiation of production of the master virus seed stock (MVSS). The 293 cell line was originally developed as a transformed cell line in 1977 by transfection of primary human embryonic kidney cells with sheared adenovirus type 5 DNA (Graham *et al.*, 1977). Our MCB was frozen at passage 5 and the vector was produced in cells at passage 12–16. 293 cells were seeded at 7.5×10^4 cells/cm² in 11×T-175 flasks (Nunc/Thermo Fisher Scientific). Three days later, cells from one of the flasks (approximately 100% confluent) were harvested by trypsinization and the cell density was determined to be 3.2×10^5 cells/cm²

(doubling time, approximately 35–40 hr). The remaining cells ($10 \times T-175$ flasks) were then infected with the VSV seed stock (1.1×10^{10} PFU/ml), using an MOI of 0.01. To initiate the infection, the culture medium was removed from each flask, and the VSV seed stock was diluted in serum-free medium, VP-SFM, and added to each flask. Approximately 24 hr after infection, the supernatant containing the VSV viral vector was harvested and centrifuged ($200 \times g$ for 15 min at 4°C) to remove cellular debris. The supernatant was then aliquoted into 240 cryovials (Corning, Corning, NY) at 1 ml each. The titer of the VSV MVSS was determined to be 9.1×10^8 PFU/ml. The assays used to characterize the MVSS are listed in Table 1. A number of assays were performed *in vitro* using indicator cell lines or in animals, including the *in vitro* viral assay, *in vivo* viral assay, and the mycoplasma assay. The viral vector was neutralized as described in Materials and Methods before being tested in these assays. However, despite preincubation with an available neutralizing serum, rVSV breakthrough was observed in the *in vitro* viral assay (only the portion of the assay performed on MRC-5 cells was free of rVSV breakthrough or evidence of other adventitious agents) and the *in vivo* viral assay (only the guinea pigs survived the assay). Thus, a conditionally negative result was reported for the *in vitro* and *in vivo* viral assays, based on the results of a subset of the cell lines or animals tested in these assays. A more

effective neutralizing antibody will need to be identified to address the issue of rVSV breakthrough in these assays before initiation of phase 2 clinical trials.

Production of pilot lot

Cell culture and infection. A 10-liter pilot lot was produced to evaluate the manufacturing procedure described in Materials and Methods. In brief, 293 cells were seeded at 7.5×10^4 cells/cm², in DMEM with 10% FBS in 10×10 -layer Cell Factories (CFs) and one single-layer Cell Factory. On day 3 (approximately 72 hr postseeding) cells were removed from the single-layer CF and counted (a cell density of 1.44×10^5 cells/cm² was determined). The culture medium was removed from the 10×10 -layer CFs and the cells were infected with the rVSV MVSS diluted at an MOI of 0.01, in the serum-free medium VP-SFM supplemented with 4 mM glutamine (Invitrogen). A volume of 900 ml of VP-SFM was used in each 10-layer CF. Approximately 24 hr after infection, cytopathic effect (CPE) was observed and the supernatant was harvested from the infected cells.

Clarification and Benzonase treatment. After the crude supernatant was clarified and treated with Benzonase, it was purified by column chromatography. Approximately 8.8 liters of supernatant was loaded onto an XK 50 column (GE Healthcare Life Sciences) with a 19-cm bed height, packed with Poros HQ-50 resin (column volume, 372 ml). The load ratio was 23.7 ml of supernatant per milliliter of resin and the linear flow rate used for the entire procedure was 1.5 cm/min. After the sample was loaded, the column was equilibrated with 4 column volumes of equilibration buffer and washed with approximately 1500 ml of 300 mM NaCl. The rVSV was eluted in a single step using approximately 550 ml of 1.2 M NaCl (an eluted peak volume of 312 ml was collected). The chromatogram is shown in Fig. 1.

