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Core bead chromatography for preparation of highly pure, infectious respiratory syncytial virus in the negative purification mode

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ABSTRACT

Respiratory syncytial virus (RSV) is an important human pathogen, and is the most frequent viral cause of severe respiratory disease in infants. In addition, it is increasingly being recognized as an important cause of respiratory disease in the elderly and immunocompromised. Although a passive prophylactic treatment does exist for high-risk neonates and children, the overall disease burden warrants the development of a safe and effective prophylactic vaccine for use in otherwise healthy newborns and children. RSV is known to be an extremely labile virus, prone to aggregation and loss of infectious titer during virus handling and preparation procedures. To date infective RSV virions have been prepared by methods which are not readily scalable, such as density gradient ultracentrifugation. In this study we describe a scalable, chromatography-based purification procedure for preparation of highly pure, infectious RSV. The purification scheme is based on core bead technology and hollow fiber tangential flow filtration (TFF) and results in a ~60% recovery of infectious virus titer. This method can be used to prepare highly purified wild type or live-attenuated vaccine strain viruses with titers as high as 1×10^8 plaque forming units per mL. A live-attenuated RSV vaccine prepared by this method was found to be immunogenic and protective *in vivo*, and its purity was 50–200-fold greater with respect to host cell dsDNA and Vero host cell proteins, than the raw feed stream. The results presented here can be considered a starting point for downstream process development of a live-attenuated vaccine approach for prevention of disease by RSV.

1. Introduction

Q2 It is estimated that human respiratory syncytial virus (hRSV) causes up to two hundred thousand deaths per year worldwide in children younger than 5 years old [1]. Approximately, 33 million children of the same age group suffer from acute lower respiratory infection due to hRSV with at least 10% of those cases being severe enough to require hospitalization [1]. The current treatment and prophylaxis landscape was recently reviewed by Gomez et al. [2]. Therapeutic intervention in humans is limited to treatment with the antiviral molecule Ribavirin, though use of this molecule is uncommon and controversial due to potential for side effects and concern about cost and efficacy [3,4]. Passive prophylaxis by a neutralizing, humanized monoclonal antibody, Palivizumab, has been shown to reduce hospitalization rates in high risk infants by 55% as compared to a placebo [5]. To date the safe and effective induction of protective immunity in humans has not been accomplished by vaccination though extensive testing of candidate vaccines has been undertaken both in animals and in humans.

In addition to subunit- and peptide-based vaccine approaches the live attenuated virus (LAV) approach has also been explored [6–10]. Temperature sensitive, gene deletion and passage-attenuated approaches have been tested in animals and in some cases in humans. A challenge to bringing such a vaccine to the market is the requirement to produce high quality, high titer material with a scalable process. Since immunogenicity of LAV candidates is dependent on the ability to infect cells, preservation of infectious titer is an important parameter to assess processes designed for purification. Numerous downstream processes for preparation of virus particles produced in cell culture have been described in the literature (reviewed by Wolf and Reichl [11]). We have previously described low shear chromatographic separation procedures for preparation of enveloped viruses such as herpes simplex virus type 2 [12] and Flaviviruses [13].

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Capto™ Core 700 (GE Healthcare Life Sciences) is a novel, core bead technology-based resin that combines size separation and binding chemistry in a single matrix, with the promise of improved process productivity for the production of large molecules such as viruses. A process was recently described for production of influenza virus from allantoic fluid, which rivals the purity achieved using methods such as zonal ultracentrifugation [14]. A more comprehensive purification scheme has been reported for preparation of Influenza A and B strains from an MDCK cell culture-produced virus [15]. Capto™ Core 700 is a layered, bead-based matrix where the surface of the bead consists of an unliganded, inactive shell with pores that have an approximate molecular weight cutoff (MWCO) of 700 kDa. At the interior of the bead is an active functionalized core with multimodal octylamine ligand designed to capture impurities which are small enough to enter the bead. Thereby, large molecules can be purified from smaller impurities in the negative purification mode.

Purification of hRSV has traditionally been performed by ultracentrifugation in either sucrose [16–19] or iodixanol [20], with recoveries of up to 60–70% infectious virus. Chromatographic purification of viral proteins from cell culture-derived RSV has been described [21,22]. A single report of an ion exchange chromatography-based purification scheme for RSV exists in the literature, though recovery of infectious virus in that case was ~1% [23]. RSV is known to be an extremely labile virus, prone to aggregation [24] and loss of infectivity during bind-and-elute chromatography [23]. Here we show that the core bead resin approach can be used to prepare highly-purified RSV that is of similar potency to crude, unpurified material when tested *in vivo*.

