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Epitope Mapping of the Hemagglutinin Molecule of a Highly Pathogenic H5N1 Influenza Virus by Using Monoclonal Antibodies▽

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We mapped the hemagglutinin (HA) antigenic epitopes of a highly pathogenic H5N1 influenza virus on the three-dimensional HA structure by characterizing escape mutants of a recombinant virus containing A/Vietnam/1203/04 (H5N1) ΔHA and neuraminidase genes in the genetic background of A/Puerto Rico/8/34 (H1N1) virus. The mutants were selected with a panel of eight anti-HA monoclonal antibodies (MAbs), seven to A/Vietnam/1203/04 (H5N1) virus and one to A/Chicken/Pennsylvania/8125/83 (H5N2) virus, and the mutants' HA genes were sequenced. The amino acid changes suggested three MAb groups: four MAbs reacted with the complex epitope comprising parts of the antigenic site B of H3 HA and site Sa of H1 HA, two MAbs reacted with the epitope corresponding to the antigenic site A in H3 HA, and two MAbs displayed unusual behavior: each recognized amino acid changes at two widely separate antigenic sites. Five changes were detected in amino acid residues not previously reported as changed in H5 escape mutants, and four others had substitutions not previously described. The HA antigenic structure differs substantially between A/Vietnam/1203/04 (H5N1) virus and the low-pathogenic A/Mallard/Pennsylvania/10218/84 (H5N2) virus we previously characterized (N. V. Kaverin et al., J. Gen. Virol. 83:2497–2505, 2002). The hemagglutination inhibition reactions of the MAbs with recent highly pathogenic H5N1 viruses were consistent with the antigenic-site amino acid changes but not with clades and subclades based on H5 phylogenetic analysis. These results provide information on the recognition sites of the MAbs widely used to study H5N1 viruses and demonstrate the involvement of the HA antigenic sites in the evolution of highly pathogenic H5N1 viruses, findings that can be critical for characterizing pathogenesis and vaccine design.

Since 1997, highly pathogenic avian H5N1 influenza viruses have caused serious outbreaks in poultry farms and markets and have caused infection in humans with >50% mortality rate (30). Between 2003 and 2007 H5N1 influenza viruses spread rapidly through Southeast Asia and emerged in Western China (3), Africa (9), Turkey (15), and Siberia (18). The rapid dissemination and ongoing evolution of avian H5N1 viruses, the possibility of future interhuman transmissibility, and the absence of anti-H5 herd immunity in humans raise concern about the pandemic potential of these viruses (13, 28) and lend new urgency to elucidation of the structure and evolution of their proteins.

The viral hemagglutinin (HA) surface glycoprotein is the primary target of neutralizing antibodies. However, few of the 16 HA subtypes have been characterized with respect to the localization and structure of their antigenic sites on the three-dimensional structure of the HA molecule. For many years the three-dimensional structure of HA was available only for the H3 subtype (29). The H3 structure was used to map antigenic sites on the H1 (1) and H2 (27) HA molecules and to begin characterizing the antigenic structure of the H5 HA molecule (17). After the X-ray crystallographic structures of H5 and H9 HA were reported (6, 7), the H5 HA molecule was antigenically mapped in greater detail (10), and mapping of H9 HA was initiated (11). The localization and fine structure of two H5 antigenic sites have been described (10). Site 1 is an exposed loop comprising HA1 residues 140 to 145 (H3 numbering here and throughout the text) that corresponds to antigenic sites A of H3 (29) and Ca2 of H1 (1), and site 2 comprises two subsites, one (HA1 residues 156 and 157) that corresponds to site B in the H3 subtype (29) and one (HA1 residues 129 to 133) that corresponds to site Sa in the H1 subtype (1).