Tangential flow ultrafiltration, formulation, and sterile filtration. The eluted material was diafiltered and further concentrated to a final volume of 105 ml and formulated by adding 11 ml of glycerol. To evaluate the final filtration step,

TABLE 1. LOT RELEASE ASSAYS, SPECIFICATIONS, AND RESULTS FOR THE RECOMBINANT VESICULAR STOMATITIS VIRUS MASTER VIRUS SEED STOCK

Assay	Specification	Result
Sterility testing (USP)	Negative	Negative
B&F assay—qualification of sterility testing	Pass	Pass
Mycoplasma (PTC)	Negative	Negative
<i>In vitro</i> adventitious viruses	Negative	Negative
<i>In vivo</i> assay for inapparent viruses	Negative	Negative
Hamster antibody production (HAP) test	Negative	Negative
Product-enhanced reverse transcriptase (PERT) assay for retrovirus	Negative	Negative
PCR-B19 parvovirus ^a	Negative	Negative
PCR-HIV1/2 (human immunodeficiency virus) ^a	Negative	Negative
PCR-HTLV I/II (human lymphotropic virus) ^a	Negative	Negative
PCR-HAV (hepatitis A virus) ^a	Negative	Negative
PCR-HBV (hepatitis B virus) ^a	Negative	Negative
PCR-HCV (hepatitis C virus) ^a	Negative	Negative
PCR-EBV (Epstein-Barr virus) ^a	Negative	Negative
PCR-CMV (cytomegalovirus) ^a	Negative	Negative
PCR-HHV6 (herpesvirus 6) ^a	Negative	Negative
PCR-HHV7 (herpesvirus 7) ^a	Negative	Negative
PCR-HHV8 (herpesvirus 8) ^a	Negative	Negative
Viral genome sequence	Report result	Result reported
Infectious titer	Report result	3.9×10^9 PFU/ml

B&F, Bacteriostasis and Fungistasis; PTC, Points to Consider; USP, U.S. Pharmacopeia.

^aPCR test panel for contaminating viral sequences.

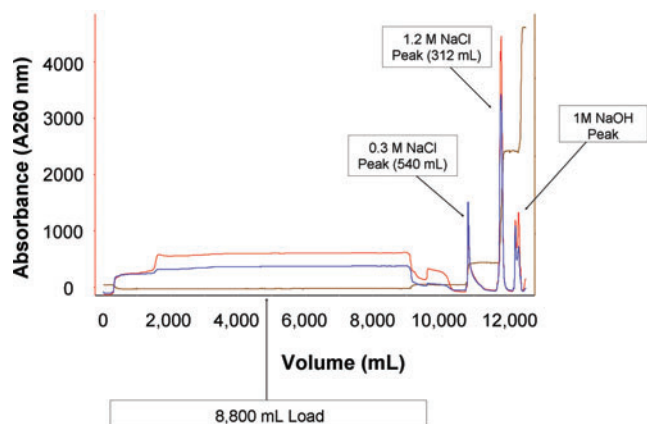


FIG. 1. Chromatogram of the recombinant vesicular stomatitis virus (rVSV) pilot lot. The culture supernatant was loaded onto the column and eluted with 1.2 M NaCl. Color images available online at www.liebertonline.com/hum.

8 ml of the formulated bulk was filtered with a Millipore 0.2- μm syringe filter, polyethersulfone (PES) membrane (surface area, 3.9 cm²). Eight 1-ml filtered samples were taken sequentially. Four samples were titered and all of these samples had a similar titer with an average titer of 1.2×10^{10} PFU/ml, indicating minimal loss in infectious titer occurs during the filtration step. Filtration of the remaining 100-ml concentrated bulk was performed with Millipore Steripak-GP-10 filters (0.2- μm PES membrane with a surface area of 100 cm²) that had been prewetted with 25 ml of Tris–NaCl–EDTA (TNE) buffer. The titer of the in-process samples and the final filtered material are summarized in Table 2.

