Efficacy of H5 Influenza Vaccines Produced by Reverse Genetics in a Lethal Mouse Model

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(See the editorial commentary by Schwartz and Gellin and the article by Stephenson et al., on pages 1207–9 and 1210–5, respectively.)

We studied the efficacy, in mice, of 2 H5 influenza vaccine viruses produced by reverse genetics. Mice were immunized with inactivated viruses and then inoculated with a human H5N1 1997 or 2003 virus or an avian H5N1 2001 virus. Vaccine viruses that we tested raised high levels of hemagglutination-inhibiting (1:160–1:1280) and virus-neutralizing (1:900–1:1900) antibodies on day 21 after a single dose of vaccine and decreased or prevented virus replication in mouse lungs; 54.5%–100% of immunized mice survived, whereas all control mice died. Protection was achieved despite antigenic differences and incomplete matching of the vaccine strain and the challenge virus. Therefore, high levels of cross-protection are predicted in the mouse model.

Highly pathogenic H5N1 avian influenza viruses are widespread and have become endemic in eastern Asia [1–3]. In the winter of 2003–2004, these viruses caused unprecedented epizootics in poultry in numerous countries in the region [3, 4]. Forty-four confirmed cases of human disease in Vietnam and Thailand, with 32 fatalities, were reported in 2004 [3–5] (http://www.who.int/csr/disease/avian_influenza/country/cases_tables_2005_02_02/en/). These events highlight the urgent need to develop vaccines for the control of emerging H5N1 influenza viruses and the prevention of infection by these viruses in humans and poultry in Asia. Human vaccines against the H5N1 viruses are not available. Avian H5 vaccine against H5N2 viruses of North American lineage has been used in Mexico since 1995 [6], but avian vaccines against Eurasian H5N1 viruses are not commercially produced.

The protection provided by influenza vaccines is based on the induction of virus-neutralizing antibodies, mainly against hemagglutinin (HA). The HA genes of highly pathogenic Asian H5N1 viruses are all of the same phylogenetic lineage, but the HAs of viruses isolated in 2003 and especially in 2004 are antigenically different from those isolated between 1997 and 2001 [1, 3, 4]. Antigenic drift in the H5 HA raises questions about the cross-protective properties of H5 HAs in human and avian vaccines. The cross-protective properties of Asian H5N1 viruses have not been studied in vitro or in animal models.

A recent development in the production of H5N1 vaccines is the use of reverse genetics [7]. Vaccine viruses obtained by using reverse-genetics techniques contain the HA and neuraminidase (NA) genes of naturally circulating isolates in a genetic background of strains with high egg-growth capacity. To remove 1 of the pathogenic properties from the vaccine virus, the basic amino acids at the cleavage site of the H5 HA are deleted [7–10]. The main benefits of using reverse-genetics techniques are that they allow a well-matched nonpathogenic H5N1 vaccine virus to be produced, and they enable rapid vaccine production [10].

The aim of the present study was to determine the cross-protective efficacy and immunogenicity of inactivated H5 influenza vaccine viruses in a mouse model. We tested 2 H5 vaccine viruses produced by reverse genetics and that contained modified HA. The candidate virus for the avian vaccine contained the HA gene of A/goose/Hong Kong/437.4/99 (Gs/HK/99), an H5N1 virus, and the NA gene of A/duck/Germany/1215/73, an H2N3 virus. This avian vaccine virus was designated ΔH5N3. The human vaccine candidate virus contained the HA and NA genes of A/Hong Kong/213/03 (HK/213/03), an H5N1 virus, and was designated ΔH5N1/03. The gene segments encoding the internal proteins in both reassortant vaccines were from A/PR/8/34 (H1N1) virus. The production and standardization of the vaccine viruses were as described elsewhere [9, 10].

