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Immunogenicity of enterovirus 70 capsid protein VP1 and its non-overlapping N- and C-terminal fragments

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Abstract

Currently no practical treatment method or effective virus vaccine is available for acute hemorrhagic conjunctivitis (AHC) caused by enterovirus 70 (EV70). Antibodies to UV-inactivated EV70 (J670/71 epidemic isolate) and to the inclusion bodies of recombinant proteins of full-length EV70 VP1 (GST-VP1m), its non-overlapping terminal fragments N138 (1–138 aa) and C170 (141–310 aa) (or GST-N138m and GST-C170) were developed in rabbits. The anti-EV70 neutralizing activities of the rabbit sera were determined by standard neutralization assays. The antibodies to UV-inactivated EV70, were immuno-reactive with EV70 capsid proteins VP1 and VP3 of four EV70 epidemic isolates (KW/97, T260/74, J670/71 and AE/72) in Western-blot analysis, and immunoprecipitated the capsid proteins VP1 and VP3 from the cell lysates of virus-infected human Chang's conjunctival (HCC) cells. The antibodies to GST-VP1m, GST-N138m and GST-C170, immunoprecipitated only the VP1 proteins of the four EV70 isolates. Anti-EV70 J670/71 antibodies and the antibodies to the three recombinant VP1 proteins were all capable of immunoprecipitating EV70 whole-virus of the four EV70 epidemic isolates grown in HCC cells. The anti-EV70 virion antibodies neutralized EV70 isolates with titers of 6000–10,000 units/ml while the antibodies to GST-VP1m, GST-N138m or GST-C170 neutralized EV70 isolates with titers of 20–320 units/ml. The results suggest that (a) immunization with bacterially produced recombinant EV70 VP1 and its non-overlapping N- and C-terminal fragments, was capable of eliciting EV70-neutralizing antibodies; (b) the neutralization titers of antibodies to the recombinant VP1 proteins were lower than that of antibodies to the UV-inactivated EV70 virions; and (c) the non-overlapping N138 and C170 fragments of EV70 VP1 both harbor independent anti-EV70 neutralization antigenic sites.

Keywords: Enterovirus 70; VP1; Recombinant protein; Neutralizing antibody; Neutralization antigenic site

1. Introduction

EV70 is the main causative agent of acute hemorrhagic conjunctivitis (AHC). Since the first report in Ghana in 1969 (Chatterjee et al., 1970), enterovirus 70 (EV70) has caused two pandemics (1969–1972, 1980–1982) (Hierholzer and Pallansch, 1989; Ishii, 1989a), many epidemics (Chatterjee et al., 1970; Kono, 1975; Reeves et al., 1986; Wolken, 1974; Wright et al., 1992; Yin-Murphy, 1984) and sporadic cases (Ishii, 1989b; Kaiwar et al., 1983) of AHC throughout the world. AHC caused by EV70 (EV70 AHC) is endemic in many tropical and subtropical countries. EV70 AHC is a highly contagious ocular infection which can affect individuals of all ages and both sexes (Langford et al., 2003; Wolken, 1974; Wright et al., 1992). The epidemics of EV70 AHC may occur recurrently on a large scale at an interval of 3–5 years (Ishii, 1989b; Uchida, 1990). Moreover, neurological complications (1:10,000) have been reported to follow EV70 AHC, and are characterized by asymmetrical flaccid motor paralysis (radiculomyelitis) similar to poliomyelitis and cranial nerve palsies as well as a dysautonomic feature (Hung, 1981; Phuapradit et al., 1976; Saenz et al., 1984; Sklar et al., 1983). These disorders can persist permanently. EV70 was reported to be inhibited or killed in vitro by a variety of disinfectants such as chlorine, iodine, phenol and different kinds of antiviral agents such as interferons, $ZnCl_2$, flavone, arilodone,

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and benzimidazoles (enviroxime and enviradone) (Langford et al., 1985, 1995; Yamazaki and Miyamura, 1989). However, these chemicals could not inactivate EV70 at levels of practical use in clinics. Therefore, there are no clinical treatment methods (chemical medicines) available at present for EV70-caused AHC. Moreover, The economic loss resulting from an EV70 AHC epidemic in a country, can be considerable, and can be comparable to the loss from a chronic disease, like tuberculosis. In India, the estimated loss for an AHC epidemic in 1981 was $219 million, while the loss for tuberculosis was $385 million in lost income and productivity (Srinivasa and D'Souza, 1987). Therefore, developing a method to prevent EV70 caused AHC or its epidemic could have practical values.