The recently defined three-dimensional HA structure of the highly pathogenic H5N1 strain A/Vietnam/1203/04 (24) differs from that of A/Duck/Singapore/3/97 (H5N3) virus (6, 7) and bears some similarity to the HA of the H1N1 human 1918 pandemic virus. Because of the continuous evolution of H5N1 viruses (4, 13, 20, 23, 28) and the emergence of new antigenic variants (8, 24), high-yield reassortant strains must be constantly redefined for vaccine production (5, 14, 25, 26), and immunologic diagnostic tests must be frequently updated (2). We therefore antigenically mapped the HA molecule of the A/Vietnam/1203/04 virus. We also characterized some mono-

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TABLE 1. Effect of amino acid changes in the HA of A/Vietnam/1203/04 (H5N1) virus on the antigenic specificity of escape mutants

Escape mutant	HA amino acid change[a]		Reactivity with MAb[b]:							
	H3 numbering	H5 numbering	VN04-2	VN04-8	VN04-9	VN04-10	VN04-13	VN04-15	VN04-16	777/1
m2(1)	S126Y, I155T	S121Y, I151T	<	0	0	0	0	0	0	0
m2(4)	R166G	R162G	<	0	0	0	0	0	0	0
m8(9)	K144E	K140E	0	<	0	0	0	<	0	−2
m9(5)	K156E	K152E	0	0	<	<	0	0	<	<
m9(6)	T160A, K193E	T156A, K189E	0	0	<	0	<	0	0	<
m10	K156E	K152E	0	0	<	<	0	0	<	<
m13(13)	S145F	S141F	0	<	0	0	<	<	0	<
m13(16)	D187N, K193E	D183N, K189E	0	0	<	0	<	−1	0	<
m15(17)	S145P	S141P	0	<	0	0	−2	<	0	<
m15(20)	G143E	G139E	0	<	0	0	0	<	0	0
m16	K156N	K152N	0	0	<	<	0	0	<	<
m777/1(2)	S145P	S141P	0	<	0	0	−2	<	0	<
m777/1(4)	S145T	S141T	0	<	0	0	0	−2	0	<

[a] Amino acid numbering is based on H3 HA and H5 HA (6).

[b] Values are the differences in $\log_2$ units between the HI titers of MAbs in reactions with the wild-type VNH5N1-PR8/CDC-RG (H5N1) virus and with its escape mutants. 0, The HI titer of the MAb does not differ from the titer with the wild-type virus. <, The HI titer at least 32-fold (5 $\log_2$) less than the titer with the wild-type virus.

clonal antibodies (MAbs) of the panel used to study the currently circulating highly pathogenic H5N1 influenza virus strains (5, 8, 23). This information about the epitopes recognized by these MAbs will expand their usefulness in studies of new H5N1 isolates.

The HA amino acid sequence of the emerging H5N1 influenza viruses is being monitored, but the antigenic HA epitopes of these viruses have never been mapped on their three-dimensional HA structures by generating and characterizing escape mutants. Our mapping revealed that the HA antigenic structure of recent H5N1 isolates differs substantially from that of a low-pathogenicity H5 strain described earlier (10) and is rapidly evolving.

MATERIALS AND METHODS

Viruses. A reverse genetics-derived influenza virus containing the HA and neuraminidase (NA) genes of A/Vietnam/1203/04 (H5N1) virus in the genetic background of the high-growth master strain A/Puerto Rico/8/34 (H1N1) (VNH5N1-PR8/CDC-RG) was kindly provided by R. Donis (Centers for Disease Control and Prevention, Atlanta, GA). The HA gene of this virus had been modified by site-specific mutagenesis to delete the multibasic amino acids at the HA cleavage site (ΔH5-PR8). This virus is not pathogenic to chickens or ferrets and can be used in minimal-biosafety level laboratories. All other H5N1 viruses were obtained from the collaborating laboratories. The mouse-adapted variant of A/Mallard/Pennsylvania/10218/84 (H5N2) virus and its escape mutants have been described (10, 22). Viruses were propagated by growth for 48 h in the allantoic cavities of 10-day-old embryonated chicken eggs at 37°C and were stored at −80°C until used.

