Journal of General Virology (2001), **82**, 2475–2484. Printed in Great Britain

Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus

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The antigenic structure of influenza A/H2N2 virus haemagglutinin (HA) was analysed using 19 monoclonal antibodies (MAbs) against the HA of A/Kayano/57. The antibodies were classified into three groups: group I had both haemagglutination inhibition and neutralization activities, group II had neutralization activity but no haemagglutination inhibition activity and group III had neither activity. Analysis of escape mutants selected by each of the group I and II antibodies identified six distinct antigenic sites: four (I-A to I-D) were recognized by group I MAbs and two (II-A and II-B) were recognized by group II MAbs. Sequence analysis of the HA genes of the escape mutants demonstrated that sites I-A, I-B and I-C form a contiguous antigenic area that contains the regions corresponding to antigenic sites A, B and D on the H3 molecule and that sites I-D and II-B are the equivalents of sites E and C, respectively, suggesting that the antigenic structure of the H2 molecule is largely similar to that of the H3 molecule. However, the H2 molecule differed from the H3 molecule in having a highly conserved antigenic site (II-A) in the stem domain. It was also found that most of the escape mutants selected by antibodies to sites I-A, I-B and I-C acquired a new glycosylation site at position 160, 187 or 131, respectively, which indicates that A/H2N2 viruses have the potential to gain at least one additional oligosaccharide on the tip of the HA, although this has never occurred during 11 years of its circulation in humans.

Introduction

Influenza A/H2N2 viruses caused a serious pandemic in 1957, circulated in the human population until 1968 and then disappeared. Thus, the history of influenza viruses of the H2 subtype in humans was short compared to the histories of currently circulating viruses of the H1 (23 years) and H3 (32 years) subtypes. The reason for the short life of A/H2N2 viruses in humans is not known. Seroarchaeological data suggest that viruses of the H2 subtype also caused the influenza pandemic between 1889 and 1890 (Mulder & Masurel, 1958). The surveillance of avian influenza has provided evidence that A/H2N2 viruses that are antigenically similar to the 1957 pandemic strain are still circulating in wild and domestic birds (Schäfer *et al.*, 1993; Makarova *et al.*, 1999). Furthermore, anti-H2 antibodies were detected only in a small percentage of the sera of adults and were undetectable in the sera of children (unpublished data). Therefore, the probability that viruses of this haemagglutinin (HA) subtype will be reintroduced into humans may not be very low.

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The HA glycoprotein is the major surface antigen of influenza virus. Earlier studies of the HA amino acid sequences of natural and laboratory-selected antigenic variants of A/H3N2 viruses identified five distinct antigenic domains (designated A–E) on the surface of the H3 molecule (Wiley *et al.*, 1981; Daniels *et al.*, 1983) and are located as follows: site A is centred around a protruding loop containing residues 133 and 137 and 140–146; site B is centred on a loop of residues 155–160 and an α-helix at residues 186–197; site C comprises the bulge around the bonded cysteine residues 52 and 277; site D is located near the interface between monomer subunits; and site E is near the bottom of the globular domain between sites A and C (see Fig. 2A). Five operationally distinct antigenic sites (designated Sa, Sb, Ca_1, Ca_2 and Cb) were also identified on the H1 subtype HA molecule (Gerhard *et al.*, 1981; Caton *et al.*, 1982), although these sites may form a large contiguous antigenic area on the surface of its globular domain (Caton *et al.*, 1982). In contrast, very little is known about the antigenic structure of the H2 subtype HA. Yamada *et al.* (1984) attempted

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to establish an operational antigenic map of the H2 subtype HA by selecting escape mutants with anti-H2 monoclonal antibodies (MAbs) and analysing their reactivity patterns, and obtained results that suggested that rather than discrete antigenic regions, the HA may have a continuum of determinants on its surface. However, none of the antigenic determinants has been located on the H2 molecule as yet.

There are several lines of evidence indicating that oligosaccharide chains modulate the antigenicity of the HA by masking the protein surface and that the addition of carbohydrate is more effective than a single amino acid substitution in changing the antigenic properties of the HA (Caton *et al.*, 1982; Daniels *et al.*, 1983; Skehel *et al.*, 1984; Schulze, 1997). Most of the A/H3N2 viruses that circulated between 1968 and 1974 had only two oligosaccharides on the tip of the HA (residues 81 and 165). However, viruses isolated in 1975 (represented by A/Victoria/3/75) had lost a glycosylation site at residue 81 and gained two new sites at residues 63 and 126. Furthermore, the 1986 isolates (represented by A/Memphis/6/86) had acquired a new carbohydrate attachment site at residue 246 and the 1997 isolates (represented by A/Sydney/5/97) had obtained two additional sites at residues 122 and 133. Thus, the A/H3N2 viruses currently circulating have six glycosylation sites on the tip of the HA, although all of them may not be used. The HA molecules of influenza A/H1N1 viruses as well as those of influenza B viruses isolated recently also possess four or five oligosaccharide chains on their tips. These data tempt one to postulate that the addition of new oligosaccharides to the head of the HA may provide influenza viruses with an increased ability to prevail in humans (Schulze, 1997). However, examination of the available HA amino acid sequences of A/H2N2 viruses revealed that none of the HA molecules had obtained a new glycosylation site on the tip and had only one carbohydrate chain at position 169 or 170 [two glycosylation sequons overlap each other at residues 169–172 (NNTS)]. The significance of the failure of A/H2N2 virus to acquire additional oligosaccharides in its epidemiology remains unknown.

