Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants

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To elucidate the structure of the antigenic sites of avian H5 influenza virus haemagglutinin (HA) we analysed escape mutants of a mouse-adapted variant of the H5N2 strain A/Mallard/Pennsylvania/10218/84. A panel of five anti-H5 monoclonal antibodies (mAbs) was used to select 16 escape mutants. The mutants were tested by ELISA and haemagglutination inhibition with this panel of anti-H5 mAbs and the HA genes of the mutants were sequenced. The sequencing demonstrated that the amino acid changes were grouped in two antigenic sites. One corresponded to site A in the H3 HA. The other contained areas that are separated in the amino acid sequence but are topographically close in the three-dimensional structure and partially overlap in the reactions with mAbs. This site corresponds in part to site B in the H3 structure; it also includes a region not involved in site B that partially overlaps site Sa in the H1 HA and an antigenic area in H2 HA. Mutants with the amino acid change K152N, as well as those with the change D126N, showed reduced lethality in mice. The substitution D126N, creating a new glycosylation site, was accompanied by an increase in the sensitivity of the mutants to normal mouse serum inhibitors. Several amino acid changes in the H5 escape mutants occurred at the positions of reported changes in H2 drift variants. This coincidence suggests that the antigenic sites described and analysed here may be important for drift variation if H5 influenza virus ever appears as a pathogen circulating in humans.

Introduction

Highly virulent H5 influenza viruses have been isolated from several recent outbreaks in poultry (Alexander et al., 1993; Bean et al., 1985; Horimoto et al., 1995; Swayne, 1997; Swayne et al., 1997). In 1997, H5N1 influenza virus was transmitted from chickens to humans in Hong Kong, causing 18 reported cases of illness including six deaths (Centers for Disease Control & Prevention, 1998). The viruses isolated from humans and from birds were very similar in their genetic content and phenotypic features, including virulence for mammals (Claas et al., 1998; Suarez et al., 1998). Avian viruses containing one or several genes similar to the genes of the highly pathogenic 1997 Hong Kong strains continue to circulate in China (Cauthen et al., 2000), and a new outbreak of lethal H5N1 influenza emerged in the Hong Kong poultry markets in May of 2001 (Webster et al., 2002).

Only a few of the 15 antigenic subtypes of influenza virus haemagglutinin (HA) have been structurally characterized. The H3 molecule has been well characterized with the use of antigenic drift variants and HA escape mutants, and the antigenic epitopes have been mapped within the molecule’s three-dimensional structure (Wiley et al., 1981). The HA antigenic sites of the H1 and H2 subtypes have been mapped...
on the H3 three-dimensional structure (Caton et al., 1982; Tsuchiya et al., 2001). The H1, H2 and H3 subtypes that circulate (or have circulated) in humans comprise a variety of HA antigenic drift variants (Murphy & Webster, 1990). In contrast, avian HA subtypes retain a relatively stable antigenic structure because of the brevity of the avian lifespan and the replication of virus in the intestinal tracts of aquatic birds (Webster et al., 1992). For this reason, selection and characterization of escape mutants is the only available means of identifying antigenic epitopes in these HA subtypes. The antigenic structure of H5 HA of the pathogenic avian A/Turkey/Ontario/7732/86 (H5N9) influenza virus was characterized by this method (Philpott et al., 1989; 1990). Five neutralizing epitopes were identified, and their location was mapped on the three-dimensional model of the H3 HA molecule. However, because only six amino acid changes were detected in these five epitopes, additional information is needed to elucidate the location and fine structure of H5 antigenic sites.

Here we describe the selection of escape mutants of a mouse-adapted variant (Smirnov et al., 2000) of non-pathogenic avian A/Mallard/Pennsylvania/10218/84 (H5N2) virus. We characterized the escape mutants by their cross-reactions with mAbs and by the amino acid changes in their HA molecules. The extent and fine structure of two antigenic sites in the H5 HA molecule are revealed. We also describe the effect of the amino acid changes in the escape mutants on the glycosylation of HA, sensitivity to normal mouse serum inhibitors and virulence in mice.