MAbs. To select and characterize escape mutants, we used a panel of seven MAbs to the HA of A/Vietnam/1203/04 (H5N1) influenza virus (VN04-2, VN04-8, VN04-9, VN04-10, VN04-13, VN04-15, and VN04-16) and MAb 777/1 to the HA of A/Chicken/Pennsylvania/8125/83 (H5N2) virus. The MAbs were prepared by a modification of the method described by Kohler and Milstein (12) as previously described (8). Briefly, female 8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with isoflurane and intranasally inoculated with 50 μl of 10^4 50% egg infective dose/mouse of recombinant virus generated at St. Jude Children's Research Hospital and carrying HA (modified multibasic amino acid at the cleavage site) and NA genes from A/Vietnam/1203/04 (H5N1) virus and internal genes from A/Puerto Rico/8/34 (H1N1) virus in phosphate-buffered saline. Six weeks later mice were intraperitoneally boosted with 15 μg of HA of concentrated, and purified virus mixed 1:1 with incomplete Freund adjuvant (Sigma-Aldrich, Inc., St. Louis, MO). Three weeks later mice were intraperitoneally boosted with the same immunogen composition, followed by intravenous injection of 10 μg of HA of concentrated and purified virus 3 weeks later.

Selection of escape mutants. As described previously (10), virus was incubated with an excess of MAb for 1 h at 20°C, and the mixture was inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 37°C. Virus was harvested and used for limiting-dilution cloning in embryonated chicken eggs.

HI test. Hemagglutination inhibition (HI) testing was performed with 0.5% chicken red blood cells by a standard method (16).

PCR amplification and sequencing. Viral RNA was isolated from allantoic fluid by using the RNeasy minikit (QIAGEN) as specified by the manufacturer. Reverse transcription and subsequent PCR was performed with primers specific for the HA gene segment (primer sequences are available upon request). PCR products were purified with a QIAquick PCR purification kit (QIAGEN). The DNA template was sequenced by using a DNA ABI Prism 3130 sequencer (Applied Biosystems) and BigDye Terminator v3.1 kit; DNA sequences were completed and edited by using DNASTAR sequence analysis software (DNASTAR, Inc.).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in the present study have been deposited in the GenBank database (accession numbers EU122394 to EU122406).

RESULTS

Selection and antigenic characterization of escape mutants. The recombinant virus containing the HA and NA genes of A/Vietnam/1203/04 (H5N1) virus (VNH5N1-PR8/CDC-RG) was used as the wild-type virus for the selection of escape mutants. The MAbs used for selection were generated against the HA glycoprotein of A/Vietnam/1203/04 virus (seven MAbs) or A/Chicken/Pennsylvania/8125/83 virus (one MAb 777/1). Thirteen mutants were selected: two mutants with each of the MAbs VN04-2, VN04-9, VN04-13, VN04-15, and 777/1 and one mutant with each of the MAbs VN04-8, VN04-10, and VN04-16. The mutants were selected in three separate experiments: in the first experiment three MAbs were used and six mutants were selected, in the second experiment four MAbs were used and six mutants were selected, and one mutant was selected in the third experiment. The mutants are designated by the MAb number and (in parentheses) the clone number: for example, the mutants m2(1) and m2(4) were selected by MAb VN04-2.

All selected escape mutants were tested by HI assay with the panel of MAbs (Table 1). The results allowed us to operationally define two epitopes: one reacting with MAbs VN04-9, VN04-10, and VN04-16 (group 1) and one reacting with MAbs

TABLE 2. Reactions of MAbs with escape mutants of A/Mallard/Pennsylvania/10218/84 (H5N2) virus in HI assay

Escape mutant	HA amino acid change[a]		Reactivity with MAb[b]:						
	H3 numbering	H5 numbering	VN04-2	VN04-9	VN04-10	VN04-13	VN04-15	VN04-16	777/1
m46(7)	N129D	N124D	<	0	0	0	0	0	0
m46(8)	K157M	K153M	0	0	0	0	−2	0	0
m55(2)	K156N	K152M	0	<	<	0	0	<	−4
m58(1)	D131N	D126N	0	−2	<	0	<	<	−1
m24B9	R144G	R140G	0	0	0	0	<	0	0
m46(7)-55	N129D, K156T	N124D, K152T	<	<	0	0	−2	<	0
m46(7)-55-24B9	N129M, K156T, P140L	N124D, K152T, P136L	<	<	−2	<	<	<	−2
m55(2)-24B9	K156N, N142K	K152T, N138K	0	<	<	0	<	<	<
m58(1)-24B9	D131N, S145P	D126N, S141P	0	−2	<	<	<	<	<

[a] See Table 1, footnote *a*.