It is known that infection of EV70 can elicit neutralizing antibodies, although the neutralization titer decreases in the human body every year post infection (Aoki and Sawada, 1992). Periodic vaccination (every 3–7 years) could induce or increase circulating anti-EV70 neutralization antibodies effective in preventing EV70-caused AHC and possibly its more severe neurological sequelae. UV-light inactivated whole EV70 can induce antibodies to the immunodominant neutralization epitopes associated with VP1 and to a lesser degree to the VP3 capsid protein, but currently there is no effective live or attenuated virus vaccine readily available for large-scale vaccinations (Langford et al., 2003). It may be easier, more cost-effective and safer to use recombinant viral proteins (Hoatlin et al., 1987) to develop an EV70 vaccine.

EV70 VP1 was targeted for anti-EV70 recombinant protein vaccine development due to its immunodominance (Lin et al., 1988). Previously we reported that the recombinant proteins of the full-length EV70 VP1 and its non-overlapping terminal fragments (N138 and C170) were successfully and highly expressed in bacteria *Escherichia coli* (Chen et al., 2004). In this study, antibodies to these recombinant proteins were developed in rabbits to examine if they were capable of neutralizing live EV70 virus. Our results indicated that immunization of rabbits with recombinant full-length protein (GST-VP1m), non-overlapping N-terminal and C-terminal recombinant peptides (GST-N138m and GST-C170, respectively) of EV70 VP1, like immunization of rabbits with inactivated EV70 whole-viruses, elicited neutralizing antibodies against epidemic isolates of EV70 (all the EV70 epidemic isolates obtained so far belong to a single serotype). These results suggest that multiple neutralization epitopes (at least two: one in N138 fragment and the other in C170 fragment) exist within the full-length EV70 VP1.

2. Materials and methods

2.1. Cells and EV70 epidemic isolates

Human lung fibroblast cells (Wi38) and human Chang's conjunctival cells (HCC) purchased from American Type Culture Collection (ATCC) were used to maintain, propagate and assay viruses. Epidemic isolates of EV70 from Japan (J670/71 and T260/74) were generously provided by Dr. Marguerite Yin-Murphy (Department of Microbiology, National University of Singapore, Singapore). An epidemic EV70 isolate from Key West, FL (KW/97) was a gift from Dr. Kenneth Dimock (Department of Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada). The EV70 epidemic isolate from Thailand (AE/72) was obtained from Dr. Chinrudee Jayavasu (Ministry of Public Health, Bangkok, Thailand). Stocks of EV70 were grown in human Wi38 cells, aliquoted and stored at −100 °C. Viruses were purified by CsCl gradient ultracentrifugation as previously described (Langford et al., 2003; Yamazaki et al., 1974).

2.2. Bacteria and recombinant protein expressions

E. coli BL21(DE3) competent cells were obtained from Novagen (Madison, WI) and *DH5α* competent cells were made as described in (Sambrook et al., 1989). The cDNAs encoding EV70 VP1 and its N138 fragment were modified by an enzyme-ligated overlapping synthetic oligo-DNA fragment (ELOSODF) method (Chen et al., 2004). The modified cDNAs of VP1m and N138m and the unmodified C170 cDNA were in-frame cloned between the EcoR I and Xho I sites of pGEX-4T-1 vector with *DH5α* cells and highly expressed in *BL21(DE3)* cells (Chen et al., 2004).

2.3. Recombinant proteins purification

Bacteria-expressed recombinant proteins were purified by GST fusion protein purification module (Pharmacia Biotech, Uppsala, Sweden) as per the manufacturer's instructions. The inclusion bodies of the recombinant proteins were purified according to the adapted Marston method (Sambrook et al., 1989).

