

H1N1 influenza virus epitopes classified by monoclonal antibodies

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Abstract. Epitopes serve an important role in *influenza* infection. It may be useful to screen universal influenza virus vaccines, analyzing the epitopes of multiple subtypes of the hemagglutinin (HA) protein. A total of 40 monoclonal antibodies (mAbs) previously obtained from flu virus HA antigens (development and characterization of 40 mAbs generated using H1N1 influenza virus split vaccines were previously published) were used to detect and classify mAbs into distinct flu virus sub-categories using the ELISA method. Following this, the common continuous amino acid sequences were identified by multiple sequence alignment analysis with the GenBank database and DNAMAN software, for use in predicting the epitopes of the HA protein. Synthesized peptides of these common sequences were prepared, and used to verify and determine the predicted linear epitopes through localization and distribution analyses. With these methods, nine HA linear epitopes distributed among different strains of influenza virus were identified, which included three from influenza A, four from 2009 H1N1 and seasonal influenza, and two from H1. The present study showed that considering a combination of the antigen-antibody reaction specificity, variation in the influenza virus HA protein and linear

epitopes may present a useful approach for designing effective multi-epitope vaccines. Furthermore, the study aimed to clarify the cause and pathogenic mechanism of influenza virus HA-induced flu, and presents a novel idea for identifying the epitopes of other pathogenic microorganisms.

Introduction

Infection by pathogenic microorganisms and viruses presents a significant threat to human life worldwide; their constant variation, evolution and spread render it difficult to prevent and control infection. HBV, one of the most infectious diseases worldwide, often varies due to the pressures of host immunity, natural selection and the use of antiviral agents. Such variations may cause changes in HBV pathogenicity, including the development of tolerance and immune escape, and have greatly hindered clinical diagnosis and treatment (1). A number of new HIV GAG subtypes have been reported due to its constant variation and accumulation (2). Continual variation in the influenza virus hemagglutinin (HA) antigen gene is the main cause of influenza outbreaks (3). This poses challenges for immunology, virology and immunopharmacology research, and for the development of vaccines against influenza and other pathogenic microorganisms.

Epitopes, also known as antigenic determinants, represent the material base of immunogen antigenicity, and is the part of an antigen recognized by the immune system. Epitopes can be classified as either conformational epitopes or linear epitopes, based on their structure and interaction with the paratope (4). The linear epitope is a section of the continual amino acid sequence of the antigen, and its interaction with the paratope predominantly depends on its primary structure. Variations in any area of the linear epitopes may lead to structural changes, a reduced antibody binding ability, and the ability to escape recognition by existing antibodies and vaccines (5).

Different subtypes of a pathogen may have a variety of antigens; thus, it is challenging to distinguish the subtype of pathogenic microorganisms, to establish immunodetection technologies, and to clarify the mechanisms of disease spread.

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Abbreviations: mAbs, monoclonal antibodies; HA, hemagglutinin; IR, inhibition rate; PDB, protein database

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Consequently, epitope prediction and utilization are of value in differential diagnosis, the prediction of variation trends, determining the mechanisms of pathogenic microorganism infection, and in the design of multi-epitope vaccines (6).

Recently, several methods of epitope prediction have been in use, the majority of which are limited to one antigen, although they still provide a satisfactory predictive capacity (7-9). X-ray diffraction requires more time and energy to identify epitope structures. To elucidate the biological profile of the epitope, multiple factors should be considered, including its location on the surface of the antigen, the flexibility, and the accessibility, although it showed a growing acceptance among this field (10-14). In addition to α -helices and β -pleated sheets, glycosylation sites are also important for prediction (15). However, the predictive accuracy of these methods is just ~60% (16). Larger protein libraries are required for phage display technology, and certain peptides have strong hydrophobicity, which influences their structure on the surface of phages. Furthermore, the predictions obtained via this method still require further verification (17). Thus, a single optimal approach is required, which is capable of predicting the epitope sequences of microorganisms comprehensively and in one pass, establishing a biological profile with the characteristics and functions of the epitopes, and modeling the behavior of these epitopes during changes to virus antigenicity. This will have an important and direct role in the design of biologically active drugs, research into pathogenic mechanisms, and the prediction of variation in certain pathogenic microorganisms.

