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Peptide mimics of a conserved H5N1 avian influenza virus neutralization site

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A panel of 52 murine monoclonal antibodies was found to recognize antigenic determinants that had been conserved among all major genetic subgroups of the H5N1 avian influenza virus prevalent since 1997. We screened a phage display library for peptides recognized by one such antibody (8H5). We analysed the specificity of 8H5 for reactive peptides presented as fusion proteins of HBc (hepatitis B core protein) and HEV (hepatitis E virus) structural protein, p239. This was then related to the specificity of the native HA (haemagglutinin) molecule by virtue of the capacity of fusion proteins to compete for 8H5 binding with different strains of H5N1 virus and the reactivity of antisera generated against fusion proteins to bind native HA molecules, and to inhibit haemagglutination and arrest infection by the virus. Nine reactive peptides of different amino acid sequences were identified, six of which were also reactive with the antibody in association with HBc and four were in association with p239. Binding occurred with the dimeric form of the four p239-fusion proteins and one of the HBc-fusion proteins, but not with the monomeric form. The HBc-fusion proteins blocked 8H5 binding with four strains of H5N1 influenza virus. Mouse antisera generated against fusion proteins bound to HA molecules, but did not inhibit haemagglutination or arrest H5N1 infection. Our findings indicate that 8H5 recognizes discontinuous sites presented by secondary and possibly higher structural orders of the peptides in spatially favourable positions for binding with the antibody, and that the peptides partially mimic the native 8H5 epitopes on the H5N1 virus.

Key words: anti-H5 monoclonal antibody, H5N1 avian influenza virus, haemagglutination, hepatitis virus, influenza, peptide mimic.

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INTRODUCTION

The H5N1 avian influenza virus normally circulates among aquatic birds, but is highly pathogenic for territorial birds and causes fatal infections in humans. H5N1 influenza viruses began to spread from late 2003, causing disease outbreaks in China, Japan, South Korea, Thailand, Vietnam, Indonesia, Cambodia, Malaysia and Laos. This resulted in extensive loss of chickens, ducks and geese [1]. Recently, H5N1 infection of humans has again been detected in many countries around the world. Of 346 confirmed cases, 213 were fatal (http://www.who.int/csr/disease/avian_influenza/country/en/). Although there is no evidence for transmission among humans to date, the virus has evolved rapidly, raising the threat that the virus may become adapted to humans, causing an influenza pandemic.

Vaccination is considered the most effective preventive measure to control influenza. Various approaches have been used to generate an H5N1 vaccine for human immunization [3–9], but efficacy of the vaccine against the pandemic strain is uncertain. Vaccines based on M2e (matrix protein 2 ectodomain) and NA (neuraminidase) might be more cross-reactive than those based on variable HA (haemagglutinin) [10–12].

We have generated over 200 mAbs (monoclonal antibodies) by successively immunizing mice with different genetic subgroups of the H5N1 virus. In line with studies using conventional ferret antisera highlighting antigenic diversity of the virus population [13,14], over 70 % of these antibodies were found to exhibit a distinct and restricted reactivity spectrum against different virus strains, but, unlike conventionally produced antisera, a substantial proportion of mAbs exhibited broad reactivity against the virus, presumably because of repeatedly boosting the antibody response of the animals to common antigenic determinants shared by different immunizing virus strains [15]. We showed further that 52 of these antibodies were capable of inhibiting haemagglutination mediated by 42 strains belonging to ten major genetic groups of H5N1 virus prevalent since 1997, exhibiting broad-spectrum neutralizing activity against these viruses, and one of these antibodies was tested and found to protect mice against these viruses even at late stages of infection [16]. These findings suggest that these broad-spectrum anti-H5N1 antibodies may recognize conserved antigenic determinants associating with neutralization sites of H5N1 virus, and, as such, these conserved determinants are potential targets for broad-spectrum intervention of H5N1 avian influenza.

The technology of epitope-based vaccine has evolved rapidly in the recent years (see [17] for a review). The immune responses to pathogens induced by native epitope and mimotope have been applied in the research of many diseases, including tumours, HIV and HCV (hepatitis C virus) [18–22]. In order to elucidate the conserved site in the HA molecules of H5N1 virus, we screened a phage display library of combinatorial 12-mer peptides for peptide reactive with a pan-H5 mAb, 8H5. The antibody has been shown to be reactive against all of the major genetic groups of H5N1 viruses prevalent since 1997, and it was found to neutralize different

Abbreviations used: gp120, glycoprotein 120; HA, haemagglutinin; HBc, hepatitis B core protein; HEV, hepatitis E virus; HI, haemagglutination inhibition; HRP, horseradish peroxidase; LB, Luria–Bertani; mAb, monoclonal antibody.

