Active immunization with a Coxsackievirus A16 experimental inactivated vaccine induces neutralizing antibodies and protects mice against lethal infection

Yicun Cai¹, Qingwei Liu¹, Xulin Huang, Daheng Li, Zhiqiang Ku, Yunfang Zhang**, Zhong Huang***

Key laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 411 Hefei Road, Shanghai 200025, China

ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form 1 March 2013
Accepted 5 March 2013
Available online 1 March 2013

Keywords:
Coxsackievirus A16
Inactivated vaccine
Neutralizing antibody
Active immunization

ABSTRACT

Coxsackievirus A16 (CA16) is one of the main pathogens that cause hand, foot and mouth disease, which frequently occurs in young children. A small percentage of patients infected with CA16 may suffer from severe neurological complications that could also lead to death. Recent epidemiological data shows the increase in both the total number and the incidence rate of severe CA16-associated cases in China, indicating that CA16 should be targeted for vaccine development. In this article, we report the immunogenicity and protective efficacy of experimental inactivated CA16 vaccines in mice. We show that immunization with β-propiolactone-inactivated whole-virus vaccines derived from two CA16 clinical isolates were able to induce CA16-specific antibody and IFN-secreting T-cell responses in mice. The resulting anti-CA16 mouse sera neutralized both homologous and heterologous CA16 clinical isolates, as well as a mouse-adapted strain called CA16-MAV, which is capable of infecting 14-day-old mice. Passive transfer of anti-CA16 neutralizing sera partially protected neonatal mice from lethal challenge by a clinical isolate CA16-G08. More significantly, active immunization of mice with the inactivated vaccines conferred complete protection against lethal infection with CA16-MAV. Collectively, these results provide a solid foundation for further development of inactivated whole-virus CA16 vaccines for human use.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Hand, foot, and mouth disease (HFMD) is an infectious disease which frequently occurs in young children. Recently, HFMD infection has been prevalent in Asia-Pacific regions, causing severe illness and fatalities (reviewed in [1]). Coxsackievirus A16 (CA16), together with enterovirus 71 (EV71), is responsible for the majority of these HFMD cases [2–5]. CA16 was first identified in 1951 [6]. It is a member of the Enterovirus genus of Picornaviridae and can be classified into two genogroups, A and B, with the latter consisting of two sub-genotypes B1 and B2 [7,8]. Its genome is a single-stranded positive-sense RNA molecule of ~7410 bases [9] and is encapsidated in isocuherald capsids composed of processed VP0, VP1 and VP3 subunit proteins [10].

CA16 infection often causes mild, self-limiting disease symptoms, including fever, mouth ulcers, rashes and blisters on the surface of the hands and feet [2,11]. Fatal cases associated with CA16 infection have been reported in the 1960s [12,13]. Recent epidemiological surveys have indicated how CA16 has become more virulent to humans, causing a significant number of recent deaths and severe cases of neurological complications [14–17]. For example, a recent report shows that, out of 92 severe HFMD cases with neurological complications, 19 were caused by CA16 infection [17]. Moreover, the co-circulation of CA16 and EV71 [8,17–19] has resulted in co-infections by the two viruses [20,21], which has been suggested to increase the severity of disease [22]. This co-infection may have led to the recombination between EV71 and CA16 [23,24], yielding a recombinant virus responsible for the large HFMD outbreak in China in 2008 [24]. Prior development of HFMD vaccines has almost exclusively focused on EV71 [25,26]. Due to the increased prevalence and severity of CA16 infection, it is now recognized that CA16 should also be targeted for vaccine development to ensure a broad and effective protection against HFMD. Indeed, several vaccine companies and academic groups in China have begun to develop CA16 vaccines, and neonatal mouse models of CA16 infection have been established for vaccine evaluation [27,28]. It has been recently shown that the passive transfer of neutralizing antibodies elicited by experimental CA16 vaccines could partially protect mice against lethal CA16 infection [27,28]. However, whether active immunization with CA16 vaccine candidates is able to induce a protective immunity has not been determined.

