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    Nicole Hartwig Trier

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Chapter 29

Enterovirus-Specific Anti-peptide Antibodies

Chit Laa Poh, Katherine Kirk, Hui Na Chua, and Lara Grollo

Abstract

Enterovirus 71 (EV-71) is the main causative agent of hand, foot, and mouth disease (HFMD) which is generally regarded as a mild childhood disease. In recent years, EV71 has emerged as a significant pathogen capable of causing high mortalities and severe neurological complications in large outbreaks in Asia. A formalin-inactivated EV71 whole virus vaccine has completed phase III trial in China but is currently unavailable clinically. The high cost of manufacturing and supply problems may limit practical implementations in developing countries. Synthetic peptides representing the native primary structure of the viral immunogen which is able to elicit neutralizing antibodies can be made readily and is cost effective. However, it is necessary to conjugate short synthetic peptides to carrier proteins to enhance their immunogenicity. This review describes the production of cross-neutralizing anti-peptide antibodies in response to immunization with synthetic peptides selected from in silico analysis, generation of B-cell epitopes of EV71 conjugated to a promiscuous T-cell epitope from Poliovirus, and evaluation of the neutralizing activities of the anti-peptide antibodies. Besides neutralizing EV71 in vitro, the neutralizing antibodies were cross-reactive against several Enteroviruses including CVA16, CVB4, CVB6, and ECHO13.

Key words Enterovirus 71, Enterovirus-specific Anti-peptide antibodies, Cross-reactive neutralizing antibody epitopes, In silico analysis of B-cell epitopes

1 Introduction

As the number of polio infections decreases, Asia is experiencing an increasing number of epidemics caused by Enteroviruses (EVs) such as Enterovirus 71(EV71) and Coxsackie virus 16(CA16). Hand, foot, and mouth disease (HFMD) infections are generally mild and are endemic in countries like China, Singapore, Taiwan and Malaysia. However, in recent years, EV71 has caused severe HFMD with associated neurological complications such as acute flaccid paralysis and brain stem encephalitis, leading to hundreds of deaths in several countries in Asia. Large HFMD outbreaks involving over 1.3 million children in China in 2013 were associated with high fatalities of 243 cases [1]. There are currently no vaccine or antiviral agent to prevent or treat serious HFMD caused by EV71. The whole virus formalin-inactivated vaccine candidate has

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gone through phase III clinical trial in 10,000 healthy children in China and appears to be promising [2]. However, practical implementation of the formalin-inactivated EV71 vaccine for clinical application will require a carefully regulated manufacturing process and the establishment of good global vaccine standards as different companies in different countries will use different vaccine strains, different cell substrates and different production processes. Although the immunogenicity of the formalin-inactivated EV71 vaccine is good, it is not long lasting and needs at least a booster dose after 6 months to remain effective. Due to antigenic variability of RNA viruses, there is also the added problem of vaccine coverage of different EV71 genotype/subgenotype strains which requires further investigations. Further difficulties involved the necessity to grow large amounts of pathogenic viruses for inactivation and the possibility of inducing inappropriate allergic and/or reactogenic responses in the host [3].

An alternative immunization approach is to identify peptide epitopes that can elicit the required immune response and to use the synthetic versions of the peptides as vaccines. Synthetic peptide vaccines are cost effective and do not carry the risk of reversion. Unlike the inactivated vaccine, it does not have a safety risk of incomplete inactivation or has components that can contribute to unwanted side effects. The use of synthetic peptide vaccines is desirable in situations when the natural protein antigen is unavailable or is difficult to prepare in large quantities. Synthetic peptide vaccines can be prepared to high purity and stored freeze-dried. This avoids the "cold-chain" requirement for storage, transport, and distribution. Rational design of synthetic peptide vaccines to include multiple epitopes from the same virus or multiple determinants from several viruses will greatly expand the usefulness of the vaccine. For example, an inactivated vaccine may not have broad protection against all the serotypes, genotypes/subgenotypes and there is a need to include several serotypes, genotypes/subgenotypes in the formulation. The inactivated Poliovirus vaccine had to include all three serotypes—*viz.* Sabin 1, 2, and 3. In several HFMD epidemics, the less virulent CA 16 virus was found to be co-circulating with EV71. A bivalent synthetic peptide vaccine can be designed to include virus-neutralizing epitopes from both EV71 and CA16 viruses. Although promising, synthetic peptide vaccines have been shown to be less immunogenic than the traditional vaccines. The challenge is to identify a synthetic peptide vaccine that can optimally stimulate both B-cell and T-cell immune responses. The added limitation to the use of a synthetic peptide vaccine is that it is mainly restricted to representing linear epitopes. If conformational B-cell epitopes are required to elicit neutralizing antibodies, peptides representing the B-cell epitopes could be assembled on a suitable backbone which represents the three-dimensional structure [4]. Despite the shortcomings of low

