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Display of enterovirus 71 VP1 on baculovirus as a type II transmembrane protein elicits protective B and T cell responses in immunized mice

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ABSTRACT

Human enterovirus 71 (EV71) has become a major public health threat across Asia Pacific. The virus causes hand, foot, and mouth disease which can lead to neurological complications in young children. There are no specific antivirals or vaccines against EV71 infection. The major neutralizing epitope of EV71 is located in the carboxy-terminal half of the VP1 protein at amino acid positions 215–219 (Lim et al., 2012). To study the immunogenicity of VP1 we have developed a baculovirus vector which displays VP1 as a type II transmembrane protein, providing an accessible C-terminus. Immunization of mice with this recombinant baculovirus elicited neutralizing antibodies against heterologous EV71 in an *in vitro* microneutralization assay. Passive protection of neonatal mice confirmed the prophylactic efficacy of the antisera. Additionally, EV71 specific T cell responses were stimulated. Taken together, our results demonstrate that the display of VP1 as a type II transmembrane protein efficiently stimulated both humoral and cellular immunities.

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1. Introduction

Human enterovirus 71 (EV71) is a major cause of hand-foot-and-mouth disease (HFMD) in children below 6 years of age. Over the last decade, HFMD has become endemic in the Asia Pacific region with outbreaks occurring in Singapore, Malaysia, Japan and China every few years (WHO, 2011). After the eradication of poliovirus, EV71 has been regarded as the most important neurotropic enterovirus and a threat to global public health (Bible et al., 2007; Qiu, 2008; Wu et al., 2010). There are no specific antivirals or vaccines for EV71 infection and prevention is mainly achieved by disrupting virus transmission by surveillance, improved hygiene and temporary closure of childcare centers and schools during outbreaks. Several patient studies showed that EV71 is cleared by both humoral and cellular immunities (Wang et al., 2003; Yang et al., 2011). Furthermore, viral loads were significantly higher in mice deficient in B cell, CD4 T-cells, or CD8 T cells (Lin et al., 2009). The involvement of B and T cell responses in the control of EV71 infection makes vaccination a feasible prevention measure. EV71 is a member of the human enterovirus A (HEV-A) species of the enterovirus genus belonging to the of the picornavirus family. The virus particle consists of a naked icosahedral capsid surrounding a single-stranded positive-strand RNA with an open reading frame coding for 11 proteins. The VP1 capsid protein harbors the main neutralizing epitopes of EV71, found on amino acids 163–177 and 208–222 in the GH loop (Foo et al., 2007b). The minimal epitope has been mapped to amino acids 215–219 (Lim et al., 2012). Being the most variable capsid protein, the VP1 sequence is used to divide EV71 into three major genogroups (denoted as A, B and C), and various subgenogroups within genogroups B (B1–B5) and C (C1–C5) (Oberste et al., 1999).

Different strategies have been adopted to produce an EV71 vaccine including inactivated virus (Bek et al., 2011; Dong et al., 2011; Ong et al., 2009), attenuated virus (Arita et al., 2007), and virus-like particles (Chung et al., 2008). As VP1 is involved in binding to host cell receptors and carries the major antigenic determinants of EV71, VP1 subunit vaccines have recently come into focus. Synthetic peptides containing linear neutralizing epitopes can elicit neutralizing antibodies and provide passive protection (Foo et al., 2007a). Subunit vaccines have a high safety profile and can be rapidly adjusted to reflect virus mutations. To enhance subunit stability and boost immune response, viral vectors are increasingly used for subunit display. EV71 VP1 protein has been expressed on

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the baculovirus surface as a fusion protein with the baculovirus envelope protein GP64. Immunization of mice with this construct resulted in a significant increase in antigen specific IgG and a high cross-neutralization titer that persisted post-immunization (Meng et al., 2011). Baculovirus surface display of foreign proteins has traditionally involved fusion to the baculovirus membrane protein GP64 (Boublik et al., 1995; Madhan et al., 2010). Draw-backs to this strategy include that only a low number of fusion proteins seem to incorporate into the viral membrane (Toivola et al., 2002). Second, there might be interference of fused GP64 with wild-type GP64 in the baculovirus budding process. Third, the orientation of the fusion partner generates a type I transmembrane protein with a distal N-terminus.

The N-terminal region of influenza A neuraminidase (NA) is sufficient to drive surface expression of a fusion protein in a mammalian expression system thereby creating a type II transmembrane protein with the N-terminus proximal to the membrane and the C-terminus distal (Xu et al., 1993). We applied the same strategy to generate recombinant baculoviruses that display VP1 on their surface in a type II transmembrane protein orientation which might make the VP1 C-terminus more accessible for immune inspection (Peterson et al., 1996). Using the amino-terminus of NA instead of full-length GP64 as a surface anchor also greatly reduces the size of the fusion protein thus enhancing the chance of membrane incorporation (Toivola et al., 2002). Together, this was anticipated to improve the immunogenicity of the displayed VP1. We here report immunogenicity studies of a VP1 protein with an accessible C-terminus displayed on baculovirus. The VP1 of EV71-Fuyang (subgenogroup C4) was fused to an N-terminal sequence of influenza neuraminidase providing a signal peptide and transmembrane domain to create a type II transmembrane protein. Recombinant baculovirus (Bac-NA-VP1), expressing NA-VP1 under the WSSV immediate-early promoter Ie1, rendered the localization of VP1 to the plasma membrane of insect cells and budded baculoviruses. After three immunizations, both humoral and cellular immune responses were activated in mice. The antisera had a high neutralization activity against the heterologous EV71-B5 strain, and passive protection resulted in a survival rate of 100% in newborn mice. Cellular immunity was observed upon stimulation with heterologous EV71 in an interferon gamma (IFNγ) enzyme-linked immunosorbent spot (ELISPOT) assay.