In the present study, we demonstrate, for the first time to our knowledge, how active immunization with an experimental inactivated CA16 vaccine can confer full protection in mice using a...
mouse model of CA16 infection. Our results provide a solid foundation for the development of inactivated whole-virus vaccines against CA16 infection in humans.

2. Materials and methods

2.1. Cells and viruses

RD, Vero, and L929 cells were grown as described previously [10]. Two CA16 clinical strains, CA16-SZ05 (GenBank ID: EU262658) [29] and CA16-G08 (GenBank ID: KC342228), were grown in Vero cells. To prepare mouse-adapted CA16, the parental strain CA16-G08 was passaged in L929 cells for three times to generate L929-adapted virus, which was subsequently used to inoculate one-day-old ICR mice via the oral route. Virus was harvested from limb muscle tissues of one dead mouse by one passage in L929 cells. The resultant virus was then injected intraperitoneally (i.p.) into a group of 7-day-old mice. Virus was isolated from muscles of one dead mouse in the i.p. injected group by one passage in L929 cells and was designated CA16-MAV (GenBank ID: KC695830). To prepare virus stock, CA16-MAV was propagated by one more passage in L929 cells. The EV71 strain G082 [30] was grown in Vero cells. All viruses were titrated for the 50% tissue culture infectious dose (TCID₅₀) using Vero cells as described previously [10,31].

2.2. Synthetic peptides

A series of 95 peptides spanning the entire amino acid sequence of VP1 of the CA16 strain SZ05 was synthesized by GL Biochem (Shanghai, China). Each peptide consisted of 15 residues with a 12 residue overlap.

2.3. Preparation of inactivated CA16 vaccines

Vero cells infected with CA16 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) without fetal bovine serum (FBS) at 37 °C until all the cells developed cytopathic effects (CPE). The culture was subjected to three freeze-thaw cycles and then clarified by centrifugation at 4500 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.22-μm filter. Virus inactivation was carried out by treating the virus-containing supernatants with a 1/2000 volume of β-propiolactone (Serva Electrophoresis, Heidelberg, Germany) for 24 h at 4 °C, followed by incubation at 37 °C for 2 h to allow for β-propiolactone hydrolysis. Subsequently, the inactivated virus was layered onto a 20% sucrose cushion for partial purification and concentration. The resulting pellets were resuspended in PBS and then layered onto 10–50% sucrose gradients for ultracentrifugation in a Beckman SW60Ti rotor at 39,000 rpm for 3 h at 4 °C. After ultracentrifugation, 11 fractions were taken from top to bottom, and assayed for the presence of CA16 by Western blot analysis using anti-VP1 antiserum as described below. Based on the Western blotting results, CA16-rich fractions were pooled and then subjected to ultracentrifugation through a 20% sucrose cushion. The resulting virus pellets were resuspended in PBS, stored at –80 °C, and subsequently used for animal immunization. The completeness of virus inactivation was tested by passage of the final vaccine preparations on Vero cells for periods of up to 2 weeks. No virus propagation was detected as indicated by the absence of cytopathic effects (CPE) during the course. The final vaccine preparations were quantified by Western blot analysis using anti-VP1 antiserum [10] as the detection antibody and using purified CA16 (provided by Hualan Biological Engineering, Inc., Henan, China) as the reference standard. The uninfected Vero cell lysate was subjected to the same purification process as the inactivated virus, and served as a negative control antigen for immunization.

2.4. Western blotting

SDS-PAGE and Western blotting analyses of protein samples were performed using anti-VP1, anti-VP3, or anti-VP0 antisera as the detection antibody as previously described [10]. For CA16 quantification, purified CA16 antigen (provided by Hualan Biological Engineering, Inc.) was used as a reference standard and the anti-VP1 was used as the detection antibody. Positive signals on the membranes were developed by chemiluminescence using the BeyoECL Plus kit (Beyotime, Shanghai, China), recorded on a LAS-4000 Luminescent Image Analyzer (Fujifilm Life Science USA, Stamford, CT, USA) and quantified using the software Multi Gauge v3.0.

