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Antigenic epitope peptides of influenza H3N2 virus neuraminidase gene based on experiments

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The neuraminidase (NA) in viral surface is one of the main subtype-specific antigen of influenza type A viruses. Neuraminidase is an enzyme to break the bonds between hemagglutinin (HA) and sialic acid to release newly formed viruses from infected cells. In this study, the H3N2 subtype virus NA genes were sequenced and NA proteins were screened for B-cell epitopes and assessed based on immunoinformatics. Based on this results, four peptides DR6, EY7, VG8 and RE8 (covering amino acid residues 151–156, 368–374, 398–405 and 428–435, respectively) of the NA protein were synthesized artificially. These peptides were used to immunize New Zealand rabbits subcutaneously to raise antisera. Experimental results showed that these four peptides were capable of eliciting antibodies against H3N2 viruses in a specific and sensitive feature, detected *in vitro* by enzyme-linked immunosorbent assay. Moreover, hemadsorption anti-releasing effects took place in three three-antisera-mixtures at a dilution of 1∶40. Alignment using NA gene database showed that amino acid residues in these four epitope peptides were substituted at specific sites in all the NAs sequenced in this study. It was suggested that these NA epitope peptides might be used in combination with HA proteins as vaccine antigens.

B-cell epitope, H3N2, immunoinformatics, neuraminidase (NA)

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The severity of influenza A virus infections varies depending on their host's acquired immunity. A novel antigenic variant of influenza A H3N2 subtype virus resulted in millions of deaths worldwide in influenza pandemic in 1968 [1]. Since then, the predominant circulating influenza A virus almost has been the influenza H3N2 subtype [2].

The two proteins in the dominant circulating H3N2 virus are the H3 hemagglutinin (HA) and N2 neuraminidase (NA) surface glycoproteins. HA protein is encoded by influenza gene segment 4 and NA is encoded by segment 6, and both proteins are subtype specific. HA binds to sialic acid (SA)-containing receptors on target cells to initiate virus infection, whereas NA cleaves sialic acid from cell receptors and from extracellular inhibitors to facilitate the release of progeny virus and to promote the spread of the infection to neighboring cells [3]. HA and NA having closely related functions must be "perfectly balanced" to allow the virus to be infectious. The original discovery of NA showed that it had a role as the determinant of "receptor-destroying" activity of free virus in hemagglutination reactions, which reinforced the focus on virion-associated NA in virion release. Later studies also reported the role of NA in cleavage of SA in the extracellular space [4]. The oseltamivir and zanamivir, two NA inhibitors, used in the current clinical treatment of

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influenza A virus infection are based on its "receptor-destroying" activity on the cell surface as oseltamivir carboxylate (the active form of Tamiflu) and zanamivir (Relenza) are SA analogs that interfere with the sialidase activity of NA [4].

Five epitope peptides have previously been identified by us in NA from the 2009 pandemic H1N1 virus with hemagglutination-inhibition antibody titers that ranged from 1∶40 to 1∶80 [5]. In this study, we characterize H3N2 viruses isolated from suspected patients' throat swabs in Guangdong province (China) and sequence the NA gene sequences from the strains. Using the SPSS (stepwise prediction and statistical screening) strategy [6], a bioinformatics (immunoinformatics) method, we predicted and screened B-cell epitopes in H3N2 virus NA. The predicted epitope peptides were synthesized artificially and rabbit antisera against the epitope peptides were collected, which contained antibodies specific to NA protein. Both the specificity and sensitivity of epitope peptides and virus strains were measured by enzyme-linked immunosorbent assay (ELISA). The objects in this study is to learn the epitope and its function of H3N2 virus NA gene. Research into and application of immunoinformatics is considered to accelerate the development of epitope-based vaccines [7].