Production of clinical lot under GMP

Cell culture and infection. The 293 cell line was expanded until sufficient cells existed for initiation of clinical lot production. Expansion of the 293 cell train is depicted in Fig. 2. Production was divided into five subbatches, each having 10 \times 10-layer CFs seeded with 293 cells. The manufacturing process for a representative subbatch is described. On day 0, cells were trypsinized, counted with a hemocytometer and trypan blue staining, and plated at 0.75×10^5 cells/cm² in 11 \times 10-layer CFs. On day 3, cells from one of the CFs were removed with trypsin and were counted (8.9×10^9 – 3.2×10^{10} cells per CF). Monolayers were approximately 85–90% confluent at the time of infection. The cells in the remaining 10 \times 10-layer CFs were then infected at an MOI of 0.01, using the rVSV MVSS (3.85×10^9 PFU/ml), by removing the growth medium from each factory and replacing it with 1 liter of VP-SFM containing the appropriate volume of rVSV MVSS. On day 4 (approximately 24 hr after infection), the viral supernatant was harvested. Samples of the crude harvest were collected for quality control (QC) testing (1 \times 26-ml and 1 \times 2-ml samples for the mycoplasma assay and 2 \times 4-ml samples for the *in vitro* viral assay), as well as for in-process testing and retention samples (40 ml). The same manufacturing process

TABLE 2. SUMMARY OF PRODUCT YIELD DURING PILOT LOT PRODUCTION

Sample ID	Volume	Infectious titer (PFU/ml)	Total infectious units (PFU)
Clarified supernatant	9 liters	2.4×10^8	2.2×10^{12}
Post-Benzonase	9 liters	6.0×10^8	5.4×10^{12}
Elution peak (1.2M)	312 ml	1.7×10^8	5.3×10^{11}
Post-UF/DF	105 ml	5.2×10^9	6.0×10^{11}
Formulated bulk	116 ml	7.2×10^9	8.4×10^{11}
Filtered (0.2 μm)	100 ml	1.2×10^{10}	1.3×10^{12}

DF, diafiltration; UF, ultrafiltration.

was repeated a total of five times for a total production of 50 liters of rVSV supernatant.

Parallel cell culture. To obtain a sample for *in vitro* viral adventitious agent testing, a parallel culture of uninfected 293 cells (2 \times T-175 flasks) from the same cell train was grown under conditions similar to those used to manufacture the clinical lot of rVSV. Cells were plated at the same density as the cells used for the production of virus (0.75×10^5 cells/cm²) with 28 ml of growth medium per flask. On day 3, the spent medium was removed from the parallel cell culture flasks and 28 ml of VP-SFM without virus was added to each flask. At the time of harvest (day 4), medium from both flasks was collected and saved (conditioned medium). Cells remaining in the flasks were removed with trypsin and then pelleted by centrifugation. The cell pellets were then resuspended in conditioned medium (described previously), aliquoted into multiple samples (4 \times 10 ml), and frozen for further QC testing.

Clarification and Benzonase treatment. The harvested crude viral supernatant was clarified with a filter train, which consisted of a Millipore Milligard LPB Opticap XL-4