Here, we investigated the antigenic structure of the HA of A/Kayano/57 (H2N2) virus by using anti-HA MAbs and the escape mutants selected by these antibodies. The results show that the H2 subtype HA has an antigenic structure largely similar to that of the H3 subtype HA, but differs from the latter in having a highly conserved neutralizing epitope in the stem domain. We also discuss the epidemiological significance of the failure of A/H2N2 viruses to increase the number of oligosaccharide chains on the globular head of the HA.

Methods

■ **Viruses and cells.** The influenza A/H2N2 virus strains used in this study were Kayano/57, Adachi/2/57, Georgia/1/63, England/12/64, California/1/66 and Berkeley/1/68. All viruses were grown in the allantoic cavities of 10-day-old embryonated hens' eggs. Virus purification was made by two cycles of centrifugation in a discontinuous CN2 gradient consisting of 30 and 60% (w/w) sucrose, as described elsewhere (Kawamura *et al.*, 1986). MDCK cells were grown in Eagle's minimal essential medium containing 10% foetal bovine serum.

■ **Monoclonal antibodies.** MAbs against the A/Kayano/57 virus HA were produced, as described previously (Hongo *et al.*, 1986; Sugawara *et al.*, 1991), using purified egg-grown virions for immunization of BALB/c mice. The isotypes of the MAbs were determined by double immunodiffusion using rabbit antisera specific for each immunoglobulin subclass (Sugawara *et al.*, 1986).

■ **Selection of escape mutants.** Serial 10-fold dilutions of the cloned parental viruses were mixed with an equal volume of a 1:10 dilution of ascites fluid containing MAb. After incubation for 30 min at room temperature, the mixture was inoculated onto a monolayer of MDCK cells and the viruses that escaped neutralization were allowed to grow under the agar overlay medium. Five to six days later, plaques were picked and treated again with a 1:10 dilution of ascites fluid containing MAb and then plaqued again. Escape mutants were used for analysis after growth in eggs. Cloned viruses were found to have greatly decreased reactivity with the MAbs used for selection, confirming that they were escape mutants.

■ **Serological assays.** Enzyme-linked immunosorbent assays (ELISA) were performed according to the method of Kida *et al.* (1982) with purified virions (2·0 μg per well) as antigens (Sugawara *et al.*, 1988). ELISA titres are expressed as the highest antibody dilution that showed an absorbance value of $> 0{\cdot}2$ at 414 nm. Haemagglutination inhibition (HI) tests were carried out in microtitre plates with a 0·5% suspension of chicken erythrocytes (Katagiri *et al.*, 1983). Neutralization (NT) tests were carried out as described previously (Sugawara *et al.*, 1986).

■ **Nucleotide sequence analysis.** Viral RNA was extracted from purified virions using the RNeasy Mini kit (Qiagen). The full-length HA gene cDNA was synthesized from viral RNA using AMV reverse transcriptase XL (Life Sciences) and an oligonucleotide primer complementary to positions 1–25 of RNA segment 4. cDNA was amplified by PCR using a plus-sense primer (positions 1–25) and a minus-sense primer corresponding to positions 1773–1751. Nucleotide sequences were determined from the PCR products by cycle sequencing using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit and an automatic sequencer ABI PRISM 310 (Applied Biosystems).

■ **Radioisotope labelling and immunoprecipitation.** Monolayers of MDCK cells were infected with stock virus at a multiplicity of about 10 p.f.u. per cell and labelled with 10 μCi/ml of [^{35}S]methionine (ARC) for 15 min at 5 h post-infection (p.i.). Cells were then disrupted in 0·01 M Tris–HCl (pH 7·4) containing 1% Triton X-100, 1% sodium deoxycholate, 0·1% SDS, 0·15 M NaCl and a cocktail of protease inhibitors (Hongo *et al.*, 1997), and immunoprecipitated, as described previously (Sugawara *et al.*, 1986), with an anti-HA MAb. The immunoprecipitates obtained were analysed by SDS–PAGE on 13% gels containing 4 M urea and processed for analysis by fluorography (Yokota *et al.*, 1983).

Results

Characterization of MAbs to the A/Kayano/57 virus HA

A total of 19 MAbs, which were specific for the HA as judged by radioimmunoprecipitation (data not shown), was obtained in this study. Based on the reactivity patterns in HI

Table 1. Characterization of MAbs against the A/Kayano/57 virus HA

Antibody group	MAb	Isotype	Antibody titre		
			ELISA*	HI	NT
I	1/119	G1	7·6	128 000	400 000
	4/79	G1	6·9	12 800	80 000
	5/77	G1	6·8	25 600	320 000
	3/186	G1	7·6	25 600	80 000
	4/148	G1	6·9	25 600	200 000
	4/11	G2a	6·5	6 400	80 000
	3/179A	G1	6·2	12 800	64 000
	3/179B	G1	6·9	25 600	400 000
	3/106	G3	4·9	6 400	16 000
	32/105	G3	5·2	160	1 600
	33/105	G3	5·5	160	1 600
II	1/87	G2a	7·5	<†	16 000
	4/68	G1	6·6	<	16 000
	4/53	G1	5·9	<	3 200
	1/153	G1	6·5	40	1 600
	3/B46	G1	6·9	40	1 600
	3/181	G3	5·2	20	400
III	2/9	G1	6·9	<	<
	3/152	G1	6·5	<	<

* Log_{10} antibody titres are recorded for ELISA.
† Titre below 20.

and NT tests, they could be classified into three different groups, I, II and III (Table 1). The 11 group I antibodies had both HI and NT activities, whereas the six group II antibodies had NT activity but little or no HI activity. The two group III antibodies had neither HI nor NT activity. The antibodies of the last group were not characterized further in this study.

