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The immunogenicity and protection effect of an inactivated coxsackievirus A6, A10, and A16 vaccine against hand, foot, and mouth disease

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ABSTRACT

Coxsackievirus belongs to the *Enterovirus* genus of the *Picornaviridae* family and is one of the major pathogens associated with human hand, foot, and mouth disease (HFMD). Historically, outbreaks of HFMD have mainly been caused by enterovirus 71 and coxsackievirus A16. Recently, coxsackieviruses A6 and A10 have been associated with increased occurrences of sporadic HFMD cases and outbreak events globally. In this study, the immunogenicity of coxsackieviruses A6, A10, and A16 (CA6, CA10, and CA16), which were inactivated by formalin or β-propiolactone (BPL) under different conditions, was evaluated as multivalent vaccine candidates. CA6 induced similar immune responses with both inactivation methods, and the immune efficacy of CA10 and CA16 was better following inactivation with BPL than with formalin. There was no sufficient cross-reactivity or cross-protectivity against heterologous strains in groups vaccinated with the BPL-inactivated (BI) monovalent vaccine. Sufficient neutralizing antibody and cell-mediated immune responses were induced in the BI-trivalent vaccinated group. These findings suggest that BI-CA6, CA10, and CA16 are potential multivalent vaccine candidates and that a multivalent vaccine is needed to control HFMD. The coxsackievirus multivalent vaccine could be useful for the development of effective HFMD vaccines.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a common disease characterized by fever, oral ulcers, and skin manifestations affecting the palms, soles, and buttocks [1]. Although HFMD is classically a mild disease, outbreaks in Asia have been associated with a high incidence of fatal cardiopulmonary and neurologic complications [2]. Outbreaks of HFMD are mainly caused by two types of enterovirus A species, enterovirus 71 (EV71) and coxsackievirus A16 (CA16). Other enteroviruses such as coxsackieviruses A6, A7, A10, A14, and B2 (CA6, CA7, CA10, CA14, and B2) may also be associated with the disease [3]. Coxsackievirus belongs to a family of nonenveloped, positive-sense single-strand RNA viruses and the *Enterovirus* genus of the *Picornaviridae* family. Recent epidemiological data indicate that infections with CA6 and CA10 have markedly increased worldwide in addition to those with CA16 [2,4]. Outbreaks of coxsackievirus have been reported in diverse countries, including China [5], Taiwan [4,6], Japan [7], France [8], Finland [9], Singapore [10], and Spain [11,12]. Although serious symptoms of coxsackievirus infections have been reported in various countries, there is no commercial vaccine. In the case of CA16, a bivalent vaccine with EV71 has been developed and studied, but it was found to not protect against CA6 or CA10 viral infections in a cell culture neutralization assay [13,14]. Based on current epidemiological data, it is reasonable to propose that not only EV71 and CA16 but also CA6 and CA10 should be candidate vaccine strains for inclusion in HFMD vaccines [14].

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There are several methods for producing inactivated virus vaccines, and the selection of the inactivating agent and conditions is critical for the preparation of inactivated vaccines [15]. Formalin and β-propiolactone (BPL) are widely used for the inactivation of licensed human viral vaccines [16]. Formalin and BPL are alkylating agents that modify the structure of nucleic acids, resulting in cross-linking between DNA and proteins [17]. A study has reported that the cross-neutralization titer differs significantly among various inactivation methods for different EV71 strains [18]. Moreover, previous studies have shown that the CA16 inactivated vaccine using BPL induces a more effective immune response than that induced using formalin [19,20].

In addition, it is very important to use appropriate animal models to evaluate vaccine candidates. An established animal model can be used to confirm the *in vivo* protective efficacy of a vaccine. The major targets of HFMD vaccine development, EV71 and CA16, already have been used to construct vaccines, which have been assessed in various animal models such as transgenic mice, neonatal mice, and gerbils [21–24]. Recently, as CA6 and CA10 epidemics have increased, neonatal mouse models have been studied for vaccine development [25,26].

In this study, we evaluated the effects of the virus inactivation method on CA6, CA10, and CA16 vaccines and confirmed the possibility of a multivalent vaccine using a neonatal mouse model.

2. Materials and methods

2.1. Cells and viruses

Human rhabdomyosarcoma (RD; WHO) and Vero (ATCC) cells were grown in Dulbecco's modified minimal essential medium (DMEM; Thermo Fisher Scientific, USA) with 10% fetal bovine serum (Corning costar, USA) and penicillin-streptomycin solution (GenDepot, USA) and incubated at 37 °C with 5% CO_2. RD cells were used to initially isolate viruses, and Vero cells were used to propagate viruses and measure titration.