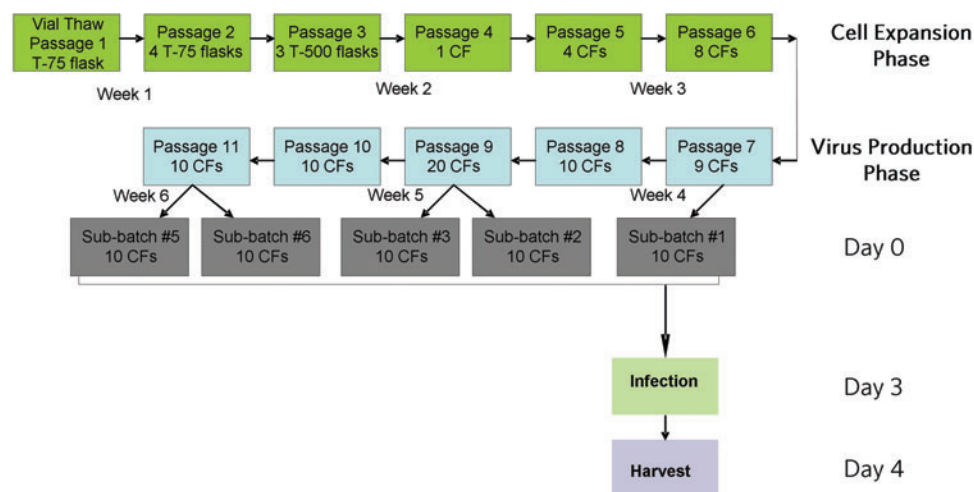


FIG. 2. Schematic representation of the expansion of the 293 cell train used for the 50-liter rVSV GMP production lot. Cells from the 293 master cell bank were thawed and expanded for the manufacture of VSV. Cells were passaged twice per week with trypsin. An additional 10-layer Cell Factory (CF) was seeded for each week's infection in order to be able to determine the number of cells in each CF (not indicated). Additional flasks (2 \times T-175) were also seeded as part of the parallel culture for each week's manufacturing production (not indicated). Color images available online at www.liebertonline.com/hum.

dual-membrane (1.2- μm /0.5- μm) filter and a second Milligard LPB Opticap XL-4 single-layer (0.2- μm) filter, for up to 10 liters of crude viral supernatant. Benzonase (50 U/ml supernatant) was added to the clarified supernatant to digest the nucleic acids. The digestion was carried out at 37°C for 4 hr. After digestion, the supernatant was held at 4°C overnight until purified by column chromatography.

Column chromatography. The Benzonase-treated supernatant was purified by anion-exchange chromatography, using a Poros HQ-50 packed column. Typically a 5 \times 20 cm column was used to process 8–10 liters of culture medium at a linear flow rate of 90 cm/hr. The column was equilibrated with 150 mM NaCl in 20 mM Tris, pH 7.5. After loading, the column was washed with \sim 4 column volumes of equilibration buffer until the absorbance returned to baseline. A second wash step was performed with \sim 3 column volumes of 300 mM NaCl in 20 mM Tris, pH 7.5. Elution was performed with 1.2 M NaCl in 20 mM Tris, pH 7.5. UV absorbance at 260 nm was used to monitor and collect the elution peak, typically between 300 and 360 ml (Fig. 3).

Buffer exchange and concentration by ultrafiltration/diafiltration. The column eluate was diluted to 2 liters with exchange buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA; pH 7.8), concentrated by tangential flow filtration (TFF) to 400 ml, and then diafiltered with 2.4 liters of exchange buffer. The product was further concentrated from 400 to 100 ml, which is approximately a 90- to 100-fold concentration compared with the original culture supernatant.

Formulation and sterile filtration. The concentrated product was formulated to 10% glycerol and stored at -80°C until final filtration and pooling of concentrated subbatches. Each subbatch of the rVSV product was sterile filtered with a Steripak-GP10 filter unit (Millipore) that had been prewetted

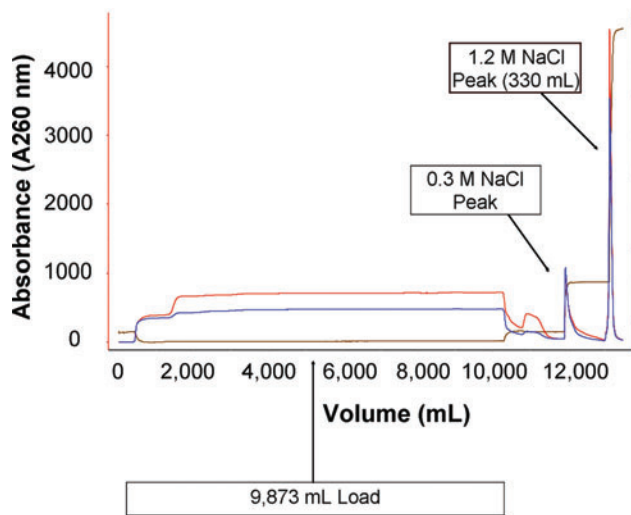


FIG. 3. Representative chromatogram of the rVSV GMP lot production. Each subbatch of culture supernatant was loaded onto the column and eluted with 1.2 M NaCl. The chromatogram shown is from one of the five subbatches and is representative of all five. Color images available online at www.liebertonline.com/hum.

