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Generation of neutralizing monoclonal antibodies against Enterovirus 71 using synthetic peptides

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ABSTRACT

Enterovirus 71 (EV71) has led to recent outbreaks of hand, foot and mouth disease (HFMD) in China, resulting in high mortality. In this study, several monoclonal antibodies were generated by immunizing mice with two synthetic peptides, SP55 and SP70, containing amino acids 163–177 and 208–222 of VP1. The specificities of the anti-EV71 peptide monoclonal antibodies were confirmed by Western blot analysis and immunocytochemistry against EV71 virus. Most importantly, we have identified a monoclonal antibody, clone 22A12, which shows strong neutralizing activity against EV71 in an *in vitro* neutralization assay. Because there is no vaccine available and treatment is very limited, mouse anti-EV71 monoclonal antibody, clone 22A12, could be a promising candidate to be humanized and used for treatment of EV71 infection.

Enterovirus 71 (EV71) has caused large outbreaks with significant mortality in China and other countries in the Asian Pacific region. EV71 is the main causative agent of hand, foot and mouth disease (HFMD) and has been associated with severe neurological diseases resulting in high mortality [1–3]. In 1995, EV71 virus was isolated from HFMD cases in Wuhan, China. In 1998, a HFMD outbreak caused by EV71 affected more than 100,000 young children in Taiwan and resulted in 78 deaths [4,5]. During the period from September to October 2000, an outbreak of HFMD occurred in Singapore, affecting mostly children below 6 years of age and resulting in four fatal cases [6]. In 2007, an outbreak of EV71 in China occurred in Shandong province with a total of 39,606 cases reported, including 14 deaths. In 2008, another outbreak started in Anhui, China, in April, and then spread to most parts of China by the end of July. Close to 500,000 young children were infected, resulting in more than 100 deaths [7]. In 2009, EV71 infection was reported in January and outbreaks were seen in many provinces of China. A total 178,680 HFMD cases, including 45 deaths were reported in June 2009 [8].

Currently, there is no approved antiviral drug for treatment of EV71 infection. Vaccine development is underway but may take many years before approval for market. Since 2000, intravenous administration of human IgG has been used in China as the last resort for treatment of severe cases of EV71 infection, with some success. However, use of human blood products also poses great potential of risk through infection by other human pathogens.

Synagis (palivizumab) is a humanized mouse monoclonal anti-RSV neutralizing antibody approved by the U.S. Food and Drug Administration (FDA) for the prevention of serious lower respiratory tract disease caused by RSV in children at high risk for RSV disease [9]. Based on this precedent, it was of great interest for us to develop neutralizing anti-EV71 monoclonal antibody for the treatment of severe hand–foot–mouth disease caused by EV71.

By immunizing groups of mice with 95 overlapping synthetic peptides spanning the VP1 capsid protein of EV71, two peptides, SP55 and SP70, containing amino acids 163–177 and 208–222 of VP1, were identified which were capable of eliciting neutralizing antibodies against EV71 [11]. SP70 was shown to be particularly potent in eliciting a neutralizing antibody titer comparable to that obtained with whole virion-immune serum [10,11].

In this study, we have used the same two peptides, SP55 and SP70, immunized mice, and generated several monoclonal antibodies against SP55 and SP70. The specificities of the anti-EV71 peptide monoclonal antibodies were confirmed by Western blot analysis against the EV71 virus. Most importantly, we identified a monoclonal antibody, clone 22A12, with strong neutralizing activity against EV71 in an *in vitro* neutralization assay. Since there is no vaccine available and treatment is very limited, mouse anti-EV71 monoclonal antibody, clone 22A12, could be a promising candidate to be humanized and used for treatment of EV71 infection.

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Materials and methods

Synthesis of peptides and preparation of immunogens. Two peptides, SP55 and SP70, containing amino acids 163–177 and 208–222 of the VP1 sequence of Enterovirus 71 strain 41 (GenBank accession No. AF316321) were synthesized at SciTech Ltd. (Beijing, China). A cysteine residue was added at the C-terminal end for conjugation. Each peptide was chemically linked to the carrier protein mcKLH through a sulfide-linker.

