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| Title            | Avirulent Avian Influenza Virus as a Vaccine Strain against a Potential Human Pandemic  |
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| Citation         | Journal of Virology, 73(10), 8303-8307<br><a href="https://doi.org/10.1128/JVI.73.10.8303-8307.1999">https://doi.org/10.1128/JVI.73.10.8303-8307.1999</a>   |
| Issue Date       | 1999-10   |
| Doc URL          | <a href="http://hdl.handle.net/2115/28140">http://hdl.handle.net/2115/28140</a>   |
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| Type             | article   |
| File Information | JV73-10.pdf   |



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## Avirulent Avian Influenza Virus as a Vaccine Strain against a Potential Human Pandemic

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Received 8 April 1999/Accepted 14 July 1999

**In the influenza H5N1 virus incident in Hong Kong in 1997, viruses that are closely related to H5N1 viruses initially isolated in a severe outbreak of avian influenza in chickens were isolated from humans, signaling the possibility of an incipient pandemic. However, it was not possible to prepare a vaccine against the virus in the conventional embryonated egg system because of the lethality of the virus for chicken embryos and the high level of biosafety therefore required for vaccine production. Alternative approaches, including an avirulent H5N4 virus isolated from a migratory duck as a surrogate virus, H5N1 virus as a reassortant with avian virus H3N1 and an avirulent recombinant H5N1 virus generated by reverse genetics, have been explored. All vaccines were formalin inactivated. Intraperitoneal immunization of mice with each of vaccines elicited the production of hemagglutination-inhibiting and virus-neutralizing antibodies, while intranasal vaccination without adjuvant induced both mucosal and systemic antibody responses that protected the mice from lethal H5N1 virus challenge. Surveillance of birds and animals, particularly aquatic birds, for viruses to provide vaccine strains, especially surrogate viruses, for a future pandemic is stressed.**

It has been well established that influenza viruses are maintained and circulate in waterfowl reservoirs (4, 26). To date, viruses of 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified in avian species (16, 26). Of these, only subtypes H1, H2, H3, N1, and N2 had been known to exist in humans in the last 100 years on the basis of serologic evidence. HAs of the pandemic human influenza A H2N2 and H3N2 viruses probably originated from avian viruses by genetic reassortment between avian and human viruses (5, 9, 17, 27, 29).

The isolation of influenza H5N1 virus from the fatal, index human case of viral pneumonia in Hong Kong in May 1997 (21, 30) on the heels of a serious outbreak of H5N1 infection on local chicken farms (2) signaled the possibility of the emergence of a new influenza pandemic virus. This was demonstrated even further when there were an additional 17 cases in November and December, 5 of which were fatal (30). There was, therefore, a critical need to set in motion preparation of a vaccine to the H5N1 virus notwithstanding the fact that, by mid-1997, preparation of the recommended vaccine to current H1, H3, and B interpandemic variants for the Northern Hemisphere winter were in hand and by late 1997 had been deployed.

Characterization studies in 1997 indicated that all eight genes of the human H5N1 virus were genetically avian and that HAs of both the avian and human H5N1 viruses contained multiple basic amino acids adjacent to the cleavage site (2, 21), indicating a highly pathogenic avian influenza virus. Moreover,

the virus was able to cause lethal infections in humans even though the receptor specificity of its HA is the same as that of avian viruses that preferentially bind to *N*-acetyl sialic acid linked to galactose by an  $\alpha$ -2,3 linkage that is found exclusively in avian tissues (12).

Because of its highly pathogenic nature, the virus could not be used to make a vaccine. It was rapidly fatal in the chicken embryo, the host used for vaccine production, the yield of virus was low when harvested early, and there was the necessity to carry out all work with a high level of biosafety. At the time, an antigenically closely related surrogate virus was not available (20). Such demanding constraints had not been present in the preparation of vaccines to the pandemic H2N2 and H3N2 viruses. In the end, the slaughter of chicken and other poultry in Hong Kong in late December 1997 seemingly averted a new pandemic. Nonetheless, exploration of alternative approaches for the production of H5N1 vaccines was necessary as an integral part of pandemic preparedness.

Here we report findings on the immunogenicity of an avirulent H5N4 virus from a migratory duck, an H5N1 reassortant virus prepared from H5N4 and H3N1 viruses from ducks, and an avirulent recombinant H5N1 virus with a genetically modified HA derived from the prototype human H5N1 virus and their potential to induce protective immunity against the pathogenic human virus.