Methods

**Viruses.** The avian H5N2 influenza virus A/Mallard/Pennsylvania/10218/84 (Mld/PA/84) was obtained from the virus depository of the Virology Department of St Jude Children’s Research Hospital (Memphis, TN, USA). The virus was adapted to mice by lung-to-lung passage, as described previously (Smirnov et al., 2000). Escape mutants of the mouse-adapted variant, which was designated Mld/PA/84-MA, were selected for study. The viruses were propagated in 10-day-old embryonated chicken eggs. The virus-containing allantoic fluid was stored at 4 °C or at −70 °C. For ELISA, virus was concentrated and partially purified by centrifuging the allantoic fluid at low speed, layering it on 20% (w/v) sucrose and pelleting the virus by centrifugation at 23,000 r.p.m. in an SW27.1 rotor at 4 °C for 90 min.

**Monoclonal antibodies.** A panel of virus-neutralizing mAbs to the HA of H5 strains was used. The mAbs 24B9 and 77B1 (kindly provided by V. Hinshaw) were obtained by priming mice with A/Turkey/Ontario/7732/66 (H5N9) (Tk/Ontario/66) virus and administering a boost dose of Tk/Ontario/66 (for 77B1) or Mld/PA/84 (for 24B9) (Philpott et al., 1989). Monoclonal antibodies to the H5N2 viruses A/Chicken/Pennsylvania/1370/83 (CK/PA/1370/83) (four mAbs) and A/Chicken/Pennsylvania/8125/83 (CK/PA/8125/83) (three mAbs) were produced by the Virology Department of St Jude Children’s Research Hospital. The mAbs are shown in Tables 1 and 2.

**Serologic methods.** Haemagglutination inhibition (HI) testing was performed by standard methods (Palmer et al., 1975). Virus neutralization tests were performed by incubating serial dilutions of mAbs with 100 50% egg infective doses (EID50) of virus for 1 h at 20 °C and inoculating each mixture into six eggs. The eggs were incubated for 48 h at 37 °C, and haemagglutination activity in the allantoic fluid was assayed. ELISA was performed essentially as described by Philpott et al. (1989). Briefly, 100 haemagglutinating units of virus were bound to each well at 4 °C in PBS, pH 7.4. Monoclonal antibodies in PBS containing 0.05% Tween 20 were added, and plates were incubated for 1 h at room temperature, washed five times with the same solution and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co.). The enzymatic activity was detected by hydrolysis with 0.03% hydrogen peroxide in the presence of 0.05% ortho-phenylenediamine in 0.15 M citrate–phosphate buffer, pH 5.0, and was quantified by measuring the absorbance at a wavelength of 492 nm. For radioimmunoprecipitation analysis, MDCK cells were infected at an m.o.i. of 10 p.f.u./cell and incubated with [125I]amino acids for 5–6 h post-infection. The cells were lysed in TNE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA) containing 0.1% SDS, 0.1% Triton X-100 and 1% sodium deoxycholate (DOC). The lysates were clarified by centrifugation at 15,000 g for 5 min; 200 µl samples of lysates were then mixed with 3 µl of mAb and incubated for 30 min at 4 °C. Protein A–sepharose (100 µl of 20% suspension in TNE buffer containing 0.1% SDS, 0.1% Triton X-100 and 1% DOC) was added and the mixtures were incubated for 30 min at 4 °C. The immune complexes were washed with the same buffer and analysed by electrophoresis in an SDS–polyacrylamide gel (Laemmly, 1970).

**Selection of escape mutants.** Single-step selection was performed as described previously (Webster & Laver, 1980). 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. Virus was harvested and used for limiting-dilution cloning in embryonated chicken eggs. The first-generation mutants (one-step escape mutants) were selected from the wild-type Mld/PA/84-MA virus. One-step escape mutants underwent further selection with the mAbs to which each mutant retained sensitivity and the resistant variants (two-step escape mutants) were cloned. The two-step mutants underwent selection with a third mAb to generate three-step escape mutants.

**PCR amplification and sequencing.** Viral RNA was isolated from virus-containing allantoic fluid using the RNeasy Mini kit (Qiagen), as specified by the manufacturer. Uni-12 primer was used for reverse transcription. PCR was performed with primers specific for the HA gene segment (primer sequences are available on request). PCR products were purified with QIAquick PCR purification kit (Qiagen). The sequencing reaction was performed by the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children’s Research Hospital. The DNA template was sequenced using rhodamine or dRhodamine dye terminator cycle-sequencing Ready Reaction Kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems) and synthetic oligonucleotides. Samples were analysed in a Perkin-Elmer Applied Biosystems model 373 or model 377 DNA sequencer. DNA sequences were completed and edited using the Lasergene sequence analysis software package (DNASTAR). Multiple sequence alignments were performed according to the methods of Nobusawa et al. (1991) and Ha et al. (2001) with GeneDoc, version 2.3, software (developed by K. B. Nikolas).

