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Formalin-inactivated vaccine provokes cross-protective immunity in a mouse model of human enterovirus 71 infection

Emily Jane Bek[a,*], Khairunnisa Mohamed Hussain[a], Patchara Phuektes[a], Chee Choy Kok[a], Qiang Gao[b], Fang Cai[b], Zhenglun Gao[b], Peter Charles McMinn[a]

[a] Infectious Diseases and Immunology, The University of Sydney, Australia
[b] Sinovac Biotech Co., Ltd., Beijing, People's Republic of China

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ABSTRACT

Human enterovirus 71 (HEV71) has emerged as a major cause of epidemics of hand, foot and mouth disease associated with severe neurological sequelae in the Asia-Pacific region. In this study, a passive protection mouse model was used to evaluate the protective efficacy of formalin-inactivated HEV71 vaccines derived from a Chinese C4 genotype strain. Pregnant mice were immunised using a prime/boost strategy and ≥50 U of vaccine protected five-day-old pups from lethal challenge with a mouse-adapted (B3 genotype) strain of HEV71. Immunised mice developed a neutralising antibody response to both the immunising C4 strain and to the mouse-adapted strain. Mice born to immunised dams showed significantly less myositis and reduced viral loads in tissues.

1. Introduction

Human enterovirus 71 (HEV71) has emerged as a significant cause of viral encephalitis in the Asia-Pacific region over the past fifteen years. In the most severe cases, onset of neurogenic pulmonary oedema is rapidly fatal for approximately 50–80% of patients [1–3]. A pattern of increased epidemic activity has been observed since 1997 [4]. The largest HEV71 epidemic reported to date occurred in China in 2008, with 4.9×10^5 cases of HFMD, 1165 cases of brainstem encephalitis and 126 deaths reported [5]. Reports posted on ProMed have documented ongoing epidemic activity in China (20090710.2478, 20100625.2121). The reports indicate that there were at least 9.9×10^5 cases of HFMD in China from 1 January 2010 to 22 June 2010 resulting in 537 deaths, 92% of which were caused by HEV71 (ProMed 20100625.2121).

HEV71 can be divided into four genotypes (A, B, C, and D) [6,7], within which virus isolates share more than 92% nucleotide sequence identity in VP1, compared to 78–83% identity between genotypes. Genotypes A and D are represented by single virus strains; the prototype BrCr for genotype A [7], and a clinical isolate from India for genotype D [8]. Genotypes B and C each have five known sub-genotypes, of which B3–B5 and C3–C5 have recently evolved in South-East Asia [8–10]. There is evidence of frequent recombination between genotypes within the human enterovirus A species [11], and it has been suggested that recent recombination events have generated a new HEV71 sub-genotype [12]. Although specific genotypes have not been linked to disease severity [13], clinical and epidemiological evidence from some outbreaks suggests sub-genotypes differ in their biological behavior and virulence. During the 1999 HFMD epidemic in Perth, Australia, sub-genotypes B3 and C2 co-circulated. C2 viruses were isolated from all identified cases of severe neurological disease and from only one case of uncomplicated HFMD [1,14]. During two discrete epidemics in Sarawak, Malaysia, in which either sub-genotype B4 or B5 viruses were predominant, B4 strains were less likely to cause central nervous system (CNS) infection than B5 strains [9].

HEV71-associated neurological disease can progress rapidly, resulting in brain damage that may not be reversible by antiviral therapeutics. The development of a vaccine suitable for a large-scale vaccination campaign is therefore essential to control this disease [10,15,16]. Several strategies have been pursued to develop an HEV71 vaccine, including non-infectious virus-like-particles (VLPs) [17,18], VP1 subunit vaccines [19–21], synthetic peptides [22,23], and DNA vaccines [24]. A cocktail of four peptides derived from the structural proteins of HEV71 genotype C4 was recently shown to be protective against lethal challenge with the same genotype virus, in a mouse model [23]. A formaldehyde-inactivated

Abbreviations: HEV71, human enterovirus 71; HFMD, hand, foot and mouth disease; CNS, central nervous system; CPE, cytopathic effect; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; $TCID_{50}$, 50% tissue culture infectious dose; HD_{50}, humane endpoint; i.p., intraperitoneal; e, embryonic day; MI, mock-immunised; NC, non-challenged; ODs, optical densities; RD, rhabdomyosarcoma; VLP, virus-like-particles.

