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# A peptide vaccine based on a B-cell epitope on the VP1 protein of enterovirus 70 induces a strong antibody response

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**Summary. –** Enterovirus 70 (EV70) is the causative agent of acute hemorrhagic conjunctivitis (AHC), for which no effective vaccine is available. This study revealed a high reactivity of the N-terminal region of EV70 VP1 (VP1-1) with an anti-EV70 mouse serum. The analysis of overlapping synthetic peptides of VP1-1 identified a B-cell epitope in this region. The E-peptide (14-ANTVESEIKAELGVI-28) showing the highest reactivity with the anti-EV70 serum induced neutralizing antibodies in mice and reduced the virus titer in the eyes, suggesting that it is a candidate vaccine against AHC caused by EV70.

**Keywords:** enterovirus 70; AHC; B-cell epitope; peptide vaccine

## Introduction

AHC is the most common eye disease in humans and is highly contagious. Enterovirus 70 (EV70 serotype of the *Human enterovirus D* species) and coxsackievirus 24 are the major causative agents of AHC (Chatterjee *et al*., 1970; Chen *et al*., 2004; Xiao *et al*., 2009). EV70-induced AHC was first reported in Ghana in 1969, and this epidemic has spread rapidly throughout the Middle East, Asia, and Oceania. Epidemics of EV70-induced AHC have reoccurred on a large scale at intervals of 3 to 5 years (Aoki and Sawada, 1992). However, the mechanism of infection and the immunological response to EV70 infection is unclear. For this reason, no therapeutic agents or effective vaccines for EV70 have been developed.

B-cell epitopes are defined as those regions on the surfaces of antigens that are recognized by, and bind to, B-cell receptors or specific antibodies. These epitopes are the main targets of pathogenesis and immunology research, as well as the targets of vaccine and diagnostic reagent development (Jiang *et al*., 2010). Synthetic peptides corresponding to B-cell epitopes have shown promise as vaccines for combating pathogenic infections. Recently, synthetic peptide vaccines have been tested against coronavirus (He *et al*., 2006), human immunodeficiency virus 1 (Cruz *et al*., 2009), influenza A virus (Wang *et al*., 2010), *Toxoplasma gondii* (Tan *et al*., 2010), and *Yersinia pestis* (Tripathi *et al*., 2006; Khan *et al*., 2008). Therefore, to determine the B-cell epitope of EV70 is an important step in developing a peptide vaccine for EV70. In this study, we attempted to identify a major immunogenic B-cell epitope of EV70 by analyzing overlapping synthetic peptides of the N-terminal region of EV70 VP1. As the peptide with the B-cell epitope was able to induce neutralizing antibodies in mice, it can be considered as a candidate peptide vaccine against AHC caused by EV-70.

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**Abbreviations:** EV70 = enterovirus 70; AHC = acute hemorrhagic conjunctivitis; VP1-1 = virus capsid protein VP1 amino-terminus; VP1-2 = virus capsid protein VP1 middle; VP1-3 = virus capsid protein carboxy-terminus

## Materials and Methods

*Virus and cells*. The EV70 J670/71 isolate was a generous gift of the Korean National Institute of Health. LLC-MK2 cells (derived from Rhesus macaque kidney epithelium) were purchased from the