Methods and results. Table 1 shows the antigenic relationships among H5N1 viruses used in the study as determined in hemagglutination inhibition (HI) tests [11]. The HA of A/Hong Kong/156/97 (HK/156/97) was antigenically similar to that of Gs/HK/99 but was different from that of HK/213/03. Analysis of HI titers according to the method of Archetti and Horsfall [12] showed that these antigenic differences were indeed sig-
Table 1. Antigenic relationship between H5N1 viruses used for immunization and challenge.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ck/Hidalgo/28159-232/95</th>
<th>HK/156/97</th>
<th>Gs/HK/99</th>
<th>Ck/HK/122/02</th>
<th>HK/213/03</th>
<th>ΔH5N1/03</th>
<th>CP 24</th>
<th>CP 46</th>
<th>CP 58</th>
<th>CP 406/7</th>
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<tbody>
<tr>
<td>Ck/Queretaro/14588-19/95</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>320</td>
<td>40</td>
<td>40</td>
<td>1600</td>
<td>800</td>
<td>400</td>
<td>6400</td>
</tr>
<tr>
<td>HK/156/97</td>
<td>40</td>
<td>10,240</td>
<td>10,240</td>
<td>1280</td>
<td>40</td>
<td>40</td>
<td>6400</td>
<td>12,800</td>
<td>3200</td>
<td>12,800</td>
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<tr>
<td>Gs/HK/99</td>
<td>20</td>
<td>2560</td>
<td>5120</td>
<td>640</td>
<td>20</td>
<td>40</td>
<td>3200</td>
<td>3200</td>
<td>1600</td>
<td>3200</td>
</tr>
<tr>
<td>Ck/HK/01-MB</td>
<td>&lt;10</td>
<td>2560</td>
<td>1280</td>
<td>640</td>
<td>20</td>
<td>40</td>
<td>3200</td>
<td>&lt;100</td>
<td>3200</td>
<td>&lt;100</td>
</tr>
<tr>
<td>HK/213/03</td>
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<td>320</td>
<td>2560</td>
<td>6120</td>
<td>320</td>
<td>1280</td>
<td>800</td>
<td>&lt;100</td>
<td>800</td>
<td>100</td>
</tr>
<tr>
<td>ΔH5N3</td>
<td>40</td>
<td>2560</td>
<td>2560</td>
<td>640</td>
<td>40</td>
<td>40</td>
<td>3200</td>
<td>6400</td>
<td>1600</td>
<td>6400</td>
</tr>
<tr>
<td>ΔH5N1/03</td>
<td>20</td>
<td>320</td>
<td>2560</td>
<td>640</td>
<td>320</td>
<td>1280</td>
<td>800</td>
<td>&lt;100</td>
<td>800</td>
<td>200</td>
</tr>
</tbody>
</table>

**NOTE.** Titers are expressed as the reciprocal of the highest dilution of serum that inhibits 4 hemagglutinin (HA) units of virus. The titers of serum samples with homologous viruses are underlined.

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significant. An avian virus, A/chicken/Hong Kong/YU822,2,01 (Ck/HK/01-MB), was antigenically more related to human H5N1/97 and avian H5N1/99 viruses than to ΔH5N1/03 (table 1). The results of the antigenic analysis also showed that the modified vaccine viruses were antigenically indistinguishable from the corresponding wild-type viruses.

Groups of 16–19 female, 6-week-old BALB/c mice (Jackson Laboratories) were immunized by subcutaneous injection of 50 μL of purified, β-propiolactone–inactivated virus preparations that contained 7 or 15 μg of viral HA in a 1:1 mixture of PBS and incomplete Freund’s adjuvant. Control groups were mock immunized with PBS and incomplete Freund’s adjuvant only. High doses of antigen with the inclusion of an adjuvant were used because of the single-dose immunization protocol. On day 21 after immunization, mice were inoculated intranasally with 50 μL of PBS-diluted allantoic fluid that contained ~55% mouse lethal dose (MLD₅₀) of HK/156/97 (0.5 × 10⁻⁵ 50% egg-infecting dose (EID₅₀), HK/213/03 (0.5 × 10⁻⁶ EID₅₀), or a highly pathogenic variant of the avian virus Ck/HK/01-MB (0.5 × 10⁻⁷ 5 EID₅₀) that is neurotropic in mice [13]. Infected mice were observed for 15 days for morbidity and mortality. Mice used in the study were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC), under an approved protocol from the Animal Care and Use Committee of St. Jude Children’s Research Hospital. All work with infected mice was performed in biosafety level 3 facilities that had been approved by the US Department of Agriculture and the Centers for Disease Control and Prevention.

