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Robust manufacturing and comprehensive characterization of recombinant hepatitis E virus-like particles in Hecolin®

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ABSTRACT

The hepatitis E virus (HEV) vaccine, Hecolin®, was licensed in China for the prevention of HEV infection and HEV-related diseases with demonstrated safety and efficacy [1,2]. The vaccine is composed of a truncated HEV capsid protein, p239, as the sole antigen encoded by open reading frame 2 and produced using *Escherichia coli* platform. The production of this virus-like particle (VLP) form of the antigen was successfully scaled up 50-fold from a bench scale to a manufacturing scale. Product consistency was demonstrated using a combination of biophysical, biochemical and immunochemical methods, which revealed comparable antigen characteristics among different batches. Particle size of the nanometer scale particulate antigen and presence of key epitopes on the particle surface are two prerequisites for an efficacious VLP-based vaccine. The particle size was monitored by several different methods, which showed diameters between 20 and 30 nm for the p239 particles. The thermal stability and aggregation propensity of the antigen were assessed using differential scanning calorimetry and cloud point assay under heat stress conditions. Key epitopes on the particulate antigen were analyzed using a panel of murine anti-HEV monoclonal antibodies (mAbs). The immuno reactivity to the mAbs among the different antigen lots was highly consistent when analyzed quantitatively using a surface plasmon resonance technique. Using a sandwich ELISA to probe the integrity of two different epitopes in the antigen, the specific antigenicity of multiple batches was assessed to demonstrate consistency in these critical product attributes. Overall, our findings showed that the antigen production process is robust and scalable during the manufacturing of Hecolin®.

1. Introduction

The hepatitis E virus (HEV), a positive, single-stranded RNA virus with a 7.2 kb genome, is the sole member of the genus *Hepevirus* within the family *Hepeviridae* [3]. HEV is a leading cause of viral hepatitis with an annual estimate of 14 million symptomatic cases of HEV infection worldwide [4]. A unique clinical feature of HEV infection is the increased severity among pregnant women with a high mortality rate of approximately 20% [5]. This unmet medical need has gained attention because the recent improvement and increased accessibility of detection assays have led to the improved diagnosis of autochthonous cases in China [6].

Vaccination with an effective prophylactic vaccine against a human pathogen is the best public health approach to prevent viral infections and control epidemics. The recombinant virus-like particle (VLP) approach was successfully employed to develop prophylactic vaccines against the hepatitis B virus and human papillomavirus. The VLP platform is an effective approach for developing prophylactic vaccines because these self-assembling bionanoparticles present multiple epitopes on their surface and

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more faithfully mimic the native virions [7]. HEV capsid proteins encoded by the ORF2 were shown to self-assemble into VLPs with the key epitopes properly presented on the VLP surface. This VLP-based vaccine was demonstrated to be safe and efficacious during a large scale clinical trial with over 110.000 enrolled volunteers [1]. The vaccine was later licensed under the trade name Hecolin® in 2011 in China [8].

Various analytical methods are important in facilitating process development and in defining the quality attributes of a vaccine during vaccine manufacturing. These methods include a combination of biophysical, biochemical and immunochemical analytical methods such as those used for the commercial human papillomavirus vaccine and hepatitis B vaccine [9–12].

For a newly developed vaccine, it is necessary to scale up the production process to meet the market demand post licensure. To demonstrate successful scale up, various methods were employed to verify the similar size and thermal stability among batches of HEV p239 produced at different scales.

To ensure the safety and quality of a vaccine, consistency was assessed across multiple lots of aqueous products. The primary sequence and secondary structure were characterized and verified using different biochemical and biophysical methods. Transmission electron microscopy (TEM) data revealed the particle morphology and particle size distribution for the particulate antigen. Particle size, a key attribute for VLP-based vaccines, was monitored in multiple lots by high performance size-exclusion chromatography (HPSEC), analytical ultracentrifugation (AUC) and dynamic light scattering (DLS) on multiple lots. The thermal stability of an antigen is another important parameter of recombinant protein-based vaccines. Differential scanning calorimetry (DSC) and cloud point methods were used to monitor the thermal stability and the aggregation propensity of the purified antigen. Most importantly, effective antigenicity is a critical attribute for a vaccine product. In this work, the sandwich enzyme-linked immunosorbent assay (ELISA) and surface plasma resonance (SPR) using five mAbs recognizing different epitopes were performed to assess the antigenicity of multiple batches of the final aqueous products. Results from these orthogonal immunochemical methods demonstrated process reproducibility and product consistency among multiple batches of antigen, including information on the integrity of different epitopes.