immunogenicity, several synthetic peptide vaccines are under development against human immunodeficiency virus (HIV1), hepatitis C virus (HCV), malaria, influenza, cytomegalovirus (CMV), and human papilloma virus (HPV) [5]. Most of the candidate peptide vaccines under development against infectious pathogens are currently in phase I and II clinical studies but some peptide vaccines against cancer have reached phase III studies. However, no human peptide-based vaccine has reached the market but strategies to improve the immunogenicity, stability, and delivery will enable some peptide vaccines to enter the human therapeutics market in the near future.

1.1 Design of Synthetic Peptide Vaccines

The development of an effective peptide vaccine will involve the identification of the immunodominant epitopes which are capable of inducing both humoral and cell-mediated immunity against the viral pathogen. Synthetic peptide vaccines can be designed to target a humoral response which is mediated by specific neutralizing antibodies or a cytotoxic immune response mediated by cytotoxic T lymphocytes ($CD8^+$ T cells) or a combination of both. Both arms of immunity are further dependent on the induction of a helper T cell response. Vaccines that induce antibody formation should contain the B-cell epitope and the T-helper epitope whereas vaccines that are designed to generate the cytotoxic response should carry the T-cell epitope and the T-helper epitope. Thus, a synthetic peptide vaccine should contain at least two antigenic epitopes, a T-helper epitope and the B-cell or the T-cell epitope [6].

This chapter focuses on the design of B-cell epitope peptide vaccines, discusses their ability to elicit antipeptide antibodies that will neutralize Enteroviruses, and confers immune protection against invading viral pathogens. Peptides chosen as immunogens should contain at least eight and not more than 20 amino acids. Peptides smaller than eight amino acids may elicit antibodies that do not recognize the native protein and peptides longer than 20 amino acids may fold incorrectly and do not represent the conformation of the native protein. The preferred length of synthetic peptides as immunogens should be ranging from 15 to 20 amino acids, targeting the externally exposed regions such as turns, connecting regions, or loops of the native protein [7]. These regions are often enriched with charged and polar amino acids [8]. Information of the native protein structure can be accessed by searching the protein NCBI database (www.ncbi.nlm.nih.gov) Uni-Prot (www.uniprot.org) and ExPaSy (us.expasy.org/tools). B-cell epitope identification can be achieved through experimental methods which are divided into either structural or functional. Structural methods include X-ray crystallography, nucleic magnetic resonance (NMR), and electron microscopy (EM) of the antigen-antibody complexes. Functional methods utilize methods such as surface plasmon resonance, mass spectrometry, as well as

immunoassays [9, 10]. B-cell epitopes can be discovered through analysis of the antigen-binding domains of an antibody that are resistant to proteolysis. Thus, an epitope can be generated whilst bound to the antibody. The epitope can be eluted from the bound antibody under non-denaturing conditions. This allows identification of linear as well as conformational epitopes [11, 12]. The antibodies used for identification of the B-cell epitope can be derived from the sera of infected patients [13].