[b] Designations are the same as in Table 1. Values are the differences in $\log_2$ units between the HI titers of MAbs in the reactions with the wild-type A/Mallard/Pennsylvania/10218/84 (H5N2) virus and with its escape mutants.

VN04-8 and VN04-15 (group 2). In contrast, the mutants selected with VN04-2 were resistant solely to VN04-02, which, therefore, could not be assigned to any epitope on the basis of the HI test. The mutants selected with MAbs VN04-13 and 777/1 (group 3) exhibited a complex pattern of specificity that overlapped both epitopes defined by group 1 and group 2 MAbs. Two mutants selected with VN04-13 differed in their reactions. Whereas mutant m13(13) failed to react with VN04-8 and VN04-15, mutant m13(16) reacted with both, but not with MAb VN04-9. Of the mutants selected by MAbs to A/Vietnam/1203/04 virus, only m2(1), m2(4), and m15(20) reacted with MAb 777/1 (Table 1).

The data demonstrated that the reactions of MAbs VN04-9, VN04-10, and VN04-16 overlapped, suggesting that these MAbs reacted with the same antigenic site, whereas MAbs VN04-8 and VN04-15 reacted with a different antigenic determinant. MAb VN04-2 had an extremely narrow specificity and therefore could not be assigned to either epitope group on the basis of HI data alone. MAbs VN04-13 and 777/1 could not be assigned to either group for the opposite reason: they failed to react with mutants selected by the MAbs belonging to either group. This result suggested that they had unusually broad epitope specificity.

Sequence analysis of the escape mutants. To identify the specific amino acids that reacted with the MAbs, we sequenced the HA gene. We found that the escape mutants carried either one or two amino acid changes in the HA1 subunit but none in the HA2 subunit.

The mutations selected by group 1 MAbs (VN04-9, VN04-10, and VN04-16) were located between positions 155 and 166, corresponding to a part of antigenic site B in H3 HA and to the site Sa in H1 HA. MAb VN04-2 also recognized this site, because it selected mutants carrying amino acid changes at positions 126, 155, and 166. The amino acid substitutions selected by group 2 MAbs (VN04-8 and VN04-15) were located in the 140-145 loop, that is, in the site corresponding to antigenic site A in H3 HA (Table 1).

Two MAbs selected escape mutants with unusual patterns of amino acid changes. MAb VN04-13 selected two mutants with amino acid changes in two widely separated areas. The mutant m13(13) carried the substitution S145F in the loop, which explained its inability to react with MAbs VN04-8 and VN04-15. The other mutant, m13(16), had no substitutions in this site but carried two mutations (D187N and K193E) in a region corresponding to a part of site B in H3 HA. The substitution K193E was clearly antigenically significant, because MAb VN04-13 failed to react with the mutant m9(6), which also carried this substitution. Further, Philpott et al. (17) have reported an amino acid change at position 193 in an H5 escape mutant.

MAb 777/1 also recognized amino acid substitutions in two distantly located antigenic sites: the region corresponding to site B in H3 HA (positions 156 and 193) and position 145 in the loop. However, both mutants selected with MAb 777/1 had a substitution at position 145: S145P in mutant m777/1(2) and S145T in mutant m777/1(4).

Clearly, epitopes reacting with MAbs VN04-13 and 777/1 overlap two antigenic sites, one corresponding to site A and one corresponding to site B in H3 HA. The other MAbs exhibited narrower specificity, selecting escape mutants with amino acid changes in sites corresponding to either antigenic site A in H3 (MAbs VN04-8 and VN04-15) or site B in H3 and site Sa in H1 (MAbs VN04-2, VN04-9, VN04-10, and VN04-16).

Each of three escape mutants—m2(1), m9(6), and m13(16)—that were selected with three different MAbs were double mutants carrying two HA amino acid changes. The HI data did not allow us to ascribe any antigenic effect to the substitutions T160A and D187N or to discern whether the antigenic change of mutant m2(1) was caused by substitution S126Y or I155T. However, all of these substitutions are located in antigenic areas, and their effect on antigenic specificity cannot be excluded.

