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Inactivated coxsackievirus A10 experimental vaccines protect mice against lethal viral challenge

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ABSTRACT

Coxsackievirus A10 (CVA10) has become one of the major causative agents of hand, foot and mouth disease (HFMD). It is now recognized that CVA10 should be targeted for vaccine development. We report here that β-propiolactone inactivated whole-virus based CVA10 vaccines can elicit protective immunity in mice. We prepared two inactivated CVA10 experimental vaccines derived from the prototype strain CVA10/Kowalik and from a clinical isolate CVA10/S0148b, respectively. Immunization with the experimental vaccines elicited CVA10-specific serum antibodies in mice. The antisera from vaccinated mice could potently neutralize *in vitro* infection with either homologous or heterologous CVA10 strains. Importantly, passive transfer of the anti-CVA10 sera protected recipient mice against CVA10/Kowalik or CVA10/S0148b infections. Moreover, active immunization with the inactivated vaccines also conferred protection against homologous and heterologous infections in mice. Collectively, our results demonstrate the proof-of-concept for inactivated whole-virus based CVA10 vaccines.

1. Introduction

Hand, foot and mouth disease (HFMD) is a common viral disease which is prevalent in the Asia-Pacific region in the last decade [1–4]. It mainly affects young children under five years old, causing rashes on palms, soles and oral cavity; a proportion of the patients could develop severe neurological complications, which may ultimately lead to death [3,5]. Thus, development of effective vaccines for control and prevention of HFMD is desirable.

Although enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) have been known to be the main causative agents of HFMD [6], infection by other members of enterovirus has more and more been reported to correlate with HFMD outbreaks in recent years [7,8]. Coxsackievirus A10 (CVA10) is a member of the *enterovirus* genus of the *picornaviridae* family, which possesses a single-stranded positive-sense RNA genome [9]. Current molecular epidemiological studies revealed that CVA10 has become one of the major causative agents of HFMD [7,10,11]. Normally, CVA10 infection causes only mild and self-limited disease symptoms, including fever, mouth ulcers, and rashes or vesicular eruptions on hands and feet, but severe complications like herpangina, onychomadesis, neuromyelitis or death could also happen [12–15]. CVA10 was first detected in 1965 during an outbreak of infectious hepatitis [16]. A nationwide HFMD outbreak caused by co-circulation of CVA10 and CVA6 occurred in Finland in 2008 [17]. Then, HFMD epidemics caused by CVA10 infection have also occurred in France in 2010 [12], Thailand in 2012 [18], India in 2009 [19] and China in 2010 [20,21]. In addition, CVA10 has also been reported to co-circulate and have potential recombination with other important HFMD associated enteroviruses including EV71, CVA16 and CVA6 [20,22]. All the evidences indicate that CVA10 has become an unnegligible HFMD causative agent that should be targeted for development of an effective HFMD vaccine.

Inactivated whole-virus based vaccines have proven effective for enterovirus as the exemplification of successful commercialization of inactivated poliovirus (PV) vaccine [23]. So far, several formalin-inactivated EV71 vaccines have finished phase III clinical trials with satisfying protection against EV71-infection related HFMD [24–26], and one of them has recently been licensed in China. Other candidate vaccines such as virus-like particle (VLP) based vaccines of EV71 or CVA16 [27–30], and inactivated CVA16 vaccines [31,32] also demonstrated good protection against infection by corresponding virus in preclinical studies, but none of them has cross protection against CVA10 [28,29]. Recently, Liu et al. reported that inactivated CVA10 could elicit neutralizing antibody response in mice [33]. However, the *in vivo* protective efficacy of inactivated CVA10 was still unknown. We aimed to develop

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inactivated vaccines against CVA10. Our studies demonstrated that inactivated CVA10 experimental vaccines could provide *in vivo* protection against lethal CVA10 challenge in a murine infection model.

2. Materials and methods

2.1. Ethics statement

All animal experiments in this study were conducted in accordance with the guide lines of the Institutional Laboratory Animal Center. The animal use protocols have been reviewed and approved by the Animal Care and Use Committee of Institut Pasteur of Shanghai (IPS).

2.2. Cells and viruses

Vero (ATCC, CRL-1586) and RD (ATCC, CCL-136) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described previously [34,35]. Three CVA10 strains were used in this study. The CVA10/Kowalik (GenBank ID: AY421767) was purchased from ATCC. CVA10/S0148b and CVA10/S0273b were isolated from fecal samples of CVA10 infected patients. The viruses were propagated on Vero cells and titrated for the 50% tissue culture infectious dose ($TCID_{50}$) using RD cells as described previously [34,36].

2.3. Polyclonal antibodies

CVA10 capsid subunit protein specific antisera were generated in house as described previously [34]. Briefly, the capsid subunit proteins, VP0 (VP0 was composed of VP2 and VP4), VP1 and VP3, of CVA10/Kowalik were expressed with 6 × His tags in *E. coli* and purified with His·Bind® resin individually. Then, the purified capsid proteins were used for immunization of Balb/c mice separately to generate antisera. The specificity of the mouse immunized sera were confirmed by ELISA and Western blot assay as described before [34].