Survival rates and morbidity in experimental and control mice showed that a single dose ΔH5N3 vaccine protected against lethal challenge with HK/156/97, Ck/HK/01-MB, or HK/213/03 virus (table 2). No significant differences in survival rates were observed between mice immunized with 7 μg and those immunized with 15 μg of HA of ΔH5N3 vaccine virus, and 90%–100% of immunized mice survived, whereas all control mice died. Both doses of ΔH5N3 vaccine prevented morbidity in immunized mice: we observed a loss of only 5%–20% of initial body weight in immunized mice. Loss of 20% body weight in mice that received 7 μg HA and were infected with HK/213/03 virus can be explained by the extremely high viral load (0.5 × 10⁻⁵ EID₅₀) in the challenge dose of ~5 MLD₅₀. Despite the decreased body weight, most of the immunized mice remained active and did not show any visual signs of disease.

A single immunization with 15 μg HA of ΔH5N1/03 protected mice against subsequent challenge with all 3 H5N1 viruses tested: survival rates were 90%–100% in immunized groups, but 100% mortality was seen in control mice (table 2). Immunization with 7 μg HA of ΔH5N1/03 vaccine protected all mice against challenge with HK/156/97 and HK/213/03 viruses but did not protect all immunized mice inoculated with the highly pathogenic Ck/HK/01-MB virus (table 2). Both doses of ΔH5N1/03 vaccine prevented morbidity in immunized mice: we recorded a loss of 5%–16% body weight and saw no visual signs of disease in immunized mice. In mice that received 7 μg HA of ΔH5N1/03 vaccine and were inoculated with Ck/HK/01-MB, we recorded severe neurological disorders in only 5 mice, which died during the experiment, but saw no clinical signs or disorders in the 6 surviving mice.

To determine viral titers in tissue, 3 mice in each inoculated group were killed on day 6 after inoculation. Previous studies have shown that H5N1 viruses are isolated from mouse lungs at similarly high titers on days 3–6; however, the titers of virus isolated from mouse brains and internal organs is low early during the infection [1, 13]. Thus, day 6 was chosen to allow the detection of virus not only in the lungs but also in the brains and internal organs of infected mice. We removed their lungs, brains, and spleens and prepared an ~10% homogenate of each tissue, as described elsewhere [13]. The EID₅₀ of virus...
in each homogenate was determined by growth in 10-day-old embryonated chicken eggs.

All of the challenge viruses replicated at high titers in the lungs of mock-immunized control mice (table 2). Immunization with the ΔH5N3 vaccine prevented the replication of Ck/HK/01-MB and HK/213/03 and substantially reduced the replication of HK/156/97 in lungs when it was administered at the higher dose, and mice immunized with the lower dose of this vaccine had significantly reduced titers of all 3 challenge viruses in the lungs (table 2). Immunization with ΔH5N1/03 vaccine at the higher dose prevented replication of all 3 viruses in the lungs and, at the lower dose, prevented the replication of HK/213/03 in lungs and resulted in significantly reduced titers of HK/156/97 and Ck/HK/01-MB in the lungs (table 2).

We detected only 1 of the tested viruses, Ck/HK/01-MB, at high titers in the brains of control mice. The higher dose of each vaccine prevented neuroinfection with this virus (table 2), which we detected in trace amounts in the brains of mice that received 7 μg HA of ΔH5N3 vaccine. The same dose of ΔH5N1/03 vaccine resulted in a reduction in titers of Ck/HK/01-MB in the brains, but this reduction was not statistically significant (table 2). No virus was detected in the spleens of immunized or control mice.