2.4. EV70 hyperimmune antiserum and antibodies to recombinant proteins

The preparation of EV70 J670/71-specific hyperimmune rabbit serum was made using UV-inactivated EV70 whole-viruses (Langford et al., 2003). The antibodies to recombinant proteins were produced in rabbits from the inclusion bodies of the recombinant proteins. Two New Zealand white rabbits (6–8 month-old; 2–3.5 kg) (Myrtle Rabbitry, Thompson Station, TN) per group were given an intramuscular injection containing 100 μg of a recombinant protein in 1.0 ml of phosphate buffered saline (PBS, pH 7.4) with Freund's complete adjuvant (50%, v/v) (Sigma, St. Louis, MO). Starting 1 month later, rabbits were boosted every 4 weeks. Booster injections were given intramuscularly and each injection contained 50 μg of the same recombinant protein in 1.0 ml of PBS with Freund's incomplete adjuvant (50%, v/v). Blood samples were collected by venipuncture 5–11 days post booster injections and the sera separated, aliquoted and stored at −20 °C. All experiments were approved by the

Institutional Animal Research Committee and performed in accordance with the Guide for Care and Use of Laboratory Animals.

2.5. Immunoprecipitation

Virus-infected cells were lysed in the cell-lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% Triton X-100, 0.1% Triton X-114, 0.1% SDS, 0.1% glycerol) and centrifuged at $12{,}000 \times g$ for 10 min to recover the supernatant. Target proteins in the above virus-infected HCC cell lysates or whole virus particles in the virus-infected HCC cell culturing media were immunoprecipitated using protein G-sepharose (Sigma) and anti-J670/71 hyperimmune antiserum or antibodies to GST or GST-fused recombinant proteins as previously described (Sambrook et al., 1989).

2.6. Western-blot analysis

Purified viruses and immunoprecipitated viruses or protein samples were boiled in sample buffer (50 mM Tris·Cl, 8% sucrose, 2% SDS, 100 mM DTT, 0.02% bromophenol blue) for 5 min and separated on 8–10% SDS polyacrylamide gel (C = 4%), and then transblotted on to Hybond-P PVDF membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Blots were probed by anti-J670/71 antibodies and subsequently the corresponding HRP-conjugated secondary antibodies (Sigma). Metal enhanced DAB substrate kit (Pierce Biotechnology, Inc., Rockford, IL) was used to visualize the positive protein bands.

2.7. Neutralization assay

The neutralizing activities of the rabbit antisera to whole-EV70 J670/71, GST and EV70 VP1's recombinant proteins GST-VP1m, GST-N138m or GST-C172, were determined in cell cultures of HCC against $100TCID_{50}$ of virus in each 96-well for each EV70 strain (Anderson et al., 1984). After incubation periods of 1 h at 37 °C or overnight at 4 °C, the reaction mixtures of EV70 with antisera were inoculated onto 96-well microplates with confluent cell sheets at 37 °C for 2–3 days, and stained with 0.2% crystal violet in 20% methanol when microscopic examination indicated 100% cell death in the virus control cultures. The titer (units) of specific neutralization activity was calculated as the reciprocal of the highest dilution of serum that inhibited the viral cytopathogenic effect by 50%.

3. Results

3.1. Antibodies to UV-inactivated whole-EV70 immunoprecipitated EV70 capsid proteins VP1 and VP3

To determine whether polyclonal antibodies to inactivated whole EV70 J670/71 (hyperimmune serum) react with the four capsid proteins of EV70, capsid proteins from different epidemic isolates of purified EV70 virus particles (Yamazaki et al., 1974) including KW/97, T260/74, J670/71 and AE/72 were probed by Western-blot analysis. Only two protein bands in all four epidemic isolates of EV70, corresponding to VP1 (34 kD) and VP3 (27 kD), reacted positively with anti-J670/71 antibodies, while the hyperimmune serum did not detect VP2 (28 kD) and VP4 (9 kD) (Fig. 1). This result suggests that anti-virion antibodies are immuno-reactive with EV70 capsid proteins VP1 and VP3 from the four tested EV70 epidemic isolates on a Western-blot.