HI testing of the anti-A/Vietnam/1203/04 MAbs with escape mutants of A/Mallard/Pennsylvania/10218/84 virus. All of the MAbs except VN04-8 reacted with the A/Mallard/Pennsylvania/10218/84 (H5N2) virus, allowing us to use single, double, and triple escape mutants of this virus generated and characterized in our previous study (10) to further examine the specificity of the MAbs. In HI testing MAb VN04-2 failed to react with mutants having the amino acid substitution N129D (Table 2). The reactions of MAbs VN04-9, VN04-10, and VN04-16 were affected by amino acid changes at position 156, which was expected on the basis of the data of the sequencing of the escape mutants selected with these MAbs (Table 1). However, MAb VN04-10 lost the ability to react with mutants carrying the K156N substitution and yet retained reactivity with those

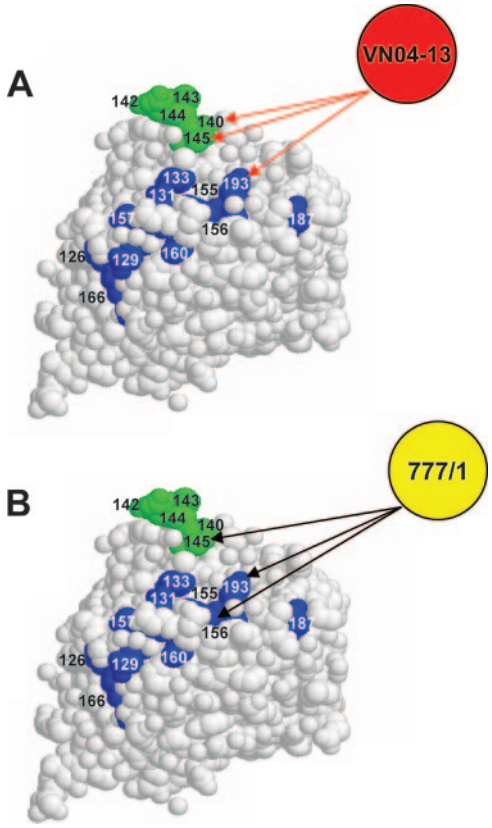

FIG. 1. Schematic representation of the antigenic sites and the epitopes that react with MAbs VN04-13 (A) and 777/1 (B) on the globular head of the HA H5 HA molecule. Images were created with RasMol 2.6, and the HA structure was obtained from the Protein Data Bank (PDB accession number 1JSM). Amino acid positions are designated in H3 numbering.

carrying K156T. Reactions of VN04-15 were affected not only by changes in the loop corresponding to site A in H3 HA (P140L, N142K, and R144G) but also by the change D131N, located in the site corresponding to site B in the H3 HA, which generates a new glycosylation site (10). Reaction of the escape mutants with MAb 777/1 was abolished by the amino acid substitution S145P and reduced by the substitution K156N, confirming the ability of this MAb to react with epitopes in two different antigenic sites.

The results obtained with escape mutants of both A/Vietnam/1203/04 (H5N1) and A/Mallard/Pennsylvania/10218/84 (H5N2) viruses allowed us to divide the MAbs into three groups on the basis of their epitope recognition. Group 1 MAbs (VN04-2, VN04-9, VN04-10, and VN04-16) react with the complex epitope comprising parts of antigenic site B of the H3 HA and site Sa of H1 HA. Group 2 MAbs (VN04-8 and VN04-15) react with the epitope corresponding to antigenic site A in H3. However, MAb VN04-15 is also sensitive to the acquisition of the glycosylation site at position 131. Group 3 MAbs (VN04-13 and 777/1) recognize amino acid changes in two distantly located antigenic sites. Figure 1 shows a schematic representation of the epitopes of the MAbs overlapping two antigenic sites.

