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Structural Characterization and Formulation Development of a Trivalent Equine Encephalitis Virus-Like Particle Vaccine Candidate

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ABSTRACT

The zoonotic equine encephalitis viruses (EEVs) can cause debilitating and life-threatening disease, leading to ongoing vaccine development efforts for an effective virus-like particle (VLP) vaccine based on 3 strains of EEV (Eastern, Western, and Venezuelan or EEE, WEE and VEE VLPs, respectively). In this work, transmission electron microscopy and light scattering studies showed enveloped, spherical, and ~70 nm sized VLPs. Biophysical studies demonstrated optimal VLP physical stability in the pH range of 7.5-8.5 and at temperatures below ~50°C. Interestingly, the individual stability profiles differed notably between the 3 VLPs. Numerous pharmaceutical excipients were screened for their VLP stabilizing effects against thermal stress. Sucrose, sorbitol, sodium chloride, and pluronic F-68 were identified as promising stabilizers and the concentrations and combinations of these additives were optimized. Candidate monovalent VLP bulk formulations were incubated at temperatures ranging from −80°C to 40°C to establish freeze-thaw, long-term (2°C-8°C) and accelerated stability trends. Good VLP stability profiles were observed at each storage temperature, except for a distinct instability observed at −20°C. The interaction of monovalent and trivalent VLP formulations with aluminum adjuvants was examined, both in terms of antigen adsorption and desorption over time. The implications of these findings on future vaccine formulation development of EEV VLPs are discussed.

Introduction

Equine encephalitis viruses (EEVs), including Eastern (EEEV), Western (WEEV), and Venezuelan (VEEV), are mosquito-borne enveloped single-stranded RNA viruses in the Alphavirus genus of the *Togaviridae* family.[1,2] These viruses measure 60 to 70 nm in diameter. EEV infections can cause debilitating diseases with moderate morbidity and potentially severe mortality in equids and humans.[3] EEV infections usually begin with nonspecific illness, such as fever, chills, myalgia, and abdominal pain, followed by, in severe cases, neurological signs indicative of encephalitis, such as confusion, seizures, coma, and paralysis.[4-6] Children and the elderly have a higher risk for developing severe disease after EEV infection. EEEV, WEEV, and VEEV have different geographic distributions and epidemiologic profiles. For example, EEEV primarily occurs in Eastern Canada and United States,[7,8] with a fatality rate of approximately 30%-70%.[9] WEEV infections are mainly reported in Western Canada and states west of the Mississippi river in the United States with an overall mortality rate of approximately 3%-7%.[1] VEEV outbreaks have occurred in Central and South America,[2] with a mortality rate of 1%-3%.

Because of the severity of the disease that can be caused by EEV infections and their potential use for bioterrorism, EEEV, WEEV, and VEEV have been identified as category B priority pathogens by the

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National Institute of Allergy and Infectious Diseases (NIAID).[10,11] Currently, there are neither known antiviral treatments nor any commercially licensed human vaccines against EEV infections. There is a live attenuated vaccine (TC-83) against VEEV for use in US military personnel; however, the low efficacy and potential side effects of the vaccine hinder its widespread use in the general population.[12,13] The Vaccine Production Program at the Vaccine Research Center at NIAID/National Institutes of Health has initiated a program to develop an effective virus-like particle (VLP)—based vaccine against EEV based on the positive safety and efficacy profile of VLPs and prior phase I clinical experience with a related Chikungunya virus VLP[14] and early nonclinical EEV studies. Three strains of EEV (EEEV, WEEV, and VEEV), are under investigation as components of a multivalent recombinant VLP vaccine product candidate.

In this work, we characterized the structural integrity and physical stability of purified recombinant VLPs of 3 strains of EEV using a variety of physicochemical techniques. A comparison of the similarities and differences between the VLPs, both intrinsic physical properties and degradation profiles versus thermal and pH stress, are presented. Furthermore, a series of pharmaceutical excipients were evaluated for their stabilizing effect on the thermal stability of these VLPs to identify stabilizers for a new bulk formulation. An optimally stable new bulk formulation was developed based on the results from excipient screening studies as well as subsequent stability assessments (storage and freeze-thaw stability). The adsorption of the 3 VLPs to aluminum salt adjuvants and the stability profile of the aluminum adjuvanted VLPs was also evaluated. In addition, the time dependence of the ability to desorb these VLPs from the adjuvant's surface after storage was also examined. The implications of these findings for future development of a stable parenteral multivalent EEV VLP vaccine formulation, both with and without aluminum adjuvants, are also discussed.

