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Coxsackievirus A 16 infection does not interfere with the specific immune response induced by an enterovirus 71 inactivated vaccine in rhesus monkeys

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ABSTRACT

Hand, foot and mouth disease is usually caused by enterovirus 71 (EV71) and coxsackievirus A 16 (CA16), which are members of the Picornaviridae family. In the present study, the characteristics of the immune response induced by an EV71 inactivated vaccine (made from human diploid cells) were explored in the presence of CA16 infection, based on the previously established neonatal rhesus monkey model. The typical clinical manifestations, including body temperature, viral viremia and virus shedding in the mouth, pharynx and feces, were characterized. A specific neutralizing antibody assay showed that the specific immune response induced by the EV71 inactivated vaccine was active against EV71 but not against CA16. No remarkable fluctuation in proinflammatory cytokine release was identified in the serum of immunized monkeys with EV71 vaccine and CA16 infections subsequently. The results showed that the specific immune response induced by the EV71 inactivated vaccine is effective against EV71 infection but is not affected by CA16 infection.

1. Introduction

In recent years, the increasing number of epidemics of hand, foot and mouth disease (HFMD) in the pediatric populations of Asia and the Pacific areas have received extensive attention because of the occurrence of severe cases of encephalitis and other symptoms of central nervous system infection with an accompanying high death rate [1–3]. Etiologic and epidemiologic studies have demonstrated that the major pathogens causing HFMD include enterovirus 71 (EV71) and coxsackievirus A 16 (CA16) [4,5]. Because EV71 infection is responsible for the majority of the infections leading to severe cases of HFMD and death [6,7], the development of an EV71 candidate vaccine has become quite essential, and progress has been made [8,9]. However, the pathogenesis of HFMD caused by EV71 and CA16 infections remains largely unclear [10,11], and the published data suggest the distinctive presence of repeated infections or frequent co-presence of EV71 and CA16 viruses in partial HFMD patients [12]. The issues regarding the specific immune response induced by an EV71 candidate vaccine include not only questions regarding the protective efficacy against EV71 infection but also whether the induced specific immune response directly contributes to an abnormal immune reaction in the presence of a CA16 infection. Investigation of cross-reactivity in the immune response induced by these two viruses has been presented and attributed to the similarity in the sequences of individual antigenic structure [13]. Furthermore, this presumed cross-reactivity may influence the administration of either EV71 or CA16 candidate vaccines, although there is an absence of immunopathological data regarding these two viral infections [14,15]. In this study, we further characterized the immune response induced by an EV71 candidate vaccine in the presence of CA16 infection using a previously established neonatal rhesus monkey model [16]. The immune response was assessed by observing the clinical and pathological manifestations in rhesus monkeys immunized with this EV71 inactivated vaccine. The results showed that CA16 infection did not affect the specific immune response induced by this EV71 inactivated vaccine and that no remarkable cross-reactivity of the immune response between EV71 and CA16 infections was observed.

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2. Materials and methods

2.1. Virus and cells

The EV71 virus FY23 strain (sub-genotype C4) originated from an epidemic in Fuyang, China in 2008 (GenBank: EU812515.1) [17]. The CA16 virus G20 strain (sub-genotype B) was isolated from the throat swab of an HFMD patient in Guangxi in 2010 (GenBank: JN590244.1) [18]. The strain was grown in Vero cells, which were maintained in DMEM (HyClone, Logan, UT, USA) with 10% FBS (Gibco, Grand Island, NY, USA).

2.2. EV71 inactivated vaccine

The EV71 inactivated vaccine was prepared as previously described [19]. Briefly, the viruses were purified, inactivated, emulsified in 1 mg/ml $Al(OH)_3$ adjuvant and assessed for antigen content (100 U/0.5 ml) using an ELISA [19].

2.3. Ethics statement

The animal experiments were designed based upon the principles expressed in the "Guide for the Care and Use of Laboratory Animals" [20] and "The Guidance to Experimental Animal Welfare and Ethical Treatment" [21]. The experimental protocols were reviewed and approved by the Yunnan Provincial Experimental Animal Management Association (Approval number: SCXK [Dian] 2011-0005) and the Experimental Animal Ethics Committee of the Institute (Approval number: YISHENGLUNZI [2013] 2).

