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Formaldehyde-inactivated human enterovirus 71 vaccine is compatible for co-immunization with a commercial pentavalent vaccine

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ABSTRACT

In this study we tested the effectiveness of a formaldehyde-inactivated EV71 vaccine and its compatibility for co-immunization with a pentavalent vaccine that contained inactivated poliovirus (PV) vaccine. The inactivated EV71 vaccine (C2 genogroup) elicited an antibody response which broadly neutralized homologous and heterologous genogroups, including B4, C4, and B5. Pups from vaccinated dams were resistant to the EV71 challenge and had a high survival rate and a low tissue viral burden when compared to those from non-vaccinated counterparts. Co-immunization with pentavalent and inactivated EV71 vaccines elicited antibodies against the major components of the pentavalent vaccine including the PV, *Bordetella pertussis*, *Haemophilus influenzae* type b, diphtheria toxoid, and tetanus toxoid at the same levels as in mice immunized with pentavalent vaccine alone. Likewise, EV71 neutralizing antibody titers were comparable between EV71-vaccinated mice and mice co-immunized with the two vaccines. These results indicate that formaldehyde-inactivated whole virus EV71 vaccine is feasible for designing multivalent vaccines.

1. Introduction

Enterovirus 71 (EV71) is a non-enveloped single positive-stranded RNA virus belonging to the *Enterovirus* genus of the *Picornaviridae* family. Most EV71 infections are mild, such as hand, foot, and mouth disease and herpangina in young children; however, central nervous system infections with life-threatening pulmonary and cardiac complications may occur [1]. EV71 has been regarded as the most important neurotropic enterovirus since the effective control of the poliovirus (PV) [2]. Increased frequencies of severe EV71 outbreaks have been reported worldwide [3–5] since it was first recognized in California in 1969 [6].

Although improved disease management has significantly reduced the mortality of severe EV71 complications [7], no specific antiviral therapy or vaccine for EV71 has been commercialized. Milrinone, a cyclic nucleotide phosphodiesterase (PDE) inhibitor [8] and intravenous immunoglobulin (IVIG) [9] have been used for the treatment of EV71 patients with autonomic nervous system dysregulation and pulmonary edema. Ribavirin, a nucleoside analogue for the treatment of several RNA viruses including hepatitis C virus and respiratory syncytial virus (RSV), exhibited an anti-EV71 effect in a mouse model [10]. We recently demonstrated that methylene blue-mediated photodynamic inactivation provided a novel way to eliminate environmentally contaminated sources of EV71 and prevent EV71 transmission and infection [11].

Several vaccine candidates have been developed and their efficacy evaluated in animals. These include inactivated whole-virus [12,13], viral protein VP1 subunit [13,14], VP1 DNA [13,15], virus-like particle (VLP) [16], as well as transgenic tomato fruit expressing VP1 protein [17]. Results from these studies and historical experience with PV vaccines [18,19] indicate that an inactivated whole-virus vaccine is feasible. Several inactivated EV71 vaccines are under development and could be readily licensed [20].

If EV71 vaccine was available, it may logically be co-administered with vaccines that are currently in use. Thus, in this study we tested the feasibility of co-immunization of an inactivated whole virus EV71 vaccine and a commercial pentavalent vaccine. We demonstrated that the two vaccines were compatible with each other and did not interfere in antibody production. At the same time they produced protection against a lethal EV71 challenge in mice.

Abbreviations: EV71, enterovirus 71; PV, poliovirus; PRP, polyribose ribitol phosphate; Hib, *Haemophilia influenza* type b; IVIG, intravenous immunoglobulin; RSV, respiratory syncytial virus; VLP, virus-like particle; CB3, coxsackie B3; CA16, coxsackie A16; ADE, antibody-dependent enhancement.

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2. Materials and methods

2.1. Cells and viruses

Rhabdomyosarcoma (RD) cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin, and streptomycin. Stock viruses of EV71/Tainan/4643/98 (GenBank accession number AF304458, C2 subgenogroup) [21], EV71/6356 (C2), EV71/7008 (B4), EV71/0318 (B4), EV71/0296 (B4), EV71/2413 (C4), EV71/3340 (C4), EV71/1757 (C4), EV71/0584 (C4), EV71/1745 (B5), EV71/0692 (B5), EV71/2838 (B5) (Department of Virology, National Cheng Kung University Hospital, Tainan, Taiwan) and mouse-adapted EV71 strain MP4 (C2) [21] were grown in RD cells. All EV71 strains were tested by monoclonal antibodies for EV71 (mAb3324), EV71/CA16 (mAb3323), CA24 (mAb3302), CB3 (mAb3306), E9 (mAb3313), PV type 1 (mAb3331), PV type 2 (mAb3332), and PV type 3 (mAb3335) (all from Chemicon, Temecula, CA) using indirect immunofluorescence staining of infected RD cell cultures.