1.2 PEPSCAN for Epitope Discovery

The "PEPSCAN" method is a fast and systematic approach for assessing whether an amino acid sequence will bind to an existing antibody. Synthetic peptides are synthesized on a solid support, usually as overlapping peptides of 9–12 mers covering the whole sequence of a given protein. The peptides are covalently bound to the solid support and the antibody binding properties are identified using antibody marked with peroxidase in an ELISA. Immunization using a recombinant VP1 protein of EV71 was shown to confer protection against lethal EV71 infection in newborn mice, indicating that VP1 contains important antigenic sites or B-cell epitopes that could elicit production of neutralizing antibodies against the virus [14]. Neutralizing synthetic peptides can be identified using either polyclonal or monoclonal antibodies. Polyclonal antisera raised against the foot and mouth disease virus (FMDV) were the first sera used to identify peptides present in the VP1 to bind to anti-peptide antibodies in the PEPSCAN analysis [15]. Short peptides are poor immunogens and need to be conjugated to carrier proteins such as keyhole limpet hemocyanin (KLH) or tetanus toxoid which provide a source of T_H epitopes. Foo et al. [16] used a PEPSCAN strategy in which 95 overlapping synthetic peptides were designed according to the primary sequence of the VP1 capsid protein of EV71. The diphtheria toxoid-conjugated synthetic peptides were injected into mice and the neutralizing activity of the anti-peptide antibodies were determined. One of the synthetic peptides, SP70, was able to elicits neutralizing titer which was only twofold lower than that elicited by a heat-inactivated whole virus [16]. When the antiserum raised against the SP70 peptide was passively administered to newborn mice, the anti-peptide antibodies were able to neutralize viruses injected into mice at a challenge dose of 1000 $TCID_{50}$ and conferred 80 % in vivo protection of EV71 sub-genogroups B2, B4, and B5. Passive protection of heterologous sub-genogroups belonging to C2 and C4 was lower at 70 % [17].

1.3 B-Cell Epitope Prediction Methods

In the absence of a native protein, computational design utilizing reliable in silico bioinformatics tools have led to B-cell epitope predictions. Most of the existing methods of computational B-cell epitope prediction does not consider the conformational structure but depend on a given protein sequence as a continuous

amino acid stretch or a linear sequence. Hopp and Woods introduced the first propensity scale method (or amino acid scale based) for predicting linear B-cell epitopes which was dependent on the chemical and physical properties of amino acids [18]. Improved propensity methods such as BepiPred which combines two propensity-scale methods with a hidden Markov model (HMM) was shown to have a statistically significant improvement in performance [19]. Machine learning methods such as ABCPred which uses recurrent artificial neural networks combined with flexible length classifiers for predicting linear B-cell epitopes were proposed by Saha and Raghava [20]. Their program was shown to achieve 66 % accuracy using a window size of 16 amino acids. In an attempt to further improve the accuracy of prediction, a support vector machine (SVM) algorithm was developed and combined with the propensity scale method. This approach was able to attain an accuracy of 72 % on a dataset of 1211 B-cell epitopes [21]. Lin et al. (2013) described the BEEPro (B-cell epitope prediction using Evolutionary information and Propensity scales), a SVM-based learning machine which uses 16 properties to predict both linear and conformational B-cell epitopes. BEEPro achieves an accuracy of 99.29 % with a sensitivity of 0.9604, a specificity of 0.9946, and a correlation coefficient of 0.9281 [22].

In silico approaches for predicting conformational epitopes can be based on the sequence or the structure or both. There are now a few structure-based epitope prediction servers that are widely used and have been validated experimentally to varying degrees. Commonly used servers include the conformational epitope predictor (CEP) which predicts surface-accessible epitopes based on the atomic positional distance between amino acids [23] and the DiscoTope developed by Andersen et al. [24] which uses a combination of amino acid statistics, spatial arrangement and surface accessibility to predict conformational B-cell epitopes. DiscoTope can be used to predict both linear and conformational epitopes. Newer algorithms that try to improve analysis and broaden targets using linear sequences when structures are unavailable include the ElliPro which can align unknown sequences in BLAST and then model the structure with MODELLER [25]. Rubinstein et al. [26] introduced Epitopia for predicting B-cell epitopes in either a three-dimensional structure or a linear sequence which are immunogenic. Epitopia server predicts epitopes based on the physic-chemical and structural geometrical features. The immunogenicity and corresponding probability scores are computed for every amino acid for a 3D structure input or for every amino acid for a sequence input. Performance of the Epitopia as a B-cell prediction tool is judged higher than the CEP, DiscoTope and ElliPro using the same data and the same assessment [26].