**Infection of mice.** White female mice weighing 8 g were lightly anaesthetized with diethyl ether and inoculated intranasally with 50 µl of serial 10-fold dilutions of virus (six mice per dilution). The mortality rate was registered for 10 days after inoculation and expressed in 50% mouse
Table 1. Operational mapping of antigenic epitopes by haemagglutination inhibition

Values are the differences between the HI titres of mAbs (reciprocals of the antibody dilutions that inhibited 8 haemagglutinating units of virus) in the reactions with wild-type mouse-adapted Mld/PA/84 (H5N2) virus and the escape mutants in log₂ units. <, HI titre at least 32-fold less than the titre with the wild-type virus.

<table>
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<th>m58(2)</th>
<th>m176/26</th>
<th>m55(1)</th>
<th>m55(2)</th>
<th>m46(7)</th>
<th>m46(8)</th>
<th>m24B9</th>
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<th>m24B9-176/26</th>
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Table 2. Binding of monoclonal antibodies with Mld/PA/84-MA escape mutants in ELISA

Percentage binding was calculated by the equation $A = 100 \times \frac{(B_{xv}/B_{pw})/(B_{xw}/B_{pw})}{},$ where $A$ is percentage binding as compared with that of wild-type virus (100%), $B_{xv}$ is binding of the mAb to test virus, $B_{xw}$ is binding of pooled mAbs to test virus, $B_{pw}$ is binding of the mAb to wild-type virus and $B_{pw}$ is binding of pooled mAbs to wild-type virus. $<, < 25\%$ binding; $+, > 75\%$ binding; $\pm$, between 25 and 75% binding.

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<th>m58(2)</th>
<th>m176/26</th>
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lethal doses (MLD<sub>50</sub>) calculated by the routine method of Reed & Muench (1932).

**Results**

### Sequential selection of escape mutants

Monoclonal antibodies cp46, cp55 and cp58 to Ck/PA/1370/83, mAb 176/26 to A/Ck/PA/8125/83 and mAb 24B9 (obtained by immunization with Tk/Ontario/66 and Mld/PA/84) were chosen to be used for the selection of escape mutants because they reacted with the Mld/PA/84-MA virus at sufficiently high titres and differed in their patterns of reaction with H5 viruses (Smirnov et al., 2001). The escape mutants were selected in three successive steps. In the first step, eight escape mutants of wild-type Mld/PA/84-MA virus (one-step mutants) were obtained. Two mutants were selected by each of the mAbs cp46, cp55 and cp58, one by mAb 24B9 and one by mAb 176/26. On the basis of the results of a preliminary HI test with the panel of mAbs (not shown), four one-step mutants – m46(7), m55(2), m58(1) and m24B9 – were chosen for use in the selection of two-step escape mutants. Six two-step escape mutants were selected. Two-step escape mutant m46(7)-55 was used for the selection of two three-step escape mutants, m46(7)-55-176/26 and m46(7)-55-24B9. Each escape mutant was designated by the mAbs sequentially used to select it. For example, the escape mutant m46(7)-55-176/26 was obtained by selection with the mAb 24B9 and one by mAb 176. The mutants are shown in Tables 1, 2 and 3.

### Operational mapping of antigenic epitopes by HI and ELISA

HI was used to test all escape mutants with the five mAbs used for their selection and with two additional mAbs, 364/1 and 77B1. The results (Table 1) were used to distribute the mAbs into groups corresponding to four antigenic determinants, three of which partially overlapped. The non-overlapping epitope (group II in Table 1) was recognized by the mAb 24B9. This epitope has been identified by Philpott et al. (1990) as corresponding to antigenic site A in the H3 HA.