### MATERIALS AND METHODS

**Viruses.** Influenza viruses A/Hong Kong/156/97 (H5N1) (HK156), A/Hong Kong/483/97 (H5N1) (HK483), A/duck/Hong Kong/836/80 (H3N1) (HK836), A/duck/Hong Kong/301/78 (H7N1) (HK301), and A/duck/Hokkaido/67/96 (H5N4) (Hok67) were propagated in the allantoic cavity of 10- to 11-day-old embryonated hen's eggs at 35°C for 48 h. Hok67 had amino acid sequences at the cleavage site of the HA typical of avirulent viruses (3). A reassortant virus (H5N1)(R513) between Hok67 and HK301 was prepared. A recombinant virus

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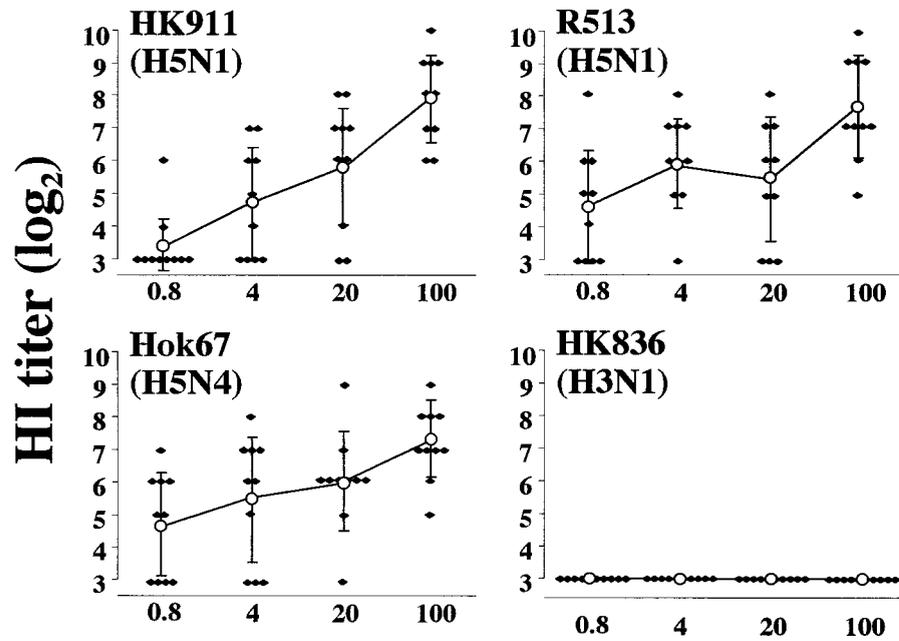
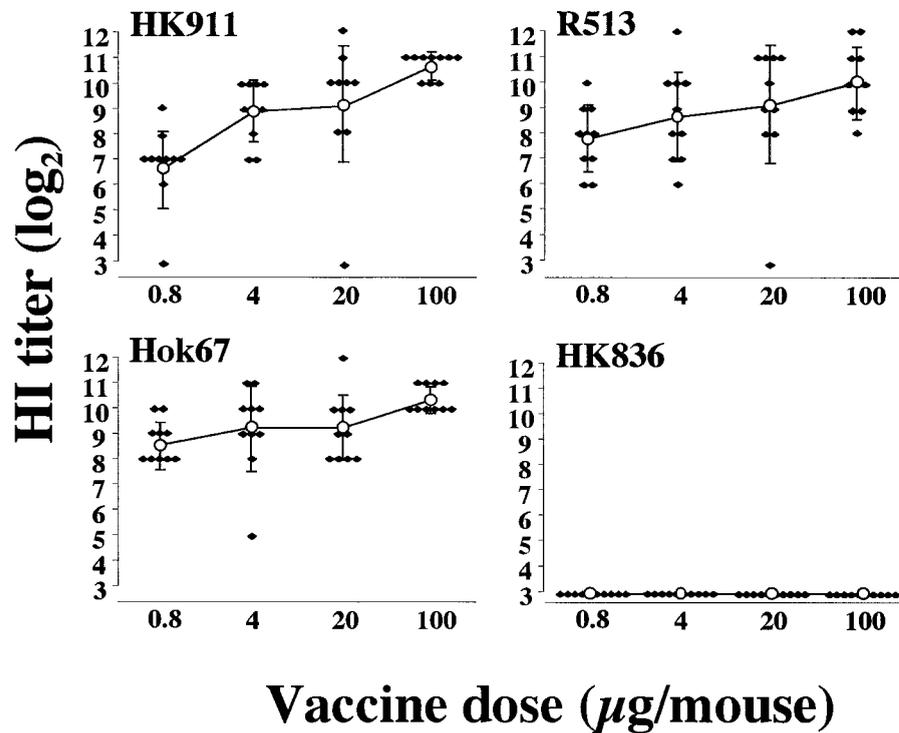
**A****B**

