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Identification and analysis of B cell epitopes of hemagglutinin of H1N1 influenza virus

Qing Feng[1,2,3] · Xiao-Yan Huang[2] · Yang-Meng Feng[1,2,3] · Li-jun Sun[1,2,3] · Jing-Ying Sun[1,2,3] · Yan Li[1,2,3] · Xin Xie[4] · Jun Hu[1,2,3] · Chun-Yan Guo[1,2,3]

Abstract

The frequent variation of influenza virus hemagglutinin (HA) antigen is the main cause of influenza pandemic. Therefore, the study of B cell epitopes of HA is of great significance in the prevention and control of influenza virus. In this study, the split vaccine of 2009 H1N1 influenza virus was used as immunogen, and the monoclonal antibodies (mAbs) were prepared by conventional hybridoma fusion and screening techniques. The characteristics of mAbs were identified by ELISA method, Western-blot test and hemagglutination inhibition test (HI). Using the obtained mAbs as a tool, the B cell epitopes of HA were predicted by ELISA blocking test, sandwich ELISA method and computer simulation method. Finally, four mAbs against HA antigen of H1N1 influenza virus were obtained. The results of ELISA and computer prediction showed that there were at least two types of epitopes on HA of influenza virus. The results of this study complemented the existing methods for predicting HA epitopes, and also provided a new method for predicting other pathogenic microorganisms.

Keywords H1N1 influenza virus · Monoclonal antibody (mAb) · ELISA blocking test · Computer prediction

Introduction

Pathogenic microorganism infection is one of the major factors that threaten the safety of human life, and the continuous variation, evolution and spread is the main reason why it is difficult to prevent and control (Gao et al. 2018). Frequent variation of influenza virus hemagglutinin (hemagglutinin, HA) antigen is the main cause of influenza pandemic

(Bedford et al. 2015, Wong and Webby 2013). Due to the high variability of HA antigen, new epidemic strains with different genotypes continue to appear, which brings great challenges to the prevention and control of influenza (Zolotarova et al. 2021). The identification of the Omicron variant (B.1.1.529.1 or BA.1) of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) in Botswana in November 2021 immediately raised alarms due to the sheer number of mutations in the spike glycoprotein that could lead to striking antibody evasion (Iketani et al. 2022).

This increasing variation leads to the emergence of new subtypes, which in turn threatens the effectiveness of existing vaccines. Therefore, antigenic epitopes are the material basis of immunogenicity, and the variation of any site on the antigenic epitopes of multi-variant proteins may change its spatial structure and reduce its binding to antibodies. New infection problems arise due to evading the protection of existing vaccines (Belongia and McLean 2019), which poses a severe test for the establishment of immune detection technology to distinguish pathogenic microorganisms and the study of disease mechanism. Therefore, the study of antigenic epitopes is of great significance for the detection and diagnosis of pathogenic microorganisms, the prediction of variation trend, the pathogenic mechanism and the design

of epitope vaccine. So far, the main methods for epitope prediction include computer simulation technology and monoclonal antibody (mAb) technology (Devi et al. 2021; Wang et al. 2022).

Yin et al. (2018) predicted the antigenic variation of H1N1 influenza virus by computer simulation technology, combined with all the variation patterns across periods, and proved the diversified mutation patterns of HA1 protein in different periods, which will help to evaluate the antigenicity of new influenza virus strains, and it will also help to quickly identify antigenic variants and influenza surveillance. Although computer simulation technology has become the mainstream of predicting antigen epitopes, the prediction results can only be used as a reference for determining the potential epitopes of each protein (Sharma et al. 2021). At the same time, most of the computer simulation predictions are conformational epitopes. Because of the flexibility of proteins, the results of computer prediction may be limited, and further experimental results are needed to confirm. The mAbs were highly specific and can specifically and sensitively bind to corresponding antigens, reflecting the structural differences and strength of antigenic epitopes (Yuan et al. 2020). It can be used as an important tool for fine analysis of antigenic epitopes, providing information for the main functions of antigenic epitopes and genetic variation related to antigen changes, which is of great benefit to the study of antigenic variation and to improve the level of vaccine design (Neu et al. 2019).

Therefore, in this study, four mAbs against HA protein of H1N1 influenza virus were prepared and screened by hybridoma technique, and these four strains of mAb were used to predict the B cell epitopes of HA. The B cell epitopes of HA were divided into two categories by blocking ELISA, double antibody sandwich ELISA and computer simulation. Combined application of several methods, the HA epitopes were studied in depth, and the results complemented the monogeneity of the existing epitopes prediction methods, laid a foundation for the development of potential influenza vaccine, and provided research ideas for elucidating the immune mechanism of influenza virus.

Material and methods

Materials

Split vaccine of H1N1 influenza virus (2009) (Chinese medicine S20090015) (numbering: A/California/07/2009×NYMC X-157), purchased from Hualan Biological Vaccine Co., Ltd.; HRP-labeled antibody prepared by our laboratory; HRP-labeled goat anti-mouse second antibody purchased from Zhongshan Jinqiao Company; total RNA extraction kit and cDNA first chain synthesis kit purchased from Beijing Tiangen Biological Company; PCR polymerase, pMD19-T vector and DNA Marker purchased from Dalian TaKaRa Company. Primer synthesis and sequencing were completed by Beijing Liuhe Huada Genome Technology Co., Ltd; Bovine serum for cell culture was purchased from Hangzhou Sijiqing Bioengineering Materials Co., Ltd. Other reagents were made in China. The crystal structure of HA refers to the PDB sequence number 3LZG of H1N1 virus (numbering: A_California_04_2009_H1N1pdm), which is 99% similar to the influenza H1N1 antigen in this study.