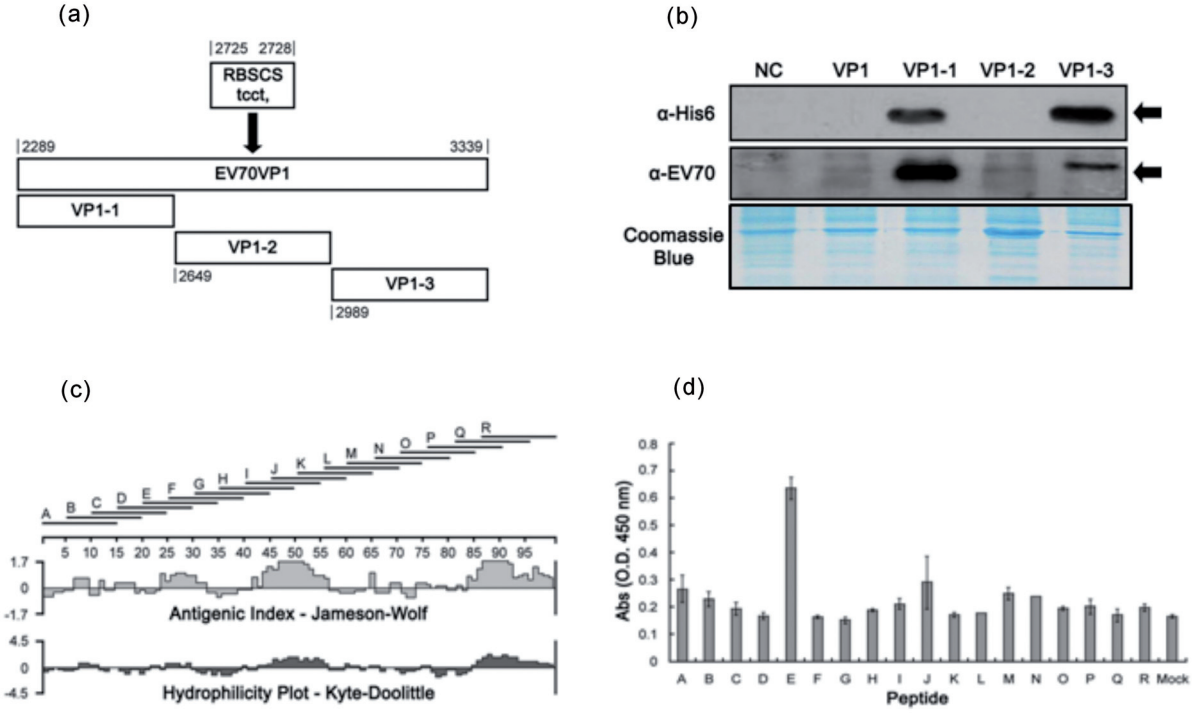


**Fig. 1**

**Identification of a B-cell epitope on the VP1 protein of EV70**

(a) Scheme of localization of the VP1 fragments on VP1. (b) Western blot analysis of His-tagged VP1 fragments with antisera to His-tag (α-His6) and EV70 (α-EV70). (c) Antigenicity and hydrophobicity prediction for the 15 aa long peptides A-R to VP1. (d) ELISA reactivity of the peptides A-R with the EV70 antisera.

American Type Culture Collection (ATCC) and used to maintain and propagate the EV70 virus. Viral titers were determined using standard 50% tissue culture infective dose ($TCID_{50}$) of EV70-infected LLC-MK2 cells. EV70 was isolated from infected LLC-MK2 after two freeze/thaw cycles and stored in aliquots at -85 °C.

*Recombinant proteins*. The full-length cDNA of EV70 VP1 was prepared by RT-PCR with primers (Table 1) that were designed using DNA sequence of the EV70 J670/71 strain. The PCR product was cloned into the pET-22b (+) (Novagen) expression vector and the resulting recombinant plasmid was verified by restriction enzyme digestion and DNA sequencing. This plasmid was used to transform *Escherichia coli* BL21 (DE3) cells for protein expression. After overnight growth in LB broth, IPTG was added in a final concentration of 1 mmol/l and incubated for 4 hrs to induce protein expression. The bacteria were then pelleted at 3,000 x g for 20 min and lysed by sonication in a buffer (40 mmol/l Tris-HCl, pH 7.5; 5 mmol/l EDTA; 0.5% Triton X-100). The lysates were centrifuged at 17,000 x g at 4°C for 20 min and the supernatant was analyzed.

*Western blot analysis*. Bacterial lysates, or cell lysates produced from EV70-infected LLC-MK2 cells, were separated by 12% SDS-PAGE and blotted to a nitrocellulose membrane. The membranes were blocked with 5% milk at 4°C for 1 hr, incubated with primary anti-EV70 or anti-6x-His antibodies (IG Therapy) overnight at 4°C, and probed with a goat anti-mouse IgG-HRP (Bethyl) secondary antibody for 1 hr at 4°C. Finally, proteins were detected by enhanced chemiluminescence (Ab Frontier).

*ELISA*. The reactivity of the EV70 antisera against the various capsid peptides was determined by a direct binding assay to identify the B-cell epitopes of EV70. A series of eighteen synthetic peptides, 15-mers in length with five residue overlaps, were used. The plates were coated with peptides (500 ng/well) and after blocking for non-specific binding, EV70 antisera produced in mice was added at dilution of 1:1000 and incubated at 37°C for 2 hrs. After three washes with PBS-T, goat anti-mouse IgG-HRP was added and incubated at 37°C for 1 hr. After washing, the reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) at 37°C for 30 min. The reaction was stopped with 2M $H_2SO_4$ and the absorbance (A492) was measured using an ELISA plate reader (Thermo Multiskan EX).

