Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide

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Abstract

Enterovirus 71 (EV71) infections could lead to high mortalities and neither vaccine nor therapeutic treatment is available. We investigated vaccination with a synthetic peptide SP70 representing a neutralizing linear VP1 epitope of EV71 strain 41 (subgenogroup B4) and passive transfer of anti-SP70 antibodies to protect suckling Balb/c mice against EV71 infectivity. When the mouse anti-SP70 antisera with a neutralizing antibody titer of 1:32 were passively administered to one-day-old suckling mice which had been challenged with a lethal dose of 1000 TCID50 per mouse, the neutralizing anti-SP70 antibodies were able to confer 80% in vivo protection. In contrast, suckling mice which did not receive any anti-SP70 antisera did not survive the viral challenge at day 21 postinfection. Histological examination and real-time RT-PCR assays revealed viral infiltration in small intestines of EV71-infected mice. Interestingly, anti-SP70 antibodies play a major role in the inhibition of EV71 replication in vivo and significantly reduced the viral titer. In conclusion, EV71-neutralizing antibodies elicited by the synthetic peptide SP70 were able to confer good in vivo passive protection against homologous and heterologous EV71 strains in suckling Balb/c mice.

Keywords: Enterovirus 71; VP1 capsid protein; Synthetic peptide; Passive protection

1. Introduction

Enterovirus 71 (EV71) is a viral pathogen within the Picornaviridae family that causes clinical diseases in humans with manifestations such as herpangina, aseptic meningitis, encephalitis, pulmonary edema and hand, foot, and mouth disease (HFMD). EV71-infected children can develop severe neurological complications that lead to rapid clinical deteriorations and even death [1–5]. A significant increase in EV71 epidemics with high mortalities has been observed throughout the Asia-Pacific region since 1997. In 1998, an epidemic of EV71 infection affected more than 100,000 young children in Taiwan and approximately 400 children were hospitalized for pulmonary edema and neurogenic shock which resulted in 78 deaths [6–8]. In October 2000, an outbreak of HFMD caused by EV71 occurred in Singapore, affecting mostly children below 6 years of age and there were four fatalities. At present, there are no specific effective antiviral drugs available and only palliative care is provided. Preventive and control measures during EV71 outbreaks are limited to public health surveillance and isolation of infected children as there is no available vaccine against EV71.

EV71 contains a positive-stranded RNA which is enclosed by capsid proteins VP1, VP2, VP3, and VP4. These proteins are presumed to be similar to the picornaviral prototype capsid which is made up of the four structural proteins arranged in 60 repeating protomeric units of an icosahedron [9,10]. In addition to protecting the viral RNA from nuclease cleavage, the capsid recognizes the receptors on the surface of host cells [11–13] and displays antigenicity [14,15]. Newborn ICR mice born to dams which were immunized with the formalin-inactivated EV71 whole virion were protected against lethal viral challenge [16]. However, this strategy involves the use of whole viral particles which may implicate safety issues...
during vaccine preparations. Another protection study has shown that the whole VP1 capsid protein is a potential vaccine candidate when it was able to confer protection against EV71 infection in suckling ICR mice, suggesting the presence of neutralizing epitope(s) within VP1 [17]. However, the neutralizing epitope(s) remained undefined. In our previous study, a synthetic peptide SP70 representing a neutralizing linear VP1 epitope of EV71 strain 41 (5865/SIN/00009) was successfully identified in eliciting high titers of neutralizing antibodies in Balb/c mice based on in vitro microneutralization assays [18]. However, passive protection of suckling Balb/c mice by anti-SP70 antibodies in an in vivo EV71 challenge study has not been reported. In this study, we demonstrated the in vivo protective efficacy of mouse antisera raised against the synthetic peptide SP70 which harbors a neutralizing linear epitope of the VP1 capsid protein of EV71 strain 41. We compared the efficacy with in vivo passive protection afforded by antisera raised against the heat-inactivated homologous EV71 strain and the synthetic peptide SP12 which represents an immunogenic but non-neutralizing linear VP1 epitope.