Preparation of monoclonal antibodies

1. Immunized animal Emulsified with Freund complete adjuvant and influenza vaccine antigen at a rate of 1:1, BALB/c mice were injected subcutaneously according to 30–50 μg. After 21 days, BALB/c mice were subcutaneously injected with Freund incomplete adjuvant and influenza vaccine antigen at a rate of 1:1, and the immune dose was the same as above. Seven days later, BALB/c mice were injected intraperitoneally with influenza vaccine. When preparing for fusion, the mice were injected intraperitoneally with influenza vaccine on the first day and began to fuse on the third day.
2. Preparation of mAb: The process of cell fusion and the screening of positive clones were carried out according to the method of reference (Guo et al. 2015). When the hybridoma cells reached 20–50% / well, the positive cells were screened by ELISA method. The positive clone hole, that is, the antibody-secreting hole was cloned by limited dilution method, and the specific antibody-secreting cell line was obtained after 3 times of cloning, which was cultured and cryopreserved. Then ascites was prepared by referring to the method of reference. First, 10-week-old BALB/c mice were injected intraperitoneally with liquid paraffin. After 7 days, 10^6 hybridoma cells were cultured and made into cell suspension and injected into the abdominal cavity of mice injected with liquid paraffin. Aseptic ascites was collected for about 7 days, identified by ELISA, subpackaged and stored at − 20 ℃.

Characterization of mAbs

1. Ig type and subtype identification of mAbs: SBA ClonotypingTM System/HRP antibody subtype identification kit was used for determination, refer to the instruction for specific methods.
2. Hemagglutination inhibition (HI) test: The 2009 H1N1 virus was successively diluted in a twofold gradient and added into a 96-well "U" shaped microreaction plate, 100 μL /well, and 0.5% chicken red blood cells were

added into each well. The cells were incubated at room temperature (20–25 ℃) for 30 min, and the hemagglutination titers of virus were determined. Four hemagglutination unit virus solutions were prepared, and the mAb was continuously diluted at a gradient of 2, 50 µL/well on a 96-well "*U*" shaped microreaction plate, then influenza virus dilution of 4 hemagglutination units (50 µL/well) was added to each well, mixed evenly, and placed at room temperature (20–25 ℃) for 30 min. Add 0.5% chicken red blood cells 50 µL to each well, mix well and place at room temperature (20–25 ℃) for 30 min. The results were observed when 4 units of virus had agglutinated red blood cells, and the maximum dilution of 100% agglutination inhibition was the hemagglutination inhibition titer of the mAb.

3. Western Blot: After SDS-PAGE electrophoresis of the H1N1 influenza virus vaccine with 12% isolated gel, the protein was transferred to nitrocellulocellulose membrane, and ascites induced by mAb cell line was diluted 1:1000 as primary antibody, and goat anti-mouse enzyme-labeled antibody was diluted at 1:2500 as secondary antibody, and finally DAB was displayed.

Localization of monoclonal antibody recognition epitopes

The improved sodium periodate method was used to label monoclonal antibody with HRP. The H1N1 antigen was packaged into a 96-well ELISA plate, then add different mAb to block, 150 µL /well, placed in 37 ℃ for 1 h, discard the liquid in the well, add HRP-labeled mAb, placed in 37 ℃ for 1 h. Finally, TMB(3, 3′, 5, 5′-Tetramethylbenzidine) was used for color rendering, and the absorbance (OD) value at 450 nm was detected. The inhibition rate was calculated, i.e. (control group—experimental group)/control group. If the value was less than 40%, the blocking mAb did not bind to the same antigenic epitope with HRP-labeled mAb. If the value was between 40 and 80%, that was, the blocking mAb was related to the antigenic epitope bound to the HRP-labeled mAb. If the value was higher than 80%, the blocking mAb and the HRP-labeled mAb bound to the same epitope (Guo et al. 2019).

Double antibody sandwich ELISA method

On a 96-well ELISA plate, four mAbs were coated with 100 µL of each well in the carbonate buffer solution of pH9.6. The coated enzyme plate was sealed with cling film and stayed overnight at 4 ℃. The next day, washed the plate with PBST for three times, 5 min/ times, added 5% skim milk for sealing, 200 µL/well, placed the plate in 37℃ incubator for 1 h. After discarding the blocking solution, washing with the same method as above, then added

H1N1 influenza virus hemagglutinin antigen with appropriate dilution, 100 µL/well, incubated for 1 h at 37 ℃, washed with the same method as before, and then added appropriate dilution HRP-labeled monoclonal antibody for 1 h, washing method was the same as before. TMB solution was used for color rendering, and P/N ≥ 2.1 was considered as the positive criterion.

Cloning of light chain and heavy chain variable region genes of monoclonal antibody

Four hybridoma cell lines secreting monoclonal antibodies against H1N1 influenza virus HA were cultured. The hybridoma cells with good growth condition were collected and the total RNA was extracted. Using the extracted total RNA as template, cDNA was synthesized by reverse transcription, which can be used as the template for subsequent gene cloning. The variable region genes of light chain and heavy chain of mouse antibody published by NCBI were compared and analyzed. A total of 27 primers were designed. The light chain variable region gene of the antibody was amplified by PCR with 8 primers (7 upstream primers + 1 downstream primer) and the heavy chain variable region gene of the antibody was amplified with 9 primers (5 upstream primers + 4 downstream primers). The products amplified by PCR were identified again by PCR (5 primers were designed for light chain downstream of amplification product and 5 primers for heavy chain upstream of amplification product), ligated, transformed, monoclonal bacteria picked, plasmid extracted and identified by restriction endonuclease digestion. After correct sequencing, the obtained nucleotide sequence was translated into amino acid sequence.

