Immune Responses in Mice Vaccinated with Virus-Like Particles of Western Equine Encephalitis Virus from an Insect Cell-Based Expression System

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ABSTRACT: Western equine encephalitis virus (WEEV) can cause lethal encephalitis in humans and equines and represents a serious public health threat in many countries. Therefore, development of efficient vaccines against WEEV remains an important challenge in the field of disease control. This study described for the first time successful production of WEEV virus-like particles (VLPs) in insect cells using recombinant baculoviruses. This well-established expression system is very suitable for production of WEEV VLPs. The immune experiment herein in mice showed that the VLPs formulated with 206-adjuvant were responsible for the stronger-VLP-specific cellular immune response, and were able to induce the secretion of IL-2, IL-4, IFN-γ and production of high titer antibodies that can effectively neutralize the WEEV pseudoviruses. The WEEV VLPs from insect cells could provide a new, safe, non-replicating and effective vaccine candidate against WEEV infections.

KEY WORDS: western equine encephalitis virus; baculoviruses; virus-like particles; Sf9 cells; cytokine; antibody

Introduction

Western equine encephalitis virus (WEEV) is a very important member of *alphavirus* that belongs to the family *Togaviridae* [1-3]. WEEV can cause severe encephalitis in humans and equines, and the case-mortality rate is between 1% and 5% [4], in addition, it should indicate that the relative high mortality is caused by aerosol infection in laboratory accidents [5-7]. The virus was first isolated from a horse's brain in 1930 in California[8]. Afterwards, outbreaks of WEEV-induced encephalitis were reported in Canada, Mexico, and Cuba. WEEV was transmitted via mosquitoes from its reservoir in wild birds to humans and horses[9, 10], but in recent years, several laboratory accidents indicated that WEEV was infectious in aerosol form[4, 5, 11, 12]. It is also a potential biological weapon, since WEEV was included on the category B Priority Pathogens [13].

The virus comprises a single stranded, positive-sense RNA genome of approximately 11.5 kb; the 5' two-thirds of the genome encoded four nonstructural proteins (nsP1-4) which are responsible for RNA replication and transcription; and the 3' one-third of the genome encodes five structural proteins, including capsid, E3, E2, 6K and E1[14, 15]. E3 and 6K are signal peptides, and E2 and E1 are involved in receptor recognition, virus attachment, membrane fusion and virion assembly, and are able to induce the production of neutralization antibodies against WEEV[13, 16].

In recent years, because there are few WEEV vaccines with high efficacy, developing a new vaccine is an important task for preventing WEEV. Virus-like particles (VLPs), mimicking the architecture and conformation of native viruses surface, have been show to be able to induce strong immune responses in animals. Since they lack the viral genome, VLPs could potentially constitute safe and effective vaccine candidates [17-19]. In addition, the baculovirus expression vector system (BEVS) has been widely adopted, which has exhibited many obvious advantages of the system, including accurate protein folding and post-translational processing to assemble the corresponding VLP [20]. At present, some baculovirus- produced subunits or VLP vaccines have been on the market for many years [21]. These products have paved the way for future licensing of new baculovirus-based vaccines.

Herein we described the generation and immunogenicity of WEEV-like particles using the BEVS in insect cells. Some alphavirus proteins and VLPs have been successfully expressed by recombinant baculoviruses[22-24]. Moreover, insect cells are readily suitable for suspension cultures and are a good match with the baculovirus system [21]. Therefore, we believed that the WEEV VLPs described here would offer a safe and effective vaccine against WEEV.