Cross-reactions of the anti-A/Vietnam/1203/04 MAbs with highly pathogenic H5N1 viruses. In HI testing, both the H5N1 viruses preceding A/Vietnam/1203/04 virus and those isolated later were resistant to some MAbs in the panel (Table 3). These strains exhibited differences from one another and from A/Vietnam/1203/04 (H5N1) virus in their reactions with the MAbs. The reaction was either slightly enhanced compared to the reaction with A/Vietnam/1203/04 (H5N1) virus, or decreased, or completely abolished. The differences did not coincide with clade and subclade division based on phylogenetic analysis of the H5 HA gene. We compared the amino acid sequences in the HA1 polypeptide chain region that showed amino acid changes in the escape mutants (positions 125 to 193) (Table 4). In several cases the amino acid changes were positioned identically in the nonreactive strains and in the escape mutants of A/Vietnam/1203/04 and A/Mallard/Pennsylvania/10218/84 viruses. The nonreactivity or reduced reactivity of the strains A/Chicken/Jogiakarta/BBVet-IX/04 and A/Duck/Laos/3295/06 with MAbs VN04-8 and VN04-15 (Table 3) may be explained by nonconservative amino acid differences between A/Vietnam/1203/04 virus and these strains in the region of the loop (Table 4). The failure of A/Duck/Laos/3295/06 to react with the MAb VN04-13 can be ascribed to the substitution S145F, which was observed in the mutants m15(17) and m58(1)-24B9 that did not react with this MAb. The strains that were nonreactive with MAb VN04-2 (Table 3) had an aspartic acid residue at position 129 (Table 4), as did the mutants

TABLE 3. Cross-reactivity of anti-A/Vietnam/1203/04 (H5N1) HA MAbs with H5N1 viruses

HA phylogeny	Virus	Reactivity with MAb[a]:						
		VN04-2	VN04-8	VN04-9	VN04-10	VN04-13	VN04-15	VN04-16
Clade 1	A/Vietnam/1194/04	−1	0	0	0	0	−1	0
	A/Hong Kong/213/03	0	+1	<	0	−2	+1	+1
Clade 2A, subclade 1	A/Chicken/Indonesia/PA/03	−3	+1	<	0	−2	0	0
	A/Chicken/Malang/BBVet-IV/04	−1	+1	<	0	−2	0	0
	A/Chicken/Jogjakarta/BBVet-IX/04	<	<	<	0	−1	<	−2
Clade 2E, subclade 2	A/Whooper swan/Mongolia/244/05	<	0	<	0	−2	0	−2
Clade 2F, subclade 3	A/Duck/Laos/3295/06	<	<	−3	0	<	<	−3

[a] Designations are the same as in Table 1. Values are the differences in $\log_2$ units between the HI titers of MAbs in the reactions with the A/Vietnam/1203/04 virus and with the other H5N1 viruses.

TABLE 4. Amino acid changes in HA of the H5N1 viruses

HA phylogeny	Virus	Amino acid at position[a]:													
		125	*129*	*133*	*142*	*144*	*145*	158	159	*160*	163	*166*	178	185	*193*
		120	*124*	*129*	*138*	*140*	*141*	154	155	*156*	159	*162*	174	181	*189*
Clade 1	A/Vietnam/1203/04	S	S	L	Q	K	S	N	S	T	T	R	V	P	K
	A/Vietnam/1194/04	S	S	L	Q	K	S	N	S	T	T	R	V	P	K
	A/Hong Kong/213/03	**N**	S	L	Q	K	S	N	**N**	**A**	T	R	V	P	**R**
Clade 2A, subclade 1	A/Chicken/Indonesia/PA/03	S	**D**	**S**	Q	K	S	N	S	**A**	T	R	V	P	**R**
	A/Chicken/Malang/BBVet-IV/04	S	**N**	**S**	Q	K	S	N	S	**A**	T	R	V	P	**R**
	A/Chicken/Jogjakarta/BBVet-IX/04	S	**D**	**S**	**L**	**R**	S	N	S	T	**I**	R	V	P	K
Clade 2E, subclade 2	A/Whooper swan/Mongolia/244/05	S	**D**	**S**	Q	**R**	S	**D**	**N**	**A**	T	R	V	P	**R**
Clade 2F, subclade 3	A/Duck/Laos/3295/06	S	**D**	**S**	Q	**T**	**P**	N	**N**	T	T	R	**I**	S	K

[a] Amino acid numbering in the top row is based on H3 HA. Amino acid numbering in the bottom row is based on H5 HA. Amino acid substitutions with respect to the HA of A/Vietnam/1203/04 (H5N1) are indicated in boldface. Amino acid positions changed in the HA of escape mutants are indicated in italics.

