PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2011, 38(10): 929~935
www.pibb.ac.cn

Characterization of Epitope Peptides of Neuraminidases of The 2009 A H1N1 Viruses Designed by Immunoinformatics*

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Abstract The neuraminidase (NA) of influenza virus is a receptor-destroying enzyme, removing sialic acid from carbohydrate chains attached to HA, and releasing the viruses from infected cells. The NA genes of the 2009 A H1N1 were sequenced, then the B-cell epitopes were predicted, screened and assessed based on immunoinformatics. Two peptides SE8 and RE6 (amino acid residues 168～175 and 428～433) of NA protein were synthesized and immunized to raise antisera in rabbits. Two antisera are capable of eliciting neutralizing antibodies against 2009 A H1N1 in the *in vitro* microneutralization assay, furthermore, the anti-releasing effects of hemagglutination existed in the antisera. Alignment with databases showed that the amino acid residues of two epitope peptides are highly conserved amongst the NA sequences of the strains isolated from the world. These findings indicate that SE8 and RE6 represents an attractive candidate for an effective synthetic peptide-based vaccine against 2009 A H1N1 viruses.

Key words 2009 A H1N1, neuraminidase (NA), B-cell epitope, immunoinformatics
DOI: 10.3724/SP.J.1206.2011.00132

The 2009 A H1N1 virus contains a combination of gene segments that has not been reported previously in swine or human influenza viruses in the USA or elsewhere [1]. Influenza pandemics occur when an influenza virus with its antigens against which there is little or no existing immunity, emerges in the human population and efficiently transmits from human to human. Avian influenza viruses often undergo reassortment events among different subtypes and swine have been found frequently with co-infections and reassortment of swine, human, and avian viruses has been reported [2]. Co-infection combined with co-habitation of swine and poultry on small family farms all over Asia, and the presence of avian as well as human receptor types in pigs have led to the "mixing vessel" conjecture that suggests that most of the inter-host reassortments are produced in pigs[2].

Hemagglutinin (HA) and neuraminidase (NA) as the matrix proteins of the 2009 H1N1 virus, are the main antigens of influenza virus and their mutations give rise to an influenza pandemic. The segments coding for the neuraminidase were distantly related to swine viruses isolated in Europe in the early 1990s and the closest isolated relatives of the neuraminidase segment have 94.4% similarity at the nucleotide level with European swine influenza A virus strains from 1992[1]. NA protein encoded by segment 6, is located in the surface of influenza viron and is of subtype specificity. NA is a receptor-destroying enzyme, cleaving terminal sialic acid from glyco-conjugates, removing sialic acid from carbo-hydrate chains

*This work was supported by a grant from The National Natural Science Foundation of China (30972757).
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Received: March 20, 2011 Accepted: May 12, 2011

attached to HA and releasing the viruses from infected cells, such as those on the viral glycoproteins and the surface of target cells in the respiratory tract.

In the Leicester of United Kingdom, the vaccine containing hemagglutinin of 2009 A H1N1 monovalent MF59-adjuvanted vaccine has been detected where the hemagglutination-inhibition and microneutralization antibody titers of 1∶40 or more were seen in 92% to 100%[3]. In this study, we characterized the 2009 A H1N1 viruses isolated from throat swabs in Guangdong and detected the strain sequences of NA genes. With the SPSS (Stepwise Prediction and Statistical Screening, SPSS) strategy[4], the predictive epitope peptides of NA protein of the 2009 A H1N1 virus were synthesized artificially and the rabbit antisera against the epitope peptides were collected, which contained specific antibodies to NA protein. The epitope peptides and their strains were detected by antisera with ELISA, identified their specificity and sensitivity[5]. It is considered that the discipline of immunoinformatics (a method of bioinformatics) is accelerating the development of vaccines composed of epitope ensembles.