1.4 Immunogenicity of Vaccine Constructs

Several excellent reviews on peptide synthesis and conjugation methods, production and characterization of antipeptide antibodies are available for referencing [27–29]. This chapter describes a multi-step algorithmic approach that utilizes both a sequence (ABCPred, BepiPred) and a structure-based Epitopia server to identify a functional peptide epitope for developing the synthetic peptide vaccine. An in silico computational analysis of the sequence and structure models was used to identify highly conserved B-cell epitopes that will elicit cross protection against all EV71 strains and other Enterovirus family members. The combined in silico approach identified three highly conserved 15-mer epitopes across multiple EV subtypes. Epitopes were discounted if their normal position lay buried within the viral coat rather than being presented on the surface. The K1 epitope was structurally more conserved with no more than 6 amino acid substitutions when compared to the VP1 sequence of other virus strains. K2 and K3 (from the VP3 protein) had less sequence conservation with other viruses and represented less exposed, more buried regions. A neutralizing peptide epitope, designated as D, was included for comparison. Other epitopes were identified from VP2 and VP4 but were not as promising as K1, K2, and K3 in terms of their conservation and antigenicity; hence, they were not evaluated as potential vaccine targets.

Following the in silico identification of several potential peptide vaccine candidates, their ability to induce production of neutralizing anti-peptide antibodies in mice was assessed. Each peptide was synthesized as a linear construct containing a universal T helper epitope from Poliovirus by standard F-moc chemistry. These peptide constructs elicited low levels of anti-peptide antibody response after the first vaccination but 6 weeks after the second dose, antipeptide antibodies against D1 and K3 were higher than those elicited by K1 and K2 peptides [30].

2 Materials

2.1 Cell Growth and Maintenance

Dulbecco's minimal essential medium containing glutamine (DMEM) supplemented with 10 % fetal calf serum and 1 % of penicillin/streptomycin (50 μg/ml).

Vero cells (African green monkey kidney cells, ATCC: CCL-81).

2.2 Synthetic Peptides

Four peptides to be evaluated as B-cell epitopes were commercially synthesized using Fmoc-solid phase peptide synthesis. Each B-cell epitope was synthesized as a linear construct containing a universal T helper epitope from Polio virus (KLFAVWKITYKDT).

2.3 Viruses

Human Enterovirus 71(EV71 isolate number CAIG 99018233), Coxsackie A16 (CVA16: isolate number CAIG 9902-2745

-4PMEK9.4.00), Coxsackie B4 (CVB4: isolate number 99039838), Coxsackie B6 (CVB6: isolate number 273370/PBO (14.09.1989), and Echovirus 13(Echo13: isolate number 28-606PMEK (07.06.1990).

2.4 Mice Immunization

BALB/c mice (6–8 weeks old).

Complete Freund's adjuvant (CFA).

Incomplete Freund's adjuvant (IFA).

2.5 Determination of Anti-peptide Antibody Titer by ELISA

1. Sodium carbonate buffer (50 mM, pH 9.6).
2. Synthetic peptide (10 μg/ml of unconjugated synthetic peptide in carbonate buffer).
3. Phosphate-buffered saline (PBS) (20 mM Na_2HPO_4 in 0.15 M NaCl, pH 7.3).
4. Phosphate-buffered saline (PBS) containing 0.05%Tween-20 and 0.5 % BSA (PBST).
5. Blocking solution: 10 mg/ml BSA in PBS.
6. Secondary antibody: Rabbit anti-mouse IgG conjugated to horseradish peroxidase.
7. Enzyme substrate: 3,3′,5,5′-Tetramethylbenzidine (TMB).
8. i-MarkMicrotiter plate reader.

2.6 Purification of Anti-peptide Antibody

Affinity chromatography columns used were Protein A-Sepharose Fast Flow columns (10 mm × 85 mm).

2.7 Neutralization Assay

96-Well plates.

Diluted anti-peptide antibodies.

3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS).