with 30 ml of the product formulation buffer. Filtered subbatches were pooled and subsequently aliquoted. A total of 196 vials were filled, 110 vials with a volume of 0.7 ml and 86 vials with a volume of 5.5 ml.

Production summary

A 50-Cell Factory clinical lot production of an oncolytic rVSV vector was successfully completed. The GMP manufacturing process consisted of five subbatches, with each subbatch being generated from 10 CFs. Each of the five subbatches was processed independently through concentration and formulation and then stored at -80°C until all five subbatches were filtered and pooled. The titration results and volumes of the in-process samples for each of the five subbatches are listed in Table 3. Final product and QC samples were also collected from the filtered, purified bulk. The titers of the purified bulk (after 0.2- μm filtration) and final product (0.7-ml and 5.5-ml vials) are listed in Table 4.

Lot release testing of the GMP lot

The assays performed on the GMP lot as well as the release test results are summarized in Table 5. To address the issue of breakthrough of rVSV vector in the infected indicator cell lines that had been observed during lot release testing of the rVSV MVSS, a parallel 293 cell culture was set up during the cell expansion and infection phase of rVSV production. This culture was used, in addition to the unprocessed bulk from the clinical lot, for the *in vitro* viral assay.

TABLE 3. VIRUS YIELD FOR CLINICAL LOT IN-PROCESS SAMPLES

Subbatch	Sample ID	Volume	Infectious titer (PFU/ml)	Total infectious units (PFU)
1	Clarified supernatant	9 liters	3.0×10^8	2.7×10^{12}
	Post-Benzonase	9 liters	1.5×10^8	1.4×10^{12}
	Elution peak (1.2M)	330 ml	1.6×10^9	0.5×10^{12}
	Post-UF/DF	116 ml	2.4×10^{10}	2.8×10^{12}
2	Clarified supernatant	9 liters	3.3×10^8	2.7×10^{12}
	Post-Benzonase	9 liters	1.5×10^8	1.4×10^{12}
	Elution peak (1.2M)	306 ml	4.7×10^9	1.4×10^{12}
	Post-UF/DF	111 ml	2.3×10^{10}	2.6×10^{12}
3	Clarified supernatant	9 liters	3.8×10^8	4.3×10^{12}
	Post-Benzonase	9 liters	2.2×10^8	2.0×10^{12}
	Elution peak (1.2M)	342 ml	4.9×10^9	1.7×10^{12}
	Post-UF/DF	117 ml	2.8×10^{10}	3.3×10^{12}
4	Clarified supernatant	9 liters	2.0×10^8	1.8×10^{12}
	Post-Benzonase	9 liters	ND	ND
	Elution peak (1.2M)	344 ml	ND	ND
	Post-UF/DF	117 ml	2.3×10^{10}	2.6×10^{12}
5	Clarified supernatant	9 liters	ND	ND
	Post-Benzonase	9 liters	ND	ND
	Elution peak (1.2M)	366 ml	ND	ND
	Post-UF/DF	125 ml	2.4×10^{10}	2.7×10^{12}

DF, diafiltration; ND, not determined; UF, ultrafiltration.