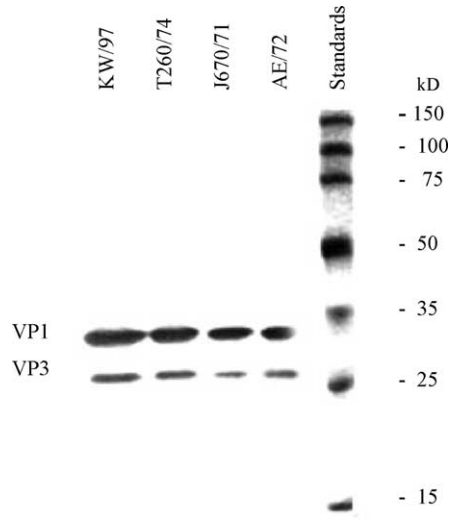

Fig. 1. Hyperimmune anti-EV70 J670/71 antiserum reacted with EV70 capsid proteins VP1 and VP3. The SDS-PAGE separated EV70 capsid proteins of purified virion particles of different EV70 epidemic isolates (KW/97, J670/71, T260/74 and AE/72) were transblotted onto a PVDF membrane, and the blot was probed with anti-EV70 J670/71 hyperimmune antiserum. The antibodies in the hyperimmune antiserum only recognized the capsid proteins VP1 and VP3 (not VP2 and VP4).

Further experiments were performed to test whether anti-J670/71 antibodies immunoprecipitate EV70 VP1 and VP3 capsid proteins. HCC cells infected by different EV70 epidemic isolates KW/97, J670/71, T260/74 or AE/72 were lysed and immunoprecipitated by anti-EV70 J670/71 antiserum. The immunoprecipitated non-denatured (native) proteins were boiled in a SDS-gel sample buffer and detected by Western-blotting with anti-EV70 J670/71 antiserum. Fig. 2 shows that the native EV70 capsid proteins VP1 and VP3 were immunoprecipitated by anti-EV70 J670/71 antibodies from the virus-infected HCC cell lysate of KW/97, T260/74, J670/71 or AE/72. This result suggests that anti-virion antibodies are also immuno-reactive with non-denatured (native) EV70 capsid proteins VP1 and VP3 from the four tested EV70 isolates.

3.2. Antibodies to UV-inactivated whole-EV70 neutralized EV70 viruses

Neutralization assay indicated that polyclonal antibodies to UV-inactivated whole EV70 J670/71, strongly neutralized

Table 1
Neutralization of different EV70 epidemic isolates by antibodies against EV70 J670/71 and EV70 VP1 recombinant proteins[a]

EV70 isolates	Hyperimmune serum against EV70 virus (J670/71)	Serum[b] against GST fusion protein		
		GST-N138m	GST-C170	GST-VP1m
KW/97	6000	240	100	320
J670/71	6000	320	260	285
T260/74	10000	20	10	80
AE/72	10000	60	20	100

[a] Neutralization titers (for every ml of serum) are the average results from the sera of two parallel rabbits immunized by the same amount of recombinant protein inclusion bodies.
[b] The sera against recombinant proteins were obtained 9 days after 4th booster immunization.

different epidemic isolates of EV70 with titers between 6000 and 10,000 units/ml (i.e., 6000 units/ml for J670/71 and KW/97; 10,000 units/ml for T260/74 and AE/72) (Table 1). The results suggest that antibodies raised against UV-inactivated EV70 J670/71 are capable of neutralizing EV70 viruses although the neutralization epitopes recognized by the polyclonal antibodies have not yet been defined.

3.3. Immunoprecipitation of EV70 VP1 proteins by antibodies against recombinant GST fusion proteins

To evaluate the potential of EV70 VP1 as an anti-EV70 recombinant protein vaccine, the antibodies to recombinant proteins were developed in rabbits. HCC cells infected by different EV70 epidemic isolates KW/97, J670/71, T260/74 or AE/72 were lysed and immunoprecipitated by the rabbit antibodies to GST, GST-VP1m, GST-N138m or GST-C170. The immunoprecipitated non-denatured (native) proteins were then boiled in a SDS-gel sample buffer and detected by Western-blotting with anti-EV70 J670/71 antiserum. Fig. 3A shows that the antisera to recombinant proteins GST-VP1m, GST-N138m and GST-C170, immunoprecipitated the EV70 VP1 proteins (but not VP3 proteins) of KW/97, T260/74, J670/71 and AE/72 from the cell lysis extracts of virus-infected HCC cells. The results suggest that (a) both the recombinant N138 and C170 fragments of EV70 VP1 are antigenic; (b) antibodies to EV70 VP1 cannot cross-react with EV70 VP3 (or the epitopes of EV70 VP1 and VP3 are different); and (c) antibodies to recombinant GST-VP1m, GST-N138m or GST-C170 EV70 capsid protein VP1 may be capable of binding or immunoprecipitating whole EV70 viruses.

