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A virus-like particle vaccine confers protection against enterovirus D68 lethal challenge in mice

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ABSTRACT

Enterovirus D68 (EV-D68) is increasingly associated with severe acute respiratory infection and acute flaccid myelitis (AFM) in children around the world. However, neither vaccines nor therapeutic drugs are available for EV-D68. Here we report the development of a virus-like particle (VLP) based experimental EV-D68 vaccine. We found that EV-D68 VLPs could be successfully generated in insect cells infected with a recombinant baculovirus co-expressing the P1 precursor and 3CD protease of EV-D68. Biochemical and electron microscopic analyses revealed that EV-D68 VLPs were composed of VP0, VP1, and VP3 capsid proteins derived from precursor P1 and were visualized as spherical particles of ~30 nm in diameter. Immunization of mice with EV-D68 VLPs resulted in the production of serum antibodies that displayed potent serotype-specific neutralizing activities against EV-D68 virus *in vitro*. Passive transfer of anti-VLP sera completely protected neonatal recipient mice from lethal EV-D68 infection. Moreover, maternal immunization with these VLPs provided full protection against lethal EV-D68 challenge in suckling mice. Together, these results demonstrate that the recombinant EV-D68 VLP is a promising vaccine candidate against EV-D68 infection.

1. Introduction

Enterovirus D68 (EV-D68), a member of the D species of the *Enterovirus* genus within the *Picornaviridae* family, was originally isolated from four pediatric patients with pneumonia and bronchiolitis in the United States in 1962 [1]. Since that time, EV-D68 infections have been identified only sporadically all around the world. For instance, only 26 cases of EV-D68 respiratory disease were documented in the United States from 1970 to 2005 [2]. However, the number of EV-D68 clinical cases increased considerably worldwide in the late 2000s [3,4]. In 2014, EV-D68 caused a large-scale outbreak of severe respiratory disease in the United States, and a total of 1153 EV-D68 infections were laboratory confirmed, including 14 fatal cases (https://www.cdc.gov/non-polio-enterovirus/about/eV-d68.html#outbreak). This number is likely to be severely underestimated, partly due to the lack of a rapid and EV-D68-specific molecular assay for the majority of clinical laboratories at that time [5]. This outbreak quickly spread to Canada, Europe and Asia, and more than 2000 cases of EV-D68 were documented in 20 countries in 2014 [6]. After the 2014 outbreak, EV-D68 continued to circulate in many countries in America, Asia, and Europe [7–11]. These epidemiological surveys indicate that EV-D68 is becoming an important etiological agent of severe respiratory illnesses. In addition, more and more studies suggest that EV-D68 infections are also associated with acute flaccid myelitis (AFM), characterized by signs including muscle weakness and paralysis [12–15]. Thus, EV-D68 has emerged as a major global threat to children's health. However, no vaccines or specific treatments are available for EV-D68.

EV-D68 is a small non-enveloped virus with a ~7.4 kb single-stranded positive-sense RNA genome, which includes a single open reading frame (ORF) and a poly(A) tail at the 3′ end [16,17]. The ORF is translated into a large polyprotein containing P1, P2, and P3 regions. The P1 region encodes the structural protein precursor

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P1, while the P2 and P3 regions contain the nonstructural proteins, such as viral protease 3CD that can efficiently cleaves P1 precursor to the three individual capsid proteins VP0 (the precursor of VP4 and VP2), VP1 and VP3. The three proteins assemble spontaneously into viral shells and concomitantly package the viral RNA genome, and finally VP0 is cleaved to form VP4 and VP2, yielding the mature EV-D68 virus [16]. The mature capsid, about 300 Å in diameter, exhibits pseudo T = 3 icosahedral symmetry and consists of 60 copies of each of VP1, VP2, VP3, and VP4 proteins, as revealed by the crystal structure of EV-D68 virus [18]. Based on phylogenetic analysis of VP1, EV-D68 is often classified into clades A, B and C, in addition to the original Fermon lineage [4]. Moreover, clades A and B can be further divided into 5 subclades (A1-A2 and B1-B3) [19].

Virus-like particles (VLPs) are empty particles assembled from recombinantly expressed virus structural proteins, which can emulate the conformation of authentic native virions but lack the viral genome. VLPs show high immunogenicity and safety, and therefore have been widely used in the development of human vaccines [20,21]. Indeed, VLPs have shown promising results as vaccine candidates against many enteroviruses, such as enterovirus 71 (EV71), coxsackievirus A16 (CA16), and coxsackievirus A6 (CA6) [22–30]. In the present study, we explored the potential of VLP as vaccine candidate against EV-D68 infection. Our results showed that EV-D68 VLPs could be successfully generated in the baculovirus-insect cell system and these VLPs could induce potent neutralizing antibody responses and provided effective protection against lethal EV-D68 infection in mice.

2. Materials and methods

2.1. Cells and viruses

RD cells (ATCC, CCL-136) were grown as described previously [31]. *Spodoptera frugiperda* Sf9 insect cells were grown in Sf-900 II SFM medium (Invitrogen, USA) at 27 °C. EV-D68 strains Fermon (GenBank ID: AY426531), US/MO/14-18947 (GenBank ID: KM851225), and US/KY/14-18953 (GenBank ID: KM851231) were purchased from ATCC and propagated in RD cells. EV71 strain EV71/G082, CA16 strain CA16/SZ05 and CA10 strain CA10/Kowalik were described previously [31–33]. All enteroviruses were titrated for the 50% tissue culture infectious dose (TCID50) using RD cells, according to the Reed–Muench method [34], and baculoviruses were titrated for TCID50 using Sf9 cells by the same method.