Production of anti-EV71 monoclonal antibodies. Balb/c mice were immunized with KLH-conjugated synthetic peptides. Two weeks after immunization, blood samples were obtained from the tail of the immunized mice and tested for titers against unconjugated peptides by ELISA. Spleens from the mice that showed the highest titers were removed, and splenocytes were fused with the mouse myeloma cell line SP2/0. Culture supernatant from individual hybridoma clones were then screened first against unconjugated peptides by ELISA, then immunoblotting against purified EV71 virus. To produce antibodies from different hybridoma clones, the clones were seeded in stationary bioreactors in DMEM (BRL-Gibco, Grand Island, NY) plus 10% low-IgG fetal bovine serum from HyClone (Logan, Utah). The Bioreactor fluids were collected every 3 days, and IgG fractions were affinity-purified using protein G agarose columns (Upstate Biotechnology, Lake Placid, NY). The concentrations of purified IgG were determined by their absorbance at OD_{280}.

Enzyme-linked immunoassay. Each well of 96-well high binding EIA plates (Corning, USA) was coated with 100 ng/well of unconjugated peptides or inactivated EV71 virus overnight at 4 °C in PBS. After two washes with PBS and blocking with 5% skim-milk–PBS for 1 h at room temperature, wells were incubated with tail bleeds (1:500 to 1:50,000 dilution) or culture supernatants (1:1) in 5% skim-milk–PBS for another hour at room temperature. After two washes, wells were then probed with HRP-conjugated goat-anti-mouse IgG Fc-specific secondary antibodies (1:2000) in 5% skim-milk–PBS for 1 h. After five washes with PBST, HRP substrate TMB solution was added and absorbencies were determined after 30 min at 450 nm with a microplate reader.

Cell culture. RD cells (rhabdomyosarcoma of human origin) for virus growth were maintained in Eagle's Minimum Essential Medium (MEM) with Earle's salts, without sodium bicarbonate and without phenol red. The preparation of this medium was as follows: MEM powder (9.7 g) was added with sodium bicarbonate, 1.5 g; fetal bovine serum, 100 ml; L-glutamine, 200 mM; Hepes, 10 mM; penicillin, 1000 U/ml; and streptomycin, 1000 μg/ml in 1000 ml of distilled water. This mixture was filtered (0.22 μm membrane), distributed into small volumes, and maintained at 4 °C. The RD cells were passaged weekly using trypsin, 0.25%, plus EDTA.

Virus growth, purification and titer determination. The EV71-Fuyang strain, provided by the Center of Disease Prevention and Control, China, was isolated from a patient with HFMD in 2008 and determined to be genotype C4 by sequence analysis. Virus stock was collected from infected RD cells 3 days post-infection at (37 °C). The virus from the tissue culture was purified by precipitation with 7% polyethylene glycol 8000 and then centrifuged on a 30% sucrose cushion at 25,000 *g* for 4 h.

Virus stocks were distributed in aliquots of 500 μl and stored at −80 °C. Virus titers were determined from the median end point of the tissue culture's infectious dose (TCID50). Serially diluted virus samples (from 10^{-2} to 10^{-9}) were added to RD cells in 96-well plates and quadruplicate samples were used at each dilution. The 96-well plates were incubated for 7 days at 37 °C, and the TCID50 values were measured by determining cytopathic effects on infected RD cells. The TCID50 values were calculated by the Reed–Muench method.

Western blot analysis. Total viral proteins of the EV71-Fuyang strain were used to study immunoreactivity of the antibodies. Briefly, viral proteins were loaded onto 12% gels, separated by SDS–PAGE, and transferred onto a nitrocellulose membrane for 2 h at 200 mA. The membrane with transferred polypeptides was immersed in 5% skim-milk in PBS at room temperature for 1 h. After two rinses with PBST, the proteins were probed with EV71 antiserum (1:1000) or anti-EV71 monoclonal antibody culture supernatants (1:3) at room temperature for 1 h. The membrane was then incubated with horseradish peroxidase-conjugated goat-anti-mouse IgG (1:2000) and immunoreaction detected with Enhanced Chemiluminescence (Promega, USA).

Neutralizing assay. Monoclonal antibodies were inactivated at 56 °C for 30 min. Each sample was added to a microtube and diluted by twofold serial dilution using fresh medium. Four hundred microliters of a 400 TCID50 virus suspension was added to each tube containing 400 μl serial diluted antibodies. After incubation at 37 °C for 1–2 h, 200 μl of serially diluted samples were added to RD cells in 96-well plates. The cultures in 96-well plates were incubated for 7 days at 37 °C, and TCI50 values were determined by quantifying cytopathic effects in infected RD cells. The 50% neutralization inhibition dose (ID50), the geometric reciprocal of the serum dilution yielding 50% reduction in the virus titer, was obtained using software ID-50 5.0.