Monoclonal antibodies (mAbs) are a subset of antibodies generated by identical immune cells with a strong monovalent affinity, in that they bind to the same epitope, with high specificity and sensitivity, and define the structure and character of epitopes (18). Such specificity can also be used as a tool to analyze the epitopes of viruses and their subtypes, provide information on the main functions of the epitopes and on genetic variations involved in changes to the epitopes, and assist research into epitope variation and improvements in vaccine design (19,20).

In the present study, mAbs from 40 previously developed anti-H1N1 influenza virus HA split vaccines had been developed and characterized (21), which were used as experimental tools to predict the epitopes of influenza virus HA proteins, after which their distribution and expression were investigated using synthesized peptides. The present study aimed to illuminate the association between variation in the influenza virus and its immunogenicity, and to develop a useful method for predicting the variable epitopes of other pathogenic microorganisms. In the present study, we just preliminary report a new method for predicting the variability epitope of influenza virus. Next, we will carry out biological functional studies on predicted different epitopes one by one, which can help us to develop epitope vaccines of influenza virus, further contribute to the diagnosis and prevention of influenza virus.

Materials and methods

Antigens. H1N1 influenza virus split vaccine (2009; SFDA Approval no.: S20090015) was obtained from Hualan Biological Bacterin Co., Ltd., (Henan, China); seasonal A1 and A3 influenza [2009; Veterinary Drug Production Approval no.: 150132145],

and H9N2 (SD696) strains were purchased from Qingdao Yebio Bioengineering Co., Ltd., (Shandong, China).

Antibodies. mAbs against the anti-H1N1 A influenza virus HA protein were prepared in our laboratory, and HRP-conjugated goat anti-mouse antibodies were provided by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., (Beijing, China).

HA protein synthetic peptides. Part of the continuous amino acid sequence of influenza virus HA was determined using DNAMAN software, and peptides were synthesized by ChinaPeptides Co., Ltd., (Shanghai, China).

ELISA analysis and classification. Indirect ELISA analyses were performed using the following: Hybridoma culture supernatant; H1N1 influenza virus split vaccine (2009); seasonal influenza viruses A1 and A3; and avian influenza viruses H5N1 and H9N2. Briefly, the 96-well plate was pre-coated with 100 μ l of each vaccine (2-5 μ g/ml). After washing three times with PBST (including 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, 2 ml of Tween-20, pH 7.2, volume adjusted to 1L with additional distilled H₂O), the plates were blocked with 200 μ l skim milk (dilution, 1:20) and incubated for 1 h at 37°C. Subsequently, 100 μ l/well supernatant aspirated from the hybridoma cell cultures for 40 mAbs was added, including the supernatant of SP2/0 as a negative control, which was incubated for 1 h at 37°C. After washing a further three times, the concentration (dilution, 1:2,500) of the HRP-labeled goat-anti-mouse IgG mAb (100 μ l/well) was added and incubated for 1 h at 37°C. Next, 100 μ l TMB-H₂O₂ chromogenic solution was added to each well and incubated for 10 min at 37°C in the dark, and terminated with H₂SO₄ solution (2 M, 50 μ l/well). Finally, the proportion of bound antibodies, which is correlated with the color intensity, was measured with an ELISA reader via absorbance at 450 nm. The ratio of each test sample (OD₄₅₀: Control OD₄₅₀) was calculated. Samples with a ratio of ≥ 2.1 were classified as exhibiting a positive reaction. Considering each test sample reaction with the five subtypes of the influenza virus, the antibodies were categorized into different groups.

Epitopes of influenza A virus HA protein prediction. In the NCBI database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>), the amino acid sequences of various influenza virus subtypes were accessed and downloaded with their GenBank IDs (Table I). Consequently, a multiple sequence alignment analysis was performed using DNAMAN software, following which the common continuous amino acid sequence (5-7 aa) between the antigens of the different groups were defined, and used to predict the epitopes of influenza A virus HA proteins. Overall, 27 candidate epitope fragments were selected, and complementary peptides were synthesized, each with a >85% purity as measured by HPLC and MS methods; these peptides were stored as freeze-dried powders at -20°C.