2. Materials and methods

2.1. Viruses

EV71 strain 41 (5865/SIN/00009) from the subgenogroup B4 was presented as the homologous strain in mice immunization and challenge studies. Representative EV71 strains (EV71/MS/7423/87) from the subgenogroup B2; 2933-Yamagata-03 from the subgenogroup B5; 1585-Yamagata-01 from the subgenogroup C2; and 75-Yamagata-03 from the subgenogroup C4 were presented as heterologous strains in challenge studies [1]. Viruses were grown in rhabdomyosarcoma (RD) cells at 37°C in minimum essential medium (MEM) (Gibco, USA) supplemented with 5% fetal bovine serum, 1% sodium pyruvate and 1.5% sodium bicarbonate. Once the cells displayed cytopathic effect (CPE), they were harvested and completely lysed by three freeze–thaw cycles. Cellular debris was removed by centrifugation at 10,000 × g for 30 min. The virus was purified by precipitation with 7% polyethylene glycol 8000 (PEG 8000) and then subjected to centrifugation on a 30% sucrose cushion at 25,000 × g for 4 h. The 50% tissue culture infective dose (TCID50) was determined in RD cells using the Reed and Muench formula [19]. Before being administered as an immunogen, the homologous EV71 strain was inactivated by heating at 56°C for 30 min and the amount of virion protein was quantified by the Bradford assay (Bio-Rad Laboratories, USA). Viability of viruses after heat inactivation was carried out by introducing viral preparations into fresh medium containing RD cells to check for CPE.

2.2. Synthetic peptides

Diphtheria toxoid-conjugated synthetic peptides representing the amino acid sequence of SP12 or SP70 were synthesized by Mimotopes Pty Ltd (Clayton, Victoria, Australia) as described previously [18].

2.3. Mice

Inbred Balb/c mice were obtained from the Centre for Animal Resources of the National University of Singapore. All institutional guidelines for animal care and use were strictly followed throughout the experiments. Groups of mice (n = 6) at different age groups, ranging from one-day-old to two-week-old, were inoculated intraperitoneally with 100 μl of the homologous EV71 strain (1000 TCID50). In another experiment, groups of mice (n = 6) at day 1 after birth were given different doses of the homologous EV71 strain (ranging from 1 TCID50 to 1000 TCID50 per mouse) via the intraperitoneal route. Mice (n = 6) in the control group were given 100 μl of phosphate buffered saline (PBS). Mice were monitored for the occurrence of mortality until three weeks postinfection. For immunization, groups of six-week-old female mice (n = 5) were intraperitoneally immunized with a 50% emulsion of Freund’s complete adjuvant containing either 50 μg of conjugated synthetic peptides or the heat-inactivated EV71 strain 41 (10 μg total protein). Two booster doses in 50% emulsions with Freund’s incomplete adjuvant were given at three weekly intervals. Sera were collected seven days after the final booster and checked for the presence of EV71-neutralizing antibodies using the in vitro microneutralization assay. Sera containing neutralizing antibodies were pooled and stored at −80°C until use. For passive protection study, groups of mice (n = 6) at day 1 after birth were injected intraperitoneally with 100 μl of EV71 (1000 TCID50 per mouse), followed by 100 μl of heat-treated (56°C, 30 min) mouse immune sera 24 h later. Suckling mice (n = 6) from control groups were either given naive sera or not given any antisera at all. Mice were monitored for body weight gain/loss and the occurrence of mortality until three weeks postinfection.

2.4. In vitro microneutralization assay

EV71-neutralizing antibodies were identified using the in vitro microneutralization assay as described previously [18]. The assay was carried out in triplicates.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Organs from mice were aseptically harvested in MEM and were homogenized for total RNA extraction using the RNeasy extraction kit (Qiagen, USA) according to the manufacturers’ instructions. The extracted RNA was then analyzed for the presence of EV71 using real-time Hybridization Probe RT-PCR for the detection of EV71 RNA as described previously [20]. Each assay was carried out in triplicates.

2.6. Immunohistochemical analysis

Cryosections with a thickness of 4 μm were made from frozen tissues and fixed on poly-L-lysine glass slides. Permeabilization of the fixed tissue sections was carried out by incubating with 0.2% Triton at room temperature for 10 min.
followed by the addition of mouse anti-EV71 monoclonal antibody (Chemicon International, USA) and further incubation for 30 min. Slides were washed three times with PBS and bound antibodies were detected using secondary biotinylated anti-mouse IgG (Zymed Laboratories, USA). The reaction was visualized by the addition of aminoethyl carbazole substrate (Zymed Laboratories, USA) to give a red colored peroxidase stain and the slide was counterstained with haematoxylin (Zymed Laboratories, USA) to generate a blue background.

