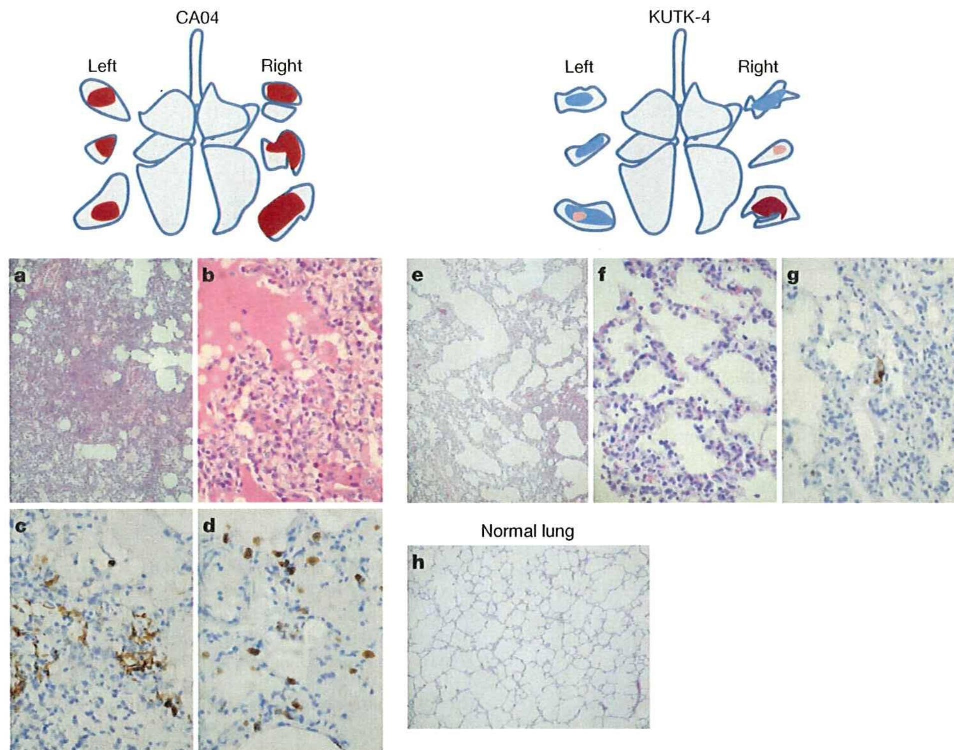

Figure 1 | Pathological examination of the lungs of infected cynomolgus macaques. a–h, Representative pathological images of CA04-infected (macaque no. 1, **a–d**), KUTK-4-infected (macaque no. 7, **e–g**) and mock-infected (**h**) lungs on day 3 after infection. One or two sections per lung lobe were examined. Representative findings are shown to depict the distribution of lesions in the sections (shown as cross-sections placed next to illustrations of each lung lobe), with or without viral antigen, as follows: brown, severe lung lesion containing moderate to many viral-antigen-positive cells; pink, mild lung lesions containing a few viral-antigen-positive cells; blue, lung lesions with alveolar wall thickening, with remaining air spaces unaffected. Original magnification: **a, e, h,** ×40; **b–d, f, g,** ×400.

KUTK-4 (Supplementary Fig. 10f and g). Thus, in all three mammalian models tested, CA04 seemed to be more pathogenic than a contemporary human H1N1 virus, KUTK-4.

Efficient human-to-human transmission is a critical feature of pandemic influenza viruses. To assess the transmissibility of CA04, naive ferrets in perforated cages were placed next to ferrets inoculated with 10^6 p.f.u. of CA04 (see Methods for detailed procedures). This experimental setting allows for aerosol transmission (that is, the exchange of respiratory droplets between the inoculated and non-inoculated ferrets) but prevents transmission by direct and indirect contact. All three contact ferrets were positive for CA04 virus on days 3 and 5 after infection (Supplementary Table 4). This transmission pattern is comparable to those of two human control influenza viruses that are known to transmit among ferrets: KUTK-4 and A/Victoria/3/75 (H3N2)[9]. By contrast, an avian influenza virus (A/duck/Alberta/35/76; H1N1) did not transmit (Supplementary Table 4).