Localization of predicted epitopes with anti-influenza virus HA mAbs. To investigate the positions of the predicted epitopes of influenza A virus HA, 27 candidate-epitope peptides

Table I. Information about the amino acid sequences of subtype influenza virus.

Name of antigens	Source of HA amino acid sequence	GenBank ID
2009 H1N1-HA	(A/reassortant/NYMCX-179A (California/07/2009xNYMC X-157)(H1N1))	ACR47014.1
H3N2-HA	Influenza A virus (A/Victoria/210/2009(H3N2))	CY121077.1
Seasonal H1N1-HA	Influenza A virus (A/Brisbane/59/2007(H1N1))	CY163864.1
H5N1-HA	Influenza A virus (A/Goose/Guangdong/1/96(H5N1))	AF144305.1
H9N2-HA	Influenza A virus (A/chicken/Shandong/6/96(H9N2))	AAAY52514.1

HA, hemagglutinin.

Table II. mAb cross-reactivity with various subtypes of influenza virus.

mAb group	No. of cell lines
Common antigens of influenza virus (2009 H1N1 and seasonal A1, A3 and avian influenza H5N1 and H9N2)	20
Common antigens of 2009 H1N1 influenza virus and seasonal influenza virus (2009 H1N1 and seasonal A1, A3)	14
Specific H1 subtype (2009 H1N1 and seasonal A1)	6
Total	40

mAB, monoclonal antibody.

were used and screened using mAbs against influenza virus HA. The process was as follows: The synthesized peptides were mixed with 40 HA mAbs, and incubated for 1 h at 37°C. A total of 100 µl mixed reagent was placed into each well of an ELISA plate pre-coated with H1N1 influenza virus HA antigens (2 µg/ml), according to standard ELISA protocols. After a 1 h incubation and three washes, the goat anti-mouse antibodies (dilution, 1:2,500) were added, and the steps of a conventional ELISA were performed. The OD₄₅₀ values for all wells were calculated from TMB coloration and an inhibition rate (IR) was calculated. The formula used to calculate the IR was as follows: $IR = (OD_{CTL} - OD_{TEST}) / OD_{CTL}$. Correlations between the antigens and the antibody binding sites were defined according to the following criteria: No correlation (IR ≤ 0.4); correlation (0.4 ≤ IR ≤ 0.8); and strong correlation (IR ≥ 0.8).

Distribution of predicted epitopes in the HA crystal structure. The PyMOL Molecular Graphics System (<http://www.PyMOL.org>) and Protein Database (PDB) were used to analyze the distribution of predicted epitopes in the HA crystal structure. Peptides recognized by mAbs against influenza virus HA proteins in the ELISA experiments were selected and analyzed. First, the PDB database was used to search for and generate a model of the HA protein X-ray crystal structure by referring to the 3LZG structure, which was produced from the A/California/04/2009 H1N1 virus HA and had a similar structure to that of the antigen in the present study. Secondly, the selected peptides' distributions were determined using PyMOL software according to the manufacturer's protocol.

Results

Specificity and cross reactivity of mAbs. ELISA reactions between 40 influenza virus HA antigen mAbs and five different influenza virus subtype vaccines were evaluated using the OD₄₅₀ ratio, and classified as positive (OD₄₅₀ ≥ 2.1) or negative (OD₄₅₀ < 2.1) reactions. According to the cross-ELISA results, all the assessed influenza virus HA antigens can be classified into three groups. Approximately half (20/40) were recognized by all five antigens, ~35% (14/40) were recognized by the antigens of 2009 H1N1 virus A, or seasonal influenza virus A1 and A3, and 6 mAbs only reacted with the antigens of H1N1 virus A and seasonal A1 (Table II).

Detection of conserved peptides in influenza virus A HA. Twenty seven common continuous amino acid sequences of influenza HA antigens detected through multiple sequence alignment analysis of the three groups using DNAMAN software (Table III). There were 9 peptides located in the conserved sequences of vaccines in group 1, 7 peptides in the conserved sequences of group 2, and 11 in group 3 (Table III).