* Corresponding author at: Infectious Diseases and Immunology, Sydney Medical School, Blackburn Building D06, The University of Sydney, NSW 2006, Australia. Tel.: +61 2 9036 9093; fax: +61 2 9351 4731.
E-mail address: emily.bek@sydney.edu.au (E.J. Bek).

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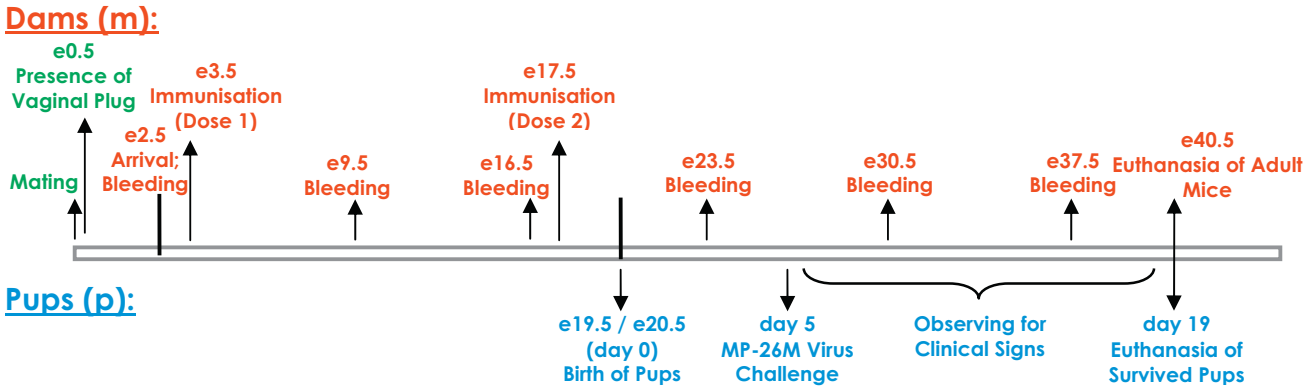

Fig. 1. Timeline of animal procedures. Procedures relating to dams are shown above the line in red, beginning with the detection of plugs the morning after mating (embryonic day e0.5). Procedures performed by the Animal Resources Centre (Canning Vale, WA, Australia) are shown in green. Procedures relating to progeny infant mice are shown below the line in blue, beginning with birth of the pups (day 0). (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

whole virus vaccine (sub-genotype B3) developed by Ong et al. [25] provided complete protection against lethal challenge of infant ICR mice with a mouse-adapted B3 strain. Antibody generated against this inactivated vaccine by ICR mice could neutralise HEV71 strains belonging to sub-genotypes B3, B4, and C1–C5. Although this model provided good evidence for the cross-reactivity of immune responses to HEV71 infection; protection of mice from lethal challenge with viruses belonging to other sub-genotypes was not evaluated. To our knowledge, the study reported here provides the first animal model data showing cross-protection induced by an HEV71 vaccine.

In this paper, we describe the protective efficacy of two formalin-inactivated HEV71 vaccines derived from the sub-genotype C4 strain of HEV71 (HEV71-C4) that is currently prevalent in China [5,26]. The mouse model used is based on a mouse-adapted strain of an Australian clinical isolate of HEV71 (MP-26M), which belongs to sub-genotype B3 [27]. The mouse-adapted virus causes acute flaccid paralysis in infant mice [17], primarily due to the development of severe skeletal muscle myositis. Newborn mice challenged with mouse-adapted virus were fully protected from lethal myositis by immunisation of their mothers during pregnancy with the formalin-inactivated vaccines.