1 Materials and methods

1.1 Viruses and cells

Ten 2009 A H1N1 viruses were isolated from Guangdong in 2009 and whose NA gene sequences had been determined (GenBank accession No.GU471691-GU471695 and GU562466-GU562469). The viruses were used for animal inoculation and antiserum production as well as viral RNA preparation. The viruses for inoculation were grown in 10 days embryonated-eggs and those for detection were grown in MDCK cell-lines. The cells were propagated in Dulbecco's modified eagle medium (DMEM, GIBCOTM, Invitrogen Corporation, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Sijiqing Corporation, China) and penicillin-streptomycin (GIBCO/BRL) in humidified 5% CO_2 atmosphere at 37℃. The other 59 NA genes were downloaded from GenBank and LANL.

1.2 Sequencing of NA genes

Two pairs of primers were designed by Primer Premier 5.0 and synthesized in accordance with the NA gene sequences of 2009 A H1N1 viruses isolated from Mexico and the United States: P-NA_{F1} (5′ ttaaaatgaatccaaaccaaaag 3′), P-NA_{R1} (5′ tacagtaaagcaagaacca 3′), P-NA_{F2}(5′ taaagtacaacggcataataacag 3′) and P-NA_{R2}(5′ gtcaatggtaaatggcaactcagc 3′). The fragment of 1 415 bp was amplified and sequenced. Total viral RNA extracted from 140 μl of viral suspension (QIAamp Viral RNA Kit, Qiagen) were added to a total reaction volume of 50 μl containing 5×QIAGEN OneStep RT-PCR buffer 10 μl (0.5 mmol/L), dNTP Mix 1 μl, Enzyme Mix 1 μl, Primer F 1 μl, Primer R 1 μl(25 μmol/L), RNA 2 μl, H_2O-RNAase-free 34 μl, which was incubated at 50℃ for 30 min and 94℃ for 5 min , and then was performed for a total of 35 cycles at 94℃ for 30 s, 45℃ for 30 s and 72℃ for 90 s (ABI GeneAmp PCR System 2700). The PCR products were purified using a QIAGEN Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced on ABI PRISM 3100 Genetic Analyzer with a ABI PRISM BigDye Terminator V 3.0 Ready Reaction Cycle Sequence Kit in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA).

1.3 Epitope prediction and screening

The DNA sequences were compiled using the Lasergene 7.1 (DNASTAR, Inc., Madison, USA) and the multiple sequence alignments were assembled with Mega 5.03 (Institute of Molecular Evolutionary Genetics, PA, USA). Based on the NA protein amino acid sequence of GD-801-2009, the epitopes of NA were predicted with analysis on the protein features by means of Hierarchical cluster, Spearman′s correlation and the quartiles with Protean 7.1 and SPSS 16.0 (SPSS, Inc., Chicago, Illinois), and screened by three steps in SPSS strategy, then assessed by the antigenic index (SPSS′AI)[4].

1.4 Homology modeling

All NA sequences isolated from different countries (69 strains), including 59 strain NA sequences downloaded from the GenBank (NCBI, USA) and 10 strain NA sequences sequenced in this study. Homology modeling was performed using a molecular simulation software SWISS-MODEL[6] and the epitope peptides were shown by YASARA in molecular structure[7], which labeled the location of each epitope peptide sequence.

1.5 Peptide synthesis

All epitope peptides were synthesized artificially, then coupled the Keyhole Limpet Hemocyanin (KLH). Some epitope peptides were needed to decorate by cysteine (C) at C-terminal or N-terminal depending on the peptide features to make it easy to couple KLH.

1.6 Antiserum preparation

Anti-virion serum was prepared using strain A/Guangdong/801/2009 (new H1N1) [for short, GD-801-2009]. Groups of 2 adult New Zealand Rabbits were immunized subcutaneously into the skin in a 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 50% emulsion with Freund′s incomplete adjuvant were given at 2 weeks intervals. Antisera were collected at the 7th day after the last immunization.