The one-step escape mutants selected by mAbs cp55 and cp46 were not cross-resistant to these two mAbs. However, the escape mutants selected by mAbs cp58 and 176/26 were resistant to both cp46 and cp55 (and to mAb 364/1, which reacted similarly to cp55). This finding suggested that the epitopes recognized by cp58 (and 176/26), by cp55 (and 364/1) and by cp46 partially overlapped. The epitopes were designated I<sub>a</sub>, I<sub>b</sub> and I<sub>c</sub>, respectively (Table 1).

The mAb 77B1 could not be definitely assigned to any of the groups. It reacted with the wild-type virus at a relatively low HI titre but reacted with some escape mutants at much higher titres. However, because the mutants selected with cp55 did not react with 77B1, it seems likely that the latter is related to group I<sub>b</sub>. The mAb 77B1 has been shown to recognize an area equivalent to site B in the H3 subtype (Philpott et al., 1990).

The two-step and three-step escape mutants reacted with mAbs in a pattern generally consistent with that of the one-
Sequence analysis of the escape mutants. However, mAb 176/26 reacted to a limited extent with two two-step escape mutants selected either with 176/26 (the mutant m24B9-176/26) or with a similar mAb, cp58 (the mutant m58(1)-24B9). It should be noted that mAb 176/26 also reacted with one-step escape mutants selected by cp58 and 176/26, although to a very slight extent, so that HI titres were too low to be reflected in Table 1 (7 log₂ units lower than the titre of the wild-type virus). Possible reasons for the anomalous reactions of mAb 176/26 are discussed with the ELISA results.

The pattern of cross-reaction of the escape mutants with mAbs in ELISA (Table 2) differed from the results of HI in several ways. The mAbs cp58, cp46, 24B9 and 77B1 reacted similarly in ELISA and in HI with one-step, two-step and three-step escape mutants. However, mAbs 176/26, cp55 and 364/1 reacted differently. In ELISA, mAb 176/26 reacted with several escape mutants that it had selected (or that the closely related cp58 had selected). The mAb cp55 also reacted with its own escape mutants, although to a lesser extent. The mAb 364/1 had not been used for the selection of mutants. However, it reacted in ELISA (but not in HI) with the mutants selected by the closely related cp55. The escape mutants that reacted in ELISA with the mAbs used for their selection were confirmed to be genuine escape mutants: they could not be neutralized by these mAbs in egg infectivity neutralization tests (not shown). One three-step escape mutant, m46(7)-55-176/26, unlike other mutants selected by the mAb 176/26, did not bind the mAb in ELISA (Table 2). This mutant also exhibited no residual HI reactivity with mAb 176/26, unlike the two-step escape mutants m24B9-176/26 and m58(1)-24B9 (Table 1). On the whole, the results of ELISA confirmed the grouping based on HI cross-reactions, although the continued binding of some mAbs to the neutralization-resistant mutants made interpretation of ELISA results more difficult than interpretation of HI results.

Sequence analysis of the escape mutants

The HA genes of the cloned Mld/PA/84-MA virus and of all escape mutants were sequenced and the encoded amino acid sequences were compared with the previously reported sequence of the Mld/PA/84-MA HA (Smirnov et al., 2000) (GenBank accession no. AF100179). The HA sequence of the cloned Mld/PA/84-MA virus used in our studies differed from the reported HA sequence in only one amino acid, A263T [H5 numbering according to Ha et al. (2001) is used here and below]. The amino acid changes in escape mutants were grouped mainly in three areas of the HA1 subunit (Table 3). The mutations conferring resistance to 24B9 (epitope II in the operational mapping) were concentrated in the area 136–141 (140–145 in the H3 sequence). With the exception of m46(7)-55-176/26, the mutants selected by the mAbs cp58 and 176/26 (epitope Ia) had a D126N change (position 131 in the H3 sequence). The mutants selected by cp55 (epitope Ia) had a change at position 152. Interestingly, the amino acid changes in two one-step escape mutants selected by cp46 (epitope Iac) occurred in different areas: N124D in m46(7) and K153M in m46(8). Fig. 1 shows the positions of the mutations identified in the escape mutants aligned with the three-dimensional structure of H5 HA (Ha et al., 2001). The amino acid changes identified by Philpott et al. (1990) are designated by asterisks.