Materials and Methods

Materials and Sample Preparation

VEEV-like particles (VEE VLPs), EEEV-like particles (EEE VLPs), and WEEV-like particles (WEE VLPs) were expressed by transient transfection of HEK293 cells (VRC293) with plasmids encoding the viral capsid protein (C) and 2 envelope glycoproteins (E1 and E2) of the native virus. These enveloped VLPs were then purified to homogeneity using a purification scheme, which includes a combination of tangential flow filtration and tandem flow-through column chromatography. Each of the 3 VLPs was supplied by NIAID (National Institutes of Health), in a 25-mM Tris buffer containing 100-mM sodium citrate, pH 8.5. The VLP solutions were shipped to University of Kansas on dry ice and stored frozen at −80°C upon receipt. The initial characterization of the VLPs was performed in the Tris/Citrate buffer described above. For the remaining studies, the VLP samples were thawed and buffer exchanged 3 times into target buffers, depending on the experiments, by overnight dialysis at 4°C using Slide-A-Lyzer® dialysis cassettes (3.5 kDa MWCO; Thermo Scientific, Rockford, IL). All reagents and excipients for preparing different formulations were purchased from Sigma-Aldrich (St. Louis, MO). Carbohydrates such as trehalose and sucrose were purchased from Pfanstiehl Inc. (Waukegan, IL). All excipients were of high purity grade (>99%).

Methods

Descriptions of many of biochemical and biophysical methods performed in this work have been described previously.[15-17] For a detailed description of the analytical characterization experimental methods, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ultraviolet (UV) absorption spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), intrinsic fluorescence spectroscopy, circular dichroism (CD), and differential scanning calorimetry (DSC), see the Supplemental Methods section. The methodology for construction of 3-index empirical phase diagrams (EPDs) for data visualization of the biophysical data sets as a function of pH and temperature has also been described previously[18] and is also presented in detail in the Supplemental Methods section. The methods for excipient screening, freeze-thaw, and storage stabilities and interaction of the VLPs with aluminum salt adjuvants are also described in Supplemental Methods section.

Results

Size Analysis of EEE, WEE, and VEE VLPs

As part of initial characterization, the size and morphology of the 3 VLPs (EEE, WEE, and VEE) were studied using TEM analysis. As seen in Figures 1a-1c, each of the 3 VLPs are enveloped, mostly spherical in shape with diameters ranging from 64 to 74 nm. These results are in good agreement with the hydrodynamic diameter values obtained by DLS (D_h, 67-72 nm) as well as with the previously reported sizes[2,19,20] of the 3 EEVs (Fig. 1d). In addition, the polydispersity values of each of the 3 VLP samples as measured by DLS varied between 3%-7% indicating that these VLP preparations were relatively homogenous.

Protein Purity and Overall Higher Order Structure of EEE, WEE, and VEE VLPs

A combination of SDS-PAGE, UV-visible absorption, CD, and intrinsic tryptophan fluorescence spectroscopy were used to evaluate the purity and overall higher order structure (HOS) of the proteins contained in the VLPs (formulated in a Tris-citrate, pH 8.0 buffer; see Materials and Methods). The 3 VLPs showed 2 or 3 protein bands as observed using SDS-PAGE under both nonreducing and reducing conditions (Fig. 2a). The protein bands between 28 and 49 kDa markers correspond to the E1 and E2 envelope proteins (theoretical MW: 47 kDa), whereas the protein band at ~29 kDa corresponds to the capsid protein (theoretical MW: 29 kDa). A separation of the E1 and E2 protein bands was apparent for EEE VLP, whereas for WEE and VEE VLPs, one band was primarily observed for the 2 envelope proteins, probably due to a similarity in MW between the E1 and E2 proteins. Overall protein purity levels (bands corresponding to VLP proteins) were estimated to be >99% by SDS-PAGE.

UV absorbance spectra were used to provide an indication of the nucleic acid and protein content of these VLP solutions. Each of the 3 VLPs showed a peak maximum of 265 nm (after light scattering correction) as shown in Figure 2b. The UV absorbance spectra of pure protein species have a peak at 280 nm, whereas those of pure nucleic acids are present at 260 nm.[21] The observed peak maximum indicates the presence of both proteins and nucleic acids in these VLP samples. Viruses with internalized nucleic acids have been shown by Porterfield and Zlotnick[22] to have similar UV absorbance spectra. In addition, because a pure protein (~100%) displays a A_{260}/A_{280} ratio of 0.57, a mixture of 90% protein and 10% nucleic acid has an A_{260}/A_{280} ratio of 1.32,[23,24] the observed $A_{260}/_{280}$ ratios of 1.25-1.40 (Fig. 2) indicate each of the 3 VLP samples contain ~10% nucleic acids (see Discussion).

CD was used to monitor the overall secondary structure of the proteins contained in each of the 3 VLPs. At 10°C, CD spectra in the far UV region (200-260 nm) showed a broad minimum between 209-220 nm suggesting a mixture of alpha and beta-sheet secondary structures (Fig. 2c). Intrinsic Trp fluorescence spectroscopy was