1.7 Peptide identification with ELISA

The levels of antibody to each synthetic peptide were measured by the enzyme-linked immunosorbent assay (ELISA)[5]. The 96-well microtiter plates were coated overnight at 4℃ with 100 μl of 0.05 mol/L phosphate buffered saline (PBS) (pH 9.6) containing 50 mg/L of unconjugated synthetic peptides or 1 hemagglutination units (HAU) (strain GD-801-2009). 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 the serial dilutions at 37℃ for 1 h and then washed three times with PBS containing 0.1% Tween 20. Detection was performed using secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) antibody (Scicrest, China, dilution 1∶6 000) at 37℃ for 1 h, followed by three washes. The reaction was revealed by the addition of the o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) and the absorbance at 450 nm was measured by an ELISA plate reader (Molecular Devices, USA). Each assay was performed three times independently. Moreover, after antigen of epitope peptides was coated to ELISA microtiter plates and washed, polylysine (50 mg/L) and glycine (1 mol/L) were added serially to each well. The cut-off value was determined for each peptide by incubation with sera from five normal rabbits (pre-immunization).

1.8 Microneutralization assay

Diluent sera were mixed with 100 $TCID_{50}$ of viruses for 1 h. Then the mixture was added onto an MDCK monolayer in the 96-well microtiter plates[8]. After 1 h incubation, the suspension was removed, and the cells were cultured in DMEM containing 5 mg/L trypsin. After incubation at 34℃ for 48 h in the presence of 5% CO_2, the number of wells with the cytopathic effects (CPEs) was counted in quintuplicate culture as setting the control. Neutralization titers were expressed as the dilution in which CPEs were observed in 50% of the wells.

1.9 Hemagglutination inhibition (HI) assays and HI-releasing

The serum samples were pretreated with receptor destroying enzyme at 37℃ overnight and then inactivated at 56℃ for 1 h. The assays were performed as described previously using a 1.0% rooster red blood cell (RRBC). For HI assay, 50 μl of a two-fold serial dilution of the antiserum samples in PBS were incubated at 4℃ for 1 h with an equal volume of 4 HAU (Hemagglutination Unit) of the virus antigen (GD-801-2009), followed by the addition of 50 μl of 1.0% RRBC[9–10]. The reciprocal of the highest dilution of the antibody showing no hemagglutination was noted as the HI titer. Observation for hemagglutination and hemadsorption -releasing was made after 30 min, 1 h, 2 h, 3 h, 4 h at 22℃, setting negative control.

2 Results

2.1 In silico epitope prediction, screening and assessment

The NA gene of GD-801-2009 (H1N1) consists of 469 amino acids encoded by 1410 nucleotides, which weights 51.6 ku with pH 6.1 of isoelectric point (IP). The ratios of basic, acidic, hydrophobic and polar amino acids in NA protein were 7.6%, 8.5%, 30.5% and 36.2%, respectively. The substitution rate with 3.41% (16/469) existed in 69 isolate NA genes in the 2009 H1N1 viruses, including $M_{015}I$, $I_{030}V$, $I_{034}P$, $S_{035}I/V$, $L_{040}F$, $V_{053}I$, $V_{080}M$, $V_{083}M$, $S_{095}G$, $V_{106}I/H$, $I_{108}V$, $N_{248}D$, $G_{249}E$, $N_{307}T$, $Q_{313}R$ and $S_{319}R$.

The epitopes of NA proteins were predicted by the hydrophilicity, the surface probability and the antigenic index, assisted by analysis on the secondary structure and the flexible regions. The epitopes were analyzed by hierarchical cluster, Spearman's correlation and the quartiles and predicted by the method of SPSS. Both epitope peptides scoring of 0.022 and 0.051 ($_{168}$SPYNSRFE$_{175}$ and $_{428}$RGRPKE$_{433}$, for short, SE8 and RE6) with the average antigenic index (AAI) were screened, shown in Table 1. The two epitope peptide sequences were conserved in 69 strain NA genes, where there was no variation in nucleotide and amino acid sequences. Both peptides were synthesized artificially in this study.