Results

Identification of mouse monoclonal antibodies against EV71

Because the mice were immunized with a mixture of KLH-conjugated SP55 and SP70, the immunospecificity of all monoclonal antibodies was tested by ELISA in wells of 96-well microtiter plates coated with the SP55 or SP70 peptides. Immunoreactivities against EV71 virus were also tested in the same experiment with wells coated with inactivated EV71 virus. Eleven monoclonal IgGs, including Clone 14F9, 18A12 and 20E11, were specific for SP55 and only one IgG, Clone 22A12, for SP70. All of the 12 clones reacted with the inactivated EV71 virus. (Table 1).

Confirmation of specificity against VP1 of EV71 virus

To further confirm that the ELISA-positive clones reacted with VP1 of EV71 virus, the total viral proteins of EV71 strain 41 were used to perform a Western blot analysis. As shown in Fig. 1, all of the 12 monoclonal antibodies detected a single band at an apparent molecular weight (MW) of 30 kDa, the expected MW of the VP1 monomer, indicating that the monoclonal antibodies reacted with VP1 specifically and showed no cross-reaction with other virus proteins (see examples in Fig. 1). Lanes 1–4 show clones of 14F9, 18A12, 20E11 and 22A12. Lane 5 is a negative control without the primary antibody.

Table 1
Identification of anti-EV71 mAbs by ELISA.

Antigen	Blank	14F9	18A12	20E11	22A12
SP55	0.047	3.437	3.229	3.318	0.130
SP70	0.050	0.226	0.135	0.215	3.174
EV71	0.053	3.316	>3.50	3.209	>3.5

Each well was coated with 100 ng of unconjugated peptides or inactivated EV71 virus in PBS, then incubated with culture supernatants (1:1) in 5% skim-milk–PBS for another hour at room temperature. After two washes, wells were probed with HRP-conjugated goat-anti-mouse IgG Fc-specific secondary antibodies (1:2000). HRP substrate TMB was added to develop the color. At the end of 30 min, absorbance was determined at 450 nm with a microplate reader.

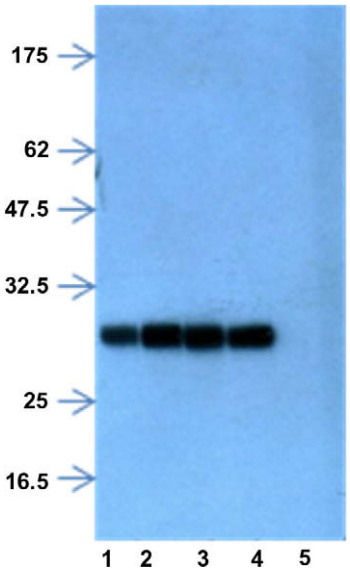

Fig. 1. Western blot analysis of anti-EV71 mAb against total viral lysates of EV71. The membrane with transferred total viral proteins was cut into strips and incubated separately with 14 different monoclonal anti-EV71 antibody clones at 2 μg/ml in 5% skim-milk–PBS. The membrane was then incubated with horseradish peroxidase-conjugated goat-anti-mouse IgG (1:2000) and immunoreactions detected with Enhanced Chemiluminecence.

Table 2
In vitro neutralization assays of anti-EV71 mAbs.

	Control virus	EV71 virus
Anti-EV71 serum	<1:4	48
14F9	<1:4	4
18A12	<1:4	<1:4
20E11	<1:4	<1:4
22A12	<1:4	16

Virus suspension containing 400 TCID50 was pre-incubated with serially diluted antibodies for 1 h before adding to RD cells in 96-well plates. The cultures in 96-well plates were incubated for 7 days at 37 °C, and the TCID50 values were measured by evaluating cytopathic effects in infected RD cells. The 50% neutralization inhibition dose (ID50), the geometric reciprocal of the serum dilution yielding 50% reduction in the virus titer, was obtained using software ID-50 5.0.