Materials and Methods

Plasmid construction and generation of recombinant baculoviruses

The 3711 bp target fragments (*c-e3-e2-6k-e1*) coding 5 proteins of WEEV (Figure 1A), including capsid (C), E3, E2, 6K and E1 (Genbank accession # NC_003908.1) was synthetically obtained (Gene Art) and equipped with pGEM-T vectors. Subsequently, the fragments were amplified using PCR with the forward primer (5′-ATCA*AGGCCT*ATGTTTCCATACCCTCAGCTGAACTT-3′) introduced a *Stu*I restriction site (in italics) at the 5′ end and reverse primer (5′-TTAC*AAGCTT*TCATCTACGTGTGTTTATAAGCATAGAGCT GC-3′) introduced a *Hin*d III restriction site (in italics) at the 5′ end, and inserted into *Stu*I / *Hin*d III site of downstream polyhedrin promoter (Pph) in the pfastBac^{TM}1 vector (Invitrogen) to construct the recombinant plasmid, after it was confirmed by using PCR and enzyme-digestion methods, designated as pfastBac^{TM}1-*c-e3-e2-6k-e1*. The pfastBac^{TM}1-*c-e3-e2-6k-e1*, as transferring plasmids, and derivatives of the Autographa californica nuclear polyhedrosis virus (AcMNPV) backbone were adopted to construct the recombinant baculoviruses according to the Bac-to-Bac baculovirus expression system [23][18]. The recombinant bacmid DNA was successfully verified by the PCR method and transfected into spodoptera frugiperda (SF9) cells to generate the recombinant baculovirus, resulting in AcMNPV-C-E3-E2-6K-E 1(Ac-C-E). Baculoviruses were multiplied in SF9 cells, which were cultured at 27°C in Grace's insect cell medium (Invitrogen) supplemented with 5% foetal bovine serum (FBS, Gibco).

Production and purification of VLPs

Sf9 cells, an insect cell lines, in the mid-logarithmic phase of growth were seeded in cell culture dishes with 100 mm diameter at 2 x 10^6 cells/ml and infected with Ac-C-E using a multiplicity of infection (MOI) of 5. The supernatants were harvested and cleared by centrifugation at 6000 g for 20 min, at 72h post-infection, then, the collected supernate was centrifuged at 35000 rpm for 2 h at 4°C. The collected precipitate was resuspended in 500 µl PBS, and the dissolved matter loaded slowly on a 20- 40-70% (w/v) discontinuous sucrose gradient. It was then centrifuged for 4 h at 35000 rpm and 4°C, a visible band between the 40% and 70% sucrose layers was collected, concentrated by centrifugation and resuspended in phosphate buffer saline, then was analyzed for VLPs by western blot or electron microscopy. VLP proteins were quantified by using the BCA Protein Assay Kit.

Western blot analysis

The purified VLPs were denatured in a gel loading buffer with SDS and β-mercaptoethanol, boiled for 5 min, and the treated samples were then stored at -20°C until processing. After the SDS-PAGE experiment, protein samples were transferred onto PVDF membranes (Millipore). Membrane blots were blocked with 5% nonfat milk in TBST (tris-buffered saline and tween 20) for 2 h at RT or overnight at 4 °C. Membranes were washed 3×10 min with TBST and then incubated for 2 h at RT with mouse monclonal antibodies against WEEV-E1 protein (Millipore), 1:2500 diluted in TBST, or with 1: 3000 diluted mouse polyclonal antibodies against WEEV E2 protein (in-house antibody). Membranes washed with TBST were treated using a goat-anti-mouse HRP-conjugated

antibody for 1h at RT. Images were obtained using a luminescent imaging system (LAS-4000; Fujifilm, Tokyo, Japan).

Immunofluorescence assay

4×10^5 Sf9 cells were seeded in a 24 well culture plate and infected with Ac-C-E for 48 h at 27℃. The culture media was removed, and subsequently, infected cells were fixed with 80% acetone in PBS for 2 h at -20℃ and washed 3×1 min using PBS, then, permeabilized with 0.5% Triton X-100 for 10 min, treated with 1: 50 diluted mouse monclonal antibodies against WEEV E1 protein or 1: 100 diluted mouse polyclonal antibodies against WEEV E2 protein for 1 h at 37℃. After another washing step, cells were incubated with 1:200 diluted goat anti-mouse FITC-conjugated antibodies (Invitrogen) for 1 h at 37℃ away from light. Finally, the cells were washed and the fluorescence intensity detected using a fluorescence microscope (Olympus IX51).