2.4. The study design in monkeys

A total of 20 healthy rhesus monkeys (1 ± 1.5 kg and 190 ± 10 days old) were housed in separate cages in a large room (BSL-2 conditions) with sufficient fresh air and natural light. A neutralizing antibody test was conducted to confirm that 18 rhesus monkeys did not have antibodies against EV71 prior to the experiment. They were randomly divided into six groups: A (vaccinated + EV71-infected), B (vaccinated + CA16-infected), C (EV71-infected), D (CA16-infected), E (vaccinated) and F (PBS).

The animals in groups A, B and E were immunized with 100 U/0.5 ml EV71 inactivated vaccine by intramuscular injection and were boosted after 28 days. Additionally, blood samples from the monkeys were tested for neutralizing antibodies at days 14, 28, 42 and 56 after the primary injection.

On day 56 after the primary immunization, all monkeys in groups A–D were exposed to either the EV71 (FY23 strain) or CA16 (G20 strain) virus (10^4 $CCID_{50}$/monkey) via the respiratory tract [16]. After viral exposure, all monkeys were monitored twice daily for clinical signs [16]. Venous blood (0.5 ml) from each monkey was collected daily using 1 ml EDTA-coated capillary tubes. Throat swabs and feces were collected daily for the routine detection of the viral load.

2.5. Extraction of viral RNA and quantitative RT-PCR amplification

Viral RNA was extracted from whole blood, fresh throat homogenate and fecal homogenate from the experimental animals using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The quantitative RT-PCR assay and FY-23 primers were previously described [16]. The sequences for the G20-specific primers are as follows: forward primer, 5′-caacccatctgtgtttgtgaaaa-3′; probe, 5′-ccgccagctcaagtgcagtccc-3′ and reverse primer, 5′-ggtatgcactagctggtgacatg-3′.

2.6. Neutralization assay

The neutralization assay was performed in accordance with standard protocols [22]. Briefly, a mixture of diluted serum and virus at a titer of 300 $CCID_{50}$/100 μl were incubated at 37 °C for 1 h, added to Vero cell culture in 96-well plates and incubated at 37 °C. The cellular pathogenic effect (CPE) of the virus was observed after one week.

2.7. IL4- and IFN-γ-specific Elispot assay

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep as previously described [23]. A Monkey IL4 and IFN-γ Elispot Kit (MABTECH Inc, Cincinnati, OH, USA) was used in accordance with the manufacturer's protocol. Briefly, the plate was blocked, seeded with PBMCs and the stimulant, either whole live FY23 or G20 strain cells ($10^4$$CCID_{50}$/well), and then incubated at 37 °C for 36 h. After incubation, the cells were removed, and the plate was developed. The colored spots were counted using

Table 1
Clinical manifestation in EV71-infected newborn rhesus monkeys.

Group	Monkeys ID	Vesicular lesions	Healthy status after virus challenge					
	Hand		Foot	Mouth	Muscle Tension	Eating	Diarrhea	
	(EV-V + EV71)	13022	N	N	N	–	–	–
A		13023	N	N	N	–	–	–
		13024	N	N	N	–	–	–
	(EV-V + CA16)	13025	N	N	N	–	–	–
B		13026	N	N	N	–	–	–
		13027	N	N	N	–	–	–
	(EV71)	13028	Y	N	Y	+	–	+
C		13029	N	Y	Y	–	–	–
		13030	N	N	Y	–	–	+
	(CA16)	13031	Y	N	Y	+	–	–
D		13032	N	Y	Y	–	–	–
		13033	N	N	N	–	–	–
	(EV-V)	13034	N	N	N	–	–	+
E		13035	N	N	N	–	–	–
		13036	N	N	N	–	–	–
	(PBS)	13037	N	N	N	–	–	–
F		13038	N	N	N	–	–	–
		13021	N	N	N	–	–	–

Note: Y, yes; N, no; +, abnormal; –, normal.

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