1 Materials and methods

1.1 Viruses and cells

Influenza A H3N2 viruses amounted to 17 strains isolated from Guangdong from 2007 to 2010; NA gene sequences from these have been determined (even numbers in GenBank accession numbers CY091826–CY091858). The viruses were used for animal inoculation and antiserum production as well as for viral RNA preparation. For inoculation, the viruses were cultured and grown in 10-d-old embryonated eggs; and for other applications, they were grown in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, USA). The cells were propagated in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Corporation, Hangzhou, China), 100 U/mL of penicillin and 100 μg of streptomycin (Invitrogen) with 5% CO_2 in humidified air at 37°C. Sequences of the other 50 reference NA genes were downloaded from GenBank and LANL databases (http://www.flu.lanl.gov).

1.2 NA gene sequencing

Three pairs of primers were designed using the Primer Premier 5.0 software and synthesized according to the NA gene sequences of influenza A H3N2 viruses isolated from 1968 to 2010. Primer sequences are as follows: P-NAF1 (5′-AGCARAAGCAGGAGT-3′), P-NAR1 (5′-TCGTGACAACTTGAGCTGGAC-3′); P-NAF2 (5′-TATCAATTTGCMCTTGGRCAGG-3′), P-NAR2 (5′-TCCCATCCACACRTCATTTCC-3′); and P-NAF3 (5′-TATCAATTTGCMCTTGGRCAGG-3′), P-NAR3 (5′-TCCCATCCACACRTCATTTCC-3′) [6]. Total viral RNA was isolated from 140 μL of viral suspension using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany). Then 2 μL of RNA was added to a total of 50 μL reaction mixture that contained 10 μL of 5× OneStep reverse transcription polymerase chain reaction (RT-PCR) buffer (Qiagen; 0.5 mmol/L), 1 μL of dNTP, 1 μL of enzyme, 1 μL of forward (F) and reverse (R) primers respectively (25 μmol/L), and 34 μL of RNase-free H_2O. Reaction mixtures were incubated at 50°C for 30 min and 94°C for 5 min, and then a total of 35 cycles at 94°C for 30 s, 45°C for 30 s and 72°C for 90 s. The PCR products were purified with a gel extraction kit (Qiagen) and sequenced on an ABI PRISM 3100 Genetic Analyzer with an ABI PRISM BigDye Terminator V3.0 ready reaction cycle sequence kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

1.3 Epitope prediction, screening and assessment

DNA sequences were compiled using the Lasergene 7.1 software (DNASTAR Inc., Madison, USA) and multiple sequence alignments were assembled with MEGA 5.03 software. The α-helix, β-fold, β-turn, coil, flexible region, hydrophilicity plot, surface probability plot and antigenic index (AI) were predicted based on the NA amino acid sequence of A/Guangdong/15/2007 (H3N2) (for short GD-15-2007). Neuraminidase proteins were screened for B-cell epitopes based on the analysis of their protein features and using hierarchical clustering methods, Spearman's correlation and quartiles using Protean 7.1 and SPSS 16.0 (SPSS Inc., Chicago, USA). Antigenic index and the average AI (AAI) values were acquired [6].

1.4 Homology modeling

The 44 NA sequences were totally included in this study, which included 17 Guangdong strain NA sequences and 27 NA sequences downloaded from the GenBank database. Homology modeling was performed using the SWISS-MODEL molecular simulation software [8]. Molecular three-dimensional structure of the epitope peptides was shown by YASARA [9], in which the location of each epitope peptide sequence was labelled.

1.5 Peptide synthesis

The epitope peptides were synthesized artificially and then coupled to keyhole limpet hemocyanin (KLH). Some epitope peptides needed to be modified with cysteine (C) at the C-terminus or N-terminus end dependent upon their peptide

features in order to ease coupling to KLH.

1.6 Antiserum preparation

The serum antibody to strain GD-15–2007 was prepared, which was propagated in 10-d-old embryonated eggs. Two adult New Zealand rabbits were immunized by injection of these mixtures subcutaneously: 50% emulsion of Freund's complete adjuvant with either 50 μg of each conjugated synthetic peptide or β-propiolactone (BPL)-inactivated flu strain (allantoic fluid hemagglutination titer of 1∶64). Four booster doses in a 50% emulsion with Freund's incomplete adjuvant were given at 2-week intervals. Antisera were collected after the last immunization on the seventh day.