Electron Microscopy

VLP samples were adsorbed onto the surface of copper 400 square mesh grids (Veco) and were stained with 1% phosphotungstic acid for 2 min. Excess dye solution was removed with filter paper and then dried naturally for 5 minutes. WEEV VLPs were detected using a JEM 1200EXII transmission electron microscope (TEM) at 80 kV with magnification×40K.

Immunoelectron Microscope

To further confirming VLPs, 100 µl of purified WEEV VLPs was added into a nitrocellulose microtube, with 400-mesh naked-nickel grids placed in the bottom in advance, and centrifuged at 35000 rpm for 20 min at 4°C, in order to improve the adsorbtion of VLPs onto the surface of grids. The VLP-coated grids were washed 2×3 in TBST (25mM Tris-Cl, 150mM NaCl, 0.05% Tween 20, pH7.2) and partly dried with filter paper, and then incubated with mouse monclonal antibodies against the WEEV-E1 protein (diluted 1: 200) or mouse polyclonal antibodies against WEEV-E2 protein (diluted 1: 400) for 30 min at 37°C. After another washing step, the grids were treated with TBS-diluted colloidal protein A-gold (PAG) particles (10~15 nm in diameter) (Sigma) for 30 min at 37°C. The grids were finally rinsed with TBST, and were stained with 1% phosphotungstic acid for 2 min. Nonimmune sera of mice were used as the control to confirm the specificity of the labeling, including incubation with VLP-coated grids and PAG particles.

Determination of WEEV VLP immunogenicity

Female BALB/c mice aged 6-8 weeks were used in this study. The mice were randomly divided into the blank group, 206-adjuvant group (206-group), VLPs group and 206 adjuvant+VLPs group (206-VLPs group). There were 10 mice in each group. WEEV VLPs was emulsified with an equal volume of a 206 adjuvant (Montanide™ ISA 206 VG adjuvant, Seppic, France). Each mouse of the former two groups was injected with 100 µl phosphate buffer saline and 206 adjuvant mix (containing equal volume of 206 adjuvant and PBS), respectively. Each mouse of the

other two groups were inoculated with 100 μl purified WEEV VLPs (15 μg VLPs) and 206 adjuvant plus VLPs emulsion (15 μg VLPs), respectively. Each mouse was intramuscularly inoculated twice at an interval of 2 weeks in the right and left quadriceps. Blood samples from mice were collected to assess immune effectiveness after inoculation by retro orbital plexus puncture.

ELISpot analysis of antigen-specific T cells.

Fourteen days after post booster immunization, to confirm the VLP-specific T cell activation induced by our immunisation regimen, an enzyme-linked immunospot assay (ELISpot) was performed and as described previously [25]. Briefly, multiscreen 96-well plates were coated at 4°C overnight with 100 μL per well of with 5 μg/ml anti-mouse gamma interferon (IFN-γ) IgG1 (clone R4-6A2, BD Biosciences) and blocked for 2 h at 37°C. The spleens of mice from each group were removed aseptically and punctured to produce a single-cell suspension. Red cells were removed using 0.83% ammonium chloride. Freshly prepared splenocytes were added at 5×10^5/well in triplicate with WEEV VLPs (5 μg/ml), PMA (50 ng/ml) and ionomycin (1 μg/ml). PMA were added to the positive-control group, whereas the negative-control group received no added stimuli. The plates were incubated for 24 h at 37°C in 5% CO2. Next, biotinylated detection antibody and streptavidin–horseradish peroxidase was added to wells, and spots were developed by using AEC (3-amino-9-ethylcarbazole) substrate solution after 20 min incubation at RT in the dark. Finally, IFN-γ spot-forming cells (SFCs) were counted. The results are expressed as the number of SFCs per 10^6 input cells. The number of VLPs-specific IFN-γ-secreting T cells was calculated by subtracting the background (no-VLPs) control value from the established SFC count.