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Table 1 Murine mAbs

Six murine mAbs were used in the present study. Five were generated against the H5N1 influenza virus [15,16] and one was generated against HEV [23], as indicated.

mAb	Ig subtype	Specificity
8H5	IgG2a	H5N1 influenza virus
4D1	IgG1	H5N1 influenza virus
8G9	IgG2b	H5N1 influenza virus
10F7	IgM	H5N1 influenza virus
2F2	IgG1	H5N1 influenza virus
8C11	IgG1	HEV

H5N1 virus in a cell-based neutralization assay [16]. We showed that some of these 8H5-reactive peptides are antigenically related to native HA molecules of the H5N1 virus.

MATERIALS AND METHODS

Virus strains

The H5N1 influenza virus strains, Chicken/Hong Kong/YU22/2002 (YU22), Rosybilled Pochard/Hong Kong/821/2002 (HK821), Chicken/Jiangxi/6151/ 2003 (6151) and Indonesia/2A/2004 (2A), were provided by State Key Laboratories of Emerging Infectious Diseases, Department of Microbiology, University of Hong Kong. The Sf21 insect cell line was purchased from Invitrogen.

Monoclonal antibodies

We used six murine mAbs in the present study. Five were generated against the H5N1 influenza virus and one was generated against HEV (hepatitis E virus) (Table 1). As described previously [15], the H5N1-specific antibodies were generated by successively immunizing Balb/c mice with five strains of H5N1 avian influenza virus representing different major genetic subgroups of the virus prevalent since 2002 and found to have caused human infection, and all four of the antibodies, including 8H5, exhibit broad but distinct H5 specificity [16]. The HEV-specific antibody was generated against a recombinant structural protein of HEV as described by Zhang et al. [23]. The antibodies were produced as ascetic fluids, purified by ammonium sulfate precipitation followed by DE52 column chromatography, and stored in aliquots at −20 °C until use. Immunoglobulin subtypes of the antibodies were determined using mAb-isotyping reagent (BD Biosciences).

Screening of phage library for 8H5-reactive peptides

A combinational 12-mer peptide phage display library (New England Biolabs) was used to screen for peptides that bind to 8H5 mAb, according to the manufacturer's instructions. Briefly, 2×10^{11} phage particles and 300 ng of mAb were mixed and incubated at room temperature (27 °C) for 20 min. Then, 50 μl of Protein A-conjugated magnetic beads (GE Healthcare) was added to the mixture, and the beads were washed three times to remove unbound phage. 8H5-bound phage was eluted and propagated in *Escherichia coli* cells (ER2738) cultured in LB (Luria–Bertani) medium. After three rounds of panning, bound phage was eluted and cloned by mixing serially diluted aliquots of phage with *E. coli* and plating the mixtures on LB/IPTG (isopropyl β-D-thiogalactoside)/X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) plates. Resulting plaques consisting of cloned phage were randomly picked and propagated and tested by ELISA for binding with 8H5 and control antibodies. Inserts encoding the peptides were isolated from 8H5-reactive phage using the phage ssDNA (single-stranded DNA) isolation reagent kit (Omega) and sequenced by Bioasia.

Fusion proteins carrying 8H5-reactive peptides

The sequences encoding the 12-mers were linked to the C-terminus of the gene encoding the HEV structural protein p239 [24] or inserted to positions 79 and 80 of the residue 1–149 fragment of HBc (hepatitis B core protein) [25,26]. The resulting chimaeric constructs were inserted to the pT0-T7 expression vector and expressed in *E. coli* as described previously [23]. p239-fusion proteins were recovered in the pellet of cell lysates, treated with 2 % (v/v) Triton X-100 at 37 °C for 30 min and then dissolved in 8 M urea. The proteins were renatured by dialysis against PBS (pH 7.45) at room temperature. The HBc-fusion protein was recovered from the supernatants of cell lysates. The fusion proteins were precipitated at 20%-satd. ammonium sulfate, resuspended in carbonate/bicarbonate buffer containing 5 % (w/v) 2-mercaptoethanol, by shaking at 37 °C for 30 min, and dialysed in phosphate buffer (pH 7.4). The resulting purified fusion proteins were kept at 4 °C until use.

Fusion proteins were examined by negative-stain electron microscopy. Protein samples were applied to a carbon-coated grid, and excess fluid was removed. Protein samples were stained with 2 % (w/v) uranyl acetate and examined using an F30 transmission electron microscope (Phillips) operating at 200 kV.

Immunoassays

ELISA for phage particles

Approx. 10^9 plaque-purified phages were added to microplates coated previously with 10 μg/ml 8H5 mAb, and control plates were coated with the same concentrations of three different antibodies (4D1, 10F7 and 8C11). After incubation at 37 °C for 1 h, plates were washed three times and incubated further at 37 °C for 30 min with a M13-phage-specific HRP (horseradish peroxidase)-conjugated antibody (GE Healthcare) at 1:5000 dilution. The plates were allowed to react with TMB (3,3′,5,5′-tetramethylbenzidine) liquid substrate system and read on a microplate reader.