m46(7) and m46(7)-55, which were nonreactive with this MAb (Table 2). The strains with amino acid changes at both position 160 and position 193 failed to react with MAb VN04-9, as had the escape mutants with amino acid substitutions at these positions. Although we could not identify any specific antigenic alteration caused by amino acid substitution T160A in mutant m9(6), it is noteworthy that this amino acid change is a reverse mutation: the change A160T occurred in several H5N1 strains after 2003 (4, 24). Obviously, amino acid changes in the antigenic sites revealed by the analysis of escape mutants are involved in the evolution of the H5 HA. It is worth noting that the distribution of antigenically significant amino acid changes in the HA of the H5N1 strains was not related to HA-based clades and subclades (Tables 3 and 4).

DISCUSSION

We have described the first characterization of the antigenic structure of the HA of a highly pathogenic H5N1 virus through the selection and study of escape mutants. The evolution of highly pathogenic H5N1 strains has to date been monitored by sequencing the genomes of isolates and using polyclonal sera and MAbs to detect antigenic changes. Our results will refine the detection of antigenically important changes in the evolving H5 HA.

The location and structure of influenza virus HA antigenic epitopes was first characterized in 1981 in the three-dimensional model of the H3 subtype (29). Four antigenic sites were described (A, B, C, and D), and a fifth (E) was later demonstrated (21). The antigenic sites of H1 and H2 subtype HAs were then characterized and mapped on the H3 HA three-dimensional structure (1, 27), and a preliminary map of the H5 HA antigenic sites was also based on the X-ray model of H3 HA (17). The locations of five H5 HA antigenic epitopes were identified, but their size and fine structure was not described, because only six amino acid changes were observed. After the X-ray crystallographic structures of H5 and H9 molecules were reported (6, 7), we mapped the antigenic sites on their surfaces by generating and analyzing escape mutants (10, 11). Amino acid changes in the escape mutants of the low-pathogenicity avian A/Mallard/Pennsylvania/10218/84 (H5N2) virus were grouped in two areas in the HA molecule: one corresponding to antigenic site A in H3 HA (a loop at the side of HA molecule) and another corresponding to a part of antigenic site B in the H3 HA molecule and partially overlapping the antigenic site Sa in H1 HA. The recent extensive spread of highly pathogenic influenza H5N1 viruses since 2003 prompted us to characterize the antigenic structure of the H5 HA using the human isolate A/Vietnam/1203/04 (H5N1), which represents clade 1 on the H5 HA phylogenetic tree.

We identified several important differences between a recent H5N1 strain and the previously described H5 HA molecules (10, 17). First, the positions of amino acid changes in the escape mutants of the A/Vietnam/1203/04 (H5N1) virus differed from those we previously described in A/Mallard/Pennsylvania/10218/84 (H5N2) mutants (10), with the exception of positions 144, 145, and 156, although they are located within the same areas on the surface of the HA molecule. Second, when the positions coincide, the amino acid changes are different (e.g., K156E and K156N). All of these observations are likely to reflect a difference in HA conformation between the more recent H5N1 strain and A/Mallard/Pennsylvania/10218/84 (H5N2) virus. The amino acid changes in positions previously unreported in escape mutants (126, 143, 155, 160, 166) that altered the reactivity with the MAbs were located both in the loop and in the part corresponding to antigenic site B of H3 HA. Interestingly, in the present study and in our previous study (10), all antigenically significant amino acid substitutions were located exclusively in areas corresponding to antigenic sites A and B of H3 HA and the antigenic site Sa of H1 HA. Not a single MAb against A/Vietnam/1203/04 (H5N1), A/Chicken/Pennsylvania/10218/83 (H5N2), or A/Chicken/Pennsylvania/8125/83 (H5N2) viruses in our studies selected an escape mutant with amino acid changes in the areas at the side of the HA molecule designated as sites 2, 3, and 4 by Philpott et al. (17). It seems plausible that the repertoire of immunocompetent antibody-producing cells (at least in mice) is directed largely against the upper surface of the H5 HA molecule.