3. Results

3.1. Age of mice and viral dosage

EV71 infection was characterized by hairless skin lesions and paralysis of limbs in infected Balb/c mice. Both clinical manifestations persisted throughout the observation period (Fig. 1). Suckling Balb/c mice from different age groups were infected intraperitoneally with the homologous EV71 strain at a dose of 1000 TCID$_{50}$ per mouse. Mice which were infected at day 1 after birth did not survive by day 11 postinfection. With mice infected at day 4 after birth, death was delayed but all mice died by day 16 postinfection. Mice which were infected at day 7 or day 10 after birth had higher survival rates of 60% and 90%, respectively, by day 21 postinfection. All mice which were infected at day 14 after birth survived throughout the experimental period (data not shown). To determine the lethal viral dosage, groups of mice at day 1 after birth were infected intraperitoneally with different doses of viruses, ranging from 1 TCID$_{50}$ to 1000 TCID$_{50}$ per mouse. With an infective dose of 1000 TCID$_{50}$ virus, all mice died by day 11 postinfection whereas a 30% survival rate was observed for those which were infected with a reduced dosage (1 TCID$_{50}$ per mouse). Mice which were not infected did not show any sign of distress and all of them survived (Fig. 2A).

3.2. Passive protection against lethal homologous EV71 strain challenge in suckling mice

To assess the efficacy of passive protection by antisera raised against the homologous EV71 whole virion (anti-EV71) and the synthetic peptide SP70 (anti-SP70), suckling Balb/c mice born to naive dams were administered with the respective antisera one day after lethal challenge with the homologous EV71 strain (1000 TCID$_{50}$ per mouse). Groups of mice that received the anti-EV71 antisera with neutralizing antibody titers of 1:64 or 1:32 survived throughout the experimental period. The anti-SP70 antisera with a neutralizing antibody titer of 1:32 provided 80% protection whereas EV71-infected suckling mice which did not receive any antisera and those which received antisera either from SP12-immunized (anti-SP12) or naive mice did not survive by day 12 postinfection (Fig. 2B). The body weight of mice that were protected against EV71 infectivity rose steadily up to an average of approximately 12 g at day 21 postinfection. At day 9 postinfection, they weighed approximately 8 g on average when compared to unprotected mice that had an average body weight of approximately 3 g before they died from EV71 infection (Fig. 2C). When the neutralizing antibody titer of anti-EV71 antisera was reduced to 1:16, the survival rate of EV71-infected suckling mice dropped to 70% and subsequently to 50% when the neutralizing antibody titer was further reduced to 1:8 (Fig. 3A). Approximately 80% of suckling mice survived when they were given the anti-SP70 antisera with a neutralizing antibody titer of 1:32 and a 50% survival rate was observed with the antisera at reduced titer of 1:16. With a reduced neutralizing antibody titer of

![Fig. 1. Viral infection of suckling Balb/c mice with a lethal EV71 dose (1000 TCID$_{50}$ per mouse). (A) Mice at day 3 postinfection are shown. The mouse on the right side is a non-infected age-matched control. (B) Mice at day 9 postinfection are shown. The mouse on the left side is a non-infected age-matched control. Limb paralysis is represented by * and skin lesion is represented by **.](image-url)
1:8, the anti-SP70 antisera were still able to confer 20% protection (Fig. 3B).

3.3. Histological examination in EV71-infected suckling mice

Histological examinations revealed EV71 infiltration in small intestines of suckling Balb/c mice infected with the homologous EV71 strain. The presence of the virus was detected with the anti-EV71 monoclonal antibody. From micrographs of the cryosections, infected mice had intestinal structures which were damaged, thus indicating extensive viral replications. Mice which received the anti-EV71 or anti-SP70 antisera had intact intestinal structures and were protected against EV71 infectivity. However, mice which received the anti-SP12 antisera showed signs of tissue damage in their small intestines. The red colored peroxidase stain indicated EV71 infiltration of intestinal tissues (Fig. 4).

3.4. Detection of EV71 by real-time RT-PCR hybridization probe assay

Organs were harvested from Balb/c mice after challenge studies and RNA was extracted from homogenized tissues for the detection of EV71. The amplicons generated from the real-time RT-PCR assay were based on 204 base pairs PCR product of the VP1 region. Quantification analysis showed that the positive control has a Ct value of 13 which represents $5 \times 10^8$ EV71 copies and every 3.3 Ct value difference
represents a 10-fold difference in viral copies. Viral RNA samples from suckling mice which did not receive any antisera and those which received the anti-SP12 antisera or naive sera indicated a Ct value of 33. This represents the presence of 50 EV71 copies in the three respective samples. However, suckling mice which received either the anti-EV71 or anti-SP70 antisera had RNA transcripts with a higher Ct value of 37, representing approximately five EV71 copies (Fig. 5).

Fig. 3. *In vivo* passive protection study conferred by different EV71-neutralizing antibody titers. Groups of Balb/c mice (*n* = 6) at day 1 after birth were infected with the homologous EV71 strain (1000 TCID₅₀ per mouse) via the intraperitoneal route. One day after infection, suckling mice were intraperitoneally administered with serially diluted pooled antisera from (A) EV71-immunized mice or (B) SP70-immunized mice. Mice in control groups were not administered with any antisera. Death was monitored until 21 days postinfection.