![Fig. 1. Location of amino acid changes in the HA of H5 escape mutants in the three-dimensional structure of A/Duck/Singapore/3/97 H5 HA (Ha et al., 2001). The amino acid changes identified by Philpott et al. (1990) are designated by asterisks.](Image)
and m176/26 and m46(7)-24B9 had additional changes in the HA2 subunit. There is no evidence that these changes affected the antigenic specificity of the mutants. The mutants m58(1) and m58(2) behaved identically in the immunological tests, although m58(1), unlike m58(2), had no amino acid change at position 48. The location of the amino acid change T185K in the m46(7)-55a mutant corresponds to site B in H3 HA (T189K in H3 numbering) (Wiley et al., 1981); however, because no antigenic differences were observed between m46(7)-55a and m46(7)-55, which did not have this mutation, there is no evidence that the T185K affects immune specificity.

To determine whether the potential glycosylation site created by the D126N mutation is glycosylated and, if so, whether the carbohydrate chain blocks mAb binding, we infected MDCK cells with the mutant m58(1) or with the wild-type virus and incubated them with or without tunicamycin (TM) (2 µg/ml) for 2 h before and for 1 h during incubation with 14C amino acids. Cells were lysed and precipitated with mAb cp58. The mAb 176/26, which selected the same mutation, 176/26, could not be used in the immunoprecipitation studies because it retained the ability to bind the D126N mutants, as revealed by ELISA. Analysis of the immunoprecipitates by polyacrylamide gel electrophoresis revealed that mAb cp58 precipitated HA in the samples infected with wild-type virus and incubated either with or without TM, but did not precipitate HA in the samples infected with m58(1) and incubated without TM (Fig. 2). The incubation of m58(1)-infected cells with TM restored the reactivity of HA with the mAb to the level observed in cells infected with the wild-type virus. These results suggest that the prevention of glycosylation by TM unmasks the antigenic epitope that reacts with cp58.

Virulence of H5 escape mutants in mice
None of the amino acid changes observed in escape mutants (Table 3) coincided with the mutations observed in the

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<tr>
<td>1</td>
<td>Mld/PA/84-MA</td>
<td>—</td>
<td>3.52 ± 0.68</td>
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<td></td>
<td>m46(7)</td>
<td>N124D</td>
<td>4.12 ± 0.79</td>
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<tr>
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<td>m46(8)</td>
<td>K153M</td>
<td>2.76 ± 0.53</td>
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<td></td>
<td>m55(2)</td>
<td>K152N</td>
<td>6.13 ± 0.81</td>
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<td>m58(1)</td>
<td>D126N</td>
<td>6.38 ± 0.82</td>
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<tr>
<td></td>
<td>m24B9</td>
<td>R140G</td>
<td>4.00 ± 0.77</td>
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<td>N124D, K152T</td>
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<td>N124D, S129L, K152T</td>
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* Expressed as log10 EID50/MLD50 ± SE × tα,ν−2, where tα is the Student’s coefficient with probability α (α = 0.95).
† Reciprocals of HI titre against 8 haemagglutinating units.

Table 4. Effect of amino acid substitutions in the antigenic epitopes of Mld/PA/84-MA on virulence in mice and sensitivity to normal mouse serum inhibitors

Fig. 2. Radioimmunoprecipitation analysis of the haemagglutinin of an escape mutant that acquired a new potential glycosylation site. MDCK cells were infected with the wild-type Mld/PA/84-MA virus (lanes 1, 2, 5 and 6) or with the m58(1) escape mutant (lanes 3, 4, 7 and 8). The cells were incubated without TM (lanes 1, 3, 5 and 7) or in the presence of TM 3–6 h p.i. (lanes 2, 4, 6 and 8) and labelled with 14C amino acids 5–6 h p.i. The cells were either lysed in electrophoresis buffer (lanes 1–4) or lysed and immunoprecipitated with mAb cp58 (lanes 5–8). Cell lysates and immunoprecipitates were analysed by SDS–polyacrylamide gel electrophoresis. NG-HA, non-glycosylated HA.
HA gene in the process of the adaptation of the A/Mallard/Pennsylvania/10218/84 virus to mice (Smirnov et al., 2000). However, some amino acid changes occurred at positions earlier associated in H5 and other subtypes with variation in the pathogenicity (Philpott et al., 1990; Gitelman et al., 1986). We performed a parallel titration of the infectivity in embryonated chicken eggs and of the ability to kill mice of the wild-type virus and the relevant escape mutants. The first experiment was performed with six one-step mutants bearing a single mutation in the antigenically relevant area and one double mutant, m46(7)-55. We found amino acid changes D126N and K152N to be associated with a sharp decline in virulence (Table 4). The double mutant m46(7)-55, having a K152T substitution, had the same degree of virulence as the wild-type virus; this finding could be ascribed to a different effect of K152T versus K152N substitution, or to a reversal of the effect of the K152T mutation by the N124D change. In the second experiment, we investigated the double and triple mutants that had additional amino acid changes, with the exception of those selected from the single mutant having the D126N substitution, since the latter would mask the effect of the other mutations on virulence. The mutants m46(7)-24B9 and m46(7)-55-176/26, which had the additional mutations R140K and S129L, respectively, were slightly but statistically significantly less virulent than were m46(7) and m46(7)-55. The decrease in virulence associated with the D126N change correlated with an increased HI titre with normal mouse serum inhibitors (Table 4). The results of the virulence experiments were confirmed by repeated comparative titrations in eggs and mice.