Locations of predicted epitopes determined using anti-influenza virus HA mAbs. The ELISA results demonstrated that 9/27 peptides were recognized by 13/40 mAbs, considering their IRs calculated with OD₄₅₀ values (Figs. 1-3). In group 1, 5 mAbs were identified by 3 peptides, designated peptides 1, 2 and 9 (Fig. 1); in group 2, 6 mAbs reacted with 4 peptides (peptides 10, 11, 15 and 16; Fig. 2); and 2 mAbs in group 3 were identified by 2 peptides (peptides 17 and 27; Fig. 3).

Table III. Peptide fragments in influenza virus HA identified subtype influenza virus mAbs.

Groups and peptides no.	Sequence of peptides	Position
Group 1: (9) ^a		
Peptide 1	LVLWGIHHP	191aa-199aa
Peptide 2	LPFQNI	307aa-312aa
Peptide 3	LATGLRN	331aa-337aa
Peptide 4	RGLFGAIAAGFIEGGW	344aa-358aa
Peptide 5	GWYGYHH	364aa-370aa
Peptide 6	STQNAID	384aa-390aa
Peptide 7	YNAELLVL	438aa-445aa
Peptide 8	ENERTLD	447aa-453aa
Peptide 9	WSYIVE	93aa-98aa
Group 2: (7) ^b		
Peptide 10	DTLCIGYHANNSTDT	17aa-32aa
Peptide 11	MNYYWTLVEPGD	244aa-255aa
Peptide 12	ATGNLVVPR	261aa-269aa
Peptide 13	GYAADLKSTQNAIDEI	377aa-392aa
Peptide 14	EINGNCF	476aa-482aa
Peptide 15	FYHKCDNT	484aa-491aa
Peptide 16	SVKNGTYD	495aa-502aa
Group 3: (11) ^c		
Peptide 17	KAILVLLYTFA	2aa-13aa
Peptide 18	SVNLEDK	46aa-53aa
Peptide 19	KLRGVAPLHLGK	60aa-71aa
Peptide 20	ESLSTASS	85aa-92aa
Peptide 21	TSSSDNGT	99aa-106aa
Peptide 22	PNHDSNKGVTA	141aa-151aa
Peptide 23	PHAGAKSFYKNLI	154aa-166aa
Peptide 24	KLSKSYINDKGKEV	177aa-190aa
Peptide 25	GSSRYSKKFKPE	219aa-230aa
Peptide 26	RYAFAMERNAGSG	269aa-281aa
Peptide 27	VVSLGAISF	544aa-552aa

^a2009 H1N1 and seasonal A1, A3 and avian influenza H5N1 and H9N2; ^b2009 H1N1 and seasonal influenza virus A1, A3; ^c2009 H1N1 and seasonal A1. mAB, monoclonal antibody; HA, HA, hemagglutinin.

Distribution of predicted epitopes in the HA crystal structure. After the predicted epitopes were located, three peptides (including 93-WSYIVE-98, 191-LVLWGIHHP-199 and 307-LPFQNI-312), located in the continuous conserved amino acid sequences in all five HA antigens, were chosen for distribution analysis. PyMOL software analysis identified the three peptides sequences in the HA crystal structure, and predicted their location in the 3D structure of HA (Fig. 4).

Discussion

Variability in the HA proteins of the influenza virus impacts on the suitability and efficacy of existing vaccines. Developing universal vaccines effective against various subtypes of