Neutralization activity by clone 22A12

As reported (10,11), mouse antiserum raised by immunizing with either SP55 or SP70 has neutralization activity against homologous and heterologous EV71 strains. To test neutralizing activities of the SP55- and SP70-specific monoclonal antibodies against EV71 virus, the purified monoclonal antibodies were analyzed in an *in vitro* microneutralization assay using RD cells infected with 103TCID50 of the EV71-Fuyang strain. Antiserum from a rabbit immunized with heat-inactivated EV71 virus served as a positive control.

Whereas the antiserum from a rabbit immunized with the heat-inactivated virus showed a titer of 1:48, the SP70-specific monoclonal 22A12 exerted complete protection of RD cells from EV71 virus infection at 1:16 and the titer for SP55-specific monoclonal 14F9 was observed at 1:4. No neutralization activity was observed with the other four SP55-specific monoclonal antibodies tested (Table 2). Our data is in agreement with earlier observations (10,11) that the antisera from SP70 immunized mice have better neutralization activity than antisera from SP55 immunized mice. SP70-specific monoclonal 22A12 had the highest neutralization activity and is the best candidate for drug development.

Discussion

Currently, there is no approved antiviral drug for treatment of EV71 infection. Vaccine development is underway, but may take many years before approval to market. Intravenous administration of human IgG has been used in China since 2007, with some success, as the last resort to treat severe cases of EV71 infection. However, use of human blood products also poses great potential of risk of infection by other human pathogens.

In this study, we have successfully generated a monoclonal antibody, clone 22A12, with strong neutralizing activity against EV71 in an *in vitro* neutralization assay. Mouse anti-EV71 monoclonal antibody, clone 22A12, could be a promising candidate to be humanized and used for treatment of EV71 infection. The anti-EV71 monoclonal antibody 22A12 used in this study is a murine antibody. Chimerization and/or humanization will be necessary to reduce human anti-mouse antibody response for therapeutic application. We have cloned the variable regions of mAb 22A12, and engineering of 22A12 is currently underway.

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References

[1] A.B. Brown, M.S. Oberste, J.P. Alexander Jr., L.K. Margery, A.P. Mark, Molecular epidemiology and evolution of Enterovirus 71 strains isolated from 1970 to 1998, J. Virol. 73 (1999) 9969–9975.
[2] A. Hagiwara, I. Tagaya, T. Yoneyama, Epidemic of hand, foot and mouth disease associated with Enterovirus 71 infection, Intervirology 9 (1978) 60–63.
[3] P.C. McMinn, An overview of the evolution of Enterovirus 71 and its clinical and public health significance, FEMS Microbiol. Rev. 26 (2002) 91–107.
[4] M. Ho, E.R. Chen, K.H. Hsu, S.J. Twu, K.T. Chen, S.F. Tsai, J.R. Wang, S.R. Shih, An epidemic of enterovirus infection in Taiwan. Taiwan Enterovirus Epidemic Working Group, N. Engl. J. Med. 341 (1999) 929–935.
[5] C.C. Liu, H.W. Tseng, S.M. Wang, J.R. Wang, I.J. Su, An outbreak of Enterovirus 71 infection in Taiwan, 1998; epidemiologic and clinical manifestations, J. Clin. Virol. 17 (2000) 23–30.
[6] K.P. Chan, K.T. Goh, C.Y. Chong, E.S. Teo, G. Lau, A.E. Ling, Epidemic hand, foot and mouth disease caused by human Enterovirus 71, Singapore Emerg. Infect. Dis. 9 (2003) 78–85.
[7] W.B. Xu, Virus surveillance on HFMD in China, in: Beijing International Symposium on HFMD, 2009.
[8] Infectious Case Report for June 2009 by Ministry of Public Health of China.
[9] The IMpact RSV Study Group, Palivizumab a humanized respiratory syncytial virus monoclonal antibody reduces hospitalization from respiratory syncytial virus infection in high-risk infants, Pediatrics 102 (1998) 531–537.
[10] S.K. Lal, P. Kumar, W.M. Yeo, A. Kar-Roy, V.T. Chow, The VP1 protein of human Enterovirus 71 self-associates via an interaction domain spanning amino acids 66–297, J. Med. Virol. 78 (2006) 582–590.
[11] D.M. Guang, W. Foo, S. Alonso, M.C. Phoon, N.P. Ramachandran, V.T.K. Chow, C.L. Poh, Identification of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides, Virus Res. 125 (2007) 61–68.