Computer simulation analysis of amino acid sites of mAb binding to HA protein

The method of operation was as follows:

1. The crystal proteins with the best amino acid sequence similarity with each monoclonal antibody were selected from the RCSB PDB protein crystal database, and used as the template for homology modeling.
2. The homology modeling software Modeler was used to construct three-dimensional structural models of the variable region of each monoclonal antibody.
3. UCSF Chimera software was used to construct the 3D structure of HA.
4. Using Rosetta software, the HA protein was docked with 4 mAbs respectively, and the binding epitopes of 4 mAbs to HA protein were determined.

Distribution of amino acid sites on the HA crystal structure

PyMOL software were used to analyze the distribution of amino acid sites on the HA crystal structure. Swiss-Pdb-Viewer was used to generate a HA protein X-ray crystal texture model, and 3LZG (Protein Data Bank [PDB]), the crystal structure of HA from A_California_04_2009_H1N1pdm, which was similar (99%) to that of the antigen under study, was used as the reference. Subsequently, the distribution of the amino acid sites was determined using PyMOL according to the manufacturer's instructions.

Results

Screening and establishment of mAb cell line

MAbs were prepared by traditional method, and four mAbs against HA protein of 2009 H1N1 influenza virus were obtained, which were named H1-4, H1-75, H1-81 and A1-6, respectively. After resuscitation and resuscitation with liquid nitrogen and cryopreservation, the antibody secretion was detected by ELISA, and the characteristics were stable and unchanged.

Results of mAb characterization

The light chain of the four mAbs was κ chain, and the heavy chain was IgG subclass, among which three mAbs were HI positive (Table 1).
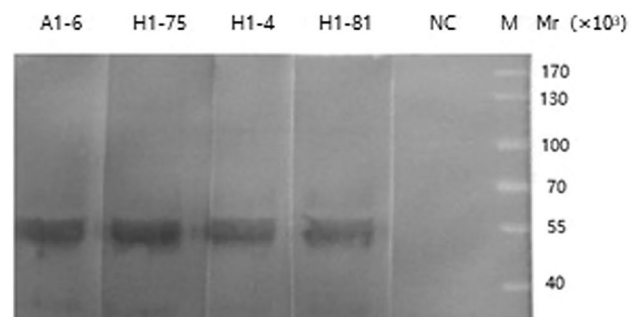
All four mAbs reacted with HA antigen

The four strains of mAb and H1N1 influenza virus split vaccines were identified by Western Blot. As shown in Fig. 1, the four mAbs showed specific reaction bands with a size of 55kD with HA of H1N1 influenza virus, indicating that

Fig. 1 Western-blot identification of the specificity of the 4 clones of mAbs with HA. *Lane 1* monoclonal antibody A1-6; *lane 2* monoclonal antibody H1-75; *lane 3* monoclonal antibody H1-4; *lane 4* monoclonal antibody H1-81; *lane 5* negative control; *lane 6* marker

the four mAbs were all mAb against HA protein of H1N1 influenza virus (Fig. 1).

Analysis of B cell epitopes on HA recognized by four mAbs

In this study, four mAbs were used as research tools to study the epitopes of HA protein on influenza virus. Four mAbs were labeled by HRP, and then competed with the other three mAbs to bind to the B cell epitopes of HA (Table 2). The inhibition rate of the interaction between H1-4 and A1-6 was more than 80%. The inhibition was obvious, indicating that the recognition sites may be the same. At the same time, the inhibition rate between H1-81 and H1-75 was also more than 80%, so it was believed that the two strains recognized the same epitopes. The epitopes on HA of influenza virus were divided into two groups by four mAbs.

However, when H1-4 was used as a competitive antibody and H1-81 as an enzyme-labeled antibody, the inhibition rate was 90%, indicating that the sites recognized may be the

Table 1 Identification of subclass and hemagglutination inhibition of mAbs against HA protein

mAbs	Ascites titer	subclass	HI
H1-4	$1:10^6$	IgG1	+
H1-75	$1:10^5$	IgG3	+
H1-81	$1:10^6$	IgG3	+
A1-6	$1:10^7$	IgG1	−

The ascites titers of 4 mAbs to HA antigen of 2009 H1N1 virus were detected by indirect ELISA. The ascites titers were all above $1:10^5$, showing good binding activity with the immunogen. In addition, "+" indicated that the mAb has hemagglutination inhibition activity, and "−" indicated that the mAb has no hemagglutination inhibition activity

Table 2 Inhibition rate between mAbs

HRP-labeled antibodies	Blocking antibodies			
	H1-4	H1-81	A1-6	H1-75
H1-4	100	46	97	39
H1-81	90	100	34	88
A1-6	81	66	100	34
H1-75	27	94	97	100

By blocking ELISA method, HA was wrapped as an antigen, and the mAbs were added as blocking antibodies, and then the mAbs labeled HRP were added one by one. The absorbance value was measured at OD450nm. According to the formula of inhibition rate, the inhibition rate between the two mAbs was greater than or equal to 80%, and the epitopes recognized by the two antibodies were consistent. If the value was less than or equal to 40%, it indicated that the identified epitopes between the two mAbs are inconsistent

same. When H1-81 was used as competitive antibody and H1-4 as enzyme-labeled antibody, the inhibition rate was 46%, and the inhibition was not obvious. The smaller the inhibition rate was, the farther the spatial position was, indicating that the recognized sites might be different. Similarly, when A1-6 was used as a competitive antibody and H1-75 as an enzyme-labeled antibody, the inhibition rate was 97%, indicating that the sites recognized by the two mAbs may be the same. When H1-75 was used as a competitive antibody and A1-6 as an enzyme-labeled antibody, the inhibition rate was 34%, indicating that the sites recognized by the two mAbs were different.