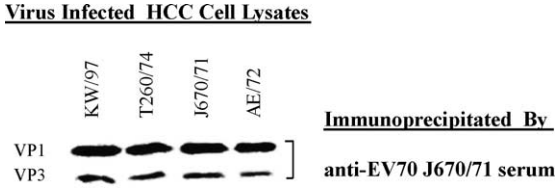

Fig. 2. Immunoprecipitation of EV70 capsid proteins VP1 and VP3 by anti-EV70 J670/71 hyperimmune antiserum. HCC cells infected by different EV70 epidemic isolates (KW/97, J670/71, T260/74 and AE/72) were lysed and immunoprecipitated by anti-EV70 J670/71 antiserum. The immunoprecipitated proteins were then separated by SDS-PAGE and further analyzed by Western-blotting with anti-EV70 J670/71 antiserum. The native (non-denatured) capsid proteins VP1 and VP3 (not VP2 and VP4) of all the four EV70 epidemic isolates were detected in the immunoprecipitated protein samples from the virus-infected HCC cell lysates.

3.4. Immunoprecipitation of EV70 virus particles by antibodies against UV-inactivated EV70 and recombinant GST fusion proteins

The anti-EV70 J670/71 antibodies strongly immunoprecipitated whole EV70 viruses of various epidemic isolates (KW/97, T260/74, J670/71 and AE/72) demonstrated in Western-blotting of viral VP1 and VP3 detected by anti-virion antibodies (Fig. 3B, lane of "EV70 Virus (J670/71)). Similarly, antibodies to recombinant GST-VP1m, GST-N138m or GST-C170, also immunoprecipitated the whole-EV70 viruses of the four epidemic isolates (Fig. 3B), but the levels of the immunoprecipitated EV70 viruses were much lower. The results suggest that (a) the antibodies to recombinant GST-VP1m, GST-N138m or GST-C170 were capable of binding the whole-virus particles of different EV70 epidemic isolates, and (b) recombinant GST-VP1m, GST-N138m or GST-C170 might be capable of inducing animals to produce neutralizing antibodies against EV70.

3.5. Neutralization of EV70 viruses by antibodies against recombinant GST fusion proteins

To examine whether the highly expressed recombinant proteins can induce rabbits to make anti-EV70 neutralizing antibodies, serum samples directed against recombinant GST-N138m, GST-C170, GST-VP1m or GST (control) were tested for the neutralization activity against different epidemic isolates of EV70 virus. Same antibody had various neutralization titers for different EV70 epidemic isolates: Antibodies to recombinant GST-N138m, GST-C170, GST-VP1m all had higher neutralization titers for J670/71 and KW/97 than for T260/74 and AE/72 (Table 1). In addition, with each boost of recombinant GST-N138m, GST-C170 or GST-VP1m (after second booster injection), the anti-EV70 J670/71 neutralization titers were increased and reached more than 250 units/ml by the 4th boost (data not shown). Anti-EV70 neutralizing antibodies (10–300 units/ml) were detected in the serum of rabbits injected with recombinant