Genetic analysis suggests that S-OIV originated in pigs[1]. However, there were no confirmed influenza virus outbreaks in Central American pigs before the reported S-OIV infections in humans. To assess S-OIV replication in pigs, we inoculated specific-pathogen-free miniature pigs, which are easier to manage, with CA04 or a classical swine influenza virus (A/swine/Hokkaido/2/81, H1N1). No signs of disease were observed (data not shown), although both viruses replicated efficiently in the respiratory organs of these animals (Supplementary Tables 5 and 6). Slightly higher titres of CA04 were detected in lungs on day 3 after infection, which is supported by pathological findings that show more apparent bronchitis and bronchiolitis in pigs infected with CA04 (Supplementary Fig. 11). The asymptomatic infection of CA04, despite efficient virus replication, might explain the lack of reports of S-OIV outbreaks in pigs before virus transmission to humans.

Antiviral compounds are the first line of defence against pandemic influenza viruses. Sequence analysis suggests that S-OIVs are resistant to ion channel inhibitors such as amantadine and rimantadine[1]. We therefore tested the licensed neuraminidase inhibitors oseltamivir and zanamivir, the experimental neuraminidase inhibitor R-125489 (the active form of CS-8958[10]) and the experimental compound T-705 (a broad-spectrum viral RNA polymerase inhibitor[11]) for their efficacy against CA04. In cell culture, CA04 was highly susceptible to all compounds tested (Supplementary Table 7), as were the human H1N1 control viruses A/Kawasaki/UTK-23/08 and KUTK-4, with the exception of the known oseltamivir resistance of KUTK-4. Comparable sensitivities were also found in an enzymatic neuraminidase inhibition assay[12] (Supplementary Table 8) and in mice (Fig. 2), consistent with observations in clinical settings.

A recent report suggested that 33% of individuals over 60 years of age had neutralizing antibodies to CA04 (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5819a1.htm; Morbidity and Mortality Weekly Report, Centers for Disease Control and Prevention), probably due to previous exposure to antigenically similar H1N1 viruses. In fact, both the human H1N1 viruses that circulated until 1957 and the classical swine virus HA gene of S-OIVs are descendants of the 1918 pandemic virus, possibly explaining their antigenic relatedness. In 1977, H1N1 viruses re-emerged that were genetically and antigenically very closely related to viruses circulating in the 1950s[13] and should thus have elicited neutralizing antibodies to CA04 among younger age groups; however, this does not seem to be the case, according to the above described report. To resolve this puzzling finding, we assessed the neutralizing activities of sera collected from a broad range of age groups against CA04 and KUTK-4. We used two sets of donor sera, collected in 1999 from residents and workers in a nursing home (donor set 1), and in April

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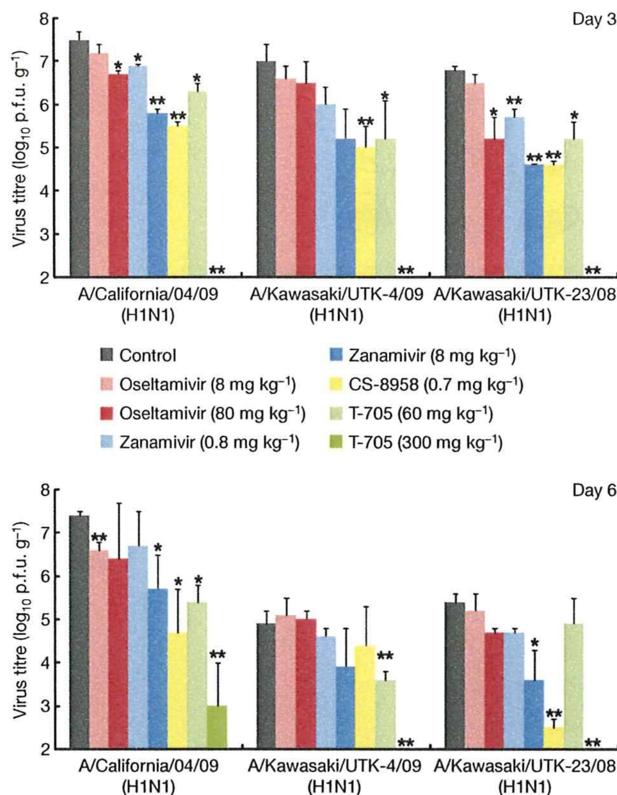

Figure 2 | CA04 sensitivity to antiviral compounds in mice. Mice were intranasally inoculated with 10^4 p.f.u. (50 μl) of CA04, KUTK-4 or A/Kawasaki/UTK-23/08 (H1N1). At 1 h after infection, mice were administered oseltamivir phosphate, zanamivir, CS-8958, T-705, or distilled water and PBS (control). Three mice per group were killed on days 3 and 6 after infection and the virus titres in lungs were determined by plaque assays in MDCK cells; results are reported as means ± s.d. The statistical significance of differences in lung virus titres of control mice and those treated with antivirals were assessed by use of the Student's *t*-test (asterisk, $P < 0.05$; double asterisk, $P < 0.01$).