Detection of antigenic epitopes recognized by mAbs by sandwich ELISA

The antigenic epitopes recognized by H1-4 and H1-81 and A1-6 and H1-75 were detected by this method. As can be seen from Table 3-A, when H1-75 mAb was first coated, then the HA antigen of influenza virus was added, and finally the HRP-labeled A1-6 mAb was added, the OD_{450nm} value was 14.18, indicating that the recognition epitopes of these two mAbs were different, or the antigen epitopes recognized by A1-6 contained the antigen epitopes recognized by H1-75. On the contrary, the antigen was coated with A1-6, then the HA antigen was added to react with it, and finally the H1-75 labeled with HRP was added. The OD_{450nm} value was 1.21, which further indicated that the antigen epitope recognized by A1-6 may contain the epitope recognized by H1-75. Similarly, it can be seen from Table 3-B that the

Table 3 Results of double antibodies sandwich ELISA

Coated antibodies	HRP-labeled antibodies		Control antibodies
A	A1-6	H1-75	SP2/0
H1-75	14.18 ± 0.06	1.40 ± 0.01	1 ± 0.01
A1-6	1.21 ± 0.02	1.42 ± 0.01	1 ± 0.03
B	H1-4	H1-81	SP2/0
H1-81	15.91 ± 0.05	1.35 ± 0.01	1 ± 0.01
H1-4	1.37 ± 0.01	1.34 ± 0.01	1 ± 0.01

By indirect ELISA, mAbs were coated, followed by the addition of H1N1 influenza virus hemagglutinin antigen, and finally the addition of HRP-labeled mAbs. The absorbance value was measured at OD450nm with P/N ≥ 2.1 as the positive standard. P was the value of the experimental group, N was the value of the control group SP2/0. (*A*). H1-75 was coated and HRP-labeled A1-6 mAb was added, and the *P/N* value was greater than 2.1 (14.18), indicating that the two mAbs recognized sites were different; otherwise, the *P/N* value was less than 2.1 (1.42), indicating that there may be inclusion relationship or steric hindrance in the epitopes recognized by the two mAbs. (*B*). Similarly, the antigenic epitopes recognized by H1-81 and H1-4 were inconsistent, and there may be inclusion relationship or steric hindrance between them

epitopes recognized by H1-4 may contain those recognized by H1-81.

Amino acid sequence analysis of light and heavy chain variable regions of four mAbs

Molecular biological methods were performed to obtain amino acid sequences of the heavy and light chain variable regions (V_H and V_L) of mAbs H1-4, H1-75, H1-81 and A1-6. The V_H and V_L sequences of the four mAbs were listed in Table 4.

Based on the above four mAbs, the complementary determining region (CDR) and framework region (FR) of the heavy chain variable region and the light chain variable region of the four mAbs were analyzed by abYsis software. V_H and V_L contained three CDR, namely CDR1, CDR2, and CDR3, and four FR, namely FR1, FR2, FR3, and FR4 (Fig. 2a–d). Comparing the CDR and FR amino acid sequences of V_H and V_L of the four mAbs, it was found that there was no significant change in FR. However, the changes of amino acids in the three CDR of V_H and V_L were obvious, especially in CDR3. These results suggested that the four mAbs come from different cell clones.

Analysis of B cell epitopes of HA by computer simulation

According to the variable region amino acid sequences of mAb light chain and heavy chain, the B cell epitopes of HA were predetermined by computer model. As shown in Table 5, the four mAbs used different amino acid residues in the variable regions of light and heavy chains respectively to recognize two different antigenic sites on HA of 2009 H1N1 influenza virus, namely 135-T, 157-G, 158-N, 186-T, 187-S, 189-D, 192-S, 193-L and 48-G, 49-V, 57-C, 69-C, 70-E, 86-S, 273-P, 274-V. PyMOL and Swiss-PdbViewer were also used to analyze the distribution of these antigenic sites on the HA crystal structure. As shown in Fig. 3, 3LZG (PDB) was used as the reference structure, and the distribution of these antigenic sites recognized by mAbs on the HA trimer crystal structure was marked in red and purple, and all of them located in the head of HA.

Discussion

Vaccination is an effective means of preventing influenza epidemics. Epitopes prediction is an important tool to study the epidemic and pathogenicity of influenza and to develop vaccines (Zhang et al. 2018). Accurate prediction of potential epitopes, selection of protective epitopes and elimination of harmful epitopes are the key to improve vaccine protection and safety (Mccarthy et al. 2021, Guo et al. 2021).

Table 4 Amino acid sequences of heavy and light chain variable regions of four mAbs