TABLE 4. VIRUS YIELD IN PURIFIED BULK AND FINAL PRODUCT

Sample ID	Volume (ml)	Infectious titer (PFU/ml)	Total infectious units (PFU)
Purified bulk	570	1.7×10^{10}	1.0×10^{13}
Final product (0.7-ml vials)	77	1.2×10^{10}	9.2×10^{11}
Final product (5.5-ml vials)	473	1.8×10^{10}	8.5×10^{12}

An SDS-polyacrylamide gel of the clinical lot purified vector is shown (Fig. 4). The morphology of the rVSV present in the clinical lot was also examined by transmission electron microscopy (TEM) and shown to be the unique bullet shape expected for rVSV (Fig. 5).

Discussion

A robust and readily scalable manufacturing process has been developed to produce rVSV vector suitable for clinical studies. The crude vector supernatant is generated by infecting adherent 293 cells at a low MOI, then harvesting the excreted rVSV vector when extensive CPE is formed. The purification process involves five steps: (1) a clarification step to remove cellular debris; (2) a nuclease treatment step to degrade host cell DNA; (3) an anion-exchange chromatographic step for purification of the vector; (4) a tangential flow ultrafiltration/diafiltration step for concentration and buffer exchange; and (5) a terminal sterile filtration step. Five independent subbatches representing 10 liters of supernatant each were produced under cGMP conditions with consistent virus yields ranging between 1.8×10^{12} and 2.8×10^{12} PFU per

TABLE 5. LOT RELEASE ASSAYS AND SPECIFICATIONS FOR THE RECOMBINANT VESICULAR STOMATITIS VIRUS CLINICAL LOT

Assay	Specification	Results
<i>In vitro</i> viral assay (parallel culture)	Negative	Negative
<i>In vitro</i> viral assay (unprocessed bulk)	Negative	Negative
Mycoplasma (PTC)	Negative	Negative
Residual host DNA	Report result	64.03 fg/ μ l
Size distribution of residual DNA	<500 bp	Pass
Residual Benzonase by ELISA	<100 ng/ml	<1 ng/ml
Vector ID (RT-PCR)	Match expected size	Pass
LAL—endotoxin	<10 EU/ml	Pass
Viral particle counts by TEM	Report result	1.6×10^{11} particles/ml
Sterility assay (USP)	Negative	Negative
B&F—Qualification of sterility testing	Pass	Pass
Infectious titer	Report result	1.7×10^{10} PFU/ml

B&F, Bacteriostasis and Fungistasis; LAL, *Limulus* amoebocyte lysate; PTC, Points to Consider; TEM, transmission electron microscopy; USP, U.S. Pharmacopoeia.

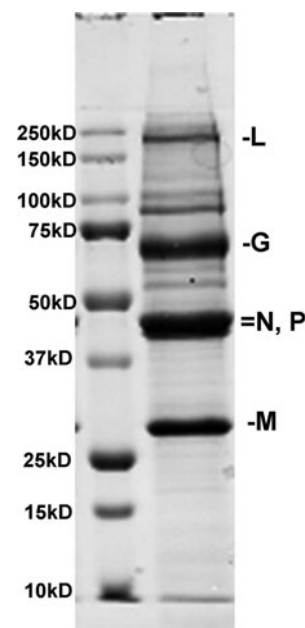


FIG. 4. A sodium dodecyl sulfate–polyacrylamide gel of the rVSV(MD51)-M3 clinical lot. Major bands corresponding to the five VSV proteins (L, G, N, P, and M) were present at the expected sizes.

10-liter subbatch. Although the diafiltration/ultrafiltration and column chromatography steps are easily scalable using existing technology, in order to scale up the infection process for licensed product manufacturing, it will be necessary to investigate the use of bioreactor technology using adherent cells on microbeads or suspension cell cultures to support the production of rVSV at high titers. Ideally, cell expansion would occur in serum-free medium to eliminate the need for a complete media exchange at the time of infection and