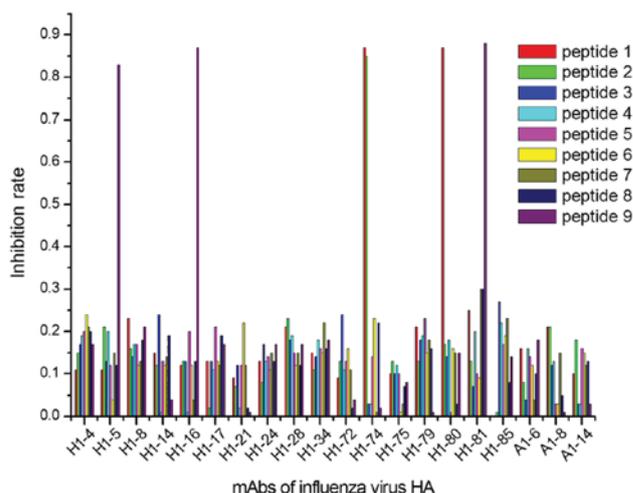


Figure 1. Positioning results of mAbs against common antigens of influenza virus A in conserved areas of the influenza virus HA protein sequence. In total, 5 mAbs (H1-5, H1-16, H1-74, H1-80 and H1-81) showed a strong interaction (IR ≥ 0.8) with 3 epitopes (peptides 1, 2, and 9). mAbs, monoclonal antibodies; HA, hemagglutinin.

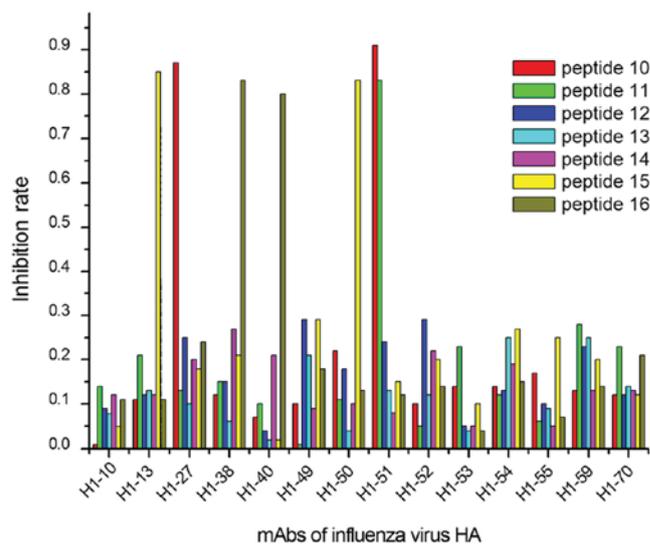


Figure 2. Positioning results of mAb against common antigens of the influenza A virus H1N1 and seasonal influenza virus in conserved areas of the influenza virus H1N1+A1+A3 and HA sequences. A total of 6 mAbs (H1-13, H1-27, H1-38, H1-40, H1-50 and H1-51) showed a strong interaction (IR ≥ 0.8) with 4 epitopes (peptides 10, 11, 15 and 16). mAbs, monoclonal antibodies; HA, hemagglutinin.

influenza is the primary approach for controlling the spread of infection (22). As epitopes are a key feature of viruses, several strategies have been successfully applied in the design and development of 'epitope-focused' vaccines (23,24), which demonstrate advantages such as high specificity, fewer side effects, simple preparation, and easy storage and transportation (25,26). These rapid and accurate strategies have become the foundation for the development of influenza virus vaccines, as well as supporting clinical diagnosis and treatment.

In the present study, we predicted the epitopes of multiple subtypes of the influenza virus HA protein using 40 previously developed mAbs, and extracted the common continuous amino acid sequences as linear epitopes. Following this, we

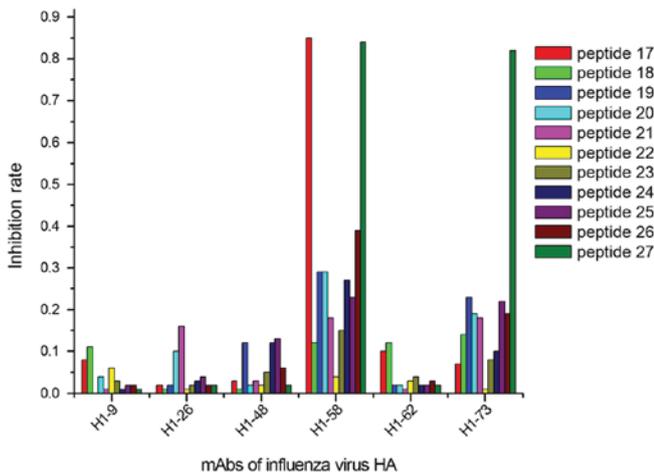


Figure 3. Positioning results of mAbs allowed the identification of the H1 subtype influenza virus HA. Additionally, 2 mAbs (H1-58 and H1-73) showed a strong interaction ($IR \geq 0.8$) with 2 epitopes (peptides 17 and 27). mAbs, monoclonal antibodies; HA, hemagglutinin.