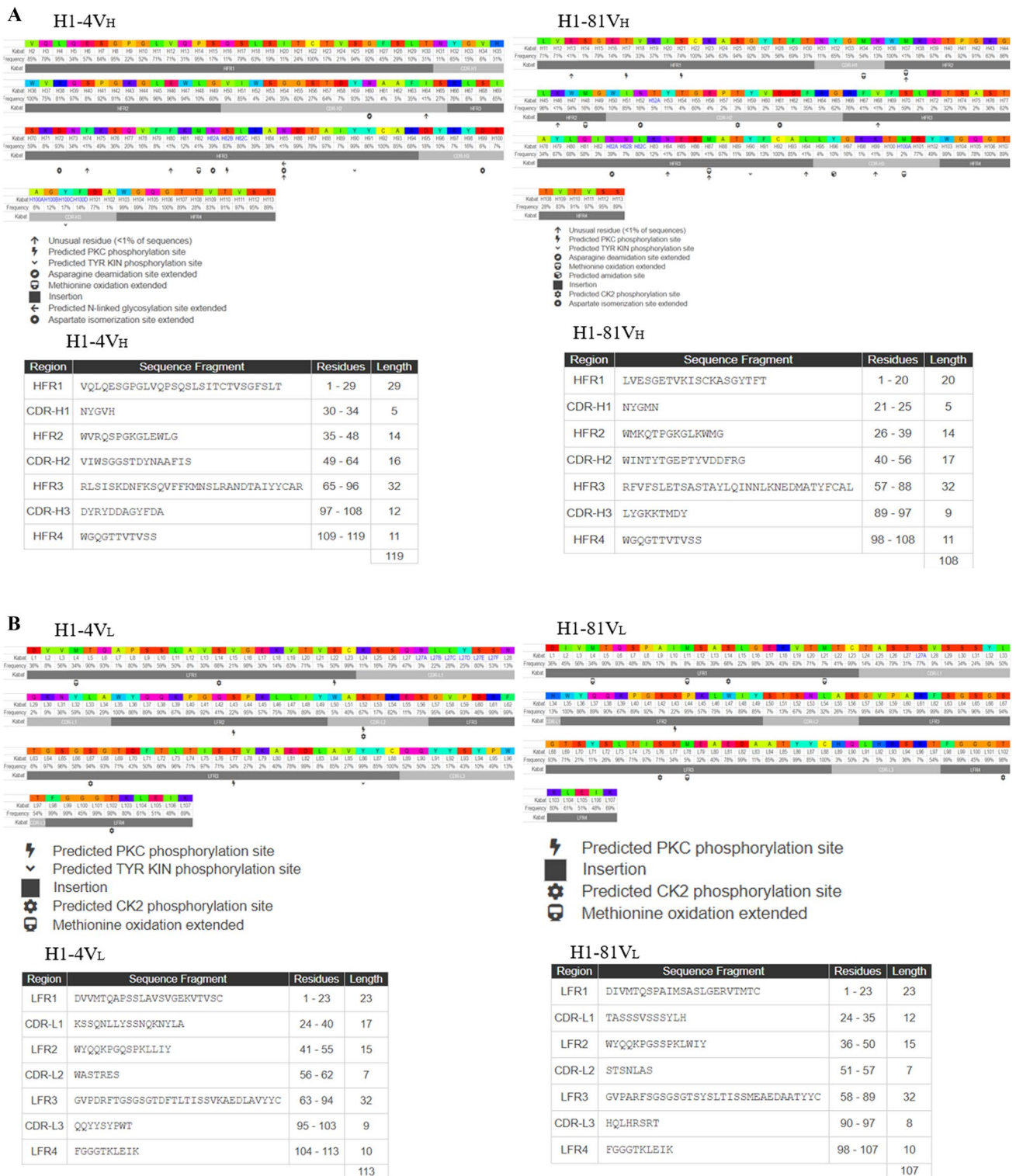| mAbs	Amino acid sequence
H1-4 V_H	VQLQESGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGSTDYNA AFISRLSISKDNFKSQVFFKMNSLRANDTAIYYCARDYRYDDAGYFDAWGQGTTVTVSS
H1-4 V_L	DVVMTQAPSSLAVSVGEKVTVSCKSSQNLLYSSNQKNYLAWYQQKPGQSPKLLIYWASTR ESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYSYPWTFGGGTKLEIK
H1-75 V_H	VQLEESGPELKKPGETVKISCKASGYSFTNYGMNWVKKTPGKDLKWMGWINTYTGEPTYA DDFKGRFAFSLESSPSAAYLQINNLKNEDMATYFCALLYGKKTMDYWGQGTTVTVSS
H1-75 V_L	DIVMTQSPAIMSASLGERVTMTCTASSSVSSSYLNWYQQKPGSSPRLWIYSTSNLASGVP PRFSGSGSGTSYSLTISSMEAEDAATYYCHQYHRSRTFGGGTKLEMK
H1-81 V_H	LVESGETVKISCKASGYTFTNYGMNWMKQTPGKGLKWMGWINTYTGEPTYVDDFRGRFVF SLETSASTAYLQINNLKNEDMATYFCALLYGKKTMDYWGQGTTVTVSS
H1-81 V_L	DIVMTQSPAIMSASLGERVTMTCTASSSVSSSYLHWYQQKPGSSPKLWIYSTSNLASGVP ARFSGSGSGTSYSLTISSMEAEDAATYYCHQLHRSRTFGGGTKLEIK
A1-6 V_H	VQLVESGPELKKPGETVKISCKASGYTFTNYGMNWVKQAPGKGLKWMGWINTNTGEPTYA EEFKGRFAFSVETSASIAYLQINNLKNEDMATYFCARGKADYWGQGTTVTVSS
A1-6 V_L	DIVMTQTAFSNPVTLGTSASISCRSSKSLLHSNGITYLYWYLLKPGQSPQLLIYQMSNLA SGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCVQNLELWTFGGGTKLEMK

According to the variable region genes of light chain and heavy chain of mouse antibody published by NCBI, a total of 27 primers were designed to amplify the variable region genes of light chain and heavy chain of 4 strains mAbs by PCR, and then translated into amino acids to provide data for computer prediction of antigen epitopes recognized by mAbs

Monoclonal antibody technology has been widely used in the study of epitopes and computer simulation technology can effectively simplify the process of epitope study (Kalita et al. 2021, Sesterhenn et al. 2020). The human monoclonal antibody C10 exhibited extraordinary cross-reactivity, potently neutralizing Zika virus (ZIKV) and the four serotypes of dengue virus (DENV1–DENV4) (Sharma et al. 2021). Ozger (Ozger et al. 2022) applied the new integrated fuzzy classification model to predict the B cell epitopes of SARS-COV-2. This prediction approach was useful for designing effective vaccines and drugs against future coronavirus families, especially SARS-COV-2 and its possible mutations.