Cytokine release assay

To determine the cytokine secretion from lymphocytes, mouse splenocytes fourteen days after post booster immunizationwere prepared as above and were plated at a density of 1×10^6 cells per well in 96-well plates, treated with WEEV VLPs (5 μg/ml)) at 37°C in 5% CO2. The culture supernatants were collected after 72-h incubation and IFN-γ, IL-2, IL-4 and IL-10 levels were analyzed by commercial mouse immunoassay ELISA kits (BD Biosciences), according to the manufacturer's instructions. The concentrations of cytokines detected in the samples were determined by using standard curves.

Analysis of ELISA for IgG antibodies

To measure IgG antibodies against the E2 protein of VLP in individual mouse serum samples fourteen days after post booster immunization, an enzyme-linked immunoabsorbent assay (ELISA) was performed using purified His-tagged E2 proteins as coated antigens. E2 proteins were home-made in *E.coil* expression system and were purified using Ni-NTA Spin Kit (Qiangen). Briefly, the purified His-tagged E2 proteins with a concentration of 5 μg/ml were coated in 96-well polystyrene microtiter plates overnight at 4 °C. After coating, the plates were blocked

with 5% non-fat milk in PBST at 37 °C for 60 min. Each serum sample was added in triplicate at serial dilution with PBST and incubated for 2 h at 37 °C. Following incubation, HRP-labeled antibodies against mouse IgG, IgG1, IgG2a or IgG3 (Sigma) at 1:5,000 diluted in PBST was added to corresponding well and incubated for 60 min at 37 °C, followed by adding a substrate of TMB (3,3′,5,5′ -tetramethyl benzidine, Sigma), and 0.5 M sulfuric acid as a stop buffer. The optical density (OD) values at 450 nm were read with an enzyme-labeled instrument (BIORAD 680), and the mean OD for each sample was calculated. The assay cut-off value was determined by counting the mean OD value of the serum samples from normal mice plus 3 standard deviations (SD) [26]. Finally, antibody endpoint titers were expressed as Log10 concentrations.

Neutralization assay

A neutralization assay was performed using the WEEV envelope glycoproteins (GP) pseudotyped HIV. To produce WEEV pseudoviruses, 293 T cells were cotransfected with plasmids carrying HIV *gag-pol* (pLP1; Invitrogen), *rev* (pREV; Invitrogen), and EGFP (enhanced green fluorescent protein) plasmids (Lentivirus shuttle plasmid), the WEEV-GP expression plasmids (pcDNA3.1-C-E vectors containing WEEV capsid, E3, E2, 6k, and E1 genes). After 48 hours post-transfection, the culture supernatants were collected and concentrated using centrifugal filter devices, and then IU (infectious units) of the pseudovirus was determined by counting the 293T cells with green fluorescent expression under the condition of 10-fold dilution series.

To evaluate the neutralization of IgG antibodies from VLP-immunized mice serum fourteen days after post booster immunization, each heat-inactivated serum sample with 2-fold dilution series was treated, resulting in 1/2 to 1/512 dilution, and then 50 µl of diluted sera was incubated with equal volume DMEM containing 500 IU of WEEV pseudoviruses in a 96-well plate for 1 h at 37 °C. After incubation, 100 µl of 293T cells (5×10^4 cells) suspension in DMEM supplemented with 10% FBS was added into each well of the plates, and were cultured at 37°C, 5% CO_2 for 72 h. Neutralization was determined according to the percentage of reduction of the number of fluorescent expression cells in sample wells, which showed the pseudovirus incubated with serum samples from immunized groups, compared with the control wells. This showed that the same dilution of per group matched preimmune serum samples was incubated with the same dose of pseudotyped virus [(the number of cells with fluorescent in control wells - the number of cells with fluorescent in sample wells)/(the number of cells with fluorescent in control wells) × 100%].

Statistical analysis

A statistical analysis was performed using the SPSS10.0 statistical software. Differences in mean values were tested by Student's t-test; p values < 0.01 or $p < 0.05$ were considered significant.

