Vaccine 33 (2015) 6604–6610

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Pros and cons of VP1-specific maternal IgG for the protection of Enterovirus 71 infection

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ARTICLE INFO

Article history:
Received 4 February 2015
Received in revised form 6 October 2015
Accepted 24 October 2015
Available online 31 October 2015

Keywords:
Enterovirus 71
VP1
Maternal IgG
Protective immunity
Pre-existing immunity

ABSTRACT

Enterovirus 71 (EV71) causes hand, foot, and mouth diseases and can result in severe neurological disorders when it infects the central nervous system. Thus, there is a need for the development of effective vaccines against EV71 infection. Here we report that viral capsid protein 1 (VP1), one of the main capsid proteins of EV71, efficiently elicited VP1-specific immunoglobulin G (IgG) in the serum of mice immunized with recombinant VP1. The VP1-specific IgG produced in female mice was efficiently transferred to their offspring, conferring protection against EV71 infection immediately after birth. VP1-specific antibody can neutralize EV71 infection and protect host cells. VP1-specific maternal IgG in offspring was maintained for over 6 months. However, the pre-existence of VP1-specific maternal IgG interfered with the production of VP1-specific IgG antibody secreting cells by active immunization in offspring. Therefore, although our results showed the potential for VP1-specific maternal IgG protection against EV71 in neonatal mice, other strategies must be developed to overcome the hindrance of maternal IgG in active immunization. In this study, we developed an effective and feasible animal model to evaluate the protective efficacy of humoral immunity against EV71 infection using a maternal immunity concept.

1. Introduction

Enterovirus 71 (EV71), together with Coxsackievirus A16, is a common cause of hand, foot, and mouth disease (HFMD), and sometimes, albeit rarely, infection with this virus can result in neurological disorders induced by inflammation in the central nervous system (CNS). In Asian countries, including Korea, Japan, and China, there have been several outbreaks of HFMD, and thus it has become an important public health problem in this region [1]. HFMD caused by EV71 infection usually occurs in infants and children under 5 years of age and shows mild symptoms including blisters or ulcers on the hands, feet, and mouth with pharyngitis. However, CNS infection with EV71 sometimes results in aseptic meningitis, encephalitis, and acute flaccid paralysis with significant fatality rates. Unfortunately, there are currently no approved vaccines or antiviral therapeutics to prevent or treat EV71 infection [2].

EV71 is a non-enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Enterovirus* in the family *Picornaviridae*. The RNA genome of EV71 consists of about 7400 nucleotides, and the viral capsid includes 4 proteins: VP1, VP2, VP3, and VP4. Based on its antigenicity and the neutralization capacity of antibodies that are raised against it, VP1 is regarded as one of the major antigenic targets for the development of a subunit vaccine to prevent EV71 infection [3]. In addition, VP1 contains the most antigenic determinants for T cell responses that reinforce humoral immunity and that are known to be critical for preventing EV71 infection [4]. In this

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http://dx.doi.org/10.1016/j.vaccine.2015.10.103
0264-410X/

regard, recent study has suggested that recombinant VP1 protein produced by *Escherichia coli* would be a good EV71 vaccine candidate because of the strong humoral and cellular immunity induced in mice [3]. Although the antigenic characteristics of recombinant VP1 proteins expressed in *E. coli* [5] and *Pichia pastoris* [6] were evaluated in mice, the protective efficacy of these vaccine candidates was tested only by passive serum transfer in newborn mice, due to the lack of appropriate EV71-infection models. Similarly, it has been difficult to evaluate the protective efficacy of other types of EV71 vaccines based on virus-like particles and inactivated virus in existing mice model [5,7,8].

Recently, randomized, double-blinded, placebo-controlled phase 3 trials of inactivated EV71 vaccines were conducted in China with healthy children, and provided promising results in terms of vaccine efficacy against infection, although they did not confer protection against HFMD or herpangina caused by CA16 or other Enterovirus serotypes [9,10]. To further compare vaccine efficacy, and to optimize these vaccines to elicit cross-neutralizing antibody responses against other subgenotype strains of EV71, we need to develop appropriate animal models. Toward this goal, we immunized pre-gravid female mice with recombinant VP1 protein, and found that the offspring of the mice had significantly higher levels of maternal IgG specific for VP1 than control mice, and that this antibody persisted for more than 6 months. In addition, the neonate mice from vaccinated female mice showed a significant level of protection against lethal, intracerebral challenge with EV71. In this study, we have demonstrated a novel model to monitor protective humoral immunity elicited by EV71 vaccination in adult mice that will be useful for further vaccine development and evaluation.

2. Materials and methods

2.1. Virus and growth condition

Non-mouse-adapted EV71 virus strain 23/Jap/98 (GenBank: AF251802.1) belonging to subgenotype B4 and EV71 strain Fuyang.Anhui.P.R.C/17.08/3 (GenBank: EU703814.1) belonging to subgenotype C4a were used in this study. The virus was inoculated into Vero cells and purified as previously reported [2].