ELISA test for fusion proteins

Purified fusion proteins (10 μg/ml in phosphate buffer, pH 7.4) were coated on to 96-well microplates at 37 °C for 2 h. The plates were blocked with ED buffer [PBS, pH 7.4, containing 2 % gelatin, 0.5 % casein and 0.1 % ProClin 300 (Sigma–Aldrich)], washed and incubated with 4 μg/ml 8H5 or control antibodies at 37 °C for 1 h. Then, 100 μl of goat anti-(mouse IgG) conjugated to HRP (1:10 000 dilution) was added to each well and incubated at 37 °C for 30 min. Colour development was as described above.

Competitive ELISA test

The 8H5 mAb at a concentration of 10 μg/ml in phosphate buffer (pH 8.0) was coated on to 96-well plates at 37 °C for 2 h. The plates were washed once, followed by blocking with ED buffer. Then, the purified fusion proteins (10 μg/ml) and H5 avian influenza virus (4HA of YU22 or HK821, 8HA of 6151 or 2HA of 2A) were added to the wells together and incubated at 37 °C for 30 min. HBc antigen protein was used as a negative control. 2F2–HRP (1:500 dilution) against H5 avian influenza virus was used as the secondary antibody.

Western blotting

The purified fusion proteins were resolved by SDS/PAGE (12 % polyacrylamide gel), transferred on to a nitrocellulose

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membrane, and reacted with 8H5 mAb at room temperature for 1 h. The membrane was then treated with goat anti-(mouse IgG) conjugated to HRP (1:10 000 dilution) and then developed with AEC (3-amine-9-ethylcarbazole) (single solution; Zymed Laboratories) to reveal protein bands.

Antisera against HBc-fusion proteins

The purified chimaeric HBc proteins were emulsified with an equal volume of complete Freund's adjuvant and primed Balb/c mice were injected subcutaneously at multiple sites at the dose of 100 μg of protein per mouse. A booster immunization using an incomplete Freund's adjuvant was given every other week. Blood was collected from the mice's eyes, and the antiserum was separated from clotted blood and stored at -4 °C for future use. All animal experiments were conducted in compliance with the humane and ethical treatment of experimental animal rules and regulations required by the Chinese government.

Flow cytometry

FACS analysis was carried out to detect the reaction of mouse antiserum with HA of YU22 virus that was expressed on Sf21 cells, using a FACScan flow cytometer (Beckman Coulter) with the FL1 detector channel. The data were analysed with Expo32 ADC Analysis software (Beckman Coulter).

The Sf21 cells were infected by recombinant baculovirus containing the HA gene of YU22 virus, incubated for 36 h, and were compared with negative control Sf21 cells without HA gene infection. Up to 10^6 cells/sample were collected, washed with PBS and blocked with goat serum for 30 min. The cell suspensions were then incubated with mouse serum for 1 h. After washing, the cell suspensions were incubated with a FITC-conjugated goat anti-(mouse Fc) secondary antibody (Sigma–Aldrich) for 30 min and washed again before FACS analysis. All procedures were performed at room temperature. Results are presented as peak graphs and the percentage of positive cells.

HI (haemagglutination inhibition) test

The HI test was performed to assess the reactivity of antiserum against H5N1 isolates, using the method described in [27]. All polyclonal antisera were treated with a receptor-destroying enzyme (Denka Seiken) before testing. The HI test was started at a 1:40 dilution for polyclonal antiserum and 1:100 dilution for mAb and adsorbed with a 0.5 % suspension of chick red blood cells.

RESULTS

Identification of 8H5-reactive 12-mer peptides

We screened for 8H5-binding peptides by panning a phage display library of combinational 12-mer peptides with the antibody. A total of 190 plaques were picked from the plates, propagated individually and tested in triplicate by ELISA for binding with 8H5 and three control antibodies, two of which (4D1 and 10F7) were other pan-H5 mAbs and one (8C11) is an HEV-specific mAb (Figure 1). Specific 8H5 binding is indicated when mean 8H5 binding exceeds that concurrently obtained with the control antibodies at least 3-fold. Table 2 showed that 69 (36 %) of 190 clones of phage (plaques) tested were 8H5-reactive. DNA sequencing of inserts from these phage isolates distinguished nine reactive peptides of different amino acid sequences. This suggests that the antibody recognizes discontinuous amino acid residues of these peptides. It was noted that four amino acid residues, namely threonine, leucine, threonine and leucine, were preserved in the same positions 2, 4, 5 and 9 respectively in amino acid sequences of six of these peptides, and that the same peptides together accounted for 63 (95 %) of 69 clones of 8H5-reactive phages isolated, including the most frequently isolated carrying peptides 124 and 125.