FIG. 1. Serum HI antibody response of mice immunized intraperitoneally. Ten mice in each group were immunized with different doses of each inactivated virus vaccine. The mice were sacrificed after 3 weeks, and serum HI antibody titers to HK483 (A) and HK156 (B) were determined. Each point represents a single mouse. Open circles and error bars represent means and standard deviations for each group. The limit of detection in this assay was  $2^4$ . Titers less than  $2^4$  were set to  $2^3$  for calculation of means and standard deviations.

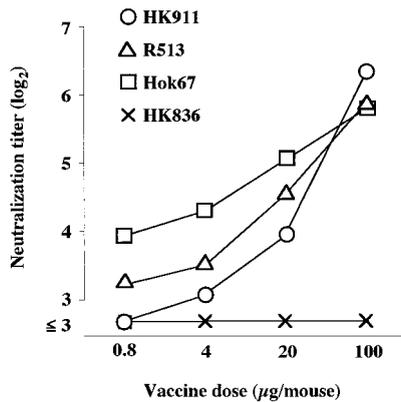


FIG. 2. Serum neutralizing antibody response of mice immunized intraperitoneally. The grouping of mice was the same as that described in the legend to Fig. 1. The sera from the 10 mice from each group were pooled and examined in neutralization tests. Each antibody titer to HK483 is expressed as the reciprocal of the serum dilution which gave 50% plaque reduction. The limit of detection in this assay was  $2^3$ .

with a modified HK156 HA gene to express avirulent type of HA (H5N1) (HK911) was generated by reverse genetics by using HK836 as a helper virus (unpublished data). The pathogenic viruses (HK156 and HK483) were handled in biosafety containment.

**Vaccines.** Virus was concentrated by high-speed centrifugation of infected allantoic fluid followed by differential centrifugation through a 10 to 50% sucrose density gradient and pelleted (10). The pellet was resuspended in phosphate-buffered saline (PBS) and treated with 0.1% formalin at 4°C for a week to inactivate the virus. Protein concentration of each of vaccines was standardized on the basis of optical densities at 280 nm and HA units. Each virus contained 100 to 200 HA units in 100 µg of purified viral proteins. Inactivation was confirmed by the absence of detectable hemagglutination activity following inoculation of the treated material into 10 embryonated eggs.

**Antibody assays.** Serum samples were examined for antibody by virus infectivity neutralization by using a plaque reduction assay with MDCK cells (6) and hemagglutination inhibition (HI) (19), using both the sera treated with receptor-destroying enzyme (Takeda Chemical Industries), and an enzyme-linked immunosorbent assay (ELISA) (6). In the ELISA, the wells were coated with disrupted HK911 obtained by treating purified virions with 0.05 M Tris-HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl at room temperature and diluted in PBS. The reactions were detected by using rabbit anti-mouse immunoglobulin A (IgA; Zymed Laboratories) and goat anti-mouse IgG (Bio-Rad Laboratories) antibodies conjugated to horseradish peroxidase.

**Immunization and protection tests.** Ten 6-week-old female ddY mice (Shizuoka Laboratory Animal Center) were immunized intraperitoneally with 0.5 ml of 100, 20, 4, and 0.8 µg of protein from inactivated viruses in PBS. Three weeks later, the mice were bled, and the sera were examined in HI and neutralization tests. The protective effects of intranasal vaccination was evaluated by intranasal inoculation with 20 µl of 100 µg of inactivated virus protein under anesthesia with sodium pentobarbital. The mice were revaccinated 2 and 3 weeks later. Control mice were given PBS under the same conditions. On the fourth week, five mice were sacrificed to obtain sera, trachea-lung washes, and nasal washes. Trachea-lung and nasal washes were collected as previously described (24). Ten mice were challenged intranasally with 10 µl of 20 50% lethal doses ( $LD_{50}$ ;  $10^{2.3}$  PFU in MDCK cells) of HK483 under anesthesia. Clinical signs were observed every 12 h for 20 days after challenge. The  $LD_{50}$  was determined by infecting five mice intranasally with 10 µl of serial 10-fold dilutions of HK483. The work was carried out in biosafety containment.