2.5. Immunization of adult mice

Prior to immunization, inactivated CA16 preparations or a similarly prepared negative control (Vero) antigen were mixed with Inject alum (Pierce, Rockford, IL) at a volumetric ratio of 1:1 according to the manufacturer’s instructions. Groups (6 animals per group) of female mice at the age of 6 weeks were intraperitoneally (i.p.) injected with 2 μg of inactivated CA16 or Vero control antigens, respectively, at weeks 0 and 1. Blood samples were collected before immunization and at week 2 when the mice were sacrificed. The sera were heat-inactivated (56 °C for 30 min) and stored at –80 °C until use.

2.6. Antibody measurement

CA16-specific IgG antibodies in the mouse sera was measured as described previously [10] with the following modifications: ELISA plates were coated with Escherichia coli-expressed VP1 protein (100 ng/well); a HRP-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO, USA) was used as the secondary antibody.

2.7. ELISPOT assay

Spleens from three immunized mice were collected for each group one week after the final immunization. The splenocytes were isolated, pooled and counted. For the ELISPOT assay, 96-well polyvinylidene fluoride plates (Millipore, Billerica, MA, USA) were pre-coated with 200 ng/well of the anti-IFN-γ antibody (eBioscience, San Diego, CA, USA) at 4 °C overnight. Plates were blocked with 200 μl/well of complete RPMI-1640 medium for 2 h at 37 °C. The plates were decanted and freshly isolated spleen cells (3 × 10⁵/well) were added to the plates. Then, 50 μl/well of complete RPMI-1640 medium with or without 10 μg/ml of the VP1 peptide pool was added to the cells, and incubated for 36 h at 37 °C and 5% CO₂. Subsequently, the plates were incubated with 0.1 μg/well of biotinylated anti-IFN-γ antibody (eBioscience) diluted in PBS buffer containing 1% FBS and 0.05% Tween-20 for 2 h, and then with alkaline phosphatase (AP)-conjugated streptavidin (MABTECH, Mariemont, OH, USA) diluted 1:1000 in PBS for 1 h. After washing the plate three times with PBST and two times with PBS, 100 μl/well of NBT/BCIP substrate (Promega, Madison, WI, USA) was added to the plates and incubated for 10–25 min for color development. The IFN-γ-secreting cell spots were imaged and counted on a CTL Immunospot reader (Cellular Technology Ltd.).

2.8. Neutralization assay

Briefly, Vero cells were seeded onto 96-well plates and cultured overnight until 70% confluency; sera samples were 2-fold serially diluted starting from 1:8 to 1:512 with DMEM containing 2% FBS. Then, 50 μl of each serum dilution was mixed with 50 μl (100 TCID₅₀) of CA16 stocks and then incubated for 1 h at 37 °C in 5% CO₂. After incubation, the serum/virus mixtures were added onto
Vero cells for infection. At three days post-infection, the cells were observed under a microscope for the presence of cytopathic effects (CPE). Neutralization titers were determined as the highest serum dilutions that could prevent cells from CPE.

2.9. In vivo passive protection assay

Groups of one-day-old suckling ICR mice were i.p. injected with 100 μl of antisera or with PBS as a control. One day later, all the mice were inoculated i.p. with $1 \times 10^7$ TCID50 of live CA16-G08. The challenged mice were monitored daily for survival and clinical score for a period of 20 days. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death.

2.10. Active immunization/protection assay

Groups of neonatal ICR mice of less than 24 h of age (after birth) were i.p. injected with 0.5 μg of the inactivated CA16 preparations in the presence of alum adjuvant. Another group of mice was injected with the Vero antigen plus alum adjuvant and served as a negative control. After one week, the mice were boosted with the same corresponding antigens. One week after the second immunization, all mice were inoculated i.p. with $2.3 \times 10^5$ TCID50 of the mouse-adapted strain CA16-MAV, and then monitored daily for survival and clinical score for 18 days. Clinical scores were graded as described above. All animal studies were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai.