Fig. 4. Detection of EV71 infection in small intestines of suckling Balb/c mice after being challenged with the homologous EV71 strain (1000 TCID₅₀ per mouse). Intestinal structures from: (A) non-infected mice as the negative control; (B) EV71-infected mice as the positive control; (C) mice receiving the anti-EV71 antisera; (D) mice receiving the anti-SP70 antisera; and (E) mice receiving the anti-SP12 antisera. Cryosections of intestinal tissues were prepared and EV71 was detected with mouse anti-EV71 monoclonal antibody and biotinylated anti-mouse IgG before visualizing with aminoethyl carbazole substrate to give a red colored peroxidase stain as positive detection and haematoxylin to give a blue background.
3.5. Passive protection afforded by the anti-EV71 or anti-SP70 antisera against heterologous EV71 strains

Groups of suckling Balb/c mice born to naive dams were challenged with heterologous EV71 strains (1000 TCID₅₀ per mouse) and antisera raised against the homologous EV71 whole virion or synthetic peptide SP70 were administered at day 2 after infection. When EV71-infected suckling mice were administered with the anti-EV71 antisera at a neutralizing antibody titer of 1:32, all of them survived. Mice infected with EV71 strains from the subgenogroup B2 or B5 had 80% survival rates when they were administered with the anti-SP70 antisera with a neutralizing antibody titer of 1:32. Upon lethal challenge with EV71 strains from the subgenogroup C2 or C4, the anti-SP70 antisera protected 70% of infected suckling mice (Table 1).

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<th>EV71 subgenogroups</th>
<th>Mouse antisera</th>
<th>Survival (%)</th>
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<tr>
<td>B²&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Anti-EV71</td>
<td>100</td>
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<td></td>
<td>Anti-SP70</td>
<td>80</td>
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<td>B⁴&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Anti-EV71</td>
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<sup>a</sup> One day after EV71 challenge (1000 TCID₅₀ per mouse), suckling mice were administered with antisera from mice immunized with the heat-inactivated EV71 strain 41 (anti-EV71) or synthetic peptide SP70 (anti-SP70) and monitored until 21 days postinfection. Each group contained six mice.

<sup>b</sup> Homologous EV71 strain.

<sup>c</sup> Heterologous EV71 strains.

4. Discussion

Although immune responses such as elevated antibody level detected soon after immunization may demonstrate the immunogenicity of a vaccine, they do not necessarily provide a reliable guide to its efficacy in priming an in vivo protective response. Successful protection against viral infectivity in animal models has long been regarded as the best test for the efficacy of any vaccine. Benefits of vaccination could be derived either through active immunization or passive protection. Passive transfer of specific antibodies has been shown to reduce the severity of viral infections, including Japanese encephalitis infection [21], varicella infection [22] and coxsackievirus infection [23]. We have previously identified a neutralizing linear VP1 epitope, denoted as SP70 within the VP1 capsid protein of EV71 strain 41, which was able to elicit high titers of mouse EV71-neutralizing antibodies against the homologous EV71 strain and five heterologous EV71 strains from subgenogroups B2, B5, C2 and C4 [18]. In the present study, we have tested the in vivo functional potential of EV71-neutralizing antibodies elicited by the synthetic peptide SP70 in mice and have shown that the anti-SP70 antisera were able to confer in vivo passive protection of up to 80% survival rate in suckling Balb/c mice which have been challenged with a lethal dose of the homologous EV71 strain. Our previous study has shown that the amino acid sequence represented by SP70 was totally conserved amongst 25 EV71 strains from subgenogroups A, B₁–B₅ and C₁–C₄, which suggested possible cross-protection against infectivity of all EV71 strains [18]. Here, we have demonstrated that the anti-SP70 antisera with a neutralizing antibody titer of 1:32 were able to confer up to 80% in vivo passive protection in suckling Balb/c mice challenged with several heterologous EV71 strains. It is interesting to note that the level of protection was almost similar to that obtained against the homologous strain. This reflects the efficacy of in vivo protection conferred by passively transferred EV71-neutralizing antibodies elicited by SP70.
have also shown that the neutralizing antibody titer plays a decisive role in the in vivo passive protection against EV71 infectivity. A significant reduction in the survival rate was correlated with suckling mice receiving antisera with progressively lower EV71-neutralizing antibody titers.