An unexpected finding was the ability of at least two MAbs to overlap widely separate antigenic sites. Of the two mutants selected by MAb VN04-13, one had an amino acid change (S145F) in the loop corresponding to site A in H3, while the

other carried 2 mutations in a region corresponding to a part of site B in H3. MAb 777/1 also reacted with two antigenic sites, as demonstrated by its reactions with escape mutants of A/Vietnam/1203/04 (H5N1) and A/Mallard/Pennsylvania/10218/84 (H5N2) viruses. With some reservations, MAb VN04-15 may also be regarded as sensitive to amino acid substitutions in two antigenic sites. However, in this case the amino acid change at position 131 was associated with the acquisition of a new glycosylation site (10). The oligosaccharide chain in this position might create steric hindrance at the epitope recognized by the MAb VN04-15 in the region of the loop.

Importantly, the MAbs overlapping two antigenic sites (VN04-13 and 777/1) were not mixed preparations. Amino acid changes that abolished recognition by these MAbs were detected in several single mutants carrying a mutation in one antigenic site, while the other site was unchanged and free to bind MAbs: the mutants, therefore, would have been neutralized by a mixture containing a MAb reactive with the unchanged site. Further, escape mutants are unlikely to be selected by a mixture of two MAbs reacting with different antigenic sites, considering the mean frequency of the mutations selected by MAbs (31).

The ability of some anti-H5 MAbs to overlap 2 distinct antigenic sites may be hypothetically explained by the architecture of the H5 HA molecule. Sites A and B are situated far apart in the three-dimensional structure of H3 HA molecule (29) but are much closer in H5 HA (10, 11). It would be premature to speculate whether this proximity may influence the selection of antigenic variants under natural conditions. However, a neutralizing antibody that reacts with two antigenic sites rather than one might offer the evolving virus broader possibilities of escape, if a mutation in either site could confer resistance to neutralization. The ability of some anti-H5 MAbs to overlap two different antigenic sites suggests that if H5 viruses were to become disseminated in the human population, the generation of drift variants of H5 HA might differ from that observed in the human H1 and H3 viruses.

The panel of MAbs we characterized is widely used to study currently circulating highly pathogenic H5N1 influenza viruses (5, 8, 23). These MAbs are available from the Biodefense and Emerging Infections Research Resources Repository (http://www.beiresources.org), and in 2007 were included into the CDC influenza reagent kit. By identifying the epitopes recognized by these antibodies, our findings allow the use of the MAbs to monitor the evolution of the antigenic sites in the currently circulating H5N1 viruses. Previous data obtained by using this panel of MAbs can be reinterpreted on the basis of our results, and the characterization of future isolates will acquire a new dimension, in which the antigenic changes that are detected can be connected with amino acid substitutions at specific antigenic sites.

The antigenic sites described here and in our earlier study (10) are significantly involved in the evolution of H5N1 influenza viruses. The sites can now be analyzed in detail. Clearly, antigenic specificity can be modulated by a greater variety of amino acid changes than previously suggested (10). The amino acid substitutions in the highly pathogenic H5N1 isolates (4, 24) occur within the antigenic sites described here and in our previous study (10), and some occur in precisely the same positions as those in the escape mutants described here and in our previous study (10). These coincidences suggest a certain similarity between the evolution of the avian H5N1 viruses and the antigenic drift of human H1 and H3 influenza virus strains.

The data presented here may be regarded as a basis for future studies in several directions. First, it will be important to explore whether the antigenic epitopes recognized by murine MAbs coincide with those recognized by human antibodies. The production of human MAbs against the H5 HA was recently reported (19). Second, since the distribution of the antigenically significant amino acid changes in the HA of H5N1 strains did not coincide with the clade and subclade grouping based on phylogenetic analysis of the H5 HA genes, the choice of a strain for vaccine preparation should take into consideration not only the selection of the proper clade and subclade but also the structure of antigenic sites of individual strains. It will be important to establish to what extent the amino acid substitutions in the antigenic sites affect immune protection. Finally, last but not least, it will be of interest to find out whether the amino acid changes in the escape mutants of the H5N1 virus have any pleiotropic effects. In our previous studies we revealed that escape mutations in the HA of a low-pathogenicity mouse-adapted H5N2 and H9N2 strains may be associated with decrease of virulence and/or the affinity to sialic receptors (10, 11). Experiments aimed at the characterization of these phenotypic features of the escape mutants generated in the present study are now in progress.

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