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Similar protective immunity induced by an inactivated enterovirus 71 (EV71) vaccine in neonatal rhesus macaques and children☆

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ABSTRACT

During the development of enterovirus 71 (EV71) inactivated vaccine for preventing human hand, foot and mouth diseases (HFMD) by EV71 infection, an effective animal model is presumed to be significant and necessary. Our previous study demonstrated that the vesicles in oral regions and limbs potentially associated with viremia, which are the typical manifestations of HFMD, and remarkable pathologic changes were identified in various tissues of neonatal rhesus macaque during EV71 infection. Although an immune response in terms of neutralizing antibody and T cell memory was observed in animals infected by the virus or stimulated by viral antigen, whether such a response could be considered as an indicator to justify the immune response in individuals vaccinated or infected in a pandemic needs to be investigated. Here, a comparative analysis of the neutralizing antibody response and IFN-γ-specific T cell response in vaccinated neonatal rhesus macaques and a human clinical trial with an EV71 inactivated vaccine was performed, and the results showed the identical tendency and increased level of neutralizing antibody and the IFN-γ-specific T cell response stimulated by the EV71 antigen peptide. Importantly, the clinical protective efficacy against virus infection by the elicited immune response in the immunized population compared with the placebo control and the up-modulated gene profile associated with immune activation were similar to those in infected macaques. Further safety verification of this vaccine in neonatal rhesus macaques and children confirmed the potential use of the macaque as a reliable model for the evaluation of an EV71 candidate vaccine.

1. Introduction

With the severe development of large outbreaks of human hand, foot and mouth disease (HFMD) in Asia-Pacific regions in recent years [1–4], increased attention has gradually been given to severe cases of HFMD with neurological symptoms and the high death rate in populations of children that is typically attributed to enterovirus 71 (EV71) infection [5–9], thus further promoting the research and development of EV71 vaccines [10–12]. Based on the pre-clinical studies of inactivated EV71 vaccines that had assessed their safety and efficacy in the suckling mouse model and/or the neonatal rhesus macaque model [13–17], three EV71 inactivated vaccines have been studied in human clinical trials [10–12]. Although the clinical trials demonstrated the safety and efficacy of these inactivated EV71 candidate vaccines [10–12], the pathogenic and immunologic mechanisms of EV71 infection in animal models that have commonly supported the assessment of the safety and efficacy of candidate vaccines and in humans remain unclear. The rationale includes the observation of EV71 natural infection only in humans; for example, despite a scarb2 transgenic mouse model showing susceptibility to infection by some EV71 isolates [18], its genetic background greatly differs from that of humans [19,20], which may be important for the study of pathogenesis in humans. Additionally, rhesus macaques, although their close genetic background to humans renders them a good non-human primate model, are costly, and some ethical issues arise regarding their use, which may limit some in-depth studies about the details of pathogenesis and the immune response during EV71 infection [21,22]. Our

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results from the recent clinical trials of an inactivated EV71 vaccine in children have provided some evidence that confirms the viability of the neonatal rhesus macaque model that was used to assess the safety and efficacy of candidate vaccines in our preclinical study [10,23]. Importantly, the analysis of immune effects elicited by these vaccines in children provides additional data for understanding the immunologic characterizations of EV71 infection observed in the neonatal rhesus macaque model. In this paper, based on the comparison of the primary safety and efficacy of an inactivated EV71 vaccine in a neonatal rhesus macaque model and on subsequent observations from phase II clinical trials in children, the safety and immunogenicity of this vaccine were systematically analyzed for further justification of the significance and application of this model for EV71 infection studies. Our results demonstrated that the neonatal rhesus macaque could be applied as an effective animal model for mimicking EV71 infection progress in humans and the corresponding immune response elicited by immunization using EV71 candidate vaccines, which would shed light on the future application of this model.

2. Materials and methods

2.1. Vaccine

The inactivated EV71 vaccine was prepared from the FY-23KB strain (C4 genotype), which was cultured in KMB-17 cells (a human diploid cell line), and with 1/4000 formalin for inactivating the virus [24]. The vaccine contained 320 EU (ELISA units; ELISA: Enzyme-linked immunoassay) of viral antigen and 0.5 mg of aluminum hydroxide suspended in 0.5 ml of buffered saline. The placebo contained 0 EU of viral antigen and the same amount of aluminum hydroxide in a similar volume of buffered saline. Both the vaccine and the placebo were tested by the National Institutes for Food and Drug Control, China.

2.2. Ethics statement

The immunogenicity and protective experiments of the EV71 inactivated vaccine in rhesus macaques were performed at the Institute of Medical Biology, Chinese Academy of Medicine Sciences. The safety evaluation of this vaccine in macaques was performed at the National Center for Safety Evaluation of Drugs, China, which passed the international certification of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The experimental protocols and the ethics were reviewed and approved by the animal management committee.