1.7 Peptide identification with ELISA

Antibody titers against each synthetic peptide and the virus were measured by ELISA [10]. In detail, 96-well microtiter plates were coated overnight at 4°C with 100 μL of 0.05 mol/L phosphate-buffered saline (PBS; pH 9.6) that contained 50 μg/mL of unconjugated synthetic peptides or 1 hemagglutination unit (HAU) (strain GD-15–2007 propagated in MDCK cells). The pre-immunization serum was used as the negative control. The plates were washed with PBS three times, blocked with 3% bovine serum albumin (BSA) and incubated with 100 μL of antiserum at serial dilutions at 37°C for 1 h and then washed three times with PBS that contained 0.1% Tween-20. Detection was performed using the secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Scicrest, Beijing, China; dilution 1∶6000) at 37°C for 1 h. Then the plates were washed three times. The color was developed with *O*-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, USA) and the absorbance was read at 450 nm wavelength with an ELISA plate reader (Molecular Devices, San Jose, USA). Each assay was performed independently three times. Epitope peptides were coated to microtiter plates and the excess washed off, then 50 μg/mL of polylysine and 1 mol/L of glycine were added in serial dilutions to each well. The cut-off value was determined for each peptide by incubation with sera from five normal rabbits (pre-immunization).

1.8 Hemagglutination-inhibition (HI) assays and HI-releasing

Using 1.0% rooster red blood cells (RRBCs), these assays were performed as described previously [11]. For HI assays, 50 μL of a two-fold serial dilutions of the antisera samples in PBS were incubated at 4°C for 1 h with an equal volume of 4 HAU of the virus antigen (GD-15–2007), followed by the addition of 50 μL of 1.0% RRBCs. The highest dilution of the antibody that showed no hemagglutination was considered to HI titer. Observation for HI and HI-releasing effect was made after 30 min, 1, 2, 3, and 4 h at 22°C.

1.9 Variation and entropy

For the purpose of analysing the amino acid variations of 44 NA proteins by MEGA 5.05, the most recent common ancestor (MRCA) was considered to be the NA gene of A/Hong Kong/1/68 (H3N2) and reference virus was taken to be the NA gene of A/Wyoming/3/2003 (H3N2) [2]. Entropy plots were made using BioEdit 7.09 software and the entropy value was taken to indicate the statistically variable probability [12].

2 Results

2.1 NA gene variation

The 469 amino acids were encoded by 1410 NA nucleotides from GD-15-2007, with a 45.4 kD weight and pH 6.1 as the isoelectric point (IP). The ratios of basic, acidic, hydrophobic and polar amino acids in the NA proteins were 9.6%, 11.1%, 29.6% and 33.9%, respectively. Taken the NA gene of A/Wyoming/3/2003 as reference, the NA gene substitution ratio was 13.4% (63/469) in 43 H3N2 viruses from 1968 to 2003, which included N93D, D147N, H150R, V194I, I215V, K221E, Y310H, L370S, N372K, S387L and Q432E in most strains and E199K, Q432E in all strains.

2.2 Epitope prediction, screening and assessment

B-cell epitopes of NA proteins were predicted by their hydrophilicity, the surface probability and the AI, assisted by analysis of their secondary structure and their flexible regions. The Spearman's correlation, Hierarchical clustering and their quartiles were used to analyze the epitopes and the SPSS strategy was used to screen and assess. The AAI score values of the epitope peptides were 0.009–0.082 (Table 1).

Before immunization to produce antibodies, DR6 was decorated by cysteine at C-terminal, and EY7 and RE-8 were decorated by cysteine at N-terminal, which made it easy to couple KLH.