2.2. Production of recombinant VP1

The gene encoding VP1 protein of EV71 was amplified by polymerase chain reaction (PCR). The PCR products were digested with NdeI and XhoI restriction enzymes and inserted into the pET28a vector (GE Healthcare). The ligation mixture was transformed into *E. coli* DH5α cells, and the construct was verified by DNA sequencing using a high-throughput DNA analyzer (Bioneer Inc., Daejeon, Korea). The recombinant plasmid containing the VP1 gene was transformed into *E. coli* BL21-CodonPlus (DE3) cells, and transformed cells were grown in medium at 37 °C. When the OD_{600} reached approximately 0.5, 0.25 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added for protein expression within 4 h. After centrifugation, the cell pellet was resuspended in lysis buffer containing 20 mM Tris–HCl pH 8.5, 100 mM NaCl, 2 mM $ZnCl_2$, and 5 mM 2-mercaptoethanol, and disrupted by ultrasonication (duration time: 1 min, duty time: 40% with 2-min cooling) using a Branson Sonifier cell disruptor (Hielscher, Sonics & Materials, Inc., Newtown, CT). The disrupted cells were centrifuged at 11,057 × *g* for 1 h. Inclusion bodies were solubilized in lysis buffer containing 8 M urea and centrifuged. The supernatant was loaded onto an IMAC Ni-charged resin column (Bio-Rad), pre-equilibrated with 8 M urea lysis buffer. The bound protein was eluted using a gradient from 100 mM to 500 mM imidazole in the same buffer. Fractions containing hexahistidine (His_6) fused VP1 were pooled and dialyzed against buffer containing 4 M urea. The concentrations of urea were gradually decreased by step dialysis against buffers containing 4 M, 2 M, 1 M, and 0.5 M urea. Finally, recombinant VP1 proteins were prepared in the buffer containing 20 mM Tris–HCl pH 8.5, 100 mM NaCl, 2 mM 2-mercaptoethanol, and 0.1 M urea for further studies.

2.3. Mice and immunization

All experiments were approved by the Institutional Animal Care and Use Committees of Kangwon National University and Ajou University. Wild-type BALB/c mice were purchased from Charles River Laboratories (Orient Bio Inc., Sungnam, Korea). All mice used in the experiments were purchased at 6 weeks of age. The mice were kept in the Animal Center for Pharmaceutical Research at Kangwon National University or the Laboratory Animal Research Center of Ajou University Medical Center. For the immunization of mice, female BALB/c mice were immunized intraperitoneally (i.p.) with 20 μg recombinant VP1 at a 1:1 ratio with alum (Thermo Scientific, Rockford, IL) as an adjuvant, and were immunized again 4 weeks later. Serum samples were obtained 10 days after each immunization for the evaluation of VP1-specific IgG.

2.4. ELISA

Immuno plates (Thermo Fisher Scientific, Roskilde, Denmark) were coated with 3 μg/mL recombinant VP1 protein in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Wells were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and serum samples were diluted in 0.1% BSA/PBS before being added to the plates. Plates were then treated with horseradish peroxidase-conjugated rabbit anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL) and incubated at 4 °C overnight. TMB as a peroxidase substrate (MOSS Inc., Pasadena, MD) was added and stopped with 0.5 N HCl. Plates were read at 450 nm on an ELISA reader (Synergy H1 Hybrid Reader, BioTek, Winooski, VT). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving an OD at 450 nm of 0.1 greater than background.

2.5. EV71 infection

At 1 week after the second vaccination with recombinant VP1, female mice were co-housed with male mice for breeding. At day 1 after birth, suckling neonates born from the vaccinated or non-vaccinated female mice were intracerebrally inoculated with 10 μL of 10^8 $TCID_{50}$/mL EV71 B4 genotype. The morbidity, body weight, and mortality of the infected mice were monitored daily for 25 days. HFMD-like illness in the mice was evaluated using a graded score (0, healthy; 1, ruffled hair; 2, weakness in hind limbs; 3, paralysis in a single hind limb; 4, paralysis in both hind limbs). Brain and hind limb tissues were harvested for histological analysis and fixed in 4% formaldehyde for 1 h at 4 °C. Fixed tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E), and viewed with a digital light microscope DS-Fi2 (Nikon, Tokyo, Japan).

2.6. Serum neutralization assay

For the neutralization assay, sera were two-folds diluted from 1:10 to 1:640, and combined with $TCID_{50}$ of EV71 C4a for 1 h at 37 °C. The final solution was added to Vero cells (3×10^4 cells/well) and incubated for up to 48 h until the final reading, which was performed with basis on a cytopathic effect [11]. The cell viability was measured by SRB assay and calculated as a percentage of each sample from untreated control [2]. All serum dilutions were tested in triplicate, and the neutralizing titers were read as the highest dilution that completely inhibited viral growth in over 50% of wells.