**Statistical analysis.** Statistical analysis of the experimental data was performed by using the two-tailed Student *t* test.

## RESULTS

**Immunogenicity of avirulent viruses.** To compare the immunogenicity of the avirulent avian viruses against that of the virulent H5N1 strains isolated from humans, mice were immunized intraperitoneally with inactivated virus vaccines and serum HI antibody responses were examined (Fig. 1). H5 viruses, a recombinant HK911 virus (H5N1) with a modified HK156 HA gene, a reassortant R513 (H5N1) virus between Hok67 (H5N4) and HK301 (H7N1), and Hok67 (H5N4) induced se-

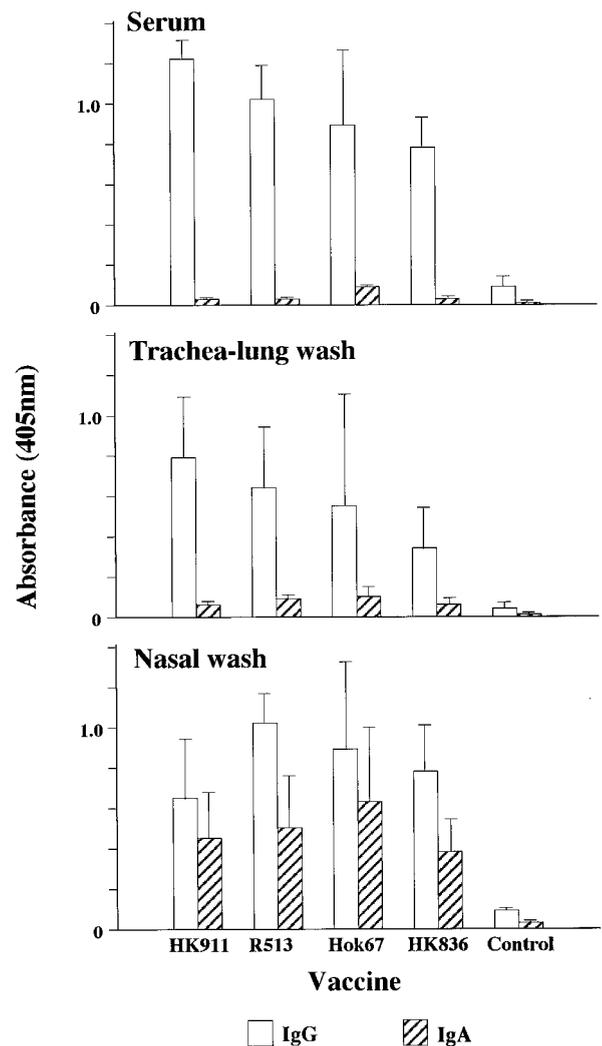


FIG. 3. Virus-specific antibodies in sera, trachea-lung washes, and nasal washes from vaccinated mice. Samples from five mice from each group were obtained before virus challenge. IgG and IgA antibodies in the samples of individual mice were detected by ELISA as described in Materials and Methods. Results are expressed as the mean absorbance  $\pm$  standard deviation of undiluted samples (trachea-lung and nasal washes) or 1:16 diluted samples (sera).

rum HI antibody responses to both HK156 and HK483 in a dose-dependent manner. Immunization with 100 µg of inactivated virus gave the highest titers, up to  $2^{11}$  and  $2^{12}$  and  $2^9$  to  $2^{10}$  for HK156 and HK483, respectively. All of these viruses induced higher HI titers to HK156 than to HK483, indicating that the antigenicity of Hok67 HA was more closely related to HK156 than to HK483. Statistically significant differences ( $P < 0.05$ ) in HI titers to the three viruses were found only between the sera of mice immunized with 0.8 µg of Hok67 and HK911. Immunization with HK836 (H3N1) did not induce any detectable serum HI antibody response to the H5N1 viruses. Neutralization tests were also carried out on pooled sera from each group (Fig. 2). The three H5 viruses induced serum neutralizing antibody titers that were in accordance with HI antibody titers. The results indicate that these avirulent viruses were sufficiently immunogenic and antigenic to elicit serum HI and neutralizing antibodies to pathogenic H5N1 viruses.