To determine antibody responses to and cross-reactivity of the H5 subtype vaccines, we collected mouse serum samples on day 21 after immunization, treated them with receptor-degrading enzyme, and performed HI (figure 1A) [11] and neutralization (figure 1B) [14] tests. The neutralization tests were done in MDCK cells [14], with H5N1 viruses used for mouse infection and the homologous H5N1 viruses. The results of these experiments revealed that both vaccines were highly immunogenic at doses of 7 and 15 μg HA. Serum samples from mice immunized with ΔH5N3 vaccine demonstrated lower HI titers with human H5N1 viruses (HK/156/97 and HK/213/03) than with the avian 2001 virus (Ck/HK/01-MB) and the homologous virus (Gs/HK/99) (figure 1A). The neutralization test showed that serum samples from mice immunized with ΔH5N3 vaccine neutralized the 1997, 2001, and 2003 H5N1 viruses at similar titers and reacted with the homologous 1999 virus at a higher titer (figure 1B). In the HI test, serum samples from mice immunized with ΔH5N1/03 vaccine reacted at lower titers with the 1997 and 1999 H5N1 viruses than with the 2001 and 2003 viruses (figure 1A); in the neutralization test, serum samples from mice immunized with ΔH5N1/03 vaccine neutralized the 1997, 1999, and 2001 viruses at similar titers (figure 1B). Significant antigenic differences (figure 1C) were observed between HK/213/03 and Gs/HK/99 in HI assays but not in neutralization tests (determined by use of the method of Archetti and Horsfall [12]). Similar calculations were not possible with the other H5 viruses, because homologous mouse serum samples were not available.

**Discussion.** The protective effect of inactivated influenza vaccines is based on the virus-neutralizing antibodies induced, mainly against HA, by immunization. The antigenic epitopes responsible for the induction and for recognition by antibodies are predominantly located in the HA1 subunit of HA. Comparison of the amino acid sequences of the HA1 subunits of the tested vaccines and challenge viruses showed that they had

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**Table 2. Mortality, morbidity, and virus replication in immunized mice.**

<table>
<thead>
<tr>
<th>Challenge virus, vaccine dose, µg HA</th>
<th>ΔH5N3 avian vaccine (HA from Gs/HK/99)</th>
<th>ΔH5N1/03 human vaccine (HA from HK/213/03)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival, maximum weight loss, Viral titer</td>
<td>Survival, Maximum weight loss, Viral titer</td>
</tr>
<tr>
<td></td>
<td>surviving/ weight loss, %</td>
<td>Lungs</td>
</tr>
<tr>
<td>HK/156/97</td>
<td>0/11 (0) 40.6 ± 1.9 5.3 ± 0.3</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>7/11 (100) 11.8 ± 10.2 3.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>15/11 (100) 8.2 ± 8.9 1.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>Ck/HK/01-MB</td>
<td>0/10 (0) 19.3 ± 11.2 5.8 ± 0.2 4.5 ± 0.2</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>9/10 (90) 4.1 ± 5.4 2.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt; 0.5 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>15/10/100 4.3 ± 8.3</td>
<td>...</td>
</tr>
<tr>
<td>HK/213/03</td>
<td>0/11 (0) 39.6 ± 3.8 4.2 ± 0.2</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>12/12 (100) 21.2 ± 7.1 0.8 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>15/10/100 9.5 ± 10.2</td>
<td>...</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SD, unless otherwise indicated.

<sup>a</sup> Survival rates were calculated after observation for 15 days.

<sup>b</sup> Viral titers were calculated as the mean of log<sub>10</sub> EID<sub>50</sub>/0.1 mL ± SD determined in the lungs and brains of 3 mice. The lower limit of virus detection was 0.1 log<sub>10</sub>/0.1 mL of tissue homogenate. Ellipses indicate that no virus was detected.

<sup>c</sup> Differences in titers between the experimental and control groups are statistically significant at <i>p</i> = .0001—.0028, according to unpaired t test.
Figure 1.  A. Hemagglutination inhibiting titers of serum samples from immunized mice. Titers are expressed as the reciprocal of the highest dilution of serum that inhibits 4 hemagglutinin (HA) units of virus. Each bar represents mean titers from 3 individual mice ± SD (error bars). Four-fold and higher differences in hemagglutination inhibition (HI) titers were considered to be significant. B. Neutralizing antibody titers of serum samples from immunized mice. Titers are expressed as the reciprocal of the highest dilution of serum that neutralizes 50% of 50 TCID₅₀ of virus in MDCK-infected cells. Each bar represents mean titers ± SD (error bars) in serum samples from 3 individual mice. C. Archetti and Horsfall [12] analysis of the HI and neutralizing titers for vaccines and homologous challenge viruses. Geometric mean, where \( r_1 \) is the titer of antiserum to virus 1 tested against virus 2 divided by the titer of antiserum to virus 1 tested against virus 1 and \( r_2 \) is the titer of antiserum to virus 2 tested against virus 1 divided by the titer of antiserum to virus 2 tested against virus 2. The value of \( r \) estimates the degree of antigenic differences between virus 1 and 2. For identical viruses, a value of \( r = 1 \), a value for \( r < 0.5 \) or \( r > 2.0 \) indicates a significant antigenic difference. D. Schematic representation of the amino acid sequences in the HA1, showing differences between the H5 viruses studied. Viruses used for the preparation of vaccine candidates are printed in bold type. Amino acid residues that are unique to the HA of each virus are marked in blue, the position at which the residues are unique in 1999 and 2003 viruses and similar in 1997 and 2001 isolates is shown in red, and residues discerning the pairs of viruses are marked in green. The nos. indicating amino acid residues that form an antigenic epitope [16] are italicized and underlined. The sequences were obtained from GenBank. Amino acids are numbered according to the HA of H5 subtype [15].