2. Materials and methods

2.1. Upstream processing (USP)

Passage-attenuated LAV was derived by forty-five serial passages on a naïve Vero cell line (L. Zhang et al., to be published elsewhere). Viruses for purification, immunization and challenge, including LAV, WT MSA-1 strain and RSV Long strain, were propagated on Vero cells grown to confluence in T-225 flasks. Cell culture incubations were performed at 37 °C, 5% CO_2, unless otherwise indicated. Vero cells were seeded at 1.8×10^7 cells/flask and grown to confluence in DMEM supplemented with 10% Fetal Bovine Serum and 2 mM L-glutamine. Flasks were aspirated and cells infected with RSV at a multiplicity of infection (MOI) of 0.001 for 1 h in 10 mL viral growth medium consisting of HyClone SFM4MegaVir (Thermo Scientific, Waltham, MA) supplemented with 2 mM L-glutamine and 1× antibiotic/antimycotic (Thermo Scientific). After 1 h the flasks were aspirated, 40 mL fresh viral growth media was added to the virus-adsorbed cells and the flasks were placed at 34 °C. At 6 days post-infection (dpi) the RSV-containing media were harvested and processed as described below.

2.2. Downstream processing (DSP)

RSV-containing cell culture media were decanted from T-225 flasks. The bulk harvest material was clarified by centrifugation at 650 × *g* for 5 min. Clarified cell culture supernatant was the starting material for further purification. In the laboratory scale studies presented here ~500 mL of virus containing material was processed at a time, whereas at the small scale ~30–60 mL of material was processed. In order to reduce the amount of Vero DNA in the process stream, the solution was adjusted to 5 mM $MgCl_2$ and treated with Benzonase® (EMD/Merck, Darmstadt, Germany) endonuclease (90 U/mL, 5 h, 25 °C, 50 rpm). The sample was then further clarified by filtration to remove cellular debris and aggregated material (0.65 μm SartoScale, SartoPure PP2, Sartorius Stedim, Göettingen, Germany). Prior to use the depth filtration manifold was sterilized by autoclaving 25 min dry cycle at 121 °C and the sample was processed at 90 mL/min by peristaltic pump (Master-Flex, Cole Parmer) without preequilibration of the membrane.

Chromatographic separation of RSV virions from contaminating host cell proteins was performed at room temperature on an ÄKTA Purifier (GEHC) located in a biosafety cabinet. A 45 mL Capto™ Core 700 column was packed into an empty XK26 housing and then equilibrated to 1× phosphate buffered saline. All chromatography steps were performed at 10 mL/min. The sample was applied to the column and virus containing material was collected as a single flowthrough (FT) fraction. Bound impurities were removed from the column by cleaning in place (CIP) with 30% isopropyl alcohol prepared in 0.5 M NaOH. FT and CIP fractions were collected manually while recording the ultraviolet absorbance at 280 nm.

Concentration and formulation of the purified virus were accomplished by ultrafiltration/diafiltration (UF/DF) using a hollow fiber tangential flow filtration (TFF) apparatus (Kros-Flo Research II, Spectrum Laboratories, Rancho Dominguez, CA). An 85 cm^2, 500 kDa MWCO polysulfone TFF module was used under low flow rate recirculation conditions to minimize shear force (130 mL/min). Transmembrane pressure (TMP) was kept below 4 psi throughout diafiltration to minimize formation of a gel layer. The virus was formulated into a buffer that allows for cryopreservation of infectivity during multiple freeze/thaw cycles (50 mM potassium glutamate, 10 mM L-histidine, 160 mM NaCl, 10% sorbitol, pH 7.4).

An additional small scale study was performed to determine if a whole cell lysate could be appropriate starting material for purification. The methods used were a scaled down version to what is described above except that in addition to clarified cell culture supernatant, whole cell lysate was assessed as the starting material for purification. To test conditions for lysate preparation, T-225 flasks of infected cells were harvested by scraping the cells from the flask. Cell disruption was accomplished either by sonication for 60 s on ice using a Branson Sonifier Cell Disruptor equipped with a microtip and set at 50% duty cycle, output level 6 (Branson Ultrasonics Corp., Danbury CT) or by microfludization using a M-110Y high pressure pneumatic, 1 *vs.* 3 passes on ice at 2500 pounds per square inch (psi) or 1 *vs.* 3 passes on ice at 5000 psi (Microfluidics Corp., Westwood, MA). Samples were then treated with benzonase endonuclease and filtered through a 0.8 μm polyethersulfone (PES, Supor®) membrane syringe filter (Pall Corp., Port Washington, NY) before loading onto a 5 mL Capto™ Core 700 column at 5 mL/min.

2.3. Characterization of purification process retains

2.3.1. Infectivity

Potency of fractions was interrogated by titration on naïve Vero cells which were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine and 1× antibiotic/antimycotic mixture. Infectivity was assessed by plaque assay as has been described previously [25]. Plaques were visualized by immunostaining with Horseradish Peroxide conjugated goat anti-RSV antibody (Abcam AB20686). Titers were determined by counting stained plaques and are expressed in plaque forming units (PFU) per mL.

2.3.2. Purity (SDS-PAGE, Western blotting, Vero HCP ELISA and Vero DNA qPCR)

RSV containing samples were resolved by 4–12% SDS-PAGE (NuPAGE, Bis-Tris, Life Technologies) after heating of the samples

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