TABLE 1. Serum HI antibody responses of mice vaccinated intranasally

| Vaccine      | HI titer ( $\log_2$ mean $\pm$ SD) to: |               |
|--------------|--|---------------|
|              | HK483                                  | HK156         |
| HK911 (H5N1) | $4.6 \pm 1.1^a$                        | $7.8 \pm 1.3$ |
| R513 (H5N1)  | $5.0 \pm 1.2$                          | $6.8 \pm 1.8$ |
| Hok67 (H5N4) | $4.8 \pm 1.5$                          | $6.8 \pm 2.0$ |
| HK836 (H3N1) | $<4.0^b$                               | $<4.0$        |
| Control      | $<4.0$                                 | $<4.0$        |

<sup>a</sup> Titers are expressed as reciprocals of serum dilution. Titers less than 4.0 were set to 3.0 for calculation of means and standard deviations.

<sup>b</sup> Titers for all five mice sacrificed were less than 4.0.

**Antibody response of intranasally vaccinated mice.** Our previous studies demonstrated that intranasal vaccination of mice with viral antigens induced both mucosal and systemic antibody responses and conferred protection against intranasal challenge with the virus (22, 24). Hence, the inactivated avirulent H5 viruses were tested for their ability to induce antibody response when inoculated by the mucosal route.

IgG and IgA antibodies in the sera, trachea-lung washes, and nasal washes of intranasally vaccinated mice were measured by ELISA (Fig. 3). In the sera and trachea-lung washes of all the vaccinated mice, only IgG antibody was clearly detected. On the other hand, both IgG and IgA antibodies were detected in the nasal washes of these mice. As with HI and neutralization tests on the sera of intraperitoneally immunized mice, no significant difference was found in the antibody levels of mice vaccinated intranasally with any of the H5 viruses. The serum HI antibody titers of mice vaccinated with H5 viruses were also uniform (Table 1). On the other hand, antibody levels in the samples of mice vaccinated with HK836 (H3N1) were slightly lower than those of mice vaccinated with the H5 viruses. This may be due simply to the presence or absence of antibodies to the neuraminidase and internal proteins, since HK911 (H5N1) virions were used as an antigen in the ELISA. The absence of detectable serum neutralizing activity in mice intraperitoneally immunized with HK836 (Fig. 2) is in accord with this view. Intranasal vaccination of mice with the inactivated H5 viruses,

therefore, induced both mucosal and systemic antibody responses without the use of any adjuvant.

**Protective effect of intranasal vaccination of mice against virus infection.** Mice vaccinated intranasally with the respective inactivated avirulent viruses were challenged intranasally with a lethal dose of pathogenic HK483 (H5N1) virus. Survival rates of the mice after virus challenge are shown in Fig. 4. While all control mice died within 9 days after challenge, 80 to 90% of mice vaccinated with H5 viruses survived without showing any disease signs. Thus, intranasal vaccination with inactivated virus conferred protective immunity on mice. It was noted that 90% of mice vaccinated with HK836 (H3N1) were also protected from the lethal challenge although they showed clinical signs, including ruffled fur, inactivity, respiratory distress, hunched posture, and depression.

## DISCUSSION

The influenza virus H5N1 incident in Hong Kong in 1997 emphasized the need to have contingency plans for the production of a vaccine in the event that the pandemic virus cannot be satisfactorily grown in the conventionally used embryonated egg. The H5N1 virus causes lethal infections in eggs. There are essentially two alternatives for a vaccine, (i) an avirulent, antigenically related virus that can be used as a surrogate for the pandemic virus and (ii) a genetically attenuated virus. Here we take stock of the immunogenicity and protective capabilities against the human virus, of three experimental vaccines, namely, a surrogate virus (Hok67), an avirulent avian H5N1 reassortant virus (R513), and an avirulent recombinant virus (HK911) generated by reverse genetics.