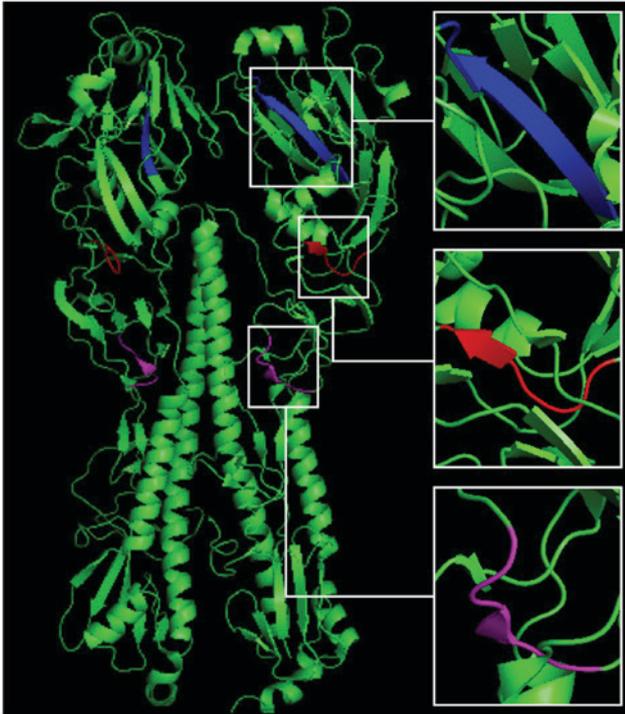


Figure 4. Distribution of three peptides in the HA crystal structure. We searched for a crystal structure of the H1N1 influenza A virus in the PDB, and used 3LZG as a reference structure. PyMOL software was used to determine the distribution of three peptides in the 3D crystal structure of HA, from which we noted that the WSYIVE peptide (red) and the LPFQNI peptide (purple) are located on the random coil domain, whereas the LVLWGIHHP peptide (blue) is situated within the β -sheet structure. HA, hemagglutinin.

determined the localization and distribution with candidate peptide analysis, to verify and confirm 9 linear epitopes of the HA protein. For five common subtypes of the influenza virus, 3 epitopes (peptides 1, 2 and 9) showed a strong association with multiple influenza viruses. Additionally, three epitopes are known to overlap with three neutralizing epitopes, HA183~195, HA127~133 and HA92~105, of the H3 subtype influenza virus HA protein, as reported by Li *et al* (27). It is also

suspected that the three peptides 1-LVLWGIHHP, 2-LPFQNI and 9-WSYIVE may stimulate organisms to produce neutralizing antibodies and promote immunogenicity, which may benefit the development of universal influenza vaccines.

In the second group, which included the 2009 influenza A virus H1N1, and the seasonal influenza A1 and A3 virus, four linear epitopes were identified. The epitopes in the conserved sequences of this group were immunodominant epitopes, capable of stimulating organisms to produce a high volume of antibodies in response. Therefore, there were more chances of mixed infection of three of them presently (28). In the third group, two linear epitopes were predicted, which are the main markers used to distinguish between the HA proteins of H1 and other subtypes. Our results suggested that only 15% (6/40) of the antibodies are produced by organisms when stimulated by epitopes in group 3, due to there being fewer common epitopes between these two subtypes of the influenza virus. To an extent, this observation may also explain the significant difference between the H1N1 influenza virus subtypes in terms of the infection frequency, pathogenicity and infection scale, among other variables (29).