Operational mapping of the A/Kayano/57 virus HA

Escape mutants resistant to each of the group I MAbs (except MAbs 32/105 and 33/105) were isolated at a frequency ranging from $10^{-4·4}$ to $10^{-6·7}$. Mutants resistant to MAbs 32/105 and 33/105 could not be isolated because their NT activity was too low. Although escape mutants resistant to group II MAbs 1/87 and 4/68 were obtained at a frequency of $10^{-5·5}$ and $10^{-2·3}$, respectively, isolation of mutants resistant to the other four antibodies of this group (MAbs 4/53, 1/153, 3/B46 and 3/181) was not possible because their NT activity was too low.

To delineate the antigenic sites recognized by the group I MAbs, a panel of escape mutants was examined for reactivity with each of the antibodies (including MAbs 32/105 and 33/105) in HI tests. The results (Table 2) show the presence of at least three non-overlapping or partially overlapping antigenic sites (I-A, I-B and I-C) defined by antibodies of this group; sites I-A and I-C are discrete from each other, whereas site I-B overlaps partially both site I-A and site I-C. MAbs 32/105 and 33/105 were both reactive with all of the escape mutants tested. Rather, their HI titres against most of the escape mutants were $\geqslant$ 10-fold higher than the titres against the parental virus. Thus, these two antibodies seemed to be directed to an antigenic site (tentatively designated I-D) distinct from sites I-A, I-B and I-C. However, the inability to select escape mutants with MAbs 32/105 and 33/105 did not allow reciprocal analysis, making the definition of the epitopes recognized by these antibodies difficult.

In Table 3, escape mutants selected by each of the two group II MAbs (MAbs 1/87 and 4/68) were examined for reactivity with six antibodies of this group in NT tests. At least two non-overlapping antigenic sites were defined by these HI-negative and NT-positive MAbs, site II-A was recognized by MAb 1/87 and site II-B was recognized by MAbs 4/68 and 1/153. MAbs 4/53, 3/181 and 3/B46 were highly reactive with all four mutants selected by either MAbs 1/87 or 4/68, which suggests that they are directed to antigenic site(s) distinct from sites II-A and II-B, although this remains inconclusive because of the inability to make a reciprocal analysis.

Reactivity of anti-HA MAbs with various human strains of A/H2N2 virus

To further characterize the epitopes recognized by anti-HA MAbs, their reactivity with various human influenza A/H2N2

Table 2. Operational mapping of the A/Kayano/57 virus HA with group I MAbs

MAb reactivity with escape mutants (EM) is graded as follows: + +, titre with mutant is ⩾ 10-fold higher than that with the parental virus; +, titre with mutant is identical to that with the parental virus; ±, titre with mutant is 8- to 32-fold less than that with the parental virus; −, titre with mutant is ⩾ 64-fold less than that with the parental virus.

			MAb reactivity										
		MAb*…	1/119	4/79		5/77	3/186	4/148	4/11		3/179A	3/179B	3/106
Antigenic site	MAb	EM†…	EM1, EM2	EM1	EM2	EM2, EM3	EM1–EM3	EM1–EM3	EM1	EM3	EM1–EM3	EM1–EM3	EM1, EM2
I-A	1/119		−	±	+	±	+	+	+	+	+	+	+
I-B	4/79		+	−	−	−	+	+	+	+	+	+	+
	5/77		+	−	+	−	±	±	+	±	±	±	±
I-C	3/186		+	−	+	−	−	−	−	−	−	−	−
	4/148		+	±	+	±	−	−	+	−	−	−	−
	4/11		+	−	+	−	−	−	−	−	−	−	−
	3/179A		+	±	−	±	−	−	−	−	−	−	−
	3/179B		+	±	−	±	−	−	−	−	−	−	−
	3/106		+	−	+	−	−	−	±	−	−	−	−
I-D	32/105		+	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	33/105		+	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

* MAb used for selection of EM.
† EM number.

Table 3. Operational mapping of the A/Kayano/57 virus HA with group II MAbs

MAb reactivity with escape mutants (EM) is graded as +, titre with mutant is identical to that with the parental virus, or −, titre with mutant is ⩾ 100-fold less than that with the parental virus.

		MAb*…	MAb reactivity 1/87	4/68
Antigenic site	MAb	EM†…	EM1, EM2	EM1, EM2
II-A	1/87		−	+
II-B	4/68		+	−
	1/153		+	−
Unassigned	4/53		+	+
	3/181		+	+
	3/B46		+	+

* MAb used for selection of EM.
† EM number.

viruses was investigated. Table 4 summarizes the reactivity of six representative A/H2N2 viruses with antibodies to antigenic sites I-B and I-C examined in HI tests. Two MAbs to site I-B (MAbs 4/79 and 5/77) were clearly different from each other in reactivity with viruses A/Adachi/2/57, A/Georgia/1/63, A/England/12/64 and A/California/1/66, indicating that site I-B contain at least two distinct epitopes. Reactivity patterns of MAbs 3/179A and 3/179B to site I-C were identical. However, the remaining four site I-C antibodies showed patterns distinguishable from each other as well as from MAbs 3/179A and 3/179B. These observations suggest the presence of at least five different epitopes in site I-C.