The inactivated coxsackievirus vaccine strains were isolated from patients with HFMD in Korea. To identify the coxsackievirus subgenotype, the homology of VP1 sequences obtained using reverse transcription polymerase chain reaction (RT-PCR) was compared with those of sequences available from the GenBank database.

2.2. Inactivated coxsackievirus vaccine

To produce a suitable vaccine for immunological evaluation, a large amount of virus was cultured in Vero cells with serum free media. And purified virus culture solution by several centrifugation and filtration with Amicon® Pro Purification System (Merck, USA) and concentrated from cell-free supernatants by ultracentrifugation at 25,000 rpm for 120 min at 4 °C for twice. The virus pellet was resuspended with PBS. Two methods were used to inactivate viruses; the first involved incubating them with 0.02% formaldehyde (Sigma, USA) at 37 °C for 5 days, and the other involved reacting them with 0.025% BPL (TCI, JAPAN) at 4 °C for 3 days.

2.3. SDS-PAGE and Western blot

Purified viruses were mixed with 5x sample buffer, boiled, and separated on 4–15% polyacrylamide gels (BioRad, USA). Proteins were showed by Coomassie blue staining or transferred onto NC membranes for Western blot analysis. Nonspecific bands were blocked with 5% skim milk solution for 1 h at room temperature and incubated overnight with primary antibodies diluted in blocking solution. The blocking solution was also used for the dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, USA). For Western blot analyses, the following primary antibodies were used: Anti-Enterovirus 71 Monoclonal antibody (MAB979, Millipore, Germany), Anti-Coxsackievirus B2 Monoclonal antibody (MAB946, Millipore, Germany).

2.4. Vaccination of mice

Female Balb/c mice (5 weeks old, six mice per group) were used in this study to evaluate the vaccine. Experimental vaccine antigens were diluted with PBS to 10 μg/50 μl. Alum ($Al(OH)_3$, Invivogen, USA) as an adjuvant was mixed with the antigen solution at a 1:1 ratio. In trivalent vaccine, each of the antigens was diluted with PBS to 10 μg/10 μl and mixed, and the alum was mixed with the antigen solution volume at a ratio of 1:1. Mice were intramuscularly (i.m.) injected with formalin or BPL (FI or BI) inactivated CA6, CA10, and CA16 individually or together as BI-trivalent according to the groups. Phosphate buffered saline (PBS) was administered to mice in the MOCK group as a negative control. Two weeks after the primary vaccination, the mice were boost-vaccinated using the same dose of the vaccines. Serum samples were collected at 4, 8, and 12 weeks post-vaccination for serology tests and stored at −80 °C. The animal experiment was performed according to National Institutes of Health (NIH) guidelines of the Institutional Animal Care and Use Committee.

2.5. Coxsackievirus-specific antibody response

The antigen-specific IgG responses were analyzed using enzyme-linked immunosorbent assay (ELISA). First, 100 ng of the coxsackievirus vaccine strains diluted in PBS was used as a coating antigen in 96-well microplates (Corning costar), which were incubated at 4 °C overnight. After blocking with 5% skim milk powder in PBS at 37 °C for 2 h, sera diluted 1:100 to 1:400 in dilution buffer (5% skim milk in PBS) were added to the microplates. After 1 h of incubation at 37 °C, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG(H + L) antibody (Novex) diluted 1: 5000 in dilution buffer was added, followed by incubation at 37 °C for 1 h. After a washing step, 3,3′,5,5′-tetra-methylbenzidine (TMB) solution (Rockland) was added as a developing agent, and 2 M H_2O_4 was added to stop the reaction. Absorbance was measured at 450 nm using a microplate reader.

2.6. Serum neutralizing assay

Vero cells were seeded in 96-well plates 1 day before the serum neutralizing assay. Twofold serially diluted test sera were mixed with 100 $TCID_{50}$ of each virus and incubated at 37 °C for 2 h. The virus-serum mixture was added to Vero cells and incubated for 5 days at 37 °C. The neutralizing titer was calculated on the basis of the number of wells showing cytopathogenic effect by using the Reed–Muench method and reported as the reciprocal titers of serum dilutions that exhibited 50% neutralization. A neutralizing titer of $>2^3$ was used as a threshold for positivity.

2.7. IFN-γ-specific ELISpot assay

A 96-well polyvinylidene difluoride-backed plate was coated with anti-IFN-γ mAb, incubated at 4 °C overnight, and blocked at 37 °C for 1 h. The splenocytes were isolated from the mice in each experimental group 1 week post-boosting and resuspended in RPMI medium containing 10% FBS. The splenocytes (1×10^6 cells) were stimulated with each vaccine strain (0.1 multiplicity of infection, M.O.I) and incubated at 37 °C for 24 h. The cells were removed, and the plates were developed using a mouse IFN-γ ELI-

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