Discussion

The sequence and three-dimensional structure of the HA antigenic epitopes has been characterized in detail only in the H3 subtype of influenza A (Wiley et al., 1981). The H3 three-dimensional model has since been used in studies of the H1 subtype (Caton et al., 1982), the H2 subtype (Tsuchiya et al., 2001) and, to a limited extent, the H5 subtype (Philpott et al., 1990). Although Philpott and others identified the antigenically significant areas of H5, the extent and structure of the relevant regions were not revealed. The relevant amino acid changes in the escape mutants selected in our studies by anti-H5 mAbs were grouped in three regions in the HA polypeptide chain. The region 136–141 corresponds to site A in the H3 structure (140–145 in H3 numbering) and to site Ca2 in H1 (Caton et al., 1982), forming a loop at the side of the HA molecule. One amino acid change in this region (position 145, H3 numbering) was demonstrated in an H5 escape mutant by Philpott et al. (1990). The amino acid changes at positions 152 and 153 (156 and 157 in H3 numbering) correspond to the area involved in the formation of site B in the H3 molecule. Philpott et al. (1990) identified a change in the H5 molecule at position 156 (H3 numbering). The H5 area 124–129, which corresponds to 129–133 in the H3 sequence, is located outside any site in the H3 HA structure recognized by virus-neutralizing mAbs (Wilson et al., 1981) but partially overlaps a region involved in the antigenic site Sa in H1 HA (Caton et al., 1982). As shown in Fig. 1, mapping of the amino acid changes on the three-dimensional structure of the H5 HA molecule (Ha et al., 2001) revealed that this region must be located near the area equivalent to site B in the H3 structure (Wilson et al., 1981). The change D126N conferred resistance to the mAb cp55, which selects escape mutants with an amino acid change at position 152 (156 in H3 numbering); this position is within the area equivalent to site B (Fig. 1). It appears likely that the region 124–129 (129–133 in H3 numbering), although not involved in the formation of site B in the H3 molecule, plays an important role in the formation of its equivalent site in the H5 subtype. The amino acid change D126N located within this region leads to the formation of a glycosylation site. A change in the equivalent position (131 in H3 numbering) in an escape mutant of H2 also formed a carbohydrate attachment site (Tsuchiya et al., 2001). The new site is glycosylated in both the H2 subtype (Tsuchiya et al., 2001) and the H5 subtype (Fig. 2), and the removal of the carbohydrate restores the reactivity with mAb. The carbohydrate chain masks the antigenic epitope in such escape mutants, obscuring the exact location of the HA polypeptide region that reacts with mAbs cp58 and 176/26. However, because the D126N change conferred resistance to mAbs cp55 and cp46 (Table 1), this region must be located within the same epitope as positions 152 and 153 (156 and 157, H3 numbering) (Table 3), that is, within the site that partially corresponds to site B in the H3 molecule.