*Animals and immune sera.* BALB/c mice (5-week-old) were immunized three times with 100 μg of recombinant peptides in Freund′s adjuvant and with 100 $TCID_{50}$ of EV70 at 2 week intervals. The antisera were collected five days after last immunization.

*Real-time PCR.* The mouse eyes were removed from infected, immunized, and challenged mice. Total RNA from the virus stocks

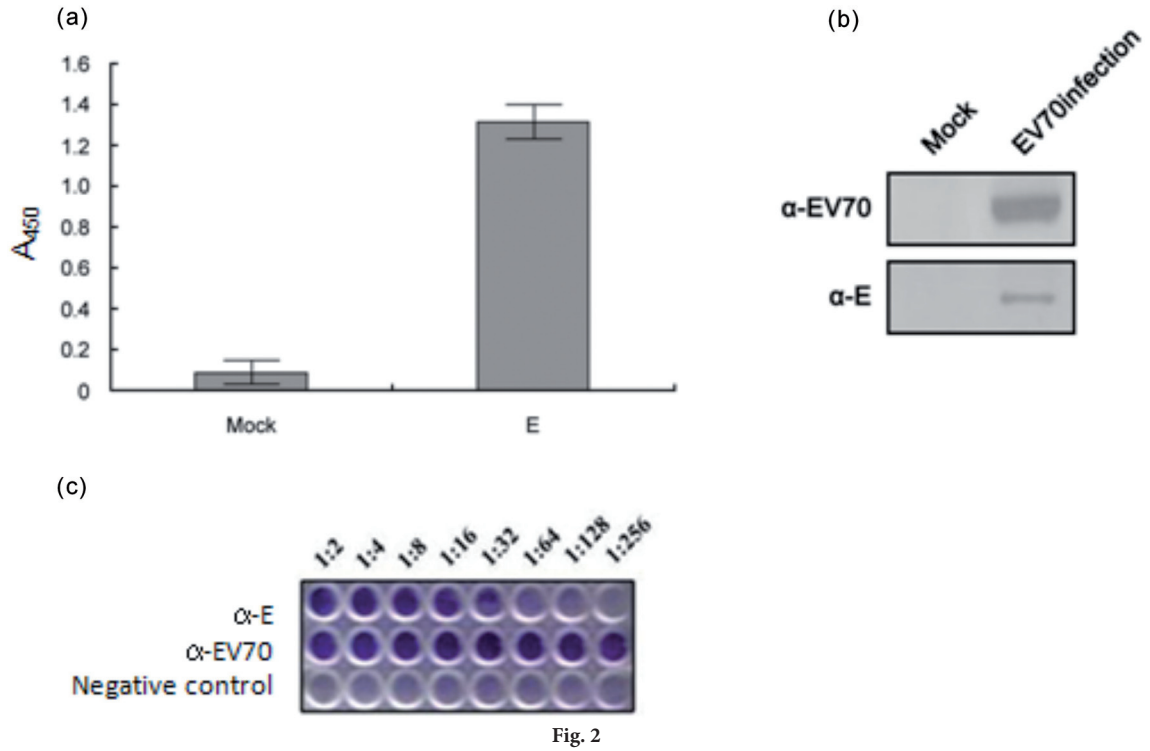


**Fig. 2**

**Immunogenicity of the E-peptide**

(a) ELISA reactivity of the E-peptide with the E-peptide antisera (E) and non-immune serum (Mock). (b) Western blot analysis of an EV70-infected cell lysate (EV70 infection) and control lysate (Mock) with the E-peptide antisera (α-E) and the EV70 antisera (α-EV70). (c) Neutralization of EV70 with α-E and α-EV70.

and mouse eye tissues were extracted with Trizol reagent (Invitrogen) and the RNA was used to synthesize and amplify cDNA by RT-PCR using RT&GO (MP Biomedical). EV70 RNA and 18S rRNA as internal control were tested by using SYBR Green (Bio-Rad) in iCycler (Bio-Rad). Quantification of a gene expression levels, expressed as relative mRNA levels compared with a control, was calculated after normalization to 18S rRNA and using delta delta Ct formula. Ct values were expressed as the mean of two independent experiments.

*Sequence alignment.* The GenBank Acc. Nos of the *Human enterovirus D* sequences used in this study are as follows: BAA04528.1, D17595.1, D17596.1, D17597.1, D17598.1, D17599.1, D17600.1, D17602.1, D17603.1, D17604.1, D17605.1, D17606.1, D17607.1, D17609.1, D17611.1, D17612.1, DQ916376.1, DQ916377.1, DQ916378.1, and DQ916379.1.