Results

Expression analysis of the WEEV structural polyprotein

The 3711 bp target fragments coding 5 proteins (C, E3, E2, 6K, E1) of WEEV (Figure 1A) were obtained by PCR (Fig. 1B), after digested, they were inserted into pfastBac^{TM}1 plasmids. After the recombinant plasmids pfastBac^{TM}1-*c-e3-e2-6k-e1* were treated with the *Stu*I and *Hin*d III restriction endonuclease and PCR method, 3711 bp target segments were exhibited in agarose gel (Fig. 1C, 1D), indicating the recombinant plasmids were correctly constructed. In addition, the about 6000 bp target-fragments (Fig. 1E), including 2300 bp (lacZ gene and "ini-att Tn7" (transposable seat point)) on baculovirus and exogenous gene fragment (3711 bp), were obtained with the recombinant AcMNPV-C-E3-E2-6K-E 1 bacmid DNA as template and M13 as primer for PCR amplification, further, the 3711 bp PCR production were displayed with the recombinant bacmid DNA as template and specific primers(Fig. 1F), therefore, these results indicated that the exogenous *c-e3-e2-6k-e1* fragments were correctly recombined to the target sites of the baculovirus genome, manifesting that the recombinant baculovirus was correctly constructed.

The purified VLPs from supernatant of cells infected with Ac-C-E was analyzed by western blot using E1 monoclonal and E2 polyclonal antibodies. Western blot analysis yielded protein bands of E1 (Fig. 2 A) and for E2 (Fig. 2 B), their sizes corresponded to predicted molecular masses of E1 (47.3 kDa), E2 (46.9 kDa), respectively. Moreover, IFA results showed that Sf9 cells infected with Ac-C-Es exhibited strong fluorescence, by contrast, there was no fluorescence in the mock cells (Figure 1C). Collectively, these results confirmed that E1 and E2 proteins were expressed by Ac-C-E-infected Sf9 cells.

Analysis of VLPs morphology

As shown in Figure 3, hollow, spherically shaped structures with a diameter of about ~50 nm were observed for the supernatants of Ac-C-Es-infected cells or purified VLPs. However, there were no such structures in the supernatants of the uninfected Sf9 cells (data not shown). Immuno-electron microscopy results showed that several gold particles located on the surface of each VLP, and at least 3-10 of the same VLPs per grid, were detectable in negative stained preparations. However, no gold particles were observed on the control grids (Figure 3).

The antigen-specific cellular immune response was induced by VLPs

In this study, we first confirmed whether WEEV VLPs could effectively induce T cell responses in mice. The production of IFN-γ by splenocytes from immunized mice was analyzed by enzyme-linked immunosorbent spot (ELISPOT) assay. Mouse spleen lymphocytes were treated with VLPs as described previously. As shown in Figure 4A, after two intramuscular vaccinations, the spot-forming cells (SFCs) from splenocytes of 206 VLPs group were significantly higher ($P < 0.01$) than that of the other groups (Blank group, 206 group and VLP group), and the SFCs from splenocytes of VLPs group was dramatically increased than that of control groups (Blank group, 206 group).

Therefore, VLPs can induced antigen-specific cellular immune response. Furthermore, this immune response was maximised by VLPs priming with 206 adjuvant.

VLPs improved cytokine production of lymphocyte from vaccinated Mice

T helper cells can secrete various cytokines that play an important role during immune regulation. To determine whether T helper cells were activated in mice immunized with VLPs, we detected cytokine IL-2, FN-γ , IL-4 and IL-10 level in the culture supernatants of mouse splenocytes after VLP incubation. As shown in Figure 4 B and 4 C, IL-2 and IFN-γ levels from 206-VLPs group were significantly higher ($P < 0.01$) than those from the other groups. The two cytokine levels of VLPs group were higher ($P < 0.05$) than those of control groups (Blank group, 206 group). Compared with control cultures supernatants, production of IL-4 from VLPs or 206-VLPs groups was markedly higher than that of control groups (Figure 4 D), and but no significant production of IL-10 was identified in any group (data not shown). Therefore, immunization with VLPs promoted FN-γ, IL-2 and IL-4 production, resulting in an increased antigen-specific T cellular immune response.