94.1%–97.2% amino acid identity. Despite this high percentage of identity, however, the HAs of HK/156/97, Gs/HK/99, and Ck/HK/01-MB are antigenically distinct from that of HK/213/03 (table 1) [1]. The amino acid differences in the HA1 subunits are summarized in figure 1D. The amino acids at positions 126, 212, and 217 differentiated the viruses isolated in 1997 and 1999 from those isolated in 2001 and 2003, and the amino acid at position 138 was unique to the 1999 and 2003 viruses and
was similar to that in the 1997 and 2001 viruses (numbering according to the HA of H5 subtype [15]). Previous studies have shown that the amino acid residues at positions 124, 126, 129, 138, and 140 are located within the antigenic epitopes of the H5 HA molecule and are important for recognition and neutralization by monoclonal antibodies [16]. The amino acid changes we observed at these residues explain the different antigenicities of the challenge viruses and show that substantial antigenic drift has occurred in the HA of H5N1 vaccines.

The results of our study have shown that immunization with vaccines containing HAs from avian Gs/HK/99 and human HK/213/03 viruses that are antigenically different can protect mice against lethal challenge with viruses of similar antigenicity, as well as with viruses that are antigenically distant. Vaccine having the HA of the human HK/213/03 virus protected mice from infection by human HK/156/97 and avian CK/HK/01-MB viruses, and vaccine having the HA of avian Gs/HK/99 virus was also highly effective against infection by these viruses. Our data lead us to suggest that, at least with regard to 1999–2003 H5N1 influenza viruses, differences in the antigenic properties of virus HAs do not affect the protective properties of these vaccine viruses; for this reason, a high level of cross-protection was achieved in our mouse model.

Our study has also shown that the ΔH5N3 and ΔH5N1/03 vaccines were highly immunogenic at single doses of 7 and 15 μg HA. Serum samples from mice immunized with either vaccine reacted at high titers with all of the challenge viruses tested. The high rate of mortality (almost 50%) among mice that received 7 μg HA of ΔH5N1/03 vaccine and were infected with Ck/HK/01-MB virus can be explained by the extremely high pathogenicity and neurotropism of the virus rather than by low efficacy of the vaccine. Antibodies from mice vaccinated with 7 μg of HA of ΔH5N1/03 vaccine reacted to Ck/HK/01-MB with titers equivalent to those of viruses for which high protection was observed (figure 1A and 1B, table 2). Immunization with 7 μg HA of ΔH5N1/03 vaccine reduced titers of this virus in the lungs but did not prevent the initiation of neuronal spreading of virus to the brain. Note that, at a dose of 7 μg HA, ΔH5N3 vaccine was more effective than ΔH5N1/03 vaccine at preventing neuroinfection with Ck/HK/01-MB virus, but both vaccine viruses were highly effective at a dose of 15 μg HA.

The ubiquity of H5N1 influenza viruses in poultry and wild birds in Asia and the extremely high fatality rate among cases of H5N1 infection in humans in Vietnam and Thailand [1–5] stress the urgent need for attention to the development of H5N1 vaccines. The present results demonstrate the cross-protective efficacy of H5N1 vaccine viruses that contain antigenically different HAs. Our data suggest that, at least in the mouse model, the vaccine strain need not match the challenge virus to achieve a high level of cross-protection.

Acknowledgments

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References