Table 2 Identification of 8H5-reactive peptides isolated from the phage display library

A total of 190 randomly picked clones of screened phage were individually tested by ELISA for 8H5 binding as described in Figure 1. DNA sequences of inserts from 69 clones of phage that were found to be reactive distinguished nine different peptides. Note that the four amino acid residues underlined, namely threonine, leucine, threonine and leucine, were preserved in positions 2, 4, 5 and 9 of six of these peptides, which together accounted for 63 of the 69 8H5-reactive phage clones.

Peptide	Amino acid sequence	Frequency of isolation
122	ETQLTTAGLRLL	9/190
123	ETPLTETALKWH	8/190
124	QTPLTMAALELF	23/190
125	DTPLTTAALRLV	17/190
128	QTPLTETALKWH	4/190
129	QTPLTMAALELL	2/190
130	HLQDGSPPSSPH	2/190
131	GHVTTLSLLSLR	2/190
133	ETPLTEPAFKRH	2/190

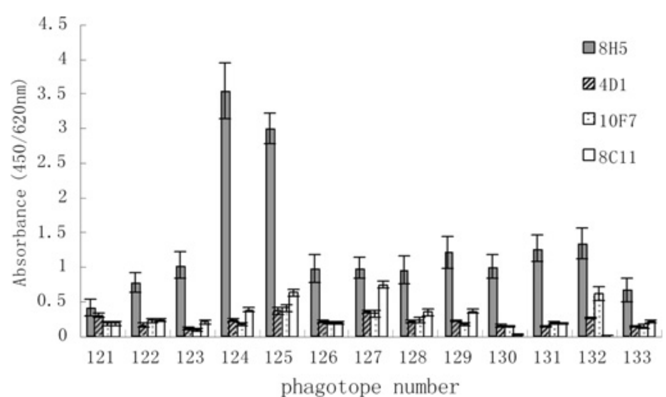

Figure 1 Identification of 8H5-reactive phagotopes

A combinatorial 12-mer phage display library was screened for 8H5-reactive phagotopes. Screened phage was cloned and tested by ELISA for binding with 8H5 and three control antibodies, consisting of two other H5-specific antibodies (4D1 and 10F7) and an HEV-specific antibody (8C11). Assays were carried out in triplicate and results are means $\pm$ S.D. Phage was considered to be 8H5-reactive when mean absorbance values obtained with this antibody exceeded at least 3-fold that of the control antibodies.

8H5 binding by 12-mer peptides associated with carrier proteins

Fusion proteins of HBc and p239 carrying different 8H5-reactive peptides were produced as described in the Materials and methods section. Preliminary studies showed that the p239-fusion proteins occurred as 23 nm particles and HBc-fusion proteins as 30 nm particles, and both were dissociated mainly into dimeric and monomeric forms after they had been subjected to SDS/PAGE (results not shown). The fusion proteins were tested for 8H5 binding by ELISA conducted under non-denaturing conditions and by Western blotting, where the fusion proteins were first subjected to SDS/PAGE. ELISA conducted under non-denaturing conditions showed that four p239-fusion proteins carrying peptides 122, 123, 125 and 129, and six HBc-fusion proteins carrying these and peptides 124 and 128 were reactive with 8H5 (Figure 2). In

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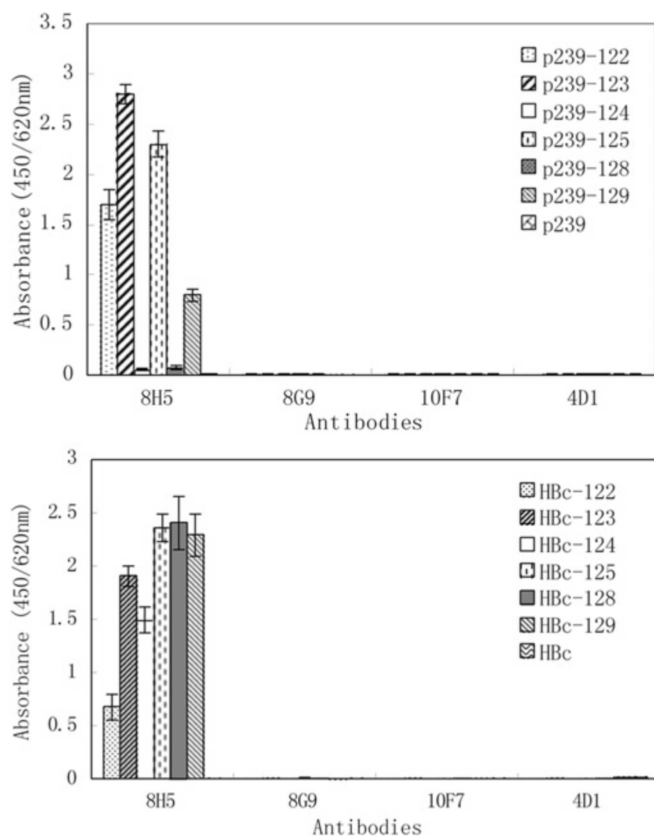

Figure 2 Binding of 8H5 and control antibodies to p239- and HBc-fusion proteins

Fusion proteins of p239 and HBc antigen carrying the different peptides, together with the parental p239 and HBc antigen, were tested by ELISA for binding with 8H5 and other pan-H5 mAbs. Assays were carried out in triplicate, and results are shown as means $\pm$ S.D. absorbance.