The reactivity of the six A/H2N2 viruses with three MAbs to antigenic site II-A or II-B was also compared by ELISA (Table 4). No strain-dependent differences in reactivity were seen with any of the three antibodies. We also observed that these MAbs were all able to neutralize A/Berkeley/1/68 as efficiently as A/Kayano/57 (data not shown). Thus, it seems likely that the neutralizing epitopes present in sites II-A and II-B, unlike those in sites I-A to I-D (Table 4, ELISA; data not shown), have been conserved completely over the period from 1957 to 1968.

Amino acid substitutions in escape mutants

To identify the amino acid changes responsible for the observed antigenic alterations (Tables 2 and 3), the HA gene sequences of 25 escape mutants were determined and their predicted HA amino acid sequences were compared with that of the parental virus (Table 5). Hereafter, all amino acid positions will be given relative to their position on the H3 molecule.

In all of the escape mutants analysed (except 3/179A-EM2), single amino acid changes were identified in their HA polypeptides. Two mutants selected by MAb 1/119 to site I-A had amino acid changes at positions 248 (T → N) or 162 (P → T). MAbs 4/79 and 5/77 to site I-B selected three mutants with a change at position 187 (D → N) and one mutant with a change at position 137 (R → Q). It should be noted that a change at residue 187, but not that at residue 137, caused the loss of reactivity with both MAbs 4/79 and 5/77 shown to recognize distinct epitopes (Table 2). It was interesting that 12 of the 13 escape mutants selected by the six antibodies to site I-C, which are directed to five different epitopes (Table 4), had the same amino acid substitution at position 131 (T → N), although one of them (3/179A-EM2) was a double mutant with an additional substitution at position 218. The only exception was mutant 4/11-EM1, which had a non-con-

Table 4. Reactivity of MAbs with various influenza A/H2N2 viruses

	HI titre								ELISA titre*		
	I-B		I-C						II-A	II-B	
Virus strain	4/79	5/77	3/186	4/148	4/11	3/179A	3/179B	3/106	1/87	4/68	1/153
Kayano/57	12 800	25 600	25 600	25 600	6400	12 800	25 600	6400	6·3	6·3	6·6
Adachi/2/57	320	25 600	25 600	25 600	6400	40	40	1600	6·3	6·3	6·3
Georgia/1/63	640	<	320	160	2560	20	80	<	6·3	6·3	6·3
England/12/64	<†	6400	40	6400	1600	<	<	<	6·6	7·2	6·6
California/1/66	<	640	<	<	<	<	<	<	6·6	6·0	6·0
Berkeley/1/68	<	<	40	80	<	<	<	<	6·6	6·6	6·3

* Log_{10} antibody titres are recorded for ELISA.
† Titre below 20.

Table 5. Amino acid substitutions detected in the HA molecules of escape mutants

Antigenic site	MAb	Escape mutant	Amino acid change	Acquisition of glycosylation site
I-A	1/119	1/119-EM1	$T^{248} \rightarrow N$	–
		1/119-EM2	$P^{162} \rightarrow T$	+
I-B	4/79	4/79-EM1	$D^{187} \rightarrow N$	+
		4/79-EM2	$R^{137} \rightarrow Q$	–
	5/77	5/77-EM2	$D^{187} \rightarrow N$	+
		5/77-EM3	$D^{187} \rightarrow N$	+
I-C	3/186	3/186-EM1	$T^{131} \rightarrow N$	+
		3/186-EM2	$T^{131} \rightarrow N$	+
	4/148	4/148-EM1	$T^{131} \rightarrow N$	+
		4/148-EM2	$T^{131} \rightarrow N$	+
	4/11	4/11-EM1	$K^{222} \rightarrow E$	–
		4/11-EM3	$T^{131} \rightarrow N$	+
	3/179A	3/179A-EM1	$T^{131} \rightarrow N$	+
		3/179A-EM2	$T^{131} \rightarrow N$ and $A^{218} \rightarrow T$	+
		3/179A-EM3	$T^{131} \rightarrow N$	+
	3/179B	3/179B-EM1	$T^{131} \rightarrow N$	+
		3/179B-EM2	$T^{131} \rightarrow N$	+
	3/106	3/106-EM1	$T^{131} \rightarrow N$	+
		3/106-EM2	$T^{131} \rightarrow N$	+
II-A	1/87	1/87-EM1	$K^{40} \rightarrow Q$	–
		1/87-EM2	$K^{40} \rightarrow R$	–
		1/87-EM3	$K^{40} \rightarrow R$	–
II-B	4/68	4/68-EM1	$T^{273} \rightarrow K$	–
		4/68-EM2	$T^{273} \rightarrow K$	–
		4/68-EM3	$T^{273} \rightarrow K$	–

servative amino acid change at position 222 (K → E). Two HI-negative MAbs, 1/87 to site II-A and 4/68 to site II-B, selected escape mutants with amino acid changes at positions 40 (K → Q or K → R) and 273 (T → K), respectively. It was impressive that new oligosaccharide attachment sites (residues 131–133, 160–162 and 187–189) were generated by any of three changes at positions 131, 162 and 187 that occurred in the majority of the mutants selected with sites I-A, I-B and I-C.