Table 1　Epitope peptides screened with SPSS

Peptide	Position	Sequence	Total AI	AAI
SE8	168～175	SPYNSRFE	0.176	0.022
RE6	428～433	RGRPKE	0.304	0.051

2.2　Homology modeling of NA protein

The neuraminidase monomer (N1) of GD-801-2009 obtained with SWISS-MODEL had shown that the 2htyF in the PDB has the highest sequence homology at amino acid residue from 83 to 467 with 91.4% identity and its X-ray resolution was 2.5Å. The final structure was further evaluated using YASARA, which is normally used to quantify the compatibility of an amino acid sequence with a 3D protein structure and especially to check the validity of a hypothetical epitope peptide structure, shown in Figure 1. The predictive epitope peptides were located in the surface of the 3D structure in molecular surface, including in the regions of residues 168～175 and 428～433.

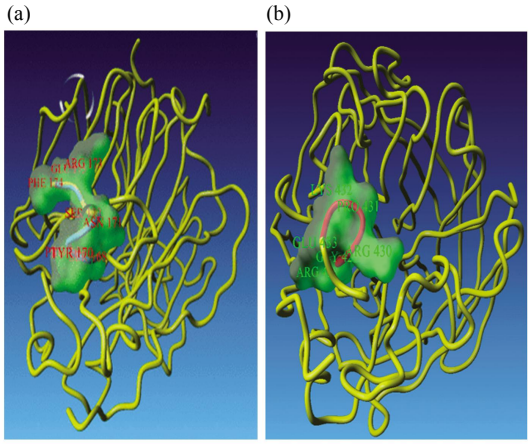

Fig. 1　Epitope peptides in 3D NA(N1) model

(a) $Peptide_{168\sim175}$. (b) $Peptide_{428\sim433}$. The epitope sequences are in molecular surface.

2.3　Sensitivity of peptide

Either the viral antigen (strain GD-801-2009 passage by MDCK cell-lines, 1 HAU, inactivated by β-propiolactone) or a peptide was coated to the 96-well microtiter plates (Jet Biofil) overnight at 4℃ with 100 μl of antiserum at dilution of 1∶100, 1∶1 000, 1∶10 000, 1∶20 000, 1∶400 000, 1∶80 000 in carbonate- bicarbonate buffer (pH 9.6). The ELISA test plate coated with the strain 2009 A H1N1 virus (GD-801-2009) was able to detect the anti-strain GD-801-2009 serum at a maximum dilution of 1∶80 000 and to detect the anti-two epitope sera at a maximum dilution of 1∶20 000. The plate coated with each epitope peptide was able to detect the corresponding anti-epitope serum at a maximum dilution of 1∶20 000 and 1∶80 000, shown in Table 2. It showed that there were high sensitivities in the test between the immunogens (including viron and peptides) and their antisera.

Table 2　Immunosensitivity and immunospecificity between epitope peptides and antisera

Immunogen	Antisera titer[1)]		
	Anti-Virion	Anti-SE8	Anti-RE6
Immunosensitivity			
Virion(2009 A H1N1)[2)]	1∶80 000	1∶20 000	1∶20 000
SE8	1∶5 000	1∶80 000	1∶100
RE6	1∶10 000	1∶100	1∶20 000
Immunospecificity			
GD-15-2007 (H1N1)	1∶80 000	<1∶100	<1∶100
GD-1-2007 (H1N1)	1∶80 000	<1∶100	<1∶100
GD-590-2006(H3N2)	1∶40 000	<1∶100	1∶100
GD-473-2008(H3N2)	1∶40 000	<1∶100	<1∶100

[1)] The diluent titer of rabbit serum before immunization against the immunogen were less than 1∶100. [2)] Strain 2009 A H1N1 was GD-801-2009.

2.4　Specificity of peptide

Antigen of the seasonal H1N1 (GD-15-2007 and GD-1-2007) and the seasonal H3N2 (GD-590-2006 and GD-473-2008) viruses by inactivation were coated to ELISA microtiter plates (96 well plate, 1 HAU), then cross-reacted with the antisera against the epitope peptides. The ELISA test plate coated with each seasonal H1N1 virus or the seasonal H3N2 virus, cross-reacted highly with the serum against GD-801-2009 (2009 A H1N1 virus) at a maximum dilution of 1∶40 000～1∶80 000, but was able to detect each anti-epitope peptide serum at a dilution of equal or lesser than 1∶100. It revealed that there were high specificities between the immunogens (including viron and peptides) and their antisera.