2. Materials and methods

2.1. Vaccine and virus strains

HEV71-C4 was provided by Sinovac Biotech Ltd., Beijing, People's Republic of China. Two formalin-inactivated EV71 vaccines manufactured by Sinovac Biotech Ltd. were provided for this study; Vac-1 (lot no. 20081104), and Vac-2 (lot no. 20091113). Both vaccines were derived by formalin inactivation of HEV71-C4.

The highly purified bulk vaccine was tested by ELISA. Briefly, polyclonal antibody (1:5000, Sinovac Biotech) was coated on 96-well microtitre plates (Shenzen Jincanhua Industries) at 4 °C overnight. After 2 h of blocking, two-fold serial dilutions of the vaccine from 1:200 to 1:3200 were added to the coated plate. After incubation at 37 °C for 70 min, the captured antigen was detected by horseradish peroxidase (HRP; Sigma–Aldrich)-conjugated monoclonal antibody (1:5000; Sinovac Biotech) for 1 h. Optical densities (ODs) were read in a microtitre plate reader (Bio-Rad) at 450 nm. ODs over 0.015 were considered positive. The minimum detectable dilution of reference product was 1:800. In order to facilitate further calculation, this OD value was defined as 4000 U/mL.

MP-26M is a mouse-adapted strain of EV71 genotype B3 [17]. Titres of the HEV71-C4 and MP-26M cell culture supernatants were determined by 50% tissue culture infectious dose ($TCID_{50}$) assays using Vero (African Green Monkey Kidney; ATCC No. CCL-81) cell monolayers [28]

In order to determine that the vaccine lots were fully inactivated, $TCID_{50}$ assays were performed on the vaccine lots prior to the inoculation of pregnant female BALB/c mice. No cytopathic effect (CPE) was observed in the $TCID_{50}$ assays after 14 days of incubation.

2.2. Serological assays

Specific pathogen-free timed pregnant female BALB/c mice (8–12 weeks old) were obtained from the Animal Resources Centre (Canning Vale, Western Australia, Australia). The first vaccine dose was delivered by intraperitoneal (i.p.) inoculation on embryonic day e3.5 (see Table 2 for details of vaccines and dosages). A second, identical vaccine dose was given 14 days later, on embryonic day e17.5. The vaccines were adjuvanted with aluminium hydroxide.

Serum samples were collected from adult female BALB/c mice by submandibular venous puncture. Serum was collected immediately prior to the first immunisation, and at weekly intervals thereafter. The timeline for animal procedures is outlined in Fig. 1. Blood samples were allowed to stand at room temperature for 1 h before centrifugation at 9000 × *g*, 4 °C for 15 min. Serum samples were inactivated at 56 °C for 30 min and stored at −20 °C until required.

2.2.1. Microneutralisation

Duplicate serum samples were diluted two-fold in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/HyClone), from 1:8 to 1:256 [20]. The sera were then mixed with an equal volume containing 10^2 $TCID_{50}$ [28] of HEV71-C4 or MP-26M virus in maintenance media (DMEM with 2% FBS (Invitrogen)), 2 mM L-glutamine (Sigma) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37 °C for 2 h. The mixtures were then added to Vero cell monolayers in 96-well plates (Greiner Bio-One). After seven days at 37 °C with 5% CO_2, neutralising antibody titres were determined as the highest dilution for which no CPE was present in either duplicate (i.e. 100% neutralisation end-point). Neutralisation data were analysed using the GraphPad Prism online software package version 5.02 (SPSS Inc.; http://www.graphpad.com).

2.2.2. Enzyme-linked immunosorbent assay (ELISA)

HEV71-C4 and MP-26M antigens were produced by infecting human rhabdomyosarcoma (RD) cells (ATCC No. CCL-136) until 3+ CPE was observed. Virus was released from cells by freeze