Pandemic influenza A viruses derive from birds, which harbor all currently known influenza A subtypes, hemagglutinins (HAs) H1 to H15 and neuraminidases (NAs) N1 to N9 [17]. When an avian HA subtype not found in humans crosses the species barrier, there is the potential for pandemics [6]. In 1997, an H5N1 virus was transmitted from birds to humans in Hong Kong and killed one-third of the people (6 of 18) it infected [2, 11]. Avian viruses containing one or several genes similar to those of the human H5N1 isolates continue to circulate in China [4, 14]. In 2001, an H5N1 virus emerged again in poultry markets in Hong Kong. In 2003, another H5N1 virus infected a single family, killing one of its members [5]. These events emphasize the continuous pandemic threat posed by the H5 virus.

Vaccination is one of the most effective ways to control influenza, provided the antigenicity of the vaccine strains matches that of the circulating strains, particularly with respect to HA. Avian influenza viruses are thought to be evolutionary static, unlike mammalian viruses [15], which suggests there is limited antigenic drift in birds. However, during the outbreak of avian influenza in Mexico in 1993–1995, the virus rapidly evolved [3, 7]. In this study, we compared HA antigenicity of the H5 viruses isolated from humans in 1997 and 2003 using a panel of monoclonal antibodies (mAbs) raised against the index H5N1 human influenza A virus, A/Hong Kong/156/97. By immunizing mice with a plasmid expressing this HA and boosting the initial immunization with cell lysates transfected with the plasmid, a total of six hybridomas producing HA-specific mAbs were established: four to the HA1 subunit with hemadsorption-inhibiting activity and two to the HA2 subunit. None of the mAbs to HA1 could bind to the HA of a recent human isolate, A/Hong Kong/213/2003, indicating that there are substantial antigenic differences between the H5N1 human influenza virus isolated in 1997 and that isolate d in 2003.

Abst ract. To assess whether the antigenic properties of H5 hemagglutinin (HA) change over time due to antigenic drift, we produced a panel of monoclonal antibodies (mAbs) against the HA of the index H5N1 human influenza A virus, A/Hong Kong/156/97. By immunizing mice with a plasmid expressing this HA and boosting the initial immunization with cell lysates transfected with the plasmid, a total of six hybridomas producing HA-specific mAbs were established: four to the HA1 subunit with hemadsorption-inhibiting activity and two to the HA2 subunit. None of the mAbs to HA1 could bind to the HA of a recent human isolate, A/Hong Kong/213/2003, indicating that there are substantial antigenic differences between the H5N1 human influenza virus isolated in 1997 and that isolate d in 2003.
We then used these mAbs in a hemadsorption-inhibition assay with chicken red blood cells. The HA-expressing cells were incubated in mAb-containing medium for 30 min at 37°C prior to use in the assay. The three HA1-specific mAbs and mAb 94F1 inhibited hemadsorption, suggesting that they may be useful for viral neutralization (Table 1). These data also confirm that mAb 94F1 recognized an epitope in HA1.

The mAbs were subsequently used to investigate the antigenicity of H5 human isolates as well as that of two highly pathogenic avian viruses representing North American (i.e., A/turkey/Ontario/7732/66 (H5N9)) and Eurasian (i.e., A/turkey/Ireland/1137/85 (H5N7)) lineages in H5 HAs. Cells expressing the individual HAs were incubated with each mAb and their interactions evaluated by means of an ABC immunodetection assay (Vector Laboratories, Burlingame, U.S.A.). Both of the HA2-specific mAbs recognized all of the HAs tested. Three of the four HA1-specific mAbs recognized the turkey/Ireland HA, while only one of these four recognized the turkey/Ontario HA. These results are consistent with the genetic similarity of these viruses: Eurasian vs. North American lineages. By contrast, none of the HA1-specific mAbs recognized the 2003 human virus. This observation is noteworthy, because the HA gene of the 2003 virus is phylogenetically more closely related to the 1997 virus than is the turkey/Ireland virus [5].

To confirm the HA antigenic difference between the 1997 and 2003 viruses totally, we used sera from immunized mice in an hemagglutination inhibition (HI) assay. These sera showed higher HI titers (64 to 256) to the homologous 1997 virus than those (4 to 128) to the 2003 virus. It is interesting to note that HA2-specific mAbs were obtained using this methodology given that HA1 is more immunogenic than HA2. HA2 is more conserved than HA1, possibly due to functional importance and/or lack of immune pressure. We examined whether our HA2-specific mAbs could recognize the HA of other subtypes, including H1, H2, and H3. Both HA2-specific mAbs recognized the HA from A/WSN/33 (H1N1), but failed not from A/mallard/New York/78 (H2N2) or A/Memphis/8/88 (H3N2).

### Table 1. Reactivity of monoclonal antibodies to HAs of H5 and other HA subtypes

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Ig subclass</th>
<th>Specificity a) Hemadsorption inhibition b)</th>
<th>Reactivity of each mAb to HAs c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HK/97 HK/2003 T/O T/I WSN (H1) MNY (H2) MEM (H3)</td>
<td></td>
</tr>
<tr>
<td>31G1</td>
<td>IgG2a (λ)</td>
<td>+ + – – – ND d)</td>
<td></td>
</tr>
<tr>
<td>61B2</td>
<td>IgG2a (λ)</td>
<td>+ + – + + ND</td>
<td></td>
</tr>
<tr>
<td>62H7</td>
<td>IgG2a (λ)</td>
<td>+ + – – + ND</td>
<td></td>
</tr>
<tr>
<td>94F1</td>
<td>IgG2a (λ)</td>
<td>+ + – + + ND</td>
<td></td>
</tr>
<tr>
<td>61E5</td>
<td>IgG2a (λ)</td>
<td>– – + – –</td>
<td></td>
</tr>
<tr>
<td>81E1</td>
<td>IgG2a (λ)</td>
<td>– + + + ND</td>
<td></td>
</tr>
</tbody>
</table>

a) Specificity was determined by using an immunoblot assay after SDS-PAGE of the HA-expressing cell lysates under reducing conditions.
b) Hemadsorption-inhibition was performed with chicken erythrocytes and the homologous virus, Hong Kong/156/97 (H5N1) (HK/97).
c) Reactivity of each mAb was determined by using an immunoenzyme assay to the HAs from Hong Kong/213/203 (H5N1) (HK/2003), turkey/Ontario/7732/66 (H5N9) (T/O), turkey/Ireland/1137/85 (H5N7) (T/I), WSN/33 (H1N1), mallard/New York/78 (H2N2) (MNY), and Memphis/8/88 (H3N2) (MEM).
d) ND, not determined.
Here, we have established six mAbs specific for the HA of the 1997 index human H5N1 virus. Of these, 4 specific for the HA1 subunit and at least 3 recognize different epitopes, as revealed by their reactivity with different H5 HAs. The lack of cross-reactivity between these mAbs and the 2003 human HA, despite the genetic similarities between the 1997 index and 2003 viruses, indicates that antigenic drift can and does occur in avian viruses during replication in land-based poultry, such as chickens.

Since the emergence of the H5N1 virus in humans in 1997, several H5 vaccines have been produced, including traditional inactivated, vector-expressed subunit vaccines and DNA vaccines [1, 8–10, 12, 13, 16]. However, the efficacy of these vaccines against the 2003 virus is questionable given the antigenic difference between the 1997 and 2003 viruses. Our findings indicate that antigenic drift must be taken into account when selecting vaccine strains, even for avian viruses that circulate among land-based poultry.

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