2. Materials and methods

2.1. Construction of recombinant bacmid DNA

The recombinant Bac-gp64-VP1 Bacmid was constructed as described (Meng et al., 2011). The Bac-NA-VP1 Bacmid with FLAG tag and Ie1 promoter was constructed in sequential steps: first, the full length VP1 gene was cloned into a modified pFastBac (Invitrogen, USA) vector containing the Ie1 promoter. Second, the NA anchor was cloned in frame before VP1. Third, the C-terminal FLAG tag was added by PCR to facilitate detection. Full length VP1 was amplified from a synthetic gene (Genscript, USA) based on the P1 sequence of the EV71-Fuyang (C4) strain (NCBI accession # EU703813.1) with the primers VP1-fw-XbaI-ATG 5′-GCtctagaATGGGAGATAGGGTGGCAGATGTAAT-3′ and VP1-rev-SphI 5′-GCgcatgcTTAAAGAGTGGTGATCGCTGTGC-3′. The PCR product was purified by gel excision, digested at 37° for 4 h with XbaI and SphI in buffer 2 (New England Biolabs, USA) and cloned into a modified pFastBac vector where the polyhedrin promoter has been replaced by the WSSVIe1 promoter as described previously (He et al., 2008). At the 3′ end of Ie1 there is a SalI restriction site left over from the original multiple cloning site of pFastBac. The vector was then transformed into *Escherichia coli*, positive colonies were sequenced, grown and purified using miniprep columns (Qiagen, Germany). Alternatively, the 36 N-terminal amino acids of NA were amplified from a synthetic gene (Genscript, China) containing the A/Brisbane/59/2007 H1N1 sequence (NCBI accession # CY030231.1) using the primers NA-pCl-Nter-fw-SalI 5′-GCgtcgacATGAATCCAAATCAAAAAATA-3′ and NA-pCl-Nter-rev-XbaI 5′-GCtctagaGTGACTAGCCCATATTGAAAT-3′. The PCR product was purified by gel excision, cloned into the gGEM-T-easy vector (Promega, USA) and transformed into competent DH10Bac *E. coli* cells (Invitrogen, USA). Positive colonies were sequenced, grown and the plasmid was purified using miniprep columns. The cycling parameters for PCR amplification by Go-Taq polymerase (Promega, USA) were: initial denaturation at 95 °C for 2 min, 25 cycles of amplification with denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Both vectors were then digested with SalI and XbaI in buffer 3 (New England Biolabs, USA) at 37 °C for 4 h. The NA fragment and linearized vector (containing Ie1 and VP1) were purified by gel excision and ligated overnight at 4 °C with T4 DNA ligase (Promega, USA). The ligated vector was again transformed into *E. coli*, positive colonies were sequenced and grown, and recombinant pFastBac-NA-VP1 was purified by column minipreps. To introduce the C-terminal FLAG tag, the NA-VP1 fusion protein was amplified with the primers PstI-Flag-NA-F 5′-GATCTGCAGGACTACAAAGACGACGACGACAAAAATCCAAATC-AAAAAATA3′ and XbaI-NA-R 5′-GCTCTAGACTCGCTAATCCATATT-GAGAT-3′ and cloned once again into the modified pFastBac vector containing the Ie1 promoter. Competent DH10Bac *E. coli* cells were then transformed with the recombinant plasmid and positive colonies were selected through two rounds of blue/white selection followed by sequencing with M13 forward and reverse primers. The recombinant bacmid DNA with in frame NA-VP1-FLAG fusion protein was isolated from white colonies according to the standard procedure (Bac-to-Bac system; Invitrogen, USA).

2.2. Generation and purification of recombinant baculoviruses

Baculovirus was propagated in *Spodoptera frugiperda* Sf9 III cell lines (ATCC, USA) which were grown at 27 °C in serum-free medium SF-900 III (Invitrogen, USA). Procedures for the generation of recombinant baculovirus were carried out according to the manufacturer's instruction (Bac-to-Bac system; Invitrogen, USA). Briefly, 10^6 Sf9-III cells were cultured in 6-well plates for 1 h. After attachment, 4 μg of recombinant beamed DNA was mixed with 10 μL Cellfectin II (Invitrogen, USA) in Grace's insect medium, unsupplemented, and then transfected into the Sf9 III cells. Transfected cells were incubated for 5 h at 27 °C and the transfection medium was replaced with fresh medium SF-900 III. After incubation for 72 h at 27 °C, the supernatant, containing recombinant viruses, was used for infecting more fresh Sf9 cells. For large scale viral production, Sf9 III cells were infected at 0.2 MOI in suspension culture having 2×10^6 cells/mL. After 4 days, the supernatant was collected and the recombinant viruses were purified by two rounds of sucrose gradient ultracentrifugation following standard protocols. The purified recombinant baculoviruses were resuspended in phosphate buffered saline (PBS) and titered by plaque assay.

2.3. Inactivation and purification of EV71 virus

Rhabdomyosarcoma (RD) cells were infected with EV71-B5 virus (NCBI accession # FJ461781.1) and grown in serum free Dulbecco's modified Eagle's medium (DMEM; Gibco, USA). 48 h post infection the cells displayed cytopathic effect (CPE). They were harvested and lysed by three freeze and thaw cycles. The 50% tissue culture infective dose ($TCID_{50}$) was determined by the Reed and Muench end-point dilution method. The virus was then

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