2.11. Statistics

Statistical significance was determined by the Student's two-tailed t-test using GraphPad Prism version 4.

3. Results

3.1. Immunogenicity of the inactivated CA16 vaccines in adult mice

Inactivated CA16 experimental vaccines were prepared from CA16-SZ05 or CA16-G08 infected Vero cells, and a control antigen was made in an identical fashion from uninfected Vero cells (Fig. 1). The identity of the viral capsid proteins was determined by Western blot using a panel of subunit protein-specific polyclonal antibodies generated in a previous study [10]. Positive bands representing VP1, VP3, VP0 and VP4 were evident for both CA16-SZ05 and CA16-G08 samples, whereas no specific signal was detected for the Vero control antigen (Fig. 1). To determine the immunogenicity, two groups of six mice were i.p. administered with 2 μg of inactivated CA16-SZ05 or CA16-G08, respectively, at weeks 0 and 1. Another group of mice was injected with the control antigen with the same regimen. At one week after the booster immunization, sera were collected from all immunized mice for antibody measurement. The inactivated CA16 antigens, but not the control antigen, elicited CA16 VP1-specific antibody responses in the immunized mice (Fig. 2A). CA16-specific T cell responses induced by the immunizations were also examined by ELISPOT. Splenocytes harvested at one week after the final immunization were analyzed for IFN-γ producing cells upon stimulation with a peptide pool derived from VP1 of CA16-SZ05. Inactivated CA16-SZ05 or CA16-G08 immunized mice showed significantly higher numbers of IFN-γ secreting cells upon stimulation with the peptide pool as compared to those of the Vero lysate-immunized or naive mice (Fig. 2B). Therefore, the immunization of mice with inactivated CA16 can elicit a virus-specific IFN-γ T-cell response, which can be recalled to respond to viral antigen exposure with rapid kinetics.

3.2. Anti-CA16 sera neutralized infection in vitro

The antisera were evaluated for their capacity to neutralize live CA16 using an in vitro microneutralization assay. Antisera against the Vero lysates did not exhibit neutralization at a 1:8 dilution (the lowest dilution tested), whereas individual antisera from both CA16-SZ05 and CA16-G08 vaccine groups neutralized their respective homologous strains with titers ranging from 64 to 256 (Fig. 3). Moreover, anti-CA16 antisera pooled for each of the groups were able to neutralize homologous and heterologous clinical isolates, as well as a mouse-adapted strain CA16-MAV; however, these were not effective for EV71 (Table 1).

3.3. Passive transfer of anti-CA16 sera partially protected mice from lethal challenge

To evaluate the protective efficacy of the neutralizing antisera, 100 μl of the anti-CA16 or the anti-Vero antisera were passively transferred into suckling mice, followed by i.p. inoculation with
1 × 10⁷ TCID₅₀ of live CA16-G08 virus. After challenge, the mice that received anti-Vero antisera gradually developed severe manifestations, including limb weakness, paralysis (Fig. 4A–B), and finally died; the final survival rate of this group was 7.1% (Fig. 4C). In contrast, the majority of the mice given the anti-CA16-SZ05 or anti-CA16-G08 antisera were protected from paralysis and death, with survival rates of 85.7% and 93.3%, respectively (Fig. 4C). These results indicate that neutralizing antibodies may play a critical role in the protection against CA16 infection.