Natural viral infections commonly lead to the production of protective neutralizing antibodies recognizing both linear and conformational epitopes of viruses. A screening study based on ELISA has shown that human sera with high titers of neutralizing antibodies against EV71 strain MS/7423/87 reacted strongly with neutralizing conformational epitopes located at the N-terminal region of VP1 but exact locations of these epitopes were not well-defined [24]. The in vivo protective efficacy of these neutralizing antibodies has not been established as well. The neutralizing linear VP1 epitope (SP70) we have previously identified was located more towards the C-terminal part of the protein and mapped in the region involving VP1 dimerization [18]. In this study, we have demonstrated that the mouse antisera raised against this neutralizing linear VP1 epitope were able to confer good in vivo passive protection to suckling Balb/c mice against EV71 infectivity.

Antisera with a neutralizing antibody titer of 1:64 from mice immunized with formalin-inactivated EV71 strain Tainan/5079/98 were able to confer 65% passive protection to suckling ICR mice following challenge with the same viral strain [16]. Subsequently, another study reported that the passive administration of antisera raised against EV71 whole virions via the intraperitoneal route also offered 70% protection to newborn ICR mice against EV71 infectivity [17]. Although a number of immunogens with good in vivo passive protective potential have been identified, the majority of candidates that have been tested experimentally relied mainly on whole viral particles. In our study, the mouse antisera which were raised against EV71 whole virions did provide higher in vivo passive protection to suckling mice against lethal EV71 challenge when compared with the anti-SP70 antisera. This might be due to higher titers of neutralizing antibodies elicited by several neutralizing epitopes located on the virus other than that represented by the synthetic peptide SP70 alone. Unlike SP70, the immunogenic but non-neutralizing linear VP1 epitope represented by the synthetic peptide SP12 elicited antibodies which were unable to neutralize EV71 infectivity and hence infected suckling mice were not protected. The passively administered antisera raised against EV71 whole virions might also contain antibodies which could induce lysis of the virus-infected host cells through activation of complement cascades or by mediating antibody-dependent cellular cytoxicity (ADCC) activities. Conventional vaccine preparations using the whole virion as an inactivated vaccine might be ideal since it offered good protection against viral infection. However, the laborious and costly procedures involved in EV71 cultivation and purification as well as safety issues are of major concerns.

It is likely that different vaccine delivery systems can present the antigen differently. It is clear, however, that the delivery system must be considered carefully when assessing the immunogenicity and protective efficacy of any vaccine candidate. In a recent study, a vaccine strategy using live-attenuated Salmonella enterica serovar Typhimurium strains to express and deliver the VP1 protein of EV71 strain 2272 was shown to protect newborn ICR mice against infection caused by the same viral strain but immunization with live-attenuated vaccines has the problem of possible reversions to wild-type virulence [25]. The use of a synthetic peptide, however, has advantages of defined chemical composition, stability, safety and reduces the complexity of manufacturing. In this study, the synthetic peptide SP70 with its highly conserved amino acid sequence was able to elicit high titers of EV71-neutralizing antibodies that confer broad immunity against homologous and heterologous EV71 strains. Due to its small size, the synthetic peptide by itself may be poorly immunogenic. To increase the immunogenicity of a synthetic peptide, it could be linked to a larger carrier protein or by forming multiple antigenic peptides [26]. The use of either natural or artificial adjuvants has been found to enhance the immunogenicity of a synthetic peptide by promoting its uptake and activating dendritic cells (DCs) to initiate the immune response [27]. In the present study, the synthetic peptide SP70 was conjugated with a diphtheria toxoid which serves as a carrier protein and may help to prolong the half-life of SP70. The addition of a 50% emulsion of Freund’s adjuvant to SP70 could have further enhanced its immunogenicity as well. The immunogenicity of a synthetic peptide could also be enhanced by designing a chimeric peptide construct incorporating both B-cell and T-cell epitopes. The neutralizing linear VP1 epitope represented by SP70 when linked to a T-helper cell epitope could be further explored as a chimeric peptide to raise antisera for in vivo protective efficacy evaluation.

In the absence of a vaccine for EV71, this study has demonstrated that the in vivo passive protection of suckling Balb/c mice by antisera raised against the synthetic peptide SP70 offers a potential therapeutic treatment for EV71 infections. The Fab region of the mouse anti-SP70 antibody which will recognize and bind to the neutralizing linear VP1 epitope can be selected as a potential candidate for humanized antibody production. Humanized monoclonal antibodies conferring good passive protection have been developed against the Venezuelan equine encephalomyelitis virus [28] and the respiratory syncytial virus [29]. A humanized monoclonal antibody which has specificity, avidity and neutralizing activity might be a viable treatment option against EV71 infection in humans.

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