## Results and Discussion

To determine the B-cell epitope of EV70 VP1, we generated full-length VP1 and three fragments of the VP1 capsid protein (VP1-1, VP1-2, and VP1-3) using a His-tagged recombinant protein expression vector, pET–22b (+), in a bacterial expression system. However, full-length VP1 and the middle fragment, VP1-2, could not be expressed because they contain sequences complementary to bacterial ribosome sequences (termed RBSCS, bacterial ribosome sequence site complementary sequence), which suppresses the expression of foreign proteins in bacteria (Fig. 1a)(Chen *et al.*, 2004). EV70 VP1-1 and VP1-3 were expressed, purified, and detected by Western blot analysis with EV70 antisera. EV70 VP1-1 strongly reacted with the anti-EV70, compared to VP1-3 (Fig. 1b), so we targeted the VP1-1 region for B-cell epitope mapping. To fine-tune the mapping of the B-cell epitope, we synthesized eighteen peptide fragments consisting of fifteen amino acids that overlapped their adjacent peptides by five residues (Fig. 1c). Among these, the E-peptide (amino acids 14-ANTVESEIKAELGVI-28 of VP1) was the most immunogenic in the ELISA with EV-70 antisera (Fig. 1d).

To confirm whether the E-peptide induces antibody production in immunized mice, E-peptide (100 μg) with complete/incomplete Freund's adjuvant was injected into the peritoneal cavities of mice in three intervals. Five days after the last immunization, the mouse serum was collected to measure the antibody titer. The E-peptide antisera reacted strongly with

E-peptide (Fig. 2a) and could detect EV70 protein in virus-infected HeLa cell lysates, as demonstrated by Western blot analysis (Fig. 2b). Anti-EV70 also reacted with virus-infected cell lysates as positive control (Fig. 2b). More importantly, 10 times the 50% tissue-culture-infective dose (10 $TCID_{50}$) of EV70 could be neutralized by a 1:32 dilution of anti-E antibodies or 1:256 dilution of anti-EV70 antibodies (Fig. 2c). These results strongly suggest that the E-peptide is the major B-cell epitope of EV70 and can potentially be used as a vaccine.

Next, we tested whether the E-peptide functions as a vaccine by protecting EV70-infected eyes *in vivo*. Five days after the final peptide vaccination, the mice were challenged with 100 $TCID_{50}$ of EV70 (Fig. 3a). The viral titers in the eyes of EV70-infected mice were determined by real-time PCR, based on a standard curve for EV70 RNA and the correlation between the Ct (cycle threshold) values and known EV70 titers ($R^2 = 0.9797$) (Fig. 3b). Five days after challenge, we assayed the Ct values of mice vaccinated with the E-peptide or EV70 as a positive control. The data were expressed as relative RNA levels compared with the average expression level in the mock group (Fig. 3c). E-peptide immunization strongly protected the mice, as did EV70 immunization, and reduced the replication of EV70 in the eyes. Interestingly, an alignment of the E-peptide sequences of several EV70 strains showed that the E-peptide contains a sequence highly conserved in other EV70 strains (Table 1). It is also similar to the EV94 sequences with the exception of a single amino acid (Table 1). Therefore, E-peptide-induced antibodies should protect against most EV70 strains and our results emphasize the potential of the E-peptide as a synthetic peptide vaccine against EV70.

Chen *et al.* (2005) have demonstrated that an N-terminal fragment (aa 1–138) of EV70 VP1, expressed by a bacterial expression system, induces more neutralizing antibodies compared to a C-terminal fragment (aa 141–310). This is similar to our data and corroborates our results. They also reported that both the N- and C-terminal fragments have independent neutralization antigenic sites (Chen *et al.*, 2005). Therefore, apparently EV70 VP1 has several independent neutralizing epitopes. Especially important is the observation that the E-peptide region within the N-terminus of VP1 is a strong B-cell epitope that can induce production of neutralizing antibodies against EV70. Thus, it could be a possible target to design an effective peptide vaccine for EV70. Vaccinations based on synthetic peptides designed to elicit specific immune responses against pathogens have several benefits. First, they are relatively simple and cost-