### 3.4. Active immunization with inactivated CA16 vaccines conferred complete protection against lethal challenge

The protective efficacy of the inactivated CA16 vaccines were further evaluated by the active immunization of suckling mice, followed by challenge with a mouse adapted CA16 strain (CA16-MAV), which could efficiently infect two-week-old mice. Groups of one-day-old mice were immunized with 0.5 µg of the inactivated CA16 or the control antigens at weeks 0 and 1, and then challenged with 2.3 × 10⁷ TCID₅₀ of CA16-MAV at week 2. In one experiment, the two CA16 vaccine groups exhibited only very minor symptoms (reduced mobility) and were completely protected from death; whereas the majority of the Vero lysate-immunized mice displayed limb paralysis during the course and finally died (Fig. 5A–B). In another challenge, the CA16-SZ05 and the CA16-G08 immunized groups were also fully protected except for one mouse in each group that were killed by the mother at 10 dpi without any prior disease symptoms. These deaths were likely accidental and thus were excluded from the calculation of the mean clinical scores and survival rates. In contrast, mice of the control (PBS) group rapidly developed severe symptoms and had an increased likelihood of death, reaching nearly 60% mortality at 8 dpi (Fig. 5C–D). These results demonstrate that active immunization with the inactivated CA16 vaccines is able to confer full protection in the mouse model of CA16 infection.

### 4. Discussion

The use of inactivated virus as vaccine against enterovirus infection has proven successful, as exemplified by world-wide application of inactivated poliovirus vaccines (reviewed in [32]). Among attempts to develop an effective vaccine for EV71 [25,26],
one of the major causative agents of HFMD, the inactivated whole-virus vaccine approach is the most advanced, with three candidates in phase 3 clinical trials [33]. Development of inactivated virus-based CA16 vaccines has recently been initiated. A recent report shows that maternal immunization with inactivated CA16 partially protected the neonatal mice born to the immunized dams against lethal infection [27]. In this study, we demonstrate, for the first time to our knowledge, that active immunization with inactivated CA16 experimental vaccines is able to provide full protection in mice against lethal challenge.

We show that immunization with inactivated CA16 may elicit virus-specific, protective antibody responses in mice. The anti-CA16 antibodies were able to neutralize infection in vitro and partially protected mice against lethal infection in vivo, indicating that neutralizing antibodies may play an essential role in in vivo protection. These results are in agreement with previous reports [27,28]. In the present study, neutralizing titers ranging from 128 to 512 (Table 1) were achieved shortly (one week) after two immunizations at weeks 0 and 1. This immunization schedule was adopted to determine whether a protective neutralizing antibody response can be elicited within 14 days, so that it could be subsequently used for active immunization of 1-day-old mice followed by challenge with mouse-adapted CA16 at the age of 14 days. The same strategy has been shown to be successful in evaluating the protective efficacy in mice of inactivated EV71 vaccine candidates in a previous study [34], in which EV71 neutralizing titers ranging from 32 to 512 were obtained within two-week immunization time. The CA16-neutralizing titers (128–512) achieved by the inactivated CA16 vaccines in the present study appear to be lower than those (1600–32,000) obtained by immunization with CA16 VLPs [28]. The drastic difference probably attributes to multiple factors, including dosage (two doses of 2 µg of inactivated whole-virus vs three doses of ∼3 µg of VLP) and total immunization time (two weeks vs eight weeks); it is unlikely due to the antigens themselves (inactivated whole-virus vs VLP). To further determine whether the two types of antigens possess distinct abilities to induce neutralizing antibodies, a side-by-side immunization study should be conducted. The presented data show that the anti-CA16 antisera neutralized both homologous and heterologous clinical isolates (both belong to B1b genotype) and a mouse-adapted CA16 strain, suggesting that the antisera may be broadly neutralizing. More CA16 clinical isolates and genotypes need to be tested to determine the breadth of neutralization by the anti-CA16 antisera. As expected, the anti-CA16 antisera did not neutralize EV71 (Table 1). Reciprocally, antisera against inactivated EV71 or EV71 virus-like particles do not seem to neutralize CA16 (unpublished data). These results underscore the need for separate antigens for EV71 or CA16 vaccine development. Although it is unlikely that a stand-alone CA16 vaccine will be commercialized, a CA16-derived antigen (for example, inactivated CA16 whole-virus) should be combined with the corresponding EV71 antigen to formulate a bivalent vaccine for dual protection against both EV71 and CA16 infections.

