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Intradermal injection of a fractional dose of an inactivated HFMD vaccine elicits similar protective immunity to intramuscular inoculation of a full dose of an $Al(OH)_3$-adjuvanted vaccine

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ABSTRACT

Enterovirus 71 (EV71) and *Coxsackievirus* A16 (CVA16) are the two major causative agents of hand, foot and mouth disease (HFMD), which erupts in the Asia-Pacific regions. A bivalent vaccine against both EV71 and CVA16 is highly desirable. In the present study, on the bases that an experimental bivalent vaccine comprising of inactivated EV71 and CVA16 induces a balanced protective immunity against both EV71 and CVA16, we compare the immunogenicity and reactogenicity of one fourth of a full dose of an intradermal vaccine administered by needle-free liquid jet injector with a full dose of an intramuscular vaccine administered by needle-syringe in monkeys. The results suggest that intradermal injection of a fractional dose of an inactivated HFMD vaccine elicits similar immunogenicity and reactogenicity to intramuscular inoculation of a full dose of an $Al(OH)_3$-adjuvanted vaccine, regardless of whether monovalent or bivalent vaccines were used. Our results support the use of an intradermal bivalent vaccine strategy for HFMD vaccination in order to satisfy the requirements and reduce the costs.

1. Introduction

Epidemics of hand, foot, and mouth disease (HFMD) in children have emerged in the Asia-Pacific regions and have been caused primarily by *Enterovirus* 71 (EV71) and *Coxsackievirus* A16 (CVA16) [1–3]. Recent clinical surveys show that EV71 and CVA16 are responsible for more than 80% of the HFMD cases in China. Therefore, a vaccine that is protective against both EV71 and CVA16 is urgently needed [4,5].

Three inactivated EV71 vaccines are commercially available in China now [6–8]. However, no efficacy of these EV71 vaccines against CVA16 or other enteroviruses was shown in the clinical trials [6]. An experimental combination vaccine comprising of inactivated EV71 and CVA16 elicits balanced protective immunity against both viruses in the mouse model [4]. However, the safety, immunogenicity and protection of the bivalent inactivated vaccine need further investigation in the clinical trials. All the commercial EV71 vaccines are inoculated intramuscularly (i.m.) till now.

It is well known that there are copious professional antigen-presenting cells (APCs) in the skin, such as Langerhans cells and dermal dendritic cells, direct delivery of immunogen to these APCs may induce robust immune responses. Moreover, needle-free intradermal (i.d.) vaccine delivery has many advantages in immunization, including increased safety for vaccinator and vaccinee, better compliance with immunization schedules, easier vaccine delivery, and reduced cost, which has been supported and recommended by the World Health Organization [9–11].

In order to develop a bivalent intradermal vaccine against EV71 and CVA16 infections, we compare in the present study the immunogenicity and protective efficacy in mice and rhesus monkeys between an i.d. vaccine and an i.m. vaccine.

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2. Materials and methods

2.1. Cells and viruses

The working seed lots of MRC-5 (ATCC NO. CCL-171) and Vero (ATCC NO. CCL-81) cells were established by Changchun Hygeia biotech Co, Ltd and granted eligible for preparation of human vaccines by the National Institutes for Food and Drug Control. Human diploid MRC-5 cells were maintained in 0.5% minimal essential medium (MEM) with 5% fetal bovine serum. Vero cells were maintained in 0.5% M199 medium with 5% fetal bovine serum. Viruses used in this study were isolated from miscellaneous clinical hand-foot-mouth disease (HFMD) samples according to the conventional methods [12] and indicated in supplementary Fig. 1 and supplementary Table 1. All viruses were titrated for the 50% cell culture infectious dose ($CCID_{50}$) using Vero cells.

2.2. Adjuvant, mediums and reagents

Aluminum hydroxide ($Al(OH)_3$) adjuvant was purchased from Pierce, Rockford, IL, USA. MEM, M199 and fetal bovine serum were purchased from Beijing skywing Technology Co., Ltd., China. *Enterovirus* 71 monoclonal antibodies (Cat. NO. 3323 and MAB979) were purchased from Millipore, UK. Neutralizing Antibody of *Coxsackievirus* A16 (Lot. NO. 201306B), Biological Standards for Neutralizing Antibody of Human *Enterovirus* 71 (Lot. NO. 201001) and *Enterovirus* 71 Antigen Quantitative Standard (first generation, Lot. NO. 201001) were purchased from the National Institutes for Food and Drug Control.

2.3. Animals

The mice (ICR and Balb/C) and rhesus monkeys (4.0 ± 0.5 kg, 2–3.5 years) were provided and housed under standard laboratory conditions by Laboratory Animal Center, Academy of Military Medical Science. Each monkey was kept in a single cage. All monkeys were kept in isolation for 7 days for health investigation before utilization. A microneutralization test was performed to confirm that the animals did not possess antibodies against EV71 or CVA16. All of the animal protocols were reviewed and approved by the Academic Animal Care and Use Committee (NO. IACUC of AMMS-2009-002).