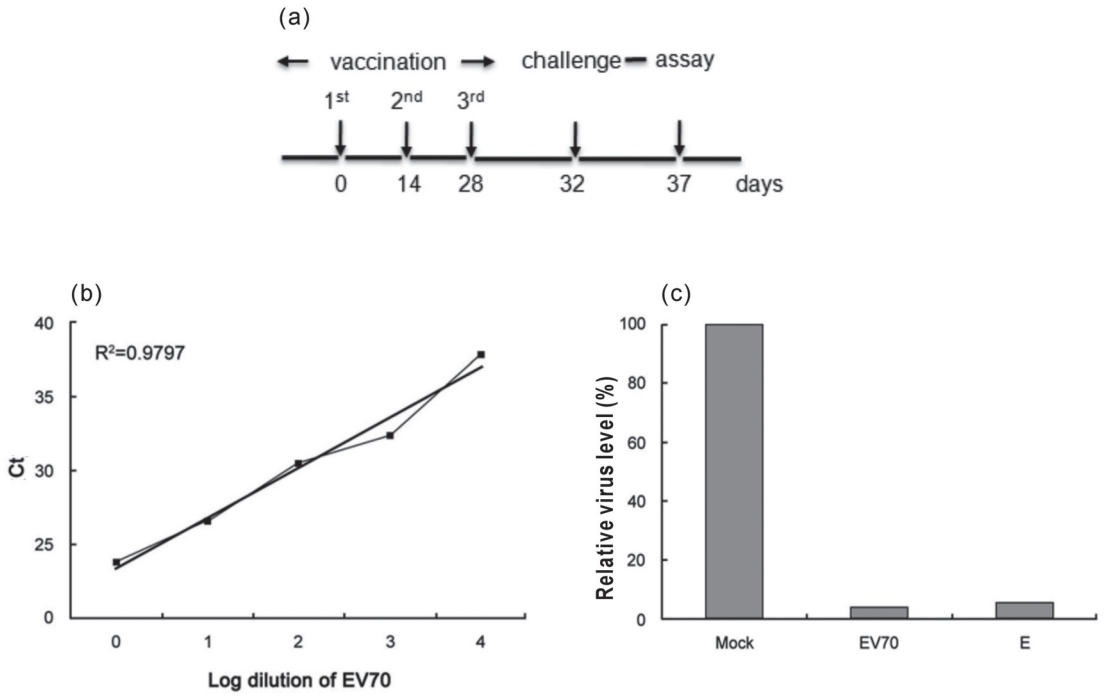


**Fig. 3**

**Protectivity of the E-peptide vaccination**

(a) Schedule of vaccination with 3 doses of the E-peptide on days 0, 14, and 28, a challenge with 100 $TCID_{50}$ of EV70 on day 32, and assay of protectivity on day 37. (b) Relationship between Ct values from real-time PCR and virus titers. (c) Relative virus levels in the eyes of mice vaccinated with EV70 (EV70), E-peptide (E) or PBS (negative control) and challenged on day 37, expressed as % of negative control.

**Table 1. Multiple alignment of amino acid sequences of the E-peptide in various strains of EV70 and EV94**

| Virus | Strain | Acc. No. | Peptide sequence | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A | N | T | V | E | S | E | I | K | A | E | L | G | V | I |
| EV70 | T260/74 | BAA04528 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | FB/73 | D17596 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | T62/73 | D17609 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | HP185/81 | D17598 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | SEC32/71 | D17607 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | I/72 | D17600 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | R20/71 | D17605 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | R6/71 | D17606 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | G10/72 | D17597 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | M51/76 | D17603 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | ENG/71 | D17595 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | V1635/81 | D17612 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | HP85/78 | D17599 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | M8/72 | D17604 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV70 | V1250/81 | D17611 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EV94 | E435 | DQ916378 1 | – | – | – | – | – | – | – | – | R | – | – | – | – | – | – |
| EV94 | E438 | DQ916379 1 | – | – | – | – | – | – | – | – | R | – | – | – | – | – | – |
| EV94 | E430 | DQ916377 1 | – | – | – | – | – | – | – | – | R | – | – | – | – | – | – |
| EV94 | E210 | DQ916376 1 | – | – | – | – | – | – | – | – | R | – | – | – | – | – | – |

effective to produce and second, it is possible to synthesize more types of peptide vaccines. This field of research is not yet well-established, but lot of work is being done to develop synthetic peptide vaccines (Wang *et al.*, 2010). After clinical trials, the peptide could be used as a vaccine in the future. Our findings provide a proof-of-concept for the potential clinical applications of peptide-based vaccines and help to increase our understanding of the pathogenesis of EV70.

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