2.5　Neutralization activity

The neutralizing activities of the antisera obtained from the SE8, RE6 or BPL- inactivated 2009 A H1N1-immunized rabbit were analyzed in an *in vitro* microneutralization assay, using MDCK cell-lines

infected with 100 $TCID_{50}$ of 2009 A H1N1 GD-801-2009. Complete protection of MDCK cells from CPE was observed with dilutions of 1∶20 of the antisera obtained from the SE8-, RE6-immunized rabbits whereas the BPL-inactivated 2009 A H1N1-antiserum gave a complete protection from CPE at dilutions up to 1∶200. Hence, these data demonstrated that the sera obtained from SE8-, RE6- or 2009 A H1N1-immunized rabbit contained neutralizing antibodies which suggest that neutralizing linear epitopes were present in synthetic peptides SE8 and RE6, as shown in Table 3; but pre-immunization sera were negative.

Table 3 Microneutralization assay of antisera

Antisera	Anti-Virion	Anti-SE8	Anti-RE6
$TCID_{50}$	1∶200	1∶20	1∶20

$TCID_{50}$ values were the diluent titers.

2.6 Hemagglutination inhibition assay and anti-releasing effects

The hemagglutination effects were observed among the U-sharp 96-well of 4 HAU of virus GD-801-2009, antisera to epitope peptides and 4 HAU 1% RBRC, but no reactivity was observed among the well of 4 HAU of virus, antiserum to virus GD-801-2009 and 1% RBRC. Reactivities of the antisera to virus or epitope peptides were tested in HI with 2009 A H1N1 virus in wells added 4 HAU of the virus. The antisera to virus GD-801-2009 reacted with the corresponding virus in dilution titer of 1∶320. No reactivity in HI was observed with any of the antisera to epitope peptides (Table 4).

Table 4 HI assay and anti-releasing effects

Antisera	HA[1)] (40 min)	HI[2)]	Anti-releasing (22℃, after 4 h)
Anti-viron	–	1∶320[3)]	±
Anti-SE8	+	–	1∶80
Anti-RE6	+	–	1∶10

[1)] HA: Hemagglutination; [2)] HI: Hemagglutination inhibition; [3)] Dilution titer of serum.

The anti-releasing effects of hemadsorption existed after 4 hours at 22℃ in the anti-SE8 serum with dilution titer of 1∶80 and in the anti-RE6 serum with dilution titer of 1∶10, while the releasing effect of hemadsorption from RRBC existed in the negative control (pre-immunization serum).

3 Discussion

Immunoinformatics developments for determining solvent accessibility, secondary structure and flexibility have also been used in recent years to predict B-cell epitopes on antigens[11]. These methods are all based on the prediction from primary structure of proteins, making the results accurate and reliable. Homology modeling is the useful method for predicting the structure of unknown proteins, and represents a new direction for structure-based epitope-prediction technology [4]. B-cell epitope in antigen is recognized or combined by B-cell antigen receptor (BCR) or by an antibody and screening of B-cell epitope is conducive to the peptide vaccine design. The discipline of immunoinformatics is accelerating the development of vaccines composed of epitope ensembles and the confirmation of these vaccines in clinical trials in humans will serve to usher in an entirely new era of epitope driven vaccine design [12–13].