All three viruses were immunogenic for human H5N1 viruses when administered intraperitoneally as shown in HI and neutralization tests. Moreover, mice vaccinated intranasally with these viruses were protected against lethal infection by the human virus. No difference was found between Hok67 and HK911 vaccines in their protective abilities. In nature, the HAs of avian influenza viruses occur in two phylogenetically distinct lineages, Eurasian and North American (26). The HAs of the H5N1 virus and the former H2N2 and H3N2 pandemic viruses belong to the Eurasian lineage as did the HAs of the three

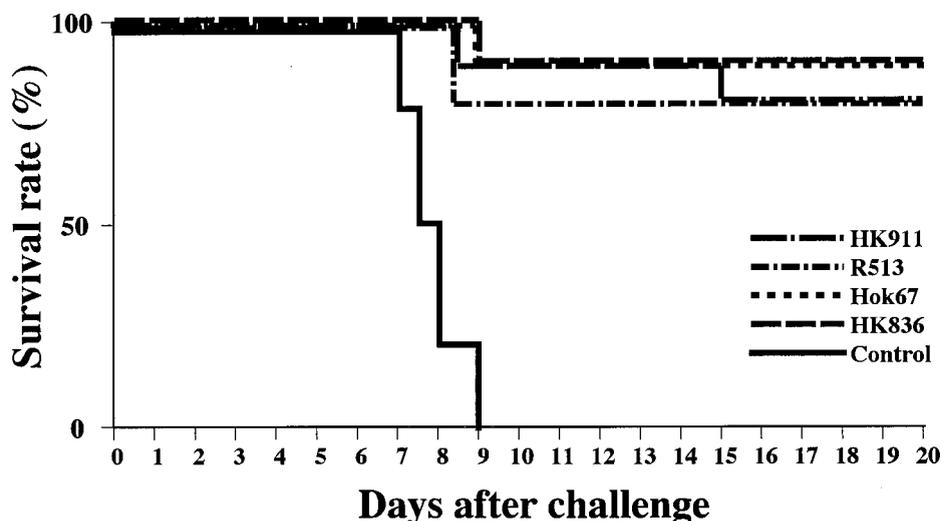


FIG. 4. Survival rates of mice after challenge with HK483. Mice were vaccinated intranasally with inactivated virus vaccines. PBS was used for control mice. All mice were challenged intranasally with 20 LD<sub>50</sub>s of HK483. Survival rates and clinical signs were observed every 12 h for 20 days.

viruses used as the vaccine starting material, thereby ensuring good HA subtype compatibility in the vaccine. Both Hok67 and HK156 belonged to the Eurasian lineage (3). The antigenicity of these H5 viruses was also closely related to that of the Eurasian avian H5 isolates (3, 20). The antigenicity of the HAs of duck influenza viruses are, in fact, highly conserved (8), and recent studies show that there is a cross-reactive immune response in mice against avian and human H3 virus infection (15).

In accord with our previous studies (22–24), the present results demonstrate that vaccination with inactivated virus by the respiratory mucosal route is a promising strategy to prevent respiratory virus infection. More importantly, such viruses are sufficiently immunogenic to induce a mucosal immune response, eliminating the use of potentially harmful adjuvants such as cholera toxin. The inactivated influenza viruses induced a mucosal immune response sufficient to interfere with or even to prevent initial infection on the mucosal surface as well as a systemic immune response, hence conferring effective protection on the mice.

While the surrogate and genetically manipulated H5 viruses were able to induce protective immunity in mice following intranasal vaccination, so did an H3N1 virus, HK836. This apparently enigmatic finding is consistent with the cross-protection afforded by NA-specific antibodies (1, 11, 14, 18), the N1 of H5N1 and H3N1 being closely related (unpublished data). Also, the sera of chickens experimentally infected with HK836 showed slight inhibition of hemagglutination activity by the human HK156 virus (unpublished data), possibly due to steric hindrance by anti-NA antibodies. It may also be possible that secretory IgA antibodies neutralized virus infectivity in the infected epithelial cells by interfering with a function of the newly synthesized viral proteins, including internal proteins (13). Since intact virus vaccine was used in this study, nasal secretory IgA antibodies to internal viral proteins should be induced in intranasally vaccinated mice. This may be an advantage of intranasal vaccination with an inactivated intact virus vaccine.

It has been hypothesized that avian influenza viruses of any HA subtype have the potential to contribute the genes for possible future pandemic strains in humans (7, 25, 28). This underlies the importance of global surveillance to isolate avian and animal influenza viruses as genetic resources for vaccine strain candidates and helps to facilitate prediction of the HA subtype of future pandemic influenza virus strains.

#### ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Culture, and Sports and the Ministry of Health and Welfare, Japan.

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