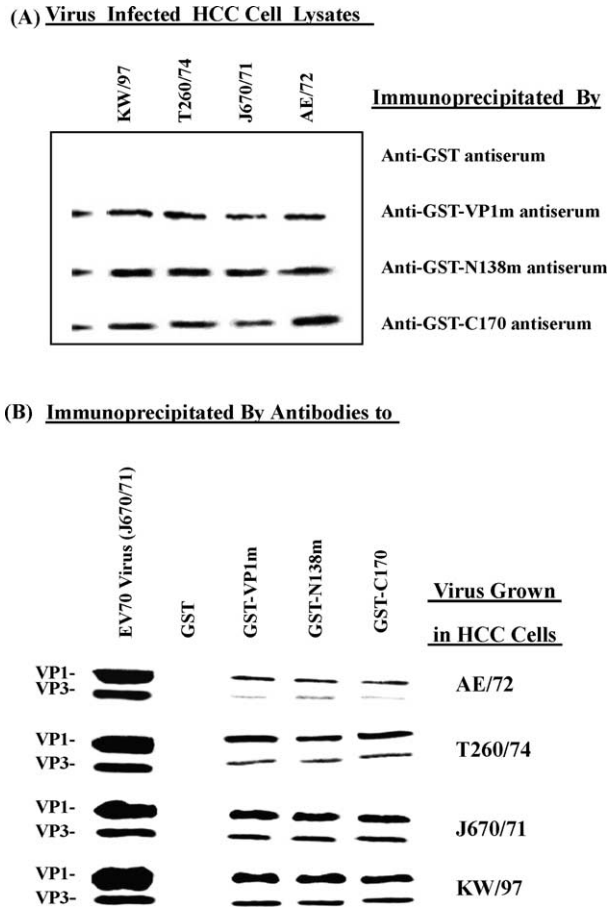

Fig. 3. Immunoprecipitation of VP1 capsid proteins and whole-viruses of four EV70 epidemic isolates by antisera directed against EV70 VP1 recombinant proteins. The four EV70 epidemic isolates (strains) used were KW/97, T260/74, J670/71 and AE/72. Immunoprecipitated proteins (native, non-denatured) or whole-viruses were denatured by boiling in a SDS-gel sample buffer, and detected by Western-blot analysis with the anti-EV70 J670/71 antiserum that detects the VP1 and VP3 capsid proteins of the four strains of EV70 viruses. (A) Immunoprecipitation of EV70 VP1. The native VP1 capsid protein of AE/72, T260/74, J670/71 or KW/97 in virus-infected HCC cell lysates, was immunoprecipitated by 1:200 diluted antiserum (obtained 9 days after 4th booster immunization) that were directed against GST, GST-VP1m, GST-N138m or GST-C170. The arrows all show the VP1 proteins in different EV70 epidemic isolates. All the antibodies to recombinant proteins GST-VP1m, GST-N138m or GST-C170 were capable of immunoprecipitating EV70 VP1 non-denatured capsid proteins in virus-infected HCC cell lysates. (B) Immunoprecipitation of EV70 whole-viruses by antisera directed against UV-inactivated EV70 and recombinant proteins. EV70 whole-viruses from the culturing medium of virus-infected HCC cells, were immunoprecipitated by 1:500 diluted hyperimmune anti-EV70 J670/71 antiserum or by 1:200 diluted antiserum (obtained 9 days after 4th booster immunization) that were directed against GST, GST-VP1m, GST-N138m or GST-C170. Antibodies to recombinant GST-VP1m, GST-N138m or GST-C170, like antibodies to UV-inactivated EV70 J670/71 whole viruses, were capable of immunoprecipitating the four strains of EV70 viruses.

protein GST-N138m, GST-C170 or GST-VP1m, suggesting that both the non-overlapping terminal peptides N138 (recombinant rN138m) and C170 (recombinant rC170) of EV70 VP1 contain neutralization antigenic sites, and that at least two neutralization epitopes exist within EV70 VP1.