2.3 Three-dimensional modeling

The N2 neuraminidase monomer of GD-15-2007 obtained

Table 1 Epitope peptides screened by stepwise prediction and statistical screening (SPSS)[a)]

Peptide	Position	Sequence	Total AI	AAI
DR6	151–156	DRTPYR	0.493	0.082
EY7	368–374	EKLRSGY	0.061	0.009
VG8	398–405	VDGRNRSG	0.443	0.055
RE8	428–435	RGRKEETE	0.441	0.051

a) AI, antigenic index; AAI, average antigenic index.

using SWISS-MODEL showed that 2aepA in the Protein Data Bank (PDB) had the highest sequence homology (94.6% identity) at amino acid residues from 82 to 469, with 2.10 Å X-ray resolution. The final structure was further evaluated by YASARA, which is normally used to quantify the compatibility of an amino acid sequence with a three-dimensional protein structure and especially to check the validity of a hypothetical epitope peptide structure (Figure 1). The peptides DR6, EY7, VG8 and RE8 were labelled on the surface of the molecular three-dimensional structure.

2.4 Epitope sensitivity

Either the virus antigen (1 HAU of GD-15-2007 passaged by MDCK cells, which was inactivated by β-propiolactone) or each epitope peptide was bound to 96-well plates overnight at 4°C with 100 μL of antiserum (dilution of 1∶100, 1∶1000, 1∶10000, 1∶20000, 1∶40000, 1∶80000) in carbonate–bicarbonate buffer (pH 9.6). The H3N2 virus GD-15-2007 in ELISA results was shown to be able to detect against the anti-strain GD-15-2007 serum at a maximum dilution of 1∶40000 and the anti-epitope serum at a maximum dilution of 1∶20000. The plates coated with each epitope peptide were able to detect the corresponding anti-epitope serum at a maximum dilution of 1∶10000 and 1∶20000 (Table 2). High sensitivities were shown in detective results between the immunogens (including viron and peptides) and their antisera. The positive was regarded in ELISA detection when the detected antiserum value was higher than the cut-off value determined by pre-immunization serum at a dilution of 1∶100.

2.5 Epitope specificity

Either the antigen seasonal inactivated H1N1 (GD-1-2007) or the antigen 2009 A H1N1 (GD-801-2009) virus was coated to ELISA microtiter plates (96-well plates, 1 HAU), then cross-reacted with antisera against the epitope peptides. The plates coated with the seasonal H1N1 virus or the 2009 H1N1 virus were able to detect each anti-epitope peptide serum at a dilution of 1∶200 or less (Table 2). The high specificities were shown in results between the immunogens and their antisera.

2.6 HI assay and anti-releasing effects

Hemagglutination was conducted in U-shaped bottom 96-well plates incubated with 4 HAU of virus GD-15-2007,