Effects of VLPs on IgG subclass production

Subsequently, we further investigated the effects of VLPs on humoral immune response. Because the WEEV E2 proteins are the primary antigens for eliciting protective immune responses against WEEV [27], we determined the total IgG levels against E2 proteins and IgG subclass (IgG1, IgG2a and IgG3) in serum samples by ELISA. The mean of the 57 sera samples from the normal female BALB/c mice was 0.11, 3*SD= 0.08. Therefore, a cut-off value of 0.19 was used in our data analysis, and all the serum samples with an OD above 0.19 were considered to be positive for E2 proteins of WEEV. As shown in Figure 5A, an analysis of total IgG titers showed that the 206-VLPs-immunized or VLPs-immunized mice generated strong IgG responses against E2 proteins, and IgG titers from 206-VLPs group were markedly higher than those from VLPs group, but there were no IgG generation in the control groups. In addition, as shown in Figure 5B, both VLPs alone and VLPs plus 206 adjuvant induced positive IgG1, IgG2a and IgG3 responses to E2 proteins. Then, we analyzed the IgG1 (Th2 polarization) and IgG2a (Th1 polarization) subclass responses induced by VLPs alone or VLPs plus 206. Comparative analysis showed that there were no significantly difference between IgG1 and IgG2a titers in VLPs-immunized mouse serum ($P > 0.05$), indicating a balanced Th1/Th2 response, but the IgG2a titers were markedly higher than IgG1 level in 206-VLPs-immunized group serum ($P < 0.05$), which indicated VLPs plus 206 generated Th1-polarized antibody response in mice.

VLPs effectively induced production of neutralizing antibodies

To evaluate whether the antibodies induced by VPLs can neutralize virus infection, we performed a pseudotype virus-neutralization assay. As show in Figure 6, serums from the 206-VLPs-immunized mice exhibited neutralizing activity of 100 % on average at 1:128 dilution, and maintained about 80% neutralizing activity at 1:256 dilution. In

addition, sera from VLP-immunized mice exhibited neutralizing activity of 100% on average at 1:64 dilution and about 80% at 1:128 dilution, but no neutralizing activity was detected in serums from the control groups. These results indicated that WEEV VLPs generated in insect cells using the BEVS can effectively induce neutralizing antibodies that can prevent WEEV virus infection.

Discussion

At present, this is the first study that recombinant baculoviruses were used to produce WEEV VLPs in Sf9 insect cells. WEEV VLP proteins were correctly processed and the VLPs provided efficient immune response in mice.

The WEEV structural cassette (C-E3-E2-6K-E1) was inserted downstream of the polyhedrin promoter of AcMNPV to form recombinant baculoviruses (Ac-C-E) and was expressed in Sf9 cells. Both western blot and IF analysis confirmed that WEEV E1 and E2 proteins were expressed successfully by Ac-C-Es-infected Sf9 cells. We observed that the VLPs are very similar to WEEV and other alphavirus morphology using electron microscopy analysis indicating E1 and E2 proteins can assemble VLP. Moreover, immunolelectron microscopy detection further showed that VLPs can specially bind E1 monoclonal antibodies or E2 polyclonal antibodies and further carry gold particles on the surface of each VLP. Taken together, these results may adequately indicate that Sf9 cells infected with Ac-C-E can generate WEEV VLPs, which appeared to be specific for cells expressing the WEEV structural polyprotein.