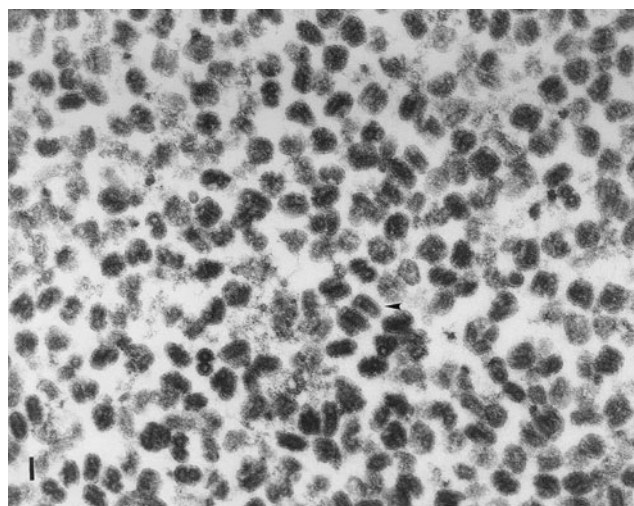


FIG. 5. Electron micrograph of rVSV particles. An aliquot of the clinical lot was submitted to a contract facility for examination and quantification of viral particles by transmission electron microscopy. A representative image shows the unique bullet shape associated with VSV. The arrowhead indicates a sample rod-shaped structure. Scale bar: 100 nm.

disposable single-use stirred tank bioreactors which are currently available in the 40–2000 liter range for use in GMP production would be ideal for virus production purposes.

Although manufacturing of the rVSV vector at the current scale was robust and did not present any significant technical challenges, characterization of the rVSV vector, both the MVSS and the clinical lot, did present some difficulties due to the lack of an effective neutralizing antibody. This may prove to be a common issue with replication competent virus products. To test the clinical product for adventitious viruses and inapparent viruses that may be present in the vector lot, the viral vector must be neutralized in order to allow detection of any contaminating viruses in the *in vitro* and *in vivo* assays. It should also be noted that complete inhibition of the infectivity of the viral vector with existing antibodies might not always be possible. A strategy for the release testing of the viral product should be formulated by assessing the available antibodies for their ability to neutralize virus, identifying indicator cell lines and animal models that may be refractory to viral vector infection, and determining the fold of dilution of the viral vector that will be required to avoid breakthrough or toxicity. If no neutralizing serum is available, effective antiserum may be prepared by immunizing rabbits or other suitable animals with the virus of interest. In addition, the effectiveness of the antiserum may be enhanced by evaluating several indicator cell lines for their sensitivity to the virus infection such that cell lines that are more refractive to the virus infection are selected for the *in vitro* viral assay.

Because an effective neutralizing antibody is a critical reagent required for release testing and product characterization of a viral product, it is important that it not be overlooked during the process development stage. It is therefore prudent that an effective antiserum to neutralize the viral infectivity or cytotoxicity be identified early during process development and a sufficient quantity of the antiserum be secured. This will avoid unnecessary lengthy delays in the release testing of the product once production is completed. It is also extremely important to keep the regulatory personnel at the Food and Drug Administration (FDA) informed regarding any difficulties encountered during lot release testing and to seek their input and guidance at all key steps in the clinical reagent development process.

Before using this material in phase 1 clinical trials, it will be necessary to submit an Investigational New Drug application to the FDA that contains all of the information regarding production and testing of the clinical grade rVSV, as well as preclinical efficacy, biodistribution, and pharmacological/toxicological testing in laboratory animals, and the draft clinical protocol. This information will also be provided to the Office of Biotechnology Activities at the National Institutes of Health. Pending FDA review, this protocol will be submitted to the Mount Sinai Institutional Review Board and Institutional Biosafety Committee for their review and approval. No patient will be enrolled in the clinical trial until all regulatory approvals are obtained.

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Author Disclosure Statement

Dr. Savio Woo is a named inventor of a patent application titled "Transgenic Oncolytic Viruses and Uses Thereof." The pending patent was filed by the Mount Sinai School of Medicine (MSSM). In the event the pending patent is licensed, Dr. Woo would be entitled to a share of any proceeds MSSM receives from the license. The outcome of this research project could affect the ability to license this patent.

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