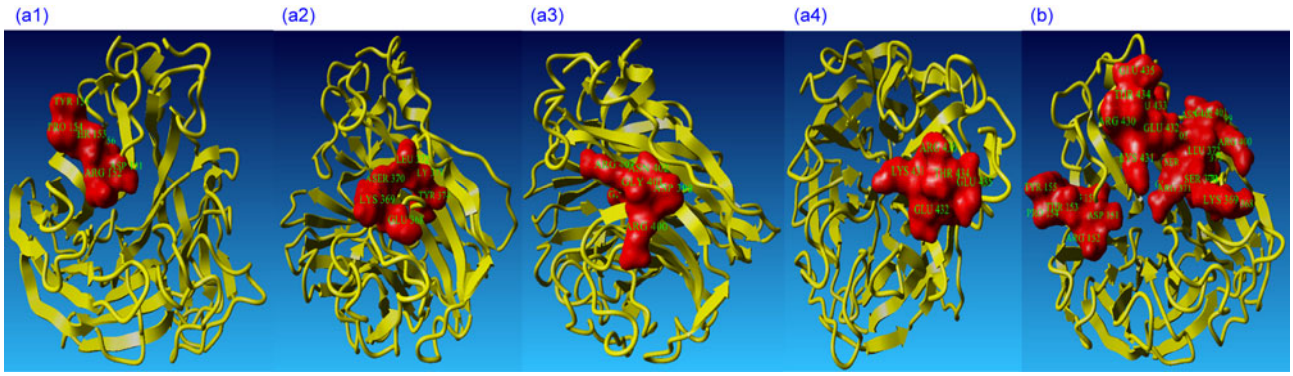

Figure 1 (Color online) Three dimensional neuraminidase (NA) (N2) models of four epitope peptides. (a) Each epitope sequences (DR6, EY7, VG8 and RE8 for a1, a2, a3 and a4, respectively) are in molecular surface; (b) four epitope sequences are labelled.

Table 2 Immunosensitivity and immunospecificity between epitope peptides and antisera

Immunogen	Antisera titer[a)]				
	Anti-virion	Anti-DC7	Anti-CY8	Anti-VG8	Anti-RE8
Immunosensitivity					
Virion (H3N2)[b)]	1∶40000	1∶20000	1∶20000	1∶20000	1∶20000
DR6	1∶10000	1∶20000	1∶100	1∶100	1∶100
EY7	1∶10000	1∶200	1∶10000	1∶100	1∶100
VG8	1∶20000	1∶100	1∶100	1∶20000	1∶100
RE8	1∶20000	1∶100	1∶100	1∶200	1∶20000
Immunospecificity					
GD-1-2007 (seasonal H1N1)	–	1∶200	1∶200	1∶100	1∶200
GD-801-2009 (2009 A H1N1)	–	1∶100	<1∶100	<1∶100	<1∶100

a) The diluent titer of rabbit serum before immunization against the immunogen was less than 1:100; b) strain H3N2 was GD-15-2007.

antisera to epitope peptides and 1% RRBCs; however no effect was observed in the plates incubated with 4 HAU of virus, antiserum to virus GD-15-2007 and 1% RRBCs. Titers of diluted antiserum to virus or each epitope peptide were measured by HI assay. Results showed that when the titer of antiserum to virus GD-15–2007 reached 1∶320, it reacted with the virus GD-15-2007 (Table 3). No HI reaction took place with any of the antisera against epitope peptides.

The hemadsorption anti-releasing effect observed after 4 h at 22°C in the three three-antisera-mixtures at a dilution of 1∶40, while the hemadsorption releasing effects of RRBCs only was seen in the negative controls (pre-immunized sera from rabbits).

2.7 Variation of epitope peptides

The reference virus was taken to be A/Kong Hong/1/68 virus. For the four predictive epitope peptide sequence regions, amino acid substitutions occurred in 44 sequences at D151V/N, I153T and H155Y for DR06, K368E, D369K/T, L370S and S372L/F for CY07, S400R/K, D401G and R402D for VG8 and R430S, Q432E, T434N and R435E for RE8 (Figure 1) and the entropy values of four peptide substitution sites were shown (Table 4).

3 Discussion

Antigenic determinants considered to be B-cell epitopes, are recognized and bound by membrane-associated receptors on the surface of B lymphocytes. The B-cell epitopes can be classified into two types: linear (continuous) and conformational (discontinuous) epitopes. Linear epitopes are short peptides that correspond to contiguous amino acid sequences within a protein, however, conformational epitopes usually are composed of several discontinuous amino acids or peptides. Immuno-informatics to determine solvent accessibility, secondary structure and flexibility has been used in recent years to screen B-cell epitopes [7,8]. Homology modeling is based on the prediction of epitopes from the primary structure of proteins, and makes the results accurate and reliable, which is a useful method to predict the structure of unknown proteins, and represents a new direction for structure-based epitope-prediction technology [5]. According to previous assays, the AI and AAI values of the epitope using SPSS did not directly correlate with the antibody activity, but they seemed to have other connections.