2.2. Proteins, inactivated EV-D68 virus and antibodies

Recombinant VP0, VP1, and VP3 proteins derived from EV-D68 strain Fermon were individually prepared in *Escherichia coli* (*E. coli*) by using a protocol described in a previous study [35]. The anti-VP0, anti-VP1, and anti-VP3 polyclonal antibodies were generated by immunizing BALB/c mice with recombinant EV-D68 VP0, VP1 and VP3 proteins, respectively. β-propiolactone-inactivated EV-D68 virus was prepared from RD cells infected with EV-D68 strain US/MO/14-18947 by using protocols identical to those described in a previous study [32].

2.3. Vector construction

The P1 gene of EV-D68 strain US/MO/14-18950 (GenBank ID: KM851228) was optimized, synthesized, and then cloned into the pFastBac™ Dual vector (pFBD, Invitrogen, USA) under the control of polyhedrin (PH) promoter, to generate plasmid pFBD-EV-D68-P1. US/MO/14-18950 strain was used as the basis for cloning viral structural protein sequence, because compared with US/MO/14-18947 strain, US/MO/14-18950 has a slightly higher degree of sequence identity to the other EV-D68 clinical strains (data not shown). To get 3CD gene, RNA was extracted from RD cells infected with EV-D68 strain US/MO/14-18947 and subsequently reverse transcribed using oligo (dT) primers and M-MLV reverse transcriptase (Promega, USA), to produce cDNA; 3CD gene fragment was amplified by PCR from the cDNA and then inserted into pFBD-EV-D68-P1 under the control of p10 promoter, resulting in a construct named pFBD-EV-D68-P1/3CD.

2.4. Generation and plaque purification of a recombinant baculovirus

The plasmid pFBD-EV-D68-P1/3CD was transformed into *E. coli* DH10Bac competent cells, to generate recombinant Bacmid DNA. The recombinant Bacmid was used to transfect Sf9 insect cells to generate the corresponding recombinant baculovirus, namely Bac-EV-D68-P1/3CD, according to the manual of the Bac-to-Bac baculovirus expression system (Invitrogen, USA).

To isolate a pure viral stock, the baculovirus Bac-EV-D68-P1/3CD was purified by one round of plaque purification. Briefly, Sf9 cell monolayers were inoculated with 10-fold serial dilutions of the baculovirus Bac-EV-D68-P1/3CD. After adsorption for 1 h, the residual baculovirus was removed, and the cells were overlayed with 1% low melting-point agarose (Promega, USA) in Sf-900 II SFM medium and then incubated at 27 °C. After 7–10 days. 5 plaques were randomly picked, amplified, titred and then separately inoculated onto Sf9 cells at the same multiplicity of infection (MOI) of 0.1 followed by culture at 27 °C for 3 days. At last, Sf9 cells from each culture were harvested, and lysed in 0.15 M PBS buffer containing 1% NP-40. The resulting cell lysates were analyzed for EV-D68 protein expression by Western blotting as described below.

2.5. Preparation of EV-D68 VLPs and control antigen

To prepare EV-D68 VLPs, Sf9 suspension cells (2×10^6/ml) were infected with the selected plaque-purified Bac-EV-D68-P1/3CD strain with the highest expression level at an MOI of 1 for 72 h. Cells were then collected and lysed in 0.15 M PBS buffer containing 1% NP-40. The lysates were clarified by centrifugation at 4000 rpm for 5 min, supplemented with 0.2 M NaCl and 10% (W/V) PEG 8000, mixed at 4 °C overnight, and then centrifuged at 12,000 rpm for 15 min. The resultant precipitates were resuspended in 0.15 M PBS buffer, and centrifuged at 12,000 rpm for 15 min to remove insoluble fractions. The clarified supernatants were then subjected to 20% sucrose cushion and 10–50% sucrose gradient ultracentrifugation by using a protocol described in a previous study [36]. After ultracentrifugation, 12 fractions were taken from the top of the sucrose gradients and then analyzed by ELISA and Western blotting as described below. The fractions that contained VLPs were pooled and concentrated by ultrafiltration using an Amicon Ultra 100 K filter (Millipore, USA). Cell lysates from uninfected Sf9 cells were subjected to the same treatments to generate the negative control antigen. Total protein concentrations of the final VLP preparation and control antigen were measured by Bradford assay and then used for immunization experiments.

2.6. Western blotting and ELISA assays

Western blotting of the cell lysates or gradient fractions was carried out as described previously [36], except that EV-D68 subunit protein-specific polyclonal antibodies were used for detection.

For indirect ELISA, 96-well microplates were coated with 1 μl/well of gradient fractions plus 49 μl of PBS buffer at 37 °C for 2 h, and then blocked with 200 μl/well of PBST containing 5% milk at 37 °C for 1 h; the plates were incubated with 50 μl/well of mouse

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