In this study, four mAbs against HA protein were prepared using 2009 H1N1 virus split vaccine as the immunogen, among which three mAbs had hemagglutination inhibition activity, which had an important role in the study of influenza vaccine (Thi et al. 2018). Among them, A1-6 had no hemagglutination inhibition activity, so it was speculated that it might not have neutralizing activity. The titers of these 4 mAbs were above 10^{-5} (Table 1), indicating that these 4 mAbs may be derived from dominant epitopes on antigens. We used these 4 mAbs as research tools to predict HA epitopes. By ELISA blocking test, the inhibition rates of H1-4 and A1-6 were found to be above 80%, and there were differences in the amino acids in the CDRs region of light and heavy chains of these two mAbs, indicating that they were derived from different cell clones. These two different mAbs recognized an epitope and divided it into one group. The inhibition rate of H1-75 and H1-81 blocking each other was also above 80%, and they could not completely

block with the previous two mAbs, so they were classified into another group (Table 2). The amino acid sequences of the variable regions of H1-4 and H1-75 light and heavy chains were compared with those of the HA crystal template by computer simulation. It was found that the amino acid sites of the two mAbs were inconsistent in recognizing 2009HA, and the four mAbs were classified into two classes (Table 5). Therefore, the results of these two methods indicate that there were at least two types of epitopes on HA of H1N1 influenza virus. It shows that the prediction results of computer analysis and ELISA block experiment can verify each other, which avoids the subjective judgment factor in computer prediction and makes the prediction result more authentic.

In addition, different stacking sequence and different inhibition rate was appeared in blocking ELISA test. As can be seen from the binding site of the monoclonal antibody to the 2009 influenza virus, the site recognized by H1-81 was located in the site recognized by H1-4, and the site recognized by H1-75 was located in the site recognized by A1-6. The results of sandwich ELISA showed that H1-4 antibody could bind to HA epitopes when H1-81 was used as a coated antibody, followed by adding HA antigen and finally adding HRP-labeled H1-4 antibody. On the contrary, H1-4 was coated with HA antigen, and then HRP-H1-81 was added, so that H1-4 antibody could not bind to the HA epitope. From the latter, it could be seen that the two antibodies recognized the same epitope. The same was true for A1-6 and H1-75 (Table 3). Therefore, it was speculated that both H1-81 and H1-75 can cause allosterism of hemagglutinin antigen, resulting in that the

A

H1-4V_H

H1-81V_H

H1-4V_H

Region	Sequence Fragment	Residues	Length
HFR1	VQLQESGPGLVQPSQSLSITCTVSGFSLT	1 - 29	29
CDR-H1	NYGVH	30 - 34	5
HFR2	WVRQSPGKGLEWLG	35 - 48	14
CDR-H2	VIWSGGSTDYNAAFIS	49 - 64	16
HFR3	RLSISKDNFKSQVFFKMNSLRANDTAIYYCAR	65 - 96	32
CDR-H3	DYRYDDAGYFDA	97 - 108	12
HFR4	WGQGTTVTVSS	109 - 119	11
			119

H1-81V_H

Region	Sequence Fragment	Residues	Length
HFR1	LVESGETVKISCKASGYTFT	1 - 20	20
CDR-H1	NYGMN	21 - 25	5
HFR2	WMKQTPGKGLKWMG	26 - 39	14
CDR-H2	WINTYTGEPTYVDDFRG	40 - 56	17
HFR3	RFVFSLETSASTAYLQINNLKNEDMATYFCAL	57 - 88	32
CDR-H3	LYGKKTMDY	89 - 97	9
HFR4	WGQGTTVTVSS	98 - 108	11
			108

B

H1-4V_L

H1-81V_L

H1-4V_L

Region	Sequence Fragment	Residues	Length
LFR1	DVVMTQAPSSLAVSVGEKVTVSC	1 - 23	23
CDR-L1	KSSQNLLYSSNQKNYLA	24 - 40	17
LFR2	WYQQKPGQSPKLLIY	41 - 55	15
CDR-L2	WASTRES	56 - 62	7
LFR3	GVPDRFTGSGSGTDFTLTISSVKAEDLAVYYC	63 - 94	32
CDR-L3	QQYYSYPWT	95 - 103	9
LFR4	FGGGTKLEIK	104 - 113	10
			113

H1-81V_L

Region	Sequence Fragment	Residues	Length
LFR1	DIVMTQSPAIMSASLGERVTMTC	1 - 23	23
CDR-L1	TASSSVSSSYLH	24 - 35	12
LFR2	WYQQKPGSSPKLWIY	36 - 50	15
CDR-L2	STSNLAS	51 - 57	7
LFR3	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	58 - 89	32
CDR-L3	HQLHRSRT	90 - 97	8
LFR4	FGGGTKLEIK	98 - 107	10
			107

Fig. 2 Sequence analysis of the H- and L-chain variable regions of the four mAbs. **a** H1-4V_H and H1-81V_H; **b** H1-4V_L and H1-81V_L; **c** H1-75V_H and A1-6V_H; **d** H1-75V_L and A1-6V_L

antibody that should bind to the same epitope cannot bind to the same site due to allosterism of antigen. Reynolds et al. proposed allosteric database, which can be used to study and analyze the structure and function of allosteric proteins (Toyoshima et al. 2022). Lam et al. predicted epitopes and found that the conformation of the epitope
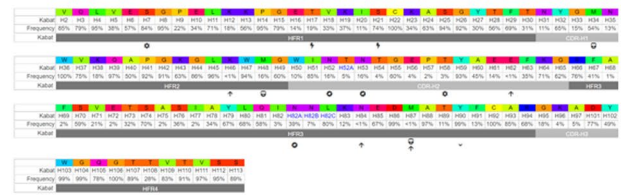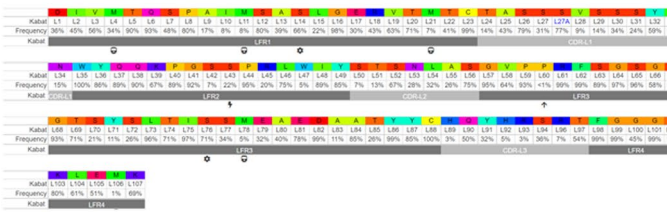
C