3 Methods

3.1 Bioinformatics Sequence Approach to Identify B-Cell Epitopes

The Picornavirus capsid protein (VP1-VP4) region was computationally analyzed for hydrophobicity, solvent accessibility, surface accessibility of residues, polarity, and spatial distance orientation relationships. The sequences were obtained from the NCBI Genbank and scored for aforementioned key antigenic attributes against the BLAST query algorithm [31]. Alignment of protein regions was compiled against multiple publicly available database sets and sorted via the clustalW alignment program [32]. Conserved sequences demonstrating homology within the protein data bank listings PDB ID: 3VBS Human Enterovirus 71; PDB ID: 1 BEV Bovine Enterovirus and PDB ID: 1HXS Mahoney Poliovirus were used to construct and verify the model.

3.1.1 Bioinformatics Structure Approach

Sequence alignment models demonstrating >40 % structural conservation with the PDB were used to generate a three-dimensional structural model for the HEV-71 (VP1 and VP3) assemblies, using the Chimera [33] interface to MODELLER [34]. The crystallographic atomic coordinates were reconstructed and uploaded to the epitopia server to estimate the rate of amino acid substitutions at each position in the alignment of homologous proteins [28].

3.2 Assessment of Immunogenicity of Peptide Vaccine Candidates

Four peptide vaccine constructs containing the universal T helper epitope from Polio virus were assessed for their ability to induce anti-peptide antibodies in mice. The vaccine constructs were designated as shown below:

K1 (KLFAVWKITYKDTLMRMKHVRAWIPRPMR).

K2 (KLFAVWKITYKDTLFHPTPCIHIPGEVRN).

K3 (KLFAVWKITYKDTLGFPTELKPGTNQFLT).

D1 (KLFAVWKITYKDTLYPTFGEHKQEKDLEYC).

Groups of six male BALB/c (6–8 weeks old) were inoculated subcutaneously at the base of the tail with each of the four peptides. Animals received 20 μg of peptide at days 0 and 28. The primary inoculation was administered with complete Freund's Adjuvant (CFA) and the subsequent inoculation with the incomplete Freund's Adjuvant (IFA). Sera were obtained from the animals 10 days after each vaccination.

3.3 Enzyme-Linked Immunosorbent Assay

The presence of anti-peptide antibody in mouse sera was detected by enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed polyvinyl microtiter 96-well plates were coated overnight with 10 μg/ml of B-cell peptide construct in 50 μl 50 mM sodium carbonate buffer at pH 9.6 and incubated at room temperature overnight. Unbound antigen was removed and unoccupied plastic surface blocked with 10 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (20 mM Na_2HPO_4 in 0.15 M NaCl, pH 7.3) at 37 °C for 1 h. After blocking, plates were incubated with serially diluted serum from individual mice starting at a 1/100 dilution in PBS containing 0.05 % Tween-20 and 0.5 % BSA at 37 °C for 1 h, and then washed two times with PBS containing 0.05 % Tween 20. One hundred microliters of a 1/2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse antibody was added to each well and allowed to incubate at room temperature for 1 h. Unbound antibody was removed, and wells washed twice with PBST and rinsed with PBS. Bound antibody was detected by the addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. The reaction was stopped after 20 min by the addition of a 1 M sulfuric acid. Color change was detected by an iMark Microplate Reader at

a wavelength of 450 nm. The assays were performed in triplicate. Antibody titers were expressed as the reciprocal of the highest analyte dilution that gives a reading above endpoint cutoff [35].

3.4 Neutralization Assay

Neutralizing activity of IgG purified from pooled sera from the immunized mice was measured using an in vitro microneutralization assay in 96-well plates (Imunoblot HB, USA). Purified IgG, (10 μg/ml) at six twofold serial dilutions was pre-incubated with an equal volume of 200$TCID_{50}$ of each virus (HEV-71, CVB4, CVB6, CA16, and Echo13) and then used to infect 80–100 % confluent Vero (CCL-81) cells. The cells were washed and then incubated at 37 °C with 5 % CO_2 until the first sign of complete CPE was observed in the virus control wells. At this point, 25 ul of 3-(4,5-dimethylthiazol2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) one-shot solution was added to all wells and an absorbance was recorded at 490 nm. The monoclonal antibodies against EV71, CA16, and CVB4 were included as positive controls. Results were read as positive if there was more than a 50 % CPE reduction as compared to naïve controls.

3.5 Statistical Analyses

Confidence interval. Statistical analyses were performed using a one-way parametric ANOVA test with 95 % confidence interval.

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