Western blotting (Figure 3), p239-fusion proteins were resolved by SDS/PAGE mainly as dimeric and monomeric forms, with the dimeric form being dominant (top-left-hand panel). HBc-fusion proteins were likewise resolved as dimeric and monomeric forms, with the latter being the more abundant (bottom-left-hand panel). 8H5 reactivity was detected in association with the dimeric form of three p239-fusion proteins (top-right-hand panel) and one of the HBc-fusion proteins (bottom-right-hand panel). It was noted that the monomeric forms of either types of fusion proteins were not reactive with the antibody.

As summarized in Table 3, nine 8H5 reactive peptides were identified from the phage display library. Six of these peptides were also reactive when presented as HBc-fusion proteins and four were reactive as p239-fusion proteins. The reactivity was detected in association with native and most probably particulate form of the fusion proteins by ELISA under non-denaturing conditions. After being dissociated into dimeric and monomeric forms during Western blotting, the reactivity was located to the dimeric form of three p239-fusion proteins and one HBc-fusion protein, whereas the monomeric form of either fusion proteins did not exhibit detectable binding with the antibody. These findings infer that recognition by 8H5 is based on folding and possibly higher structural orders of these peptides.

Mimicry of native 8H5-binding sites by 12-mer peptides

The HBc-fusion proteins were characterized further according to their capacity to compete with avian influenza viruses for binding with 8H5 (Figure 4). In these experiments, 10 μg/ml of the fusion proteins was mixed with different strains of H5N1 virus, and the mixture was added to microplates coated previously with 1 μg/ml 8H5, and, after incubation for 30 min, the amount of virus bound was determined by ELISA using another anti-H5N1 mAb (2F2) labelled previously with HRP. Compared with results obtained concurrently with viruses alone, the presence of fusion proteins was found to reduce the amount of virus bound to 8H5-coated plates by 25–85 %, whereas HBc alone did not affect virus binding.

To analyse further the relationship between 8H5 specificity of peptides and native HA, mouse antisera were raised against HBc-fusion proteins. Antigenic specificity of the antisera was evaluated by cross-titration against p239 and different p239-fusion proteins (Table 4). The results show that each antiserum was reactive against a p239-fusion protein carrying the homologous peptide and variously reactive against the other fusion proteins, but was not reactive against the parental p239. Similarly to Figure 2, 8H5 was reactive with p239-bearing peptides 122, 123, 125 and 129, but not those bearing peptides 124 and 129. Antisera against fusion proteins carrying peptides 123 or 128 exhibited broad cross-reactivity against heterologous peptides, and anti-122 was the least cross-reacting. These findings suggest that the antisera are reactive against homologous peptides carried by the respective fusion proteins and variously cross-reacting against other heterologous peptides. However, the cross-reactivity patterns of anti-peptide sera did not discriminate between fusion proteins that are reactive and not reactive with 8H5.

Nevertheless, the antisera were reactive against Sf21 cells transfected with the HA gene of the YU22 strain of H5N1 virus, but not the parental cells, whereas antisera raised against HBc alone was not reactive against either the transfected or the parental cells (Figure 5). On the other hand, the antisera did not cause haemagglutination inhibition mediated by H5N1 avian influenza virus, nor neutralization (results not shown).

DISCUSSION

We have generated 52 broad-spectrum H5-specific mAbs, which exhibit HI reactivity against all ten major genetic groups of H5N1 avian influenza virus prevalent since 1997, and virtually all of these antibodies tested also showed broad-spectrum neutralizing activities against these viruses [15,16]. One of these antibodies was shown further to protect mice against different groups of the virus, even when the antibody was administered after the infection has disseminated to different organs [16]. The sites recognized by these neutralizing and/or HI antibodies are referred to as neutralization epitopes or HI epitopes, and, as such, they constitute potential targets for intervention of a wide variety of H5N1 avian influenza viruses. The structure of the neutralization or HI sites is not known. They are operationally defined as those which are recognized by neutralizing or HI antibodies, and binding of the latter to the sites would arrest infection or effect HI, presumably by blocking binding of the virus to its receptors on host cells or to the red blood cells.