H1-75V$_H$
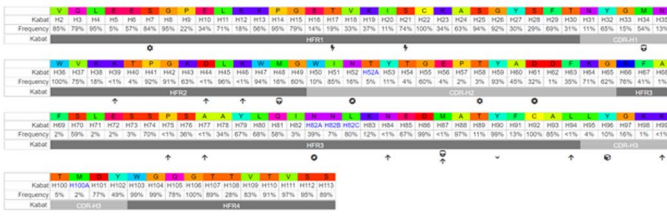
A1-6V$_H$

↑ Unusual residue (<1% of sequences)
⚡ Predicted PKC phosphorylation site
∨ Predicted TYR KIN phosphorylation site
⊙ Asparagine deamidation site extended
⊟ Methionine oxidation extended
⊜ Predicted amidation site
▪ Insertion
✿ Predicted CK2 phosphorylation site
○ Aspartate isomerization site extended

↑ Unusual residue (<1% of sequences)
⚡ Predicted PKC phosphorylation site
∨ Predicted TYR KIN phosphorylation site
▪ Insertion
✿ Predicted CK2 phosphorylation site
⊙ Asparagine deamidation site extended
⊟ Methionine oxidation extended

H1-75V$_H$

Region	Sequence Fragment	Residues	Length
HFR1	VQLEESGPELKKPGETVKISCKASGYSFT	1 - 29	29
CDR-H1	NYGMN	30 - 34	5
HFR2	WVKKTPGKDLKWMG	35 - 48	14
CDR-H2	WINTYTGEPTYADDFKG	49 - 65	17
HFR3	RFAFSLESSPSAAYLQINNLKNEDMATYFCAL	66 - 97	32
CDR-H3	LYGKKTMDY	98 - 106	9
HFR4	WGQGTTVTVSS	107 - 117	11
			117

A1-6V$_H$

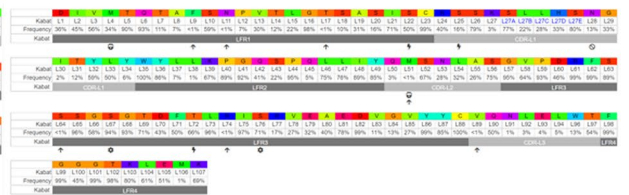| Region	Sequence Fragment	Residues	Length
HFR1	VQLVESGPELKKPGETVKISCKASGYTFT	1 - 29	29
CDR-H1	NYGMN	30 - 34	5
HFR2	WVKQAPGKGLKWMG	35 - 48	14
CDR-H2	WINTNTGEPTYAEEFKG	49 - 65	17
HFR3	RFAFSVETSASIAYLQINNLKNEDMATYFCAR	66 - 97	32
CDR-H3	GKADY	98 - 102	5
HFR4	WGQGTTVTVSS	103 - 113	11
			113

D

H1-75V$_L$

A1-6V$_L$

↑ Unusual residue (<1% of sequences)
⚡ Predicted PKC phosphorylation site
▪ Insertion
✿ Predicted CK2 phosphorylation site
⊟ Methionine oxidation extended

↑ Unusual residue (<1% of sequences)
⚡ Predicted PKC phosphorylation site
⊘ Asparagine deamidation site
▪ Insertion
✿ Predicted CK2 phosphorylation site
⊟ Methionine oxidation extended

H1-75V$_L$

Region	Sequence Fragment	Residues	Length
LFR1	DIVMTQSPAIMSASLGERVTMTC	1 - 23	23
CDR-L1	TASSSVSSSYLN	24 - 35	12
LFR2	WYQQKPGSSPRLWIY	36 - 50	15
CDR-L2	STSNLAS	51 - 57	7
LFR3	GVPPRFSGSGSGTSYSLTISSMEAEDAATYYC	58 - 89	32
CDR-L3	HQYHRSRT	90 - 97	8
LFR4	FGGGTKLEMK	98 - 107	10
			107

A1-6V$_L$

Region	Sequence Fragment	Residues	Length
LFR1	DIVMTQTAFSNPVTLGTSASISC	1 - 23	23
CDR-L1	RSSKSLLHSNGITYLY	24 - 39	16
LFR2	WYLLKPGQSPQLLIY	40 - 54	15
CDR-L2	QMSNLAS	55 - 61	7
LFR3	GVPDRFSSSGSGTDFTLRISRVEAEDVGVYYC	62 - 93	32
CDR-L3	VQNLELWT	94 - 101	8
LFR4	FGGGTKLEMK	102 - 111	10
			111

Fig. 2 (continued)

changed when the antigen reacted with the antibody, so the epitope was flexible (Nonet et al. 2022). In addition, amino acid residues with large surface polarity and strong hydrophilicity usually participate in the formation of epitopes,

so other experimental methods should be used to further verify (Manser et al. 2021).

At the same time, H1-4 and H1-81 as well as A1-6 and H1-75 found in this study adopted different stacking

Table 5 Key amino acid sites of the four mAbs recognizing the HA epitope

mAbs	Antibody binding site	2009 HA binding epitopes
H1-4	H: 25-G, 26-F, 27-S, 29-T, 30-N, 101-D	135-T, 157-G, 158-N, 186-T, 187-S, 189-D, 192-S, 193-L
H1-81	L: 62-S, 63-G, 64-V, 65-P H: 16-G, 17-Y, 18-T, 20-T, 21-N L: 57-S, 58-G, 59-V, 60-P, 61-A	135-T, 157-G, 158-N, 186-T, 187-S, 189-D, 192-S, 193-L
H1-75	H: 25-G, 26-Y, 27-S, 31-Y, 101-K, 106-Y L: 56-A, 57-S, 58-G, 59-V	48-G, 49-V, 57-C, 69-C, 70-E, 86-S, 273-P, 274-V
A1-6	H: 25-G, 26-Y, 27-T, 30-N, 31-Y, 97-R, 103-W, 104-G L: 53-I, 54-Y, 55-Q, 56-M	48-G, 49-V, 57-C, 69-C, 70-E, 86-S, 273-P, 274-V