As identified in ELISA experiments, 13/40 anti-influenza virus HA antigens were positioned at 9 epitopes. In group 1, we synthesized 9 peptides after analyzing the common sequences of the human and avian influenza viruses using DNAMAN software and 20 mAbs against epitopes common to both viruses; we positioned 5 antigens to 3 epitopes. Li *et al* (27), used an *E. coli* model to demonstrate that rabbits and mice are immune to recombinant multi-epitope peptides specific to three neutralizing epitopes, HA183~195, HA127~133 and HA92~105, from the H3 subtype of influenza virus HA, and neutralizing antibodies with high titer were produced. This indicated that, in the first group, among peptides able to identify multiple subtypes of the influenza virus, 1-LVLWGIHHP and 9-WSYIVE could potentially stimulate organisms to produce neutralizing antibodies, which would benefit the development of universal influenza vaccines. In the second group, we designed 7 peptides complementary to the antigen-conserved areas of 14 mAbs. We positioned 4 epitopes to 6 mAbs. Epitopes in the conserved areas of the 2009 influenza A virus H1N1, and the seasonal influenza A1 and A3 viruses were immunodominant, and stimulated organisms to produce an abundance of antibodies in response. In the third group, 2 antigens were positioned to 2 epitopes. Epitopes corresponding with these antibodies are the primary markers used to distinguish between the HA proteins of H1 and other subtypes.

Influenza virus HA proteins include 562-566 amino acids and consist of a HA1 spherical head (319-328aa) and a HA2 bacilli-form stalk (221-222aa). HA1 includes 8 anti-parallel β -laminated structures, including a receptor-binding domain (RBD) and 5 antigenic determinants: A, B, C, D and E (30). The RBD domain is composed of a helix at site 190, and of rings at sites 130 and 220; one of the predicted epitopes, verified by peptide 191-LVLWGIHHP-199, was located near this domain (Fig. 4), indicating that the current method was effective and reliable, and could be used to investigate the mechanisms underlying the spread of influenza, its genetic variation, and in the development of epitope-specific vaccines.

To predict the epitopes of influenza HA proteins, we used the antigen-antibody reaction method. Multiple reactivity

modes were observed, including the one-to-one mode, the one-to-many mode (H1-74 reacted with peptides 1 and 2; H1-51 reacted with peptides 10 and 11; H1-58 reacted with peptides 17 and 27), and the unresponsive mode. Two findings were notable: First, two peptides that react with the same antibody were close to the 3D structure of HA, and formed a conformational epitope, although they were separated by a long sequence in the primary structure; second, 40 mAbs were obtained using the split influenza virus vaccine, and these immunogens can induce organisms to produce the same antibodies as those induced by natural pathogens. Synthesized peptides, for which the design and utilization were based on the primary sequence of the protein, were used for linear epitope prediction and identification.

These short peptides can be also used as good immunogens to research different subtypes of influenza virus epitope vaccines. Li *et al.* (31) applied short-peptide immunization to the mice directly, and screened the prepared mAbs. In order to enhance immunogenicity, connection of polypeptides and macromolecular protein can also be used. Gong *et al.* (32) coupled the short peptides P1~P6 of the chemically synthesized influenza virus H3N2 sequence with the Keyhole Limpet Hemocyanin (KLH) carrier protein in order to increase the immunogenicity of the polypeptide, and induced a strong humoral immune response. We have previously linked 9 different polypeptides with KLH one by one, obtaining high titer and high affinity polyclonal antibodies after immunizing mice. Polyclonal antibodies were then tested for their neutralizing activity and cross-reactivity with human tissues. These experiments are underway.

In conclusion, the present study identified 9 linear epitopes of the influenza HA protein via traditional mAb and antigen interaction analysis, and verified these using ELISA and 3D structure location analyses with synthesized peptides. The results provide a novel, effective and reliable method for investigating the mechanisms underlying the spread and variation of influenza virus and other pathogenic microorganisms, in addition to improving the development of epitope-focused vaccines.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CG, HZ, XX and JH conceived and designed the experiments; CG, HL, PY, HH, JS, YL, QF, XZ, DL and ZW performed the experiments; CG, HZ and JH analyzed the data; CG, HZ, YL,

PY and LS made data interpretation and critical manuscript revisions; CG and HZ wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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