To characterize VLP-induced immune responses, we immunized mice with the purified WEEV VLPs, formulated with 206 adjuvant. As described above, WEEV VLP was found to be effective as an immunogen. ELISpot data showed that VLPs significantly induced the antigen-specific cellular immune response when analyzed the frequencies of IFN-γ-producing cells. Furthermore, cytokine release assay for antigen-specific $CD4^+$ and $CD8^+$ T cells producing cytokines confirmed that WEEV VLPs can generated significant level of both CD4 + and CD8+ T cell responses for Th1cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4). Regarding the effects of VLPs on modulating humoral immune responses, our results showed that WEEV VLPs can cause markedly strong IgG response against WEEV E2 proteins in mice, moreover, our IgG subclass data indicated that VLPs alone induced more balanced Th1/Th2 immune responses, but VLPs plus 206 generated Th1-polarized antibody response in mice suggesting the induction of a strong Th1-biased immune response. Importantly, sera from 206-VLP and VLP groups were able to exhibit neutralizing WEEV pseudovirus activity of 100% at 1:64 dilution. This is high in range to the neutralizing titers of sera from mice immunized with SIN/ WEEV strain CO92-1356 chimera vaccine, which was shown to neutralize 80% of the WEEV infectivity at about 1:60 dilutions as reported by Atasheva et al [28] [2]. Therefore, the results suggested that WEEV VLPs were capable of inducing the production of effective

neutralization antibody against WEEV. Collectively, in present experiment, 206-adjuvant is a kind O/W type adjuvant based on purified mineral, the immune efficacies of 206-VLP group were better than ones of VLPs group, which indicated that the adjuvant induced not only humoral immune responses but also strong cellular responses for assisting WEEV VLPs.

To our knowledge, there are few reports of *alphavirus* VLPs generated using the baculovirus expression vector system (BEVS). Only previous vaccination studies showed the successful production of Chikungunya virus (CHIKV) virus-like particles (VLPs) in insect cells using BEVS and immunization with CHIKV VLPs induced strong immune responses in mice [22]. Our results are consistent with the immune effect of CHIKV VLPs. More importantly, WEEV VLPs have unique advantages comparing with inactivated vaccine and recombinant WEEV vaccine [28-31]. First, the morphology and structure of the VLPs were found to be consistent with those of the parental virus. Second, VLPs were found to mimic their antigenic epitopes and so retain strong immunogenicity. Finally, these particles may be safer and have fewer side effects than parental virus because they are formulated without nucleic acids. All these findings indicate the potential of VLPs as a new vaccine.

It was confirmed that WEEV spreads not only by mosquito bite but also by aerosol exposure. Unfortunately, the latter has caused several laboratory accidents in which personnel exposed to WEEV resulted in 40% fatalities [4]. Therefore, there is a huge potential danger when researchers perform work with highly dangerous viruses similar to WEEV, which suggests that operations must be conducted in biosafety level 3 containment laboratories with select agent access. It is also recommended that researchers be vaccinated with WEEV vaccine. However, neither a human vaccine nor antiviral drugs are currently available for the prevention and treatment of WEEV. Recently, it has been suggested that a pseudovirus presenting native, neutralizing epitopes on its surface may offer a stable, safe source of alternatives to the live virus (which presents a high level of danger in assays) [32, 33]. Consequently, we chose pseudoviruses with WEEV-GP to detect neutralization antibodies against WEEV and didn′t perform the challenge assay. Even so, to a great extent, our results showed WEEV VLPs produced in Sf9 cells are immunogenic in mice, which could provide an important basis for the development of novel WEEV vaccine.

Although the study results firstly showed that WEEV VLPs were generated in insect cells and displayed effective immunogenicity in mice, in the interest of obtaining the yield of high-quality, processed, effective VLP products, we will optimize the WEEV-VLP production condition involving the time of Ac-C-E-infected insect cells, MOI, cell culture temperature, and so on, which may improve effective VLPs assembly and release from insect cells to enhance their immunogenicity. Interestingly, VLPs are also highly versatile and amenable to manipulation during production to incorporate immune-stimulatory molecules [34-38], which may be applied to the design of the WEEV VLP vaccines to increase induction of protective immune responses. Furthermore, investigations will further

optimize the VLP vaccine dose, its formulation with other adjuvants, and the immunization procedure for achieving rapid and long-term immune protection against WEEV infections in animal.

Author contributions

HuaLei WANG contributed design of the study. JinZhu MA performed the experiments. SongTao YANG and XianZhu XIA analyzed the data. XueXing ZHENG and HongXia WU wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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