To examine whether carbohydrate attachment sites produced by amino acid substitutions occurring at positions 131, 162 and 187 are used for glycosylation, MDCK cells infected with each of three mutants (1/119-EM2, 5/77-EM2 and 3/186-EM1) were labelled with [^{35}S]methionine for 15 min at 5 h p.i. in the presence or absence of 1 μg/ml of tunicamycin (TM), a specific inhibitor of *N*-linked glycosylation (Takatsuki *et al.*, 1971). Cells were then immunoprecipitated with MAb 4/68 to site II-B (reactive with all of the mutants selected by group I MAbs) and the resulting precipitates were analysed by SDS–PAGE. Fig. 1(A) shows that the HA molecules of two mutants (1/119-EM2 and 3/186-EM1) synthesized in the absence of TM migrated slightly more slowly than that of the parental virus, whereas non-glycosylated HA (NG-HA) molecules of these mutants synthesized in the presence of TM showed an electrophoretic mobility indistinguishable from that of the parental virus, indicating that the new glycosylation sites at positions 131 and 160 are both used. In contrast to the HA molecules of 1/119-EM2 and 3/186-EM1, the HA of 5/77-EM2 exhibited an electrophoretic mobility virtually identical to that of the parental virus. However, the NG-HA of this mutant migrated slightly faster than that of the parental virus, raising the possibility that the conformational change in the HA caused by the amino acid substitution at position 187 (D → N) may have resulted in an increase in electrophoretic mobility. Thus, it seems likely that the HA of 5/77-EM2, although it has an additional oligosaccharide chain at position 187, co-migrated with the parental virus HA because of its increased mobility caused by a D → N change at position 187.

Previously, Skehel *et al.* (1984) provided evidence that the addition of a new oligosaccharide chain to the HA of A/H3N2 virus, caused by an amino acid substitution at position 63, blocked sterically the access of a MAb to a region near residue 63. To examine the possibility that our escape mutants, which acquired a new oligosaccharide at position 131, 160 or 187, also escaped from neutralization by the selecting antibody through this mechanism, the HA molecules of three representative mutants (1/119-EM2, 5/77-EM2 and 3/186-EM1) synthesized in the presence or absence of TM were tested for reactivity with the MAbs used for their selection. As shown in Fig. 1(B), the glycosylated HA molecules of 5/77-EM2 and 3/186-EM1 were not precipitated at all with MAbs 5/77 and

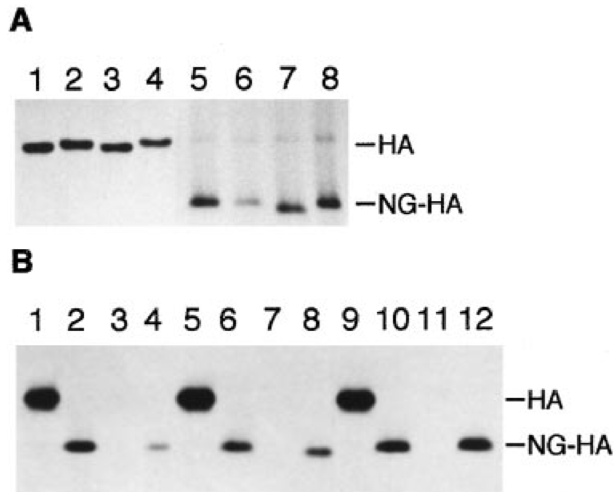

Fig. 1. Immunoprecipitation analysis of the HA molecules of escape mutants that acquired a new glycosylation site at position 131, 160 or 187. (A) MDCK cells infected with parental virus (lanes 1 and 5), 1/119-EM2 (lanes 2 and 6), 5/77-EM2 (lanes 3 and 7) or 3/186-EM1 (lanes 4 and 8) were labelled with [^{35}S]methionine for 15 min at 5 h p.i. in the absence (lanes 1–4) or presence (lanes 5–8) of TM. Cells were immunoprecipitated with MAb 4/68 to site II-B and the resulting precipitates were analysed by SDS–PAGE. (B) MDCK cells infected with parental virus (lanes 1, 2, 5, 6, 9 and 10), 1/119-EM2 (lanes 3 and 4), 5/77-EM2 (lanes 7 and 8) or 3/186-EM1 (lanes 11 and 12) were labelled with [^{35}S]methionine for 15 min at 5 h p.i. in the absence (lanes 1, 3, 5, 7, 9 and 11) or presence (lanes 2, 4, 6, 8, 10 and 12) of TM. Cells were then immunoprecipitated with MAb 1/119 (lanes 1–4), 5/77 (lanes 5–8) or 3/186 (lanes 9–12) and the resulting precipitates were analysed by SDS–PAGE.