Seven epitopes of N2 NA protein were identified in a previous assay as follows: site 153, 197–199, 328–336, 339–347, 367–370, 400–403 and 431–434; the enzyme active site of NA protein was found to include amino acid sites 119, 156, 178, 179, 198, 222, 227, 274, 277, 294 and 425 [13]. In this study, four epitopes (peptides 151–156, 368–374, 398–405 and 428–435) were determined that covered previously reported epitope sites (153, 367–370, 400–403 and 431–434), but had changes in length and peptide sequence to some degree. Therefore their antigenicity needed re-identification, as one amino acid at position 153 was too short alone to act as a sole epitope but might form part of conformational epitope. The four epitope sequences in this study were different from previous three epitope sequences, but four epitopes functioned the similar biological effects [12]. The specific function of each epitope sequence is worthy of further identification.

The epitopes as the centre of anti-infectious immunity, related to viral antigen and their antibodies still are the highlights of infectious disease control and prevention, but it is also needed that development of an immunoinformatics method combined with comprehensive analysis on subsequent experiments. Four synthetic peptides, DR6, EY7, VG8 and RE8, were designed based on the primary sequence of the NA protein determined by SPSS analysis. Analysis of the sequences of the NA genes from 44 virus strains showed that these four epitopes were located on surface of the NA protein and formed part of the three-dimensional structure. The KLH-conjugated synthetic peptides were injected into New Zealand rabbits, and then biological activities of the antisera and the corresponding antigens were determined. These four synthetic peptides showed clear sensitivities to the corresponding antisera and had distinct specificities compared with other subtype viruses *in vitro*. Although the diluent titers of the epitope peptide antibodies were lower than those of the virion (1∶10000 vs 1∶20000), these results were still clinically and biologically

Table 3 Hemagglutination-inhibition (HI) assay and anti-releasing effects

Antisera	HA[a)] (at 40 min)	HI	Anti-releasing effects (22°C, after 4 h)
Anti-viron	–	1∶320[b)]	±
Antisera mixture A[c)]	+	–	1∶40
Antisera mixture B	+	–	1∶40
Antisera mixture C	+	–	1∶40

a) HA, hemagglutination. b) Diluent titer of serum. c) Antisera mixture A, anti-DR6, anti-EY7 and anti-VG8; antisera mixture B, anti-DR6, anti-EY7 and anti-RE8; antisera mixture C, anti-DR6, anti-VG8 and anti-RE8.

Table 4 Entropy values of the influenza H3N2 virus neuraminidase site from years 1968 to 2010

Peptide	Position	Entropy	Peptide	Position	Entropy
DR6	151	0.392	VG8	400	0.545
	153	0.183		401	0.551
	154	0.078		402	0.211
	155	0.302			
EY7	368	0.183	RE8	430	0.134
	369	1.001		431	0.496
	370	0.723		434	0.432
	372	0.760		435	0.575

significant.

The NA molecule, especially its epitopes, is located on the outer surface of the influenza virion and spans the lipid layer. NA acts as an enzyme to cleave SA 2,6- or 2,3-glycosidic bonds from the HA molecules at the virus surface, and this action results in release of virus particles from the host cells. Therefore, the new released virion is capable to infect other host cells, which results in the spread of virus infection. The mechanism of antisera anti-releasing effects might be that the virus cleaving activity had been influenced by the antisera attaching to epitope peptides of a virion [5,14]. The higher entropy values of the four peptide sites in this study indicated that the epitope amino acid sites were bore on the natural or immunological pressure. As NA protein has an important biological role in influenza, epitope mutations might influence the make-up of the dominant circulating influenza virus, and therefore epidemic and vaccine selection.

Nowadays, the HA protein is usually the influenza vaccines against epidemic and pandemic influenza viruses. However, because NA has an important function in influenza pathogenesis and spreading, their B-cell epitopes might be candidates for influenza vaccines. It was believed that the combination of HA and NA (or with epitope peptides) as a vaccine benefited the effects of influenza vaccination [7].

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