According to the Declaration of Helsinki and the principles of National Good Clinical Practice (GCP), the clinical protocols and the ethics were reviewed and approved by the Guangxi Clinical Trial Base and Human Subjects Review Committee of the Institute of Medical Biology and were registered at ClinicalTrials.gov (NCT01512706). The study was conducted in Guangxi Province by the Guangxi Centers for Disease Control and Prevention (CDC).

2.3. Study design

Animal immunization and challenge: In total, 42 1- to 1.5-month-old neonatal rhesus monkeys were used in this study, among which forty (20 males and 20 females) were divided equally into the following 4 groups: a phosphate-buffered saline (PBS) group, an $Al(OH)_3$ placebo group (0 EU), a 160 EU vaccine group and a 320 EU vaccine group, and the other two neonatal rhesus macaques were used as normal controls (without immunization or challenge). These neonatal rhesus macaques were isolated and tested for antibodies against EV71 before the experiments. After 22 days, the neonatal rhesus macaques were administered their treatments via intramuscular injection of vaccine in the lateral thigh at days 0, 28 and 56 (Fig. 1). Blood samples were obtained at 0, 56 and 84 days (Fig. 1). Then, the EV71 challenge infection (with wild type EV71 strain [EV71-FY22, GenBank: EU913466.1], $10^{4.5}$ $CCID_{50}$/macaque; $CCID_{50}$: 50% cell culture infective dose) of immunized and control animals was administered to the 24 experimental monkeys (in the 160 EU, 320 EU and $Al[OH]_3$ groups) via nasal spray at 168 days post-immunization. Blood specimens (0.5 ml) were obtained throughout the entire infection period. All clinical symptoms and adverse events were monitored throughout the entire experimental period [25,26]. In the three experimental groups, 2 monkeys from each group were sacrificed by deliberate anesthetic overdose, and their organs were harvested on days 4, 7, 10 and 14 post-infection [25].

Clinical trials: In total, 360 6- to 60-month-old healthy children (Table S1) whose parent or legal guardian provided written informed consent and who were part of a random blinding phase II clinical trial were divided equally into three groups: the placebo (0 EU) group, 160 EU vaccine group and 320 EU vaccine group. These children were inoculated intramuscularly with either the vaccine or a placebo at days 0, 28 and 56 (Fig. 1). All adverse events were monitored throughout the entire experimental period [10]. Blood samples were obtained on days 0, 56 and 84 post-immunization (Fig. 1). Susceptible participants were considered those who had a titer of the antibody against EV71 that was lower than 1:8 before immunization.

2.4. Laboratory analysis

Neutralizing antibodies: The neutralizing antibody assay was performed according to a standard protocol [13,24]. Briefly, heat-inactivated sera at different dilutions were mixed with medium containing 100 $CCID_{50}$ of EV71, incubated at 37 °C for 1 h and inoculated into a Vero cell suspension (10^5 cells/ml) in a 96-well plate. The cellular pathogenic effect (CPE) was observed within 7 days post-infection.

ELISPOT assay: The IFN-γ-specific ELISPOT assay was conducted as described previously [23]. Briefly, the plates were coated with anti-IFN-γ antibody overnight at 4 °C, washed three times with PBS and blocked at 37 °C for 1 h. Then, peripheral blood mononuclear cells (PBMCs) that had been isolated using Lymphoprep medium (Ficoll-Paque PREMIUM; GE Healthcare, Piscataway, NJ, USA) and stimulating peptides were added, and the mixture was incubated at 37 °C for 24 h. Next, the cells were removed, and the colors were developed by incubating the mixture in nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate. The spots were quantified using an automated ELISPOT reader.

Viral load: RNA was extracted from the various tissues and organs from neonatal rhesus macaques, and viral loads were measured by real-time RT-PCR assay as described previously [27]. Briefly, total RNA was extracted from fresh samples using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). For quantification, a single-tube RT-PCR assay was performed using the TaqMan one-step RT-PCR Master Mix in a 7500 Fast Real-Time RT-PCR system (Applied Biosystems, Foster City, CA, USA).

Histopathological analysis: Tissue samples were obtained on days 4, 7, 10 and 14 post-infection. Histopathological examination was performed as described previously [26]. Briefly, samples were fixed in 10% formalin in PBS, dehydrated in ethanol gradients and embedded in paraffin for further hematoxylin and eosin (H–E) staining and evaluated under a light microscope.

Microarray assay: Total RNA was obtained from the PBMCs from the challenged neonatal rhesus macaques only with $Al(OH)_3$ injected and from the children immunized with the vaccine.