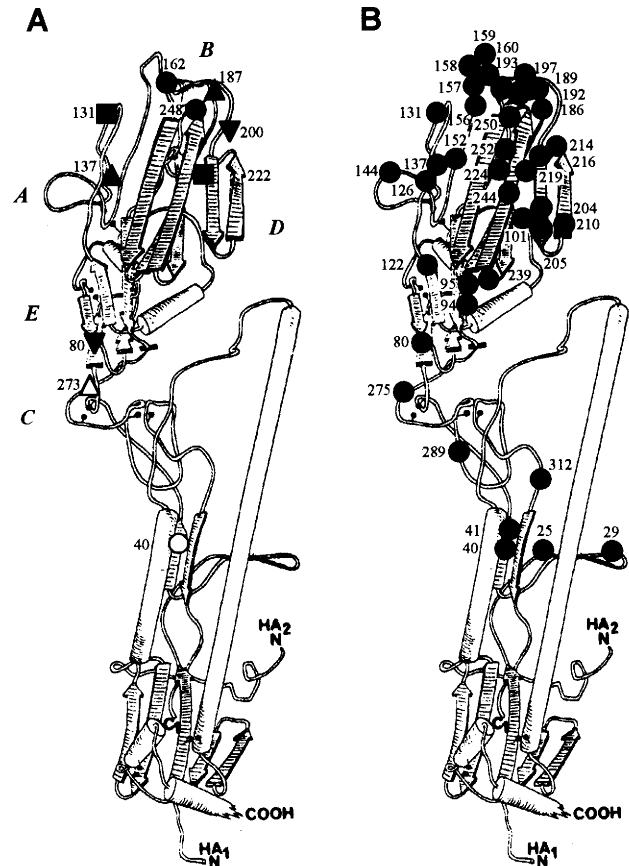

Fig. 2. (A) Location of amino acid substitutions in the escape mutants of A/Kayano/57 virus selected by MAbs to site I-A (●), I-B (▲), I-C (■), I-D (▼), II-A (○) or II-B(△). The positions of amino acid substitutions are shown on the three-dimensional structure of the H3 subtype HA (Wilson *et al.*, 1981). Sites A–E indicate the positions of the five antigenic sites identified on the H3 molecule. (B) Locations of amino acid changes on the HA1 subunit found among the following 20 A/H2N2 virus strains: Kayano/57 (AB056699), Albany/7/57 (AF270720), Chile/6/57 (AF270728), Davis/1/57 (AF270719), El Salvador/2/57 (AF270716), Japan/305/57 (J02127), Leningrad/134/57 (AF270717), RI/5+/57 (L20408), Singapore/1/57 (L11142), Albany/6/58 (AF270723), Malaya/16/58 (AF270724), Krasnodar/101/59 (L11134), Ohio/2/59 (AF270727), São Paulo/3/59 (AF270725), Victoria/15681/59 (AF270726), Ann Arbor/6/60 (AF270721), Berlin/3/64 (L11126), Izumi/5/65 (D13579), Berkeley/1/68 (L11125) and Korea/426/68 (L11133). GenBank accession numbers are given in parentheses.

3/186, respectively, whereas the NG-HA molecules of these mutants were precipitated efficiently with these antibodies, supporting the notion that the epitopes recognized by MAbs 5/77 and 3/186 are masked by new oligosaccharides added to positions 187 and 131, respectively. In contrast, the amount of the NG-HA of mutant 1/119-EM2 precipitated with MAb 1/119 was very low, suggesting that the failure of this mutant to react with MAb 1/119 is caused primarily by the conformational change in the HA induced by the P → T change at position 162 itself, although the masking effect of an oligosaccharide chain added to position 160 may also be involved.

In Fig. 2(A), the positions of amino acid changes (except a change at residue 218), which were detected in the 25 escape mutants selected by MAbs to antigenic sites I-A, I-B, I-C, II-A and II-B, are shown on the three-dimensional structure of the H3 molecule. Residue 218 seems unlikely to be related to resistance to neutralization by site I-C MAb 3/179A, since three mutants selected by this antibody contain a mutation at residue 131 in common, but only one of them (3/179A-EM2) contains a mutation at residue 218. Two amino acid substitutions at positions 162 and 248, which were detected in the mutants selected by site I-A MAb 1/119, occurred in the upper part of the globular head of the HA corresponding to antigenic site B in the H3 subtype HA. Residues 137 and 187, whose changes were found in the mutants resistant to neutralization by site I-B MAbs 4/79 and 5/77, were located in a protruding loop corresponding to antigenic site A of the H3 subtype HA and on the N-terminal exterior face of an α-helix at residues 187–196 belonging to site B, respectively. The change at residue 131, which was detected in 12 of the 13 escape mutants selected by six site I-C MAbs, occurred in an external loop corresponding to antigenic site A of the H3 molecule. One of the MAbs to site I-C (MAb 4/11) selected a mutant (4/11-EM1) with a change at residue 222, which is remote from residue 131 in the HA monomer and falls into a region corresponding to site D (or site B). Amino acid residue 40, whose substitution occurred in all of the three mutants selected