The present study aims to identify peptides that mimic the neutralization site recognized by one of the broad-spectrum anti-H5 mAbs, 8H5. This was achieved by repeatedly panning a combinatorial 12-mer peptide phage display library with this antibody. The screened phage was tested individually by ELISA to determine the reactivity of each with 8H5. Three antibodies were included as controls in these studies; two were broad-spectrum anti-H5 mAbs, similar to 8H5, and one is specific for HEV, unrelated to the anti-H5N1 avian influenza virus antibodies. Phage

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Table 3 8H5 reactivity of 12-mer peptides associated with different carriers

Peptides carried by phage particles or particulate forms of p239 or HBc were tested for 8H5 binding by ELISA (see Figures 1 and 2) and that of the peptides carried by the monomeric and dimeric forms of p239 and HBc was tested by Western blotting (see Figure 3). [+] indicates positive binding results; [−] negative binding results; nd, not detected.

Carrier		8H5 binding with peptides						
Type	Structure	122	123	124	125	128	129	130,131,133
Phage	Particulate	[+]	[+]	[+]	[+]	[+]	[+]	[+]
p239	Particulate	[+]	[+]	[−]	[+]	[−]	[+]	[−]
	Dimeric	[+]	[+]	[−]	[+]	[−]	[−]	nd
	Monomeric	[−]	[−]	[−]	[−]	[−]	[−]	nd
HBc antigen	Particulate	[+]	[+]	[+]	[+]	[+]	[+]	[−]
	Dimeric	[−]	[−]	[−]	[+]	[−]	[−]	nd
	Monomeric	[−]	[−]	[−]	[−]	[−]	[−]	nd