The antigenic sites for antibody recognition were predicted by computer simulation, in which H1-4 bound to amino acid sites on 2009 H1N1 virus through the antibody binding sites in the table above, as well as H1-75, H1-81 and A1-6
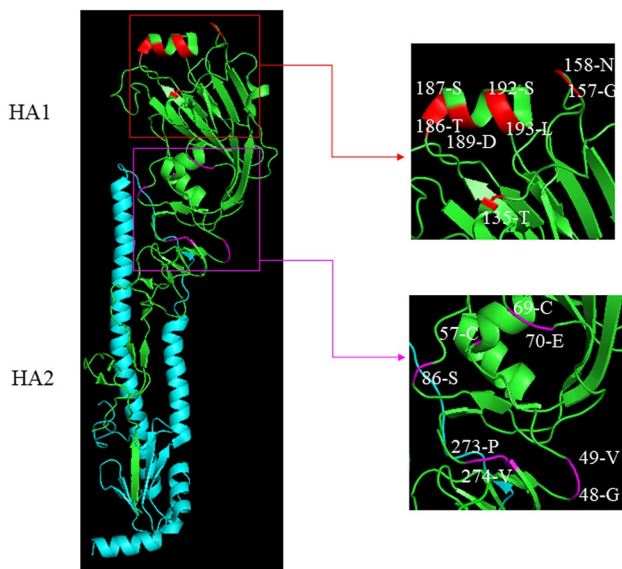
Fig. 3 Localization analysis of four mAbs against the HA antigen that recognize HA epitopes

sequences, resulting in different inhibition rates and the exclusion of antigen allosteria, which may also be due to the occurrence of steric hindrance. In recent years, steric hindrance has been widely and deeply studied in different research fields, explaining the interaction mechanism between atoms, molecules and proteins. Influenza A viruses cause seasonal epidemics and occasional but devastating pandemics and are a major public health problem. Du et al. (2021) developed A new type of anti-avian Influenza A virus (IAV) drug CBS1194 using steric hindrance effect, and CBS1194 showed a significant inhibitory effect at the early stage of virus infection. Escape mutant analyses and in silico docking further revealed that CBS1194 fits into a pocket near the fusion peptide, causing steric hindrance that blocks the low-pH induced rearrangement of HA. Nasrin et al. (2020) developed a tunable biosensor that regulates the distance between CdZnSeS/ZnSeS fluorescent quantum dots (QDs) and gold nanoparticles (AuNPs) using local surface plasmon resonance (LSPR) technology. The continuous binding of viruses on the peptide chain induced steric hindrance of LSPR behavior, which was used for influenza virus detection according to the fluorescence quenching index of quantum dots. Chen et al. (2019) reported a new mechanism of protection mediated by influenza hemagglutinin stalk-reactive antibodies, i.e., inhibition of neuraminidase activity by steric hindrance, blocking access of neuraminidase to sialic acids when it abuted hemagglutinin on whole virions.

In conclusion, the four mAbs that recognized two types of epitopes were predicted by computer to recognize epitopes on HA. The four mAbs bound to sites on HA of 2009 influenza virus using different amino acid combinations in light and heavy chains. There were two binding modes, which were as follows: 48-G, 49-V, 57-C, 69-C, 70-E, 86-S, 273-P, 274-V and 135-T, 157-G, 158-N, 186-T, 187-S, 189-D, 192-S, 193-L. By comparing the specific amino acid sites, it was found that the amino acid sites of HA binding mAbs were all distributed in the head of HA structure (Fig. 3), and the cluster of antigens in the head determined the antigenicity of the virus (Zost et al. 2021).

These antigenic sites of the H1N1 influenza virus, defined as Sa, Sb, Ca1, Ca2, and Cb. Liu et al. created mutant viruses to compare species-specific immune advantages of antigen sites in clinically relevant hemagglutinin. The results showed that Sb, Ca2 and Sa sites were immune dominant sites (Liu et al. 2018). Wilson isolated nine recombinant monoclonal antibodies from mouse memory B cells, all of which reacted to the HA1 region of HA but showed seven different binding footprints, each targeting four known antigenic sites (Wilson et al. 2015). Matsuzaki et al. screened 599 escape mutants using 16 monoclonal antibodies against influenza A H1N1 virus hemagglutinin and identified amino acid residues on Sa, Sb and Ca2 antigens by sequencing the mutated HA gene (Matsuzaki et al. 2014). The epitopes found in this study were different from the above results.

HA three-dimensional structure was composed of three HA monomers formed by non-covalent connection, which

was in the shape of a head–stem structure. The HA monomer was cleaved by protease in host cells into two subunits, HA1 (16-345aa) and HA2 (346-519aa). HA1 formed a spherical head domain, which contained receptor binding sites and five major antigen sites, and was the main antigenic determinant recognized by neutralizing antibodies (Kuenstling et al. 2018). The researchers successfully expressed HA(1-330 aa) and HA(63-286 aa) globulin domains with different lengths in Escherichia coli. After immunizing animals, they could induce the body to produce a high level of neutralizing antibodies, which had a protective effect in vivo against virus attack (Khurana et al. 2010; Taylor et al. 2011). Kuenstling used prokaryotic expression methods to express HA head fragments (HA11-326aa and HA153-269aa), and results showed that both fragments could induce neutralizing antibody production (Kuenstling et al. 2018). Therefore, the two epitopes found in this study were both within the range of HA1 and are expected to be candidate epitopes for influenza vaccine, which are worthy of further study.

Declarations

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