As the kernel of anti-infectious immunity, the epitopes related to viral antigen and their antibodies still are the highlights of infectious disease control and prevention, but developing the method of immunoinformatics combined with comprehensive analysis on subsequent experiments is needed. SPSS as a method of immunoinformatics, was used in this study and two synthetic peptides, SE8 and RE6, were designed according to the primary sequence of the NA protein. Analyzing the NA genes of 69 strains, both epitopes were conserved in amino acid sequences and were located in surface of NA protein in 3D structure. The KLH-conjugated synthetic peptides were injected into rabbits and the biological activities of the antisera and the corresponding antigens were determined. As to this study, both synthetic peptides demonstrate obviously their sensitivities to their corresponding antisera and displayed distinctly their specificities to other subtype viruses *in vitro*. ELISA, as a traditional assay, usually is a rapid and useful in reactivity between antigen and antibody *in vitro*; neutralization assay is used to identify the unknown pathogen with the known antibody-specific or the unknown antibody with the known antigen-specific in cell level or animal level. In this study, both synthetic peptides were shown to elicit the neutralizing antibodies against the 2009 A

H1N1 virus. Although the diluent titers in epitope peptides were lower than those in virion (1∶20 *vs* 1∶200), it still was clinically and biologically significant in identification. Altogether, these data remarkably suggest that synthetic peptides represent a promising approach for 2009 A H1N1 vaccine development[8].

The NA molecule presents its main part at the outer surface of influenza virion and spans the lipid layer. NA acts as an enzyme, cleaving sialic acid 2, 6 or 2, 3 glucosidic bond from the HA molecule at the viral surface, resulting in releasing the viral particles from host cells. The new virion released is capable to infect other host cells, bringing about spreading of patient's infection. The mechanism of antisera neutralization in this study might be that the antisera attaching to epitope peptides of a virion had influenced the viral adsorption and penetration into MDCK cells [14]. A novel peptide inhibits the influenza virus replication by preventing the viral attachment to the host cells [15]. In this study, both antisera against epitope peptides were capable to inhibit releasing hemadsorption effects *in vitro*, which indicated that the antisera had influenced on the NA enzyme releasing the viral particles from RRBCs. The hemadsorption site serves to enhance the catalytic efficiency of NA and they suggest that, in addition to changes in the receptor-binding specificity of the hemagglutinin, alterations of the NA are needed for the emergence of pandemic influenza viruses [9]. We might deduce that the antisera to epitope peptides are theoretically capable to decrease or end the influenza infection in patients or to alleviate the clinical symptoms of patients while the anti-peptide antisera were sprayed into patient′s nasal cavity

The HA protein usually acts as a candidate of influenza vaccine against influenza epidemic and pandemic. However, as to this study, these observations suggest that both synthetic peptides contain B-cell epitopes neutralizing influenza CPE and act as a candidate of influenza vaccine against influenza epidemic and pandemic. In spite of different viewpoints on epitope vaccine, more experimental practices in screening excellent epitope peptide vaccine are necessary. It is better to combination of both vaccinal HA and NA protein, including the NA epitope peptides.

Acknowledgments We thank HOU Nian-Mei, ZHANG Xin, ZHOU Jie, MENG Rui-Qi, YANG Xing-Fen, WU De, KE Chang-Wen, ZHENG Huan-Ying for their technical contribution.

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用免疫信息学技术设计新型 H1N1 流感神经氨酸酶抗原表位肽 *

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摘要 神经氨酸酶(NA)是一种具有唾液酸酶活性的流感毒粒表面糖蛋白，切断病毒 HA 与细胞膜上神经氨酸残基之间的连接，使病毒能够从宿主细胞表面释放．检测了新型 H1N1 流感 NA 基因核苷酸序列，用免疫信息学方法预测、筛选和鉴定 B 细胞表位；人工合成 NA 抗原多肽 SE8 和 RE6，并免疫新西兰兔制备抗血清．两种抗血清具有体外中和新型 H1N1 流感病毒能力(微量中和实验)，而且还具有拮抗血凝释放作用．根据 2009 年全球毒株 NA 基因序列检测和比对结果，发现多肽 SE8 和 RE6 序列未变异．实验揭示，多肽 SE8 和 RE6 可以作为新型 H1N1 流感候选多肽疫苗．

关键词 新型 H1N1 流感，神经氨酸酶(NA)，B 细胞表位，免疫信息学
学科分类号 R392 **DOI**: 10.3724/SP.J.1206.2011.00132

* 国家自然科学基金资助项目(30972757).
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收稿日期：2011-03-20，接受日期：2011-05-12