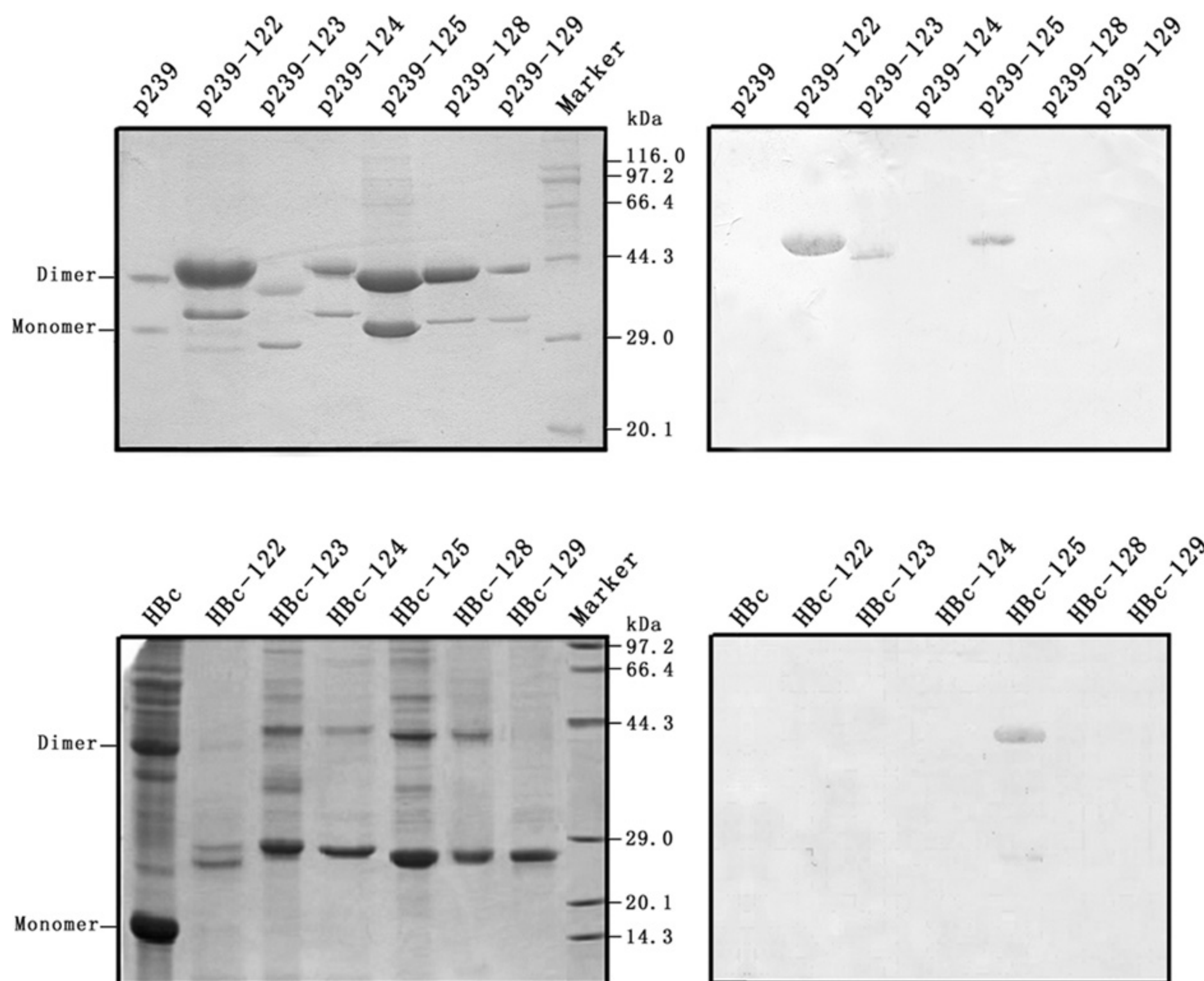

Figure 3 Western blot analysis of p239- and HBc-fusion proteins

Fusion proteins of p239 (top panels) and HBc (bottom panels) described in Figure 2 were subjected to SDS/PAGE (12 % polyacrylamide gel) and Coomassie-stained (left-hand panels), transferred on to a nitrocellulose membrane, and reacted with 8H5 mAb (right-hand panels). The amounts of samples loaded to the gel was 5–20 μg of E2 and E2-fusion protein and 6–20 μg of HBc and HBc-fusion protein. Molecular masses are indicated in kDa.

was considered to be specifically reactive with 8H5 when the mean level of binding with this antibody exceeded at least 3-fold the values obtained concurrently with all three control antibodies. Peptides reactive with 8H5 were identified by sequencing the respective inserts from the phage clones, expressed and purified as HBc-fusion and HEV structural protein (p239). Antibody binding to the fusion proteins was determined by ELISA under non-denaturing conditions, where the fusion proteins occurred in particulate form, and by Western blotting after fusion proteins had been resolved mainly into dimeric and monomeric forms by SDS/PAGE. The relationship between the 8H5-specificity of the peptides and native H5 molecule was assessed according to the capacity of the fusion protein to compete with H5N1 virus for binding with the antibody and that of antisera generated

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Table 4 Cross-titration of anti-peptide sera

Anti-peptide sera generated by immunizing mice with HBc-fusion proteins carrying different peptides and 8H5 were tested by ELISA in triplicate against p239 and p239-fusion proteins carrying these peptides. Binding is shown as mean absorbance values. Note that anti-peptide sera were reactive against homologous peptides and variously reactive against heterologous peptides, but not against p239, whereas 8H5 was reactive with p239-fusion proteins bearing four of the peptides, similarly to Figure 2.

	Anti-peptide sera (mean absorbance)						
Peptide	122	123	124	125	128	129	8H5
122	1.6	0.337	0.06	0.199	0.361	0.06	1.7
123	0.007	1.0	0.012	0.023	0.695	0.009	2.8
124	0.023	0.134	1.4	0.078	0.308	0.199	0.06
125	0.186	0.971	0.128	1.2	1.844	0.297	2.7
128	0.007	0.421	0.095	0.359	0.9	0.113	0.03
129	0.195	0.965	2.525	1.01	1.902	2.7	0.8
p239	0.0005	0.003	0.008	0.004	0.006	0.007	0.004

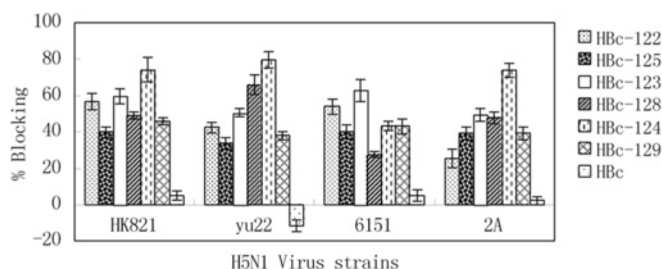

Figure 4 Fusion proteins compete with H5N1 virus for 8H5 binding

A 10 μg/ml concentration of HBc carrying different peptides or the parental HBc protein was mixed with H5N1 avian influenza virus (HK821 and YU22 at 4HA, 6151 at 8HA and 2A at 2HA). The mixtures were added to microtitre plates coated previously with 1 μg/ml 8H5. The plates were incubated for 30 min and washed, and the amount of viruses bound to 8H5 was determined using another anti-H5N1 mAb, 2F2–HRP. Experiments were carried out in triplicate and results are means $\pm$ S.D. Mean residual virus bound, [R], was calculated, where R = mean virus bound (absorbance) in the presence of fusion protein/mean virus bound in the absence of fusion protein. Mean percentage blocking of virus binding by fusion protein = 100−R.