Here we report the comprehensive characterization of the antigen in Hecolin®, p239, a truncated version of the only HEV capsid protein encoded by the second of ORFs in the HEV genome. This multifaceted antigen characterization demonstrated that the recombinant p239 particle antigen can be manufactured at a commercial scale in a consistent and robust manner. The quantitative assessment of a large range of biochemical, biophysical and immunochemical parameters provided guidance for process improvement or scale-up and the assurance of vaccine quality for the p239 VLP-based vaccine Hecolin®.

2. Materials and methods

2.1. Recombinant p239 VLPs

The gene encoding p239 was cloned into the pTO-T7 expression vector. The active ingredient of the HEV vaccine, recombinant p239, was expressed in *Escherichia coli* at various scales and purified to greater than 99% purity as previously described [13]. The concentration of p239 was determined using a BCA assay.

2.2. Monoclonal antibodies (mAbs)

The anti-HEV murine mAbs 8C11, 8H3, 13D8, 12A10, and 16D7 (all being IgGs) have been reported previously [14,15]. The conjugation of the horseradish peroxidase (HRP) to the 8H3 mAb was carried out using a standard sodium periodate ($NaIO_4$) conjugation method as previously described [14].

2.3. SDS-PAGE for molecular weight and purity determination

The analysis of proteins using SDS-PAGE was performed using the Laemmli method with minor modifications. Briefly, 30 μg/well or 1 μg/well of heated protein was loaded onto the separating gel, which was stained with Coomassie Brilliant Blue after electrophoresis. The molecular weight (MW) of the p239 protein was determined based on a calibration curve created from the known MWs of a set of protein standards (TAKARA Biotechnology CO., Dalian, China).

2.4. LC–MS and MALDI-TOF MS

The p239 aqueous product samples were subjected to reducing and alkylating conditions prior to tryptic digestion. The MWs of the peptide fragments and their amino acid compositions were determined using the TripleTOF 5600 System fitted with a Nanospray III source (AB SCIEX, Concord, Ontario, Canada) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). The data acquisition was carried out using an ion spray voltage of 2.2 kV, a curtain gas of 20 psi, a nebulizer gas of 6 psi and an interface heater temperature of 150 °C. The data was processed using Protein Pilot Software v. 4.0 (AB SCIEX, Foster City, CA).

The mass spectra of the peptide mixtures were obtained using a Reflex III MALDI-TOF mass spectrometer (Burker Daltonik, Bremen, Germany) with α-cyano-4-hydroxy cinnamic acid as a matrix prior to the MS analysis.

2.5. Isoelectric point determination

The whole column imaging method imaged capillary isoelectric focusing (icIEF) was used to determine the pI of the purified p239. The icIEF was conducted on the CEInfinite system (Advanced Electrophoresis Solutions Ltd., Ontario, Canada), which is an iCE280 Analyzer, using a 50 mm cIEF cartridge. The IEF solution (0.35% methylcellulose and 8% ampholyte, pH 3–10), catholyte (0.1 M NaOH in 0.1% methylcellulose), anolyte (0.1 M phosphoric acid in 0.1% methylcellulose) and p*I* markers were all provided by the vendor. The final running samples contained 0.5 mg/ml protein from different p239 lots. The sample running temperature was 25 °C and the protein signal was detected at 280 nm. The p*I* of the major peak in each lot was calculated based on its relative distance from the p*I* 4.14 marker and p*I* 7.90 marker.

2.6. Particle size analysis

2.6.1. Transmission electron micrograph (TEM)

The p239 antigen was diluted to 0.1 mg/mL, applied to a carbon-coated grid, and stained with 2% uranyl acetate after the removal of excess fluid. The samples were examined using a JEM2100HC transmission electron microscope (JEOL, Tokyo, Japan) operating at 200 kV to characterize the morphology of the p239 particles. The particles were selected, and their diameters were measured using the IPWIN Application 6.0 software (Media Cybernetics Inc., Sarasota, FL). The distribution of sizes was calculated using the GraphPad Prism software (GraphPad Software, San Diego, CA).

2.6.2. Dynamic light scattering (DLS)

The hydrodynamic size distributions of the p239 particles were measured using a DynaPro-MS/X DLS system (Protein Solutions, Joplin, MO). The samples (15 μL) were loaded into a 1.5 mm pathlength, 12 μL quartz cuvette. The back-scattered light from the

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