4. Discussion

Isolated capsid protein VP1 or its synthetic peptides of picornaviruses other than EV70, such as poliovirus (Chow et al., 1985; Chow and Baltimore, 1982) and FMDV (Meloen et al., 1979), have been shown to experimentally induce neutralizing antibodies. Full-length or fragmental recombinant proteins of VP1 can also induce animals to raise neutralizing antibodies to some picornaviruses (Hoatlin et al., 1987; Shire et al., 1984; Wang et al., 2003). This implies that isolated or recombinant EV70 VP1 may have the similar potential to induce neutralizing antibodies. Our results show that the recombinant proteins of full-length EV70 VP1, its terminal fragments N138 and C170 were all capable of inducing rabbits to produce neutralizing antibodies against different epidemic isolates of EV70. The amino acid sequence conservation may account for this result because the amino acid homology of EV70 VP1 between J670/71 and T260/74 isolates is 98%, and the amino acid homology of EV70 J670/71 VP1 with the VP1 proteins of other tested EV70 epidemic isolates (NCBI accession #BAA04515-04532) is between 97 and 99% (Takeda et al., 1994). However, the reason why antibodies to recombinant GST-N138m, GST-C170 and GST-VP1m all had higher neutralization titers for J670/71 and KW/97 than for T260/74 and AE/72 needs to be determined in the future. On the other hand, the neutralization titers of the antibodies to these recombinant proteins, are much lower (20–30 folds lower) than that induced by UV-inactivated purified native virions of EV70 (J670/71) (Langford et al., 2003). This is consistent with the results seen between poliovirus 1 VP1 protein and its inactivated whole virions (Chow and Baltimore, 1982). The rabbit anti-EV70 J670/71 hyperimmune serum was previously shown to be immuno-reactive with recombinant GST-N138m, GST-C170 and GST-VP1m (Chen et al., 2004). In this investigation, the hyperimmune serum not only reacted with, but also immunoprecipitated the VP1 and VP3 capsid proteins of the four tested EV70 epidemic isolates, which is consistent with the reactivity of neutralizing monoclonal antibodies to EV70 with the capsid proteins VP1 and VP3 (Wiley et al., 1990). The results suggest that the anti-virion serum has corresponding antibodies to the epitopes on EV70 VP1, VP3 and maybe VP1–VP3 complex. Therefore, the above neutralization titer differences in this study may be due to the presence of anti-EV70 VP3 neutralizing antibodies and/or anti-EV70 VP1–VP3 complex antibodies in the anti-EV70 J670/71 hyperimmune serum.

The neutralization antigenic sites of polioviruses have been well mapped (Buttinelli et al., 2001; Minor et al., 1986; Murdin and Wimmer, 1989; Reynolds et al., 1991). Poliovirus has been found to have four kinds of neutralization antigenic sites: N-Ag I, N-Ag II, N-Ag IIIA and N-Ag IIIB (Murdin and Wimmer, 1989). Although different serotypes of poliovirus have different constitutions for its neutralization antigenic sites, most of these four sites require parts of the VP1 capsid protein (Buttinelli et al., 2001; Minor et al., 1986; Murdin et al., 1989; Reynolds et al., 1991) as key components. In ad-

dition, the formation of N-Ag I just requires amino acids of 89–100 in the VP1 of poliovirus 1, 2 and 3 (Icenogle et al., 1986; Minor et al., 1986; Page et al., 1988; Roivainen and Hovi, 1987). EV70 VP1 has about 41–42% homology with the VP1 of poliovirus 1, 2 and 3, implying that the neutralization antigenic sites of EV70 VP1 may have similar feature(s). The antibodies to recombinant GST-N138m, GST-C170 and GST-VP1m only immunoprecipitated the VP1 but not VP3 proteins of the four tested EV70 epidemic isolates (KW/97, T260/74, J670/71 or AE/72), suggesting that the epitope(s) of EV70 VP1 is different from the epitope(s) of EV70 VP3. Moreover, the neutralization activity of antiserum to GST-N138m against EV70 epidemic isolates suggests that EV70 VP1 has a neutralization antigenic site(s) similar to the N-Ag I of poliovirus 1, 2 and 3. However, the antiserum to GST-C170 can also independently neutralize EV70 epidemic isolates, suggesting that EV70 VP1 C-terminal has a neutralization antigenic site(s) independent of the neutralizing antigenic site(s) found in the EV70 VP1 N-terminal half. Therefore, both the N-terminal and the C-terminal fragments (N138 and C170) of EV70 VP1 have independent neutralization antigenic site(s).

In summary, the expressed full-length GST-VP1m and the non-overlapping N-terminal and C-terminal peptides (GST-N138m and GST-C170) of EV70 VP1, like whole EV70 virions, were immunogenic and capable of eliciting antibodies to neutralize EV70 viruses. Moreover, the neutralization titer of anti-EV70 virion antibodies is higher than the titers of anti-EV70 VP1 recombinant proteins' antibodies. The capability of inducing neutralizing antibodies by both whole-EV70 virus and EV70 VP1 recombinant proteins may enable us to develop a practical vaccine in the future for the prevention of EV70 caused AHC or its epidemic.

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