2.4. Preparation of inactivated CVA10/Kowalik and CVA10/S0148b

CVA10/Kowalik and CVA10/S0148b were propagated separately on Vero cells as described previously [31,37]. Briefly, 80% confluent Vero cells were infected with CVA10/Kowalik or CVA10/S0148b at an M.O.I = 0.01. 72 h after infection, the cells and medium were harvested and subjected to 3 cycles of frozen-thaw when 70–80% of cells showed cytopathic effects (CPE). For virus inactivation, β-propiolactone was added with a ratio of 1:2000 and incubated at 4 °C for 12 h followed by 2 h at 37 °C for hydrolysis of remaining β-propiolactone. Cell debris was removed before purification of the virus by 20% sucrose cushion ultracentrifugation (SW28 Ti rotor, 131,000*g*, 4 °C, 3 h) and 10–50% sucrose gradient ultracentrifugation (SW60 Ti rotor, 205,000*g*, 4 °C, 2.5 h) on a Beckman centrifuge. Mock infected Vero cells were collected and purified in the same way and used as negative control in this study. The purified CVA10 and control antigen were quantified by Bradford assay.

2.5. SDS-PAGE and Western blot

SDS-PAGE and Western blot analysis of the purified inactivated viruses or control antigen were performed as described previously [31,37] except that the membranes were probed by anti-CVA10-VP0, -VP1, or -VP3 polyclonal antibodies.

2.6. Electron microscopy

Electron microscopy was performed as described previously [38]. Briefly, purified viruses were diluted to 0.1 mg/ml and subjected to negative staining with 0.75% uranyl formate, and subsequently observed under a Tecnai G2 Spirit transmission electron microscopy operated at 120 kV. Images were recorded at ×100,000 microscope magnification.

2.7. Mouse immunization

ICR and Balb/c mice used in this study were purchased from Shanghai Laboratory Animal Center and cared for in accordance with the institutional guidelines. Prior to immunization, the antigens were mixed thoroughly with Alhydrogel® (Invivogen, USA) adjuvant to make the experimental vaccines. A single immunization dosage contained 10 μg antigens (Inactivated-CVA10 vaccines or control antigen) and 0.5 mg aluminum hydroxide in a final volume of 100 μl. Three groups (6 mice per group, 6-week-old) of female Balb/c mice were intraperitoneally (i.p.) injected with purified CVA10/Kowalik, CVA10/S0148b or control antigen at weeks 0, 2 and 4, respectively. Blood samples from each mouse were collected at two weeks after the last immunization. Sera were isolated from the blood and incubated at 56 °C for 30 min to inactivate the complement.

2.8. Antibody response measurement

Antigen-specific antibodies in sera from immunized mice were measured by ELISA assay as described before [27,31]. Briefly, 96-well ELISA plates were coated with the mixture of CVA10-VP0, -VP1 and -VP3 capsid proteins (100 ng/well of each protein), 50 ng/well of CVA10/Kowalik, or 50 ng/well of CVA10/S0148b in PBS buffer, respectively. The diluted (1/100 or 1/64,000) sera were added to the wells (50 μl per well) and incubated at 37 °C for 2 h followed by detection with a HRP-conjugated anti-mouse IgG antibody (Sigma, USA). After color development, the absorbance was determined at 450 nm in a 96-well plate reader.

2.9. Microneutralization assay

Neutralization assay was performed as described before [31,37] with small modifications. Briefly, the serum samples collected from each mouse were 2-fold diluted (starting from dilutions of 1:4 or 1:16, as indicated in figure legend) using DMEM containing 2% FBS. Then, 50 μl of diluted serum was mixed with 50 μl (100 $TCID_{50}$) of CVA10/Kowalik, CVA10/S0148b or CVA10/S0273b in 96-plates, respectively, and incubated at 37 °C for 1 h. Then, 2×10^4 RD cells (100 μl) were added to each well of the 96-well plate and cultured in a 37 °C incubator with 5% CO_2. Three days later, the cells were observed under a microscope for presence of cytopathic effects (CPE). Neutralization titers were determined as the highest serum dilution that could fully prevent cells from CPE.

2.10. Passive immunization and virus challenge

The *in vivo* protective efficacy of the inactivated vaccine was evaluated by passive immunization/challenge assay. Three groups of female Balb/c mice (8-week old, 6 mice per group) were immunized with the inactivated vaccines or control antigen as described above. All the immunized mice were allowed to mate at four weeks after the last immunization. Suckling mice born to the immunized dams were i.p. injected with 2.32×10^5 $TCID_{50}$ of CVA10/Kowalik or 5.34×10^5 $TCID_{50}$ of CVA10/S0148b at 7-day-old. Then, the challenged mice were monitored daily for survival and clinical score for a period of 15 days. Clinical scores were graded as follows: 0,

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