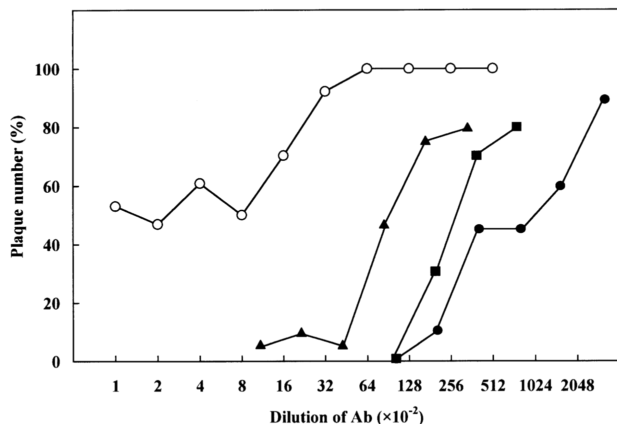

Fig. 3. Neutralization of parental virus (○), 4/79-EM1 (●), 4/148-EM1 (▲) and 4/11-EM1 (■) by MAb 32/105 to antigenic site I-D. Serial 2-fold dilutions of the ascites fluid containing MAb 32/105 were mixed with an equal volume (100 μl) of the suspension containing 100 p.f.u. of the virus to be tested. After incubation for 30 min at room temperature, the mixture (100 μl) was inoculated onto MDCK cell monolayers for plaque titration.

by MAb 1/87 to site II-A, was located in the middle of the stem region where a neutralizing epitope has not been identified in either the H1 subtype HA (Caton *et al.*, 1982) or the H3 subtype HA (Wiley *et al.*, 1981). The amino acid change at position 273, detected in all of the three mutants selected by site II-B MAb 4/68, was found in the bulge around the disulfide-bonded cysteine residues 52 and 277, which correspond to site C on the H3 subtype HA.

Further characterization of site I-D MAbs

As mentioned above, the HI and NT activities of MAbs 32/105 and 33/105 to site I-D were very low compared to those of the antibodies to sites I-A, I-B and I-C when the parental virus was used for the assays (Table 1). However, the HI titres of site I-D antibodies against almost all of the escape mutants (except 4/79-EM2) selected by antibodies to sites I-B and I-C were $\geqslant$ 10-fold higher than the titres against the parental virus (Table 2). To determine whether site I-D MAbs can neutralize these mutants more efficiently than the parental virus, the NT activity of MAb 32/105 against each of three representative mutants (4/79-EM1, 4/148-EM1 and 4/11-EM1) was compared with that against the parental virus, according to the procedures described in Fig. 3. The results in Fig. 3 show that although the parental virus was neutralized by MAb 32/105, neutralization was incomplete even at the lowest antibody dilution tested (1:100); the escape mutants, however, were all neutralized completely at antibody dilutions from 1:4000 to 1:10000. This observation raised the possibility that the use of these escape mutants may allow us to obtain antigenic variants resistant to neutralization by site I-D antibodies. Therefore, we attempted to isolate such variants by using MAb 32/105 as the selecting antibody and the seed stock of 4/11-EM1 as a parent virus and succeeded in isolating five escape mutants that were completely resistant to neutralization by MAb 32/105 (data not shown). Comparison of the deduced HA amino acid sequences of these mutants with that of 4/11-EM1 showed that four of the five mutants had a single amino acid change at position 80 (I → F), although the remaining one was a double mutant with an additional change (T → A) at position 200. Residue 80 is located near the bottom of the globular head corresponding to antigenic site E on the H3 molecule (Fig. 2A).

Discussion

We analysed the antigenic structure of the HA of A/Kayano/57 (H2N2) virus by using 17 anti-HA MAbs with NT activity and obtained evidence for the presence of at least six non-overlapping and partially overlapping antigenic sites on the HA; four sites (I-A to I-D) were recognized by group I MAbs and two sites (II-A and II-B) were recognized by group II MAbs. Sequence analysis of the HA genes of escape mutants showed that site I-A MAbs recognize a region equivalent to antigenic site B on the H3 subtype HA. The majority of the mutants selected by antibodies to site I-B or I-C possessed mutations that created a new glycosylation site at position 187 or 131, respectively. It was also found that each of these two newly introduced oligosaccharides prevents sterically the access of antibodies to most of the epitopes present in site I-B and/or site I-C. Thus, amino acids involved in the formation of antigenic epitopes in site I-B or I-C, which are covered by a carbohydrate chain added to position 187 or 131, remain mostly undetermined. However, the locations of residues 187 and 131 on the HA, in addition to those of residues 137 and 222 (whose changes occurred in mutants 4/79-EM2 and 4/11-EM1 selected by MAbs to sites I-B and I-C, respectively), suggested that site I-B presumably corresponds to antigenic sites A and B on the H3 subtype HA and site I-C to sites A and D (or B). Moreover, operational mapping demonstrated a considerable degree of overlap between sites I-A and I-B as well as between sites I-B and I-C. It seems likely, therefore, that sites I-A, I-B and I-C on the H2 molecule may form a contiguous antigenic area that contains the regions corresponding to sites A, B and D on the H3 molecule. The HA gene sequence analysis of several escape mutants isolated using site I-D MAbs as the selecting antibody and mutant 4/11-EM1 as a parent virus revealed that site I-D is the equivalent of site E on the H3 molecule. Additionally, a site II-B MAb selected escape mutants with a change at residue 273, located in an area corresponding to site C on the H3 molecule. Taken together, these observations suggest that the antigenic domains corresponding to five antigenic sites (A–E) on the H3 molecule are all present on the H2 molecule. Thus, the antigenic structure of the H2 molecule seems to be similar to that of the H3 molecule. However, the H2 molecule possesses an antigenic site that does not have an equivalent in the H3 molecule; a site II-A MAb selected mutants with a mutation at

residue 40, located in the middle of the stem region. Previously, Okuno *et al*. (1993) generated a unique anti-H2 MAb, designated C179, which neutralized all human influenza virus strains of the H1 and H2 subtypes and showed that residue 318 in the HA1 subunit and residue 52 in the HA2 subunit affect the antibody recognition of C179. Interestingly, these two amino acids are both located in the middle of the stem region very close to residue 40. Neutralizing epitopes have not been identified in the stem region of either the H3 subtype HA (Wiley *et al*., 1981) or the H1 subtype HA (Caton *et al*., 1982).