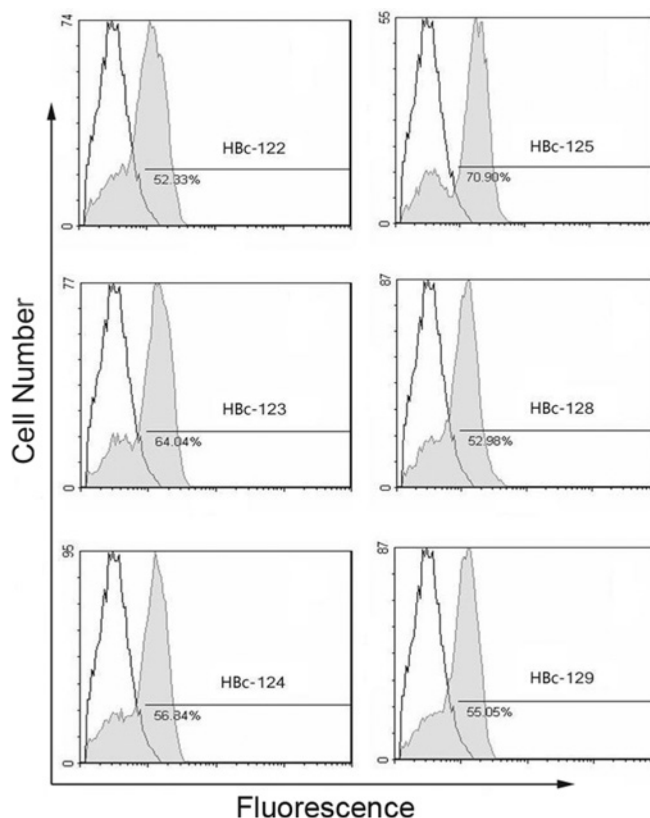

Figure 5 Reactivity of HBc-fusion protein antisera against HA of H5N1 virus

Sf21 cells expressing HA of YU22 strain of H5N1 virus were reacted with antisera generated against different HBc-fusion proteins as indicated (shaded) or anti-HBc (not shaded) and examined by flow cytometry.

against the fusion proteins to bind native HA molecule and block haemagglutination and arrest infection by the virus.

Results show that 69 of 190 randomly tested clones of the screened phage are 8H5-reactive. DNA sequencing of inserts from the reactive phage clones identified nine peptides of different amino acid sequences. It was noted that six peptides which accounted for 95 % (63/69) of all peptides identified share the same feature that they all have the amino acid residues threonine, leucine, threonine and leucine preserved in the same positions, 2, 4, 5 and 9, in the respective sequences. The same six peptides, but not the other three, were reactive with 8H5 when they were associated with native form of HBc, and four of which were reactive in association with native form of p239. Western blotting shows that 8H5 reactivity of three peptides was preserved in the dimeric form p239 and that of one peptide was preserved in dimeric form of HBc, but the reactivity of the peptides was abrogated after the fusion proteins had been dissociated into their respective monomeric forms. These findings suggest that 8H5 recognizes discontinuous amino acid residues presented by secondary, or possibly higher, structural orders of the peptides in spatially favourable positions for binding to occur, and it is possible that these may include some or all of the amino acid residues threonine, leucine, threonine and leucine locating to positions 2, 4, 5 and 9 respectively of the peptides. This interpretation would be consistent with the findings that each of the reactive peptides have distinct amino acid sequences, recognition of which by 8H5 depends on the type of carrier molecule and their respective tertiary and higher structural orders, and that binding did not occur with the monomeric form of the fusion proteins.

The relationship between 8H5-specificity of the native HA molecule and the peptides was investigated. The results show that HBc-fusion proteins bearing the peptides competed with different strains of H5N1 virus for 8H5 binding and that anti-peptide sera bind to native HA molecules expressed by Sf21 cells; they did not inhibit haemagglutination nor neutralize infection (results not shown) caused by the virus. Saphire et al. [28] and Menendez et al. [29] recently compared the X-ray crystallography of the immune complexes consisting of gp120 (glycoprotein 120) of HIV1 and an antibody produced against the native protein, which could cross-react with gp120 of HIV2, with that consisting of an antibody generated against the peptide mimic of the cross-reacting epitope, which binds with HIV1, but did not cross-react with HIV2. The authors showed that the native cross-reacting epitope is not identical with that identified by the antibody generated against the peptide mimics and suggest that the incomplete mimicry of the cross-reacting gp120 epitope could account, at least in part, for the functional deficiency of the anti-peptide. Incomplete mimicry of functional epitopes, such as the 8H5-neutralization epitope and the HIV1- and HIV2-cross-reacting epitopes, appears to be a common difficulty encountered in previous studies, resulting in functionally defective antibodies raised against the peptide mimics [30,31]. Nevertheless, our results suggest that the 8H5-reactive peptides and the native neutralization epitope

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recognized by the antibody might share certain structural features, to the extent that the antisera produced against these peptides also specifically bind to the HA molecule. Our findings suggest further it is possible that the amino acid residues threonine, leucine, threonine and leucine located at positions 2, 4, 5 and 9 respectively of all of these reactive peptides might constitute a part of the neutralization epitope recognized by 8H5.

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