2.4. Miscellaneous HFMD samples collection and virus isolation

Various HFMD samples were collected and processed according to the recommended methods [12–15] and stored at −80 °C for virus isolation. The processed samples were added into MRC-5 cells at 5% CO_2, 37 °C for 5 days to observe the cell morphology. The typical cytopathic cultures were harvested, preserved at −80 °C, passaged and assayed further according to the conventional methods [12–15].

2.5. Enterovirus identification

The isolates were determined by qRT-PCR using an EV71 or CVA16 RNA quantitative detection kit (Acon Biotech Co., Ltd., China) and indirect immunofluorescence according to the provided specifications. Viral RNA was extracted from 250 μl of fresh cell or tissue homogenate by using the Qiagen RNeasy minikit according to the manufacture's protocol (Qiagen, Germany). The viral RNA was eluted in a final volume of 20 μl and determined by qRT-PCR. The viral VP1 fragments were sequenced using a commercially available kit (Takara, Dalian, China). The obtained sequences were analyzed using the DNAMAN software (Version 5.2.2) and compared with the EV71 or CVA16 sequences available on the NCBI website [12].

2.6. Screening the candidate vaccine or challenge strains

The viral titer, immunogenicity, biological and immunological characters, virulence of each *Enterovirus* strain were determined by the conventional methods [12] and indicated in supplementary Fig. 1. Six typical *Enterovirus* strains with good viral titer and immunogenicity were purified by plaque formation and screened for further vaccine researches in detail. The virulent *Enterovirus* strain was chosen for the establishment of animal models according to the conventional methods [4,16,17]. The virulent EV71 strain BJ09/07 (GenBank accession No. JQ319054) and CVA16 strain CVA16 BJ08/07 (GenBank accession No. JX068833) were isolated in MRC-5 cells, adapted in 1-day-old ICR mice, propagated and determined in Vero cells, respectively.

2.7. Preparation of the experimental inactivated EV71 and CVA16 vaccines

The infected MRC-5 cells grown in 40-cellstack (Nalge Nunc International, Rochester, NY) by 11th passage of HBLF812/CHN/8/10 or 10th passage of SDLC494/CHN/9/10 were collected respectively, centrifuged at 4000*g* for 30 min and treated with β-propiolactone (Serva Elec-trophoresis, Heidelberg, Germany). Each mixture was incubated at 37 °C for 72 h and shaked automatically at 4-h intervals. After viral inactivation, the virus titer was determined by a microtitration assay in order to confirm the complete inactivation. Each inactivated mixture was concentrated more than 100-fold by performing ultrafiltration (Pellicon3 Ultracel, Millipore, UK). The concentrated virus was subsequently purified by sucrose cushion and sucrose gradient ultracentrifugation using a zonal rotor in a Hitachi CP80 ultracentrifuge according to the conventional methods [15,18], followed by being loaded onto and passed through Sepharose 6-FF in an ÄKTA purifier 100 system (GE Healthcare, Sweden).

The protein concentration of the final virus elution was measured by the Lowry method and the purity of the viral proteins was determined by SDS-PAGE and Coomassie brilliant blue stain (Beyotime Institute of Biotechnology, China) according to the provided specifications. Western blotting using antibody against EV71 or CVA16 was performed to identify viral proteins in the final elution, according to the standard protocol. The purity of the harvested virus was further analyzed with HPLC with TSKG4000SW (TosoHaas, Tokyo, Japan) as the molecular sieving matrix and stored at 4 °C until utilization [12].

The intramuscular (i.m.) vaccine contained 1 μg of inactivated EV71, CVA16, or bivalent viral antigen adsorbed to 0.5 mg of $Al(OH)_3$ and suspended in 0.5 ml of phosphate buffered saline (PBS). The intradermal (i.d.) vaccine contained 0.25 μg of inactivated EV71, CVA16, or bivalent viral antigen and suspended in 0.1 ml of PBS. The same volume of $Al(OH)_3$ or PBS was served as a negative control for intramuscular or intradermal immunization, respectively.

2.8. Determination of viral virulence for mice

The virulence of each virulent *Enterovirus* strain that cultured in Vero cells was determined in 7-day-old ICR mice by the conventional methods [16,19]. Especially, to establish the mouse models, the virulence of EV71 BJ09/07 or CVA16 BJ08/07 was determined in details in the mice (10 mice per group). Briefly, LD_{50} was determined by intraperitoneally (i.p.) injecting 1×10^2, 1×10^3, 1×10^4, 1×10^5 or 1×10^6 $CCID_{50}$ of each *Enterovirus* strain (the lethal EV71 BJ09/07 or CVA16 BJ08/07 strain) per mouse into

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