The positions of amino acid substitutions that were found in the HA1 subunits of 20 human A/H2N2 viruses isolated between 1957 and 1968 are shown in Fig. 2(B). Clearly, most of the substitutions occurred in the areas corresponding to antigenic sites A, B and D on the H3 molecule (sites I-A, I-B and I-C on the H2 molecule), as has been first pointed out by Schäfer *et al*. (1993). As Klimov *et al*. (1996) reported more recently, however, a few changes also occurred in sites C (corresponding to site II-B) and E (corresponding to site I-D). These observations support again the notion that the H2 molecule has an antigenic structure similar to that of the H3 molecule. Changes were also found at residues 40 and 41 located in antigenic site II-A. However, both of these replacements occurred only in a single strain and were not detected in the subsequently isolated strains.

Most of the escape mutants selected by MAbs to sites I-A, I-B and I-C were found to acquire a new oligosaccharide at position 160, 187 or 131, respectively. It also became clear that a carbohydrate chain added to position 131 limits the antibody recognition of all of the five different epitopes identified in site I-C and that the chain added to position 187 not only masks two distinct epitopes in site I-B completely, but also partially or completely blocks the access of antibodies to five different epitopes in site I-C (Tables 2, 4 and 5), which confirms the previously documented idea (Daniels *et al*., 1983; Schulze, 1997) that the addition of carbohydrates is much more effective than a single amino acid substitution in altering the antigenic properties of the HA. As described earlier, there is circumstantial evidence that suggests that influenza A (H1N1 and H3N2) and B viruses that have acquired additional oligosaccharide chains on the HA tips are more likely to prevail in the human population (Daniels *et al*., 1983; Schulze, 1997). The presence of an increased number of oligosaccharides on the HA tips of these viruses may promote their growth in humans by masking the antigenic sites efficiently.

The present study demonstrated that the 1957 strain of A/H2N2 virus had the potential to acquire at least one additional carbohydrate chain at position 131, 160 or 187. However, examination of the available HA amino acid sequences of human influenza A/H2N2 viruses showed that A/H2N2 viruses have never acquired a new carbohydrate attachment site on the tip of the HA during 11 years of circulation in humans and have only one conserved glycosylation site at position 169 or 170, which raises the possibility that the H2 subtype HA, unlike the H3 and H1 subtype HA molecules, might have a unique structural characteristic that does not allow the creation of new glycosylation sites on the tip. Although no differences were seen in the ability to grow in either eggs or MDCK cells between the parental virus and the escape mutants that had a new oligosaccharide at position 131, 160 or 187 (data not shown), it is still possible that these mutants may have the decreased ability to replicate in humans. Moreover, our preliminary experiments with site-specific mutagenesis showed that the H2 protein mutated to possess two or three oligosaccharide chains at positions 131, 160 and 187 had drastically decreased receptor-binding and membrane-fusing activities. Here, we observed that site I-D MAbs neutralized mutants with an additional oligosaccharide at position 131 or 187 much more efficiently than the parental virus, although its mechanism remains obscure. This suggests that site I-D antibodies might play a role in limiting the spread of such mutants in the human population by strongly inhibiting their growth. Although the reason why the number of oligosaccharides on the tip of the H2 subtype HA did not increase over a period of 11 years must be studied in the future, the failure of A/H2N2 viruses to employ this effective strategy for evading immune pressures might be one of the causes for their short survival time in humans.

Okuno *et al*. (1993) demonstrated previously that a neutralizing epitope was present in the middle of the stem region of the A/H2N2 virus HA and showed that the C179 antibody to this epitope cross-reacted with all human influenza virus strains of the H1 and H2 subtypes (but not with strains of the H3 subtype). The present study also showed the existence of an antigenic site (II-A) in the stem region of the H2 subtype HA and demonstrated that antibodies to this site were cross-reactive with all of the A/H2N2 viruses examined, although they did not react with the A/H1N1 viruses (data not shown). Furthermore, we found that site II-B MAbs, like site II-A MAbs, were highly cross-reactive. It should also be noted that three of the 19 MAbs generated in this study were directed to sites II-A and II-B, suggesting that the immunogenicity of these antigenic sites was not very weak, at least in mice. It is possible, therefore, that these cross-reactive antibodies may be produced in considerable amounts in humans, particularly in those infected repeatedly with A/H2N2 virus, which may inhibit significantly the spread of antigenic variants in the human population. This might be another cause for the short life of human A/H2N2 viruses.

We thank Dr Nancy J. Cox (Centers for Disease Control and Prevention) and Dr Yoshinobu Okuno (Osaka Prefectural Institute of Public Health) for providing influenza A/H2N2 virus strains. This work was supported by a research fellowship from the Japanese Society for the Promotion of Science for Young Scientists, a grant-in-aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and a research grant provided by the Japanese Ministry of Health, Labour and Welfare.

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Received 30 March 2001; Accepted 7 June 2001