We also show that immunization with inactivated CA16 induced an IFN-γ T-cell response that could be rapidly recalled upon exposure to viral antigens (Fig. 2B); this suggests that this T-cell response may be able to provide additional protection. Although the exact contribution of the T-cell response in vivo protection remains to be determined, our results nevertheless indicate that inactivated CA16 experimental vaccines can elicit balanced antibody and T-cell responses in mice.

The yield of inactivated vaccines is an important factor that needs to be taken into consideration in vaccine development. In the present study, the yield of inactivated CA16 was very low, approximately 0.3 mg/l of Vero cell culture, probably due to the severe virulence of CA16 in Vero cells. Selection of high-yield

---

**Fig. 4.** Passive transfer of anti-CA16 sera partially protected neonatal mice against lethal challenge. Three groups of one-day-old suckling mice were i.p. injected with anti-Vero (n = 14), anti-S205 (n = 14), or anti-G08 (n = 15), respectively. One day later, all the mice were i.p. inoculated with 1 × 10^7 TCID50 of live CA16-G08, and subsequently monitored on a daily basis for clinical manifestation and survival scores. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death. (A) Representative mice with different antisera treatment at 14 days post-infection. The mouse on the left side received anti-Vero and the one on the right side received anti-G08. Limb paralysis is indicated by arrows. (B) Mean clinical scores. (C) Survival rates. The data are representative of two independent experiments.
CA16 strains or optimization of culture conditions and process parameters should be conducted to significantly improve CA16 yield, as demonstrated by studies on inactivated EV71 virus [35,36].

In the present study, the in vivo protective efficacy of the inactivated CA16 vaccines was assessed using two mouse models. The first model is based on a clinical strain CA16-G08, which caused ~93% mortality in one-day-old mice (Fig. 4C). Pretreatment with the inactivated CA16 mouse sera protected the majority of the recipient mice from death (Fig. 4C), demonstrating the protective function of the neutralizing antisera. However, i.p. injection of CA16-G08 into 14-day-old mice does not cause death or apparent disease symptom (data not shown), indicating that 14-day-old mice are insensitive to CA16-G08 infection. The second model is based on a mouse-adapted strain, CA16-MAV, which is originally derived from CA16-G08. The identity of CA16-MAV was confirmed by several lines of evidence: first, the amino acid sequence of VP1 protein of CA16-MAV is 98% and 98.3% identical to that of CA16-G08 and CA16-SZ05, respectively, but only 70.4% and 70% to that of EV71-BrCr (GenBank# U22521) and EV71-SH2H98 (GenBank# AF302996) strains, respectively; second, CA16-MAV can be positively detected by RT-PCR (data not shown) using a pair of CA16-specific primers described previously [37]; third, CA16-MAV, but not EV71, can be neutralized by the anti-CA16-SZ05 and the anti-CA16-G08 mouse sera (Table 1). The CA16-MAV was able to efficiently infect 14-day-old mice, resulting in severe symptoms and nearly 80% mortality (Fig. 5). Using this model, we were able to demonstrate that active immunization with the inactivated CA16 vaccines could protect 14-day-old mice from lethal CA16-MAV infection. The availability of these two CA16 infection models will greatly enhance future vaccine and antiviral drug development for CA16.

Collectively, the presented data demonstrates how the active immunization with inactivated whole-CA16 virus vaccines may elicit the production of neutralizing antibodies and fully protect mice against lethal challenge. These results provide a solid foundation for the development of inactivated CA16 as a component of a multivalent human vaccine against HFMD.

Acknowledgments

We thank Drs Bing Sun, Qi Jin and Wei Liu for providing the CA16 viruses. We also thank Dr. Andy Tsun for excellent editorial contribution. This work was supported by a grant (#KSCX2-YW-BR-2) from the Chinese Academy of Sciences “100 Talents” program.

Conflict of interest: Q. Liu, and Z. Huang have submitted a patent application regarding the mouse-adapted CA16 strain, CA16-MAV.

References