2.2. Inactivated EV71 vaccine

EV71 virus particles were purified using sucrose gradient centrifugation. Briefly, after disruption and low speed centrifugation, a bulk culture of EV71/MP4 (250 mL) was precipitated overnight with polyethylene glycol (42%, PEG 8000; ACROS, Geel, Belgium) at 4 °C. PEG was dissolved by washing 4 times (15,000 × *g*, 20 min, 4 °C) with TES buffer (0.01 M Tris-based buffer supplied with 0.002 M EDTA and 0.15 M sodium chloride) and chloroform. The concentrated virus suspension (1–2 mL) was loaded onto a sucrose gradient (40% and 65% in an ultra-centrifuge tube 355630, Beckman, Palo Alto, CA) for ultra-centrifugation (SW41-Ti rotor, Beckman) at 80,000 × *g* for 4 h. Virus titers and the protein concentration of the fraction containing the virus were determined using plaque and a colorimetric assay (Bicinchoninic acid protein assay kit, Sigma–Aldrich, St. Louis, MO), respectively. The purity of the virus was analyzed by SDS–PAGE followed by western blotting using hyperimmune sera. The virus suspension was stored at −80 °C after dialysis (MWCO 20 kDa, Millipore, Bedford, MA) with phosphate-buffered saline (PBS). To prepare the inactivated EV71 vaccine, the purified virus preparation was resuspended in PBS (15 mL, ~40 μg viral protein per milliliter final concentration) and was incubated with formaldehyde (36.5%, Sigma–Aldrich) at a final dilution of 1/1000 in 37 °C for 2 h. The absence of infectious virus was confirmed by plaque assays and by passage of treated virus sample on RD cells for periods up to 2 weeks. The inactivated virus suspension was mixed with adjuvant (Imject Alum; Pierce, Rockford, IL) at a concentration of 40 μg of viral protein and 660 μg of alum to 1 mL. Within 30 min after preparation, the vaccine was given to mice.

2.3. Pentavalent vaccine

Each dose (0.5 mL) of the pentavalent vaccine (PEDIACEL, Sanofi Pasteur, Toronto, Canada) contained: 20 μg of pertussis toxoid, 20 μg of filamentous haemagglutinin, 5 μg of fimbrial agglutinogens 2 + 3, 3 μg of pertactin, 15 Lf of diphtheria toxoid, 5 Lf of tetanus toxoid, 10 μg of purified polyribose ribitol phosphate (PRP) capsular polysaccharide of *Haemophilus influenza* type b covalently bound to 20 μg of tetanus protein, 40 D-antigen units of poliovirus type 1 (Mahoney), 8 D-antigen units of poliovirus type 2 (MEFI), 32 D-antigen units of poliovirus type 3 (Saukett), and 1.5 mg of aluminum phosphate.

2.4. Immunization and sampling

Eight-week-old, female BALB/c mice (specific pathogen-free, Laboratory Animal Center, National Cheng Kung University College of Medicine, Tainan, Taiwan) were used. The mice were randomly divided into five groups with 10 animals per group. They were subcutaneously (s.c.) injected at the tail root with either 500 μL of inactivated EV71 vaccine (IEV group), 30 μL of pentavalent vaccine (PentaV group), 500 μL of inactivated EV71 vaccine, 30 μL of pentavalent vaccine (PentaV + IEV group), 500 μL of PBS (PBS group), or 500 μL of PBS containing 0.1% formaldehyde and 330 μg of alum (Adjuvant group). All animals were boosted after 3 and 7 weeks, and blood samples were collected via the submandibular artery [22] every two weeks after immunization. The sera were heat-inactivated (56 °C for 30 min) and stored at −80 °C until use.

2.5. Vaccine efficacy

Maternal immunization with a lethal challenge to 1-day-old pups was used to test the efficacy of the vaccines. Adult female mice were immunized with the vaccines as described above. Seven days after the first booster, the animals were allowed to mate. Lethal challenges were performed on 1-day post-natal pups. After fasting for 4 h, the pups were given an intraperitoneal (i.p.) injection of EV71/MP4 (1.6×10^3 pfu/40 μL). All mice were observed daily for weight change for 10 days after inoculation. The thigh muscle was aseptically removed from live animals at the end of the experiment and moribund animals (those with no weight gain) before death. These muscle samples were stored at −80 °C. When the pups were killed, blood samples were collected from the dams by axillary dissection under pentobarbital sodium anesthesia (80 mg/kg, i.p., Nembutal; Abbott Laboratories, North Chicago, IL).

2.6. Titration of neutralization antibody and tissue viral load

Neutralization titer (NT) of the sera collected from the animals was determined using a microassay. Briefly, 20 μL of serial serum dilutions (from 2^2 to 2^{10}) were mixed with 20 μL of a 100-fold tissue culture infectious dose 50% ($TCID_{50}$) EV71/MP4 in a 384-well plate. RD cell suspensions (final concentration 3×10^3 cells) were added 2 h later. After incubation for 2 days at 35 °C, the NT was determined as the highest dilutions of serum needed to inhibit virus growth. Mouse hyperimmune serum with an NT of 2^8 against EV71 was used as a positive control in all the assays. The hyperimmune serum was generated in adult mice immunized with live EV71 (2×10^6 pfu per 200 μL, two s.c. and one i.p. injections at 3-week intervals). For viral titration, tissue homogenates (10%, w/v) were clarified using centrifugation and the supernatants were inoculated onto monolayers of RD cells. The cells were inspected daily for a minimum of 14 days for cytopathic effect (CPE). Viral titers were expressed as pfu per gram of tissue. The lower limit of virus detection was 20 pfu.

2.7. Enzyme-linked immunosorbent assay

Concentrations of serum IgG antibodies against PV, *Bordetella pertussis*, and the PRP of *H. influenzae* type b were measured by a sandwich ELISA (RE56921, RE56141, and RE56351, IBL, Hamburg, Germany) according to the manufacturer's instruction with a brief modification. Serum antibody responses to diphtheria and tetanus toxoids were assayed using a two-step ELISA (RE57431 and RE57441, IBL). Sera were diluted 10- or 20-fold with diluent buffer and added to 96-well plates coated with the three types of PV, Bordetella antigens, the PRP of *H. influenzae* type b, diphtheria toxoid, or tetanus toxoid, incubated at room temperature for 1 h. After the washings, peroxidase-conjugated rabbit anti-mouse IgG (1:5000; Abcam, Cambridge, UK) or peroxidase-conjugated diph-