We observed several unusual reactions between mAbs and escape mutants. The escape mutants that had the amino acid change D126N were resistant to infectivity neutralization by mAb 176/26 but continued to bind the mAb in ELISA (Table 2). H3 mutants with residual binding capacity for the mAb used for selection have been described (Daniels et al., 1987), but they were receptor-binding variants whose antigenic specificity was not altered. This explanation is unlikely to apply to our D126N mutants, because the mAb cp58 selects such mutants but does not react with them in ELISA. The two-step mutants with the D126N change reacted with mAb 176/26 (but not with cp58) in HI, although to a low titre (Table 1). It seems plausible that the epitope recognized by 176/26 includes two parts, one of which is important for infectivity neutralization and is recognized by both cp58 and 176/26, and one that is inaccessible to cp58 and is able to bind 176/26 with no effect on infectivity. The retention of antibody-binding ability by escape mutants is probably not exclusive to the mutants selected with mAb 176/26. The mAb cp55 also binds its escape mutants (Table 2). The mAb 364/1, although not used for the selection of escape mutants, may have similar features, because it behaves like cp55 with respect to the cp55 escape mutants (Tables 1 and 2). Therefore, H5 escape mutants may have a characteristic tendency to acquire a resistance to
infectivity neutralization by a mAb while retaining the ability to bind the antibody molecule.

The mAb 77B1 exhibited higher HI titres with D126N escape mutants than with the wild-type virus. The strain Tk/Ontario/66, against which mAb 77B1 had been raised, has an asparagine residue at this position (Philpott et al., 1990). Thus, the increase in titre may reflect the presence in the mutant (but not in the wild-type virus) of an epitope identical to that of the virus used for immunization.

The reduced virulence of the escape mutants in mice, at least of the mutants having D126N and K152N substitutions (Table 4), is most likely due to the mutations selected by the mAbs in the HA rather than to random co-selection of the mutations in other viral genes. First, the loss of virulence associated with the D126N change occurred in two escape mutants selected independently with different mAbs, making a random effect of a co-selected mutation unlikely. Secondly, we previously showed that an amino acid change in this position in a mouse-adapted H1N1 virus eliminated a glycosylation site and correlated with the acquisition of virulence in mice (Gitelman et al., 1986). A correspondence between reduced virulence in mice and the appearance of new glycosylation sites has also been shown in viruses of the H3 subtype (Reading et al., 1997). In this case, as with our D126N mutants, the loss of virulence was associated with an enhanced reaction with mouse serum inhibitors. Another amino acid change associated with decreased virulence in mice, K152N (Table 4), is located at the same position as a substitution that attenuates the virulence of an H5 escape mutant in birds (Philpott et al., 1990). This fact argues against a random co-selection of mutations in other viral genes as the cause of the reduced virulence of the K152N escape mutant in mice. The mutations in the H5 escape mutants that attenuate their virulence may be regarded as pleiotropic, and their occurrence can presumably influence immune selection under natural conditions.

We were unable to select mutants that had amino acid changes in the regions equivalent to the H3 sites C and E or at position 120 (H3 numbering), a site described by Philpott et al. (1990). Tsuchiya et al. (2001) recently reported that mAbs reacting with the sites in the H2 molecule equivalent to the H3 sites C and E neutralized infectivity but did not react in H1. We used exclusively high-titre HI-reactive mAbs, and this choice of mAbs could have limited the diversity of the selected escape mutants. However, our findings provide detailed information about the sites equivalent to the H3 A and B regions. In the studies of Philpott et al. (1989, 1990), mAb 24B9 selected a mutant with an S141P change. In our study, this mAb selected a series of mutants with changes at positions 136, 138, 140 and 141. Thus, in H5 HA, the loop forming site A must extend at least from position 136 to position 141. The H5 site that is equivalent to site B of H3 appears to be more complex in H5 than in H3. We found that this site contains not only the region present in the H3 site B but also the region 124–129, which partially overlaps site Sα of H1 (Caton et al., 1982). It is noteworthy that all mAbs obtained by immunization with Ck/PA/1370/83 and Ck/PA/8125/83 viruses reacted with this complex site (Tables 1 and 2). Therefore, the cells that produce antibodies against this site appear to predominate in the mouse immune repertoire. Interestingly, only one amino acid change in our H5 escape mutants coincided with the position (131 in H3 numbering) of a mAb-selected mutation in the closely related H2 HA (Tsuei et al., 2001). However, amino acid changes in the H5 escape mutants occurred at several positions (131, 144, 156, 157 and 189 in H3 numbering) that coincided with substitutions in H2 drift variants (Tsuei et al., 2001). It seems plausible that the antigenic sites described and analysed here may play an important role in drift variation if the H5 virus appears as a pathogen in human circulation.

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References


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