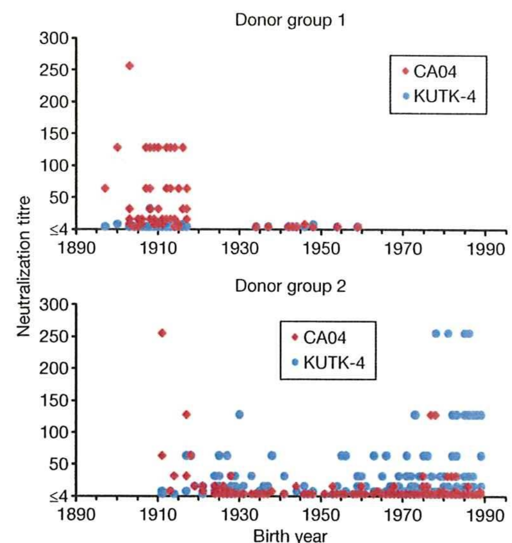

Figure 3 | Neutralization activities in human sera against viruses. Human sera of donor groups 1 (collected in 1999) and 2 (collected in April and May of 2009) were subjected to neutralization assays with CA04 and KUTK-4. Because the sera of donor group 1 were collected in 1999, little neutralization activity was expected against KUTK-4, which was isolated in 2009.

2009 from workers and patients in a hospital (donor set 2). High neutralizing activity against KUTK-4 was detected for many sera in donor set 2 (Fig. 3), but not for sera in donor set 1, probably because these sera were collected before the emergence of the current human H1N1 viruses. Interestingly, with few exceptions, no appreciable neutralizing antibodies against CA04 were found for individuals born after 1920; however, many of those born before 1918 had high neutralizing antibody titres (individual neutralizing antibody titres are shown in Supplementary Table 9). These data indicate that infection with the 1918 pandemic virus or closely related human H1N1 viruses, but not infection with antigenically divergent human H1N1 viruses circulating in the 1920s to 1950s, and again since 1977, elicited neutralizing antibodies to S-OIVs.

Our findings indicate that S-OIVs are more pathogenic in mammalian models than seasonal H1N1 influenza viruses. In fact, the ability of CA04 to replicate in the lungs of mice, ferrets and non-human primates, and to cause appreciable pathology in this organ, is reminiscent of infections with highly pathogenic H5N1 influenza viruses[14], as acknowledged in a recent report by the World Health Organization (http://www.who.int/wer/2009/wer8421/en/index.html). We therefore speculate that the high replicative ability of S-OIVs might contribute to a viral pneumonia characterized by diffuse alveolar damage that contributes to hospitalizations and fatal cases where no other underlying health issues exist (http://www.who.int/wer/2009/wer8421/en/index.html). In addition, sustained person-to-person transmission might result in the emergence of more pathogenic variants, as observed with the 1918 pandemic virus (reviewed in ref. 15). Furthermore, S-OIVs may acquire resistance to oseltamivir through mutations in their NA gene (as recently witnessed with human H1N1 viruses[16]), or through reassortment with co-circulating, oseltamivir-resistant seasonal human H1N1 viruses. Collectively, our findings are a reminder that S-OIVs have not yet garnered a place in history, but may still do so, as the pandemic caused by these viruses has the potential to produce a significant impact on human health and the global economy.

METHODS SUMMARY

Viruses and cells. All swine-origin H1N1 viruses were isolated and passaged in MDCK cells to produce viral stocks. The viruses and their passage histories are described in Methods. All experiments with S-OIVs were performed in approved enhanced biosafety level 3 (BSL3) containment laboratories.

MDCK cells and MDCK cells overexpressing the β-galactoside α2,6-sialyltransferase I gene[17] were maintained in Eagle's minimal essential medium (MEM) containing 5% newborn calf serum. Human airway epithelial (HAE) cells were obtained from residual surgical tissue trimmed from lungs during the process of transplantation. The bronchial specimens were dissected and enzymatically digested, and monolayers of HAE cells were isolated, cultured and differentiated as previously described[18].

Animals. Five- and six-week-old female BALB/c mice (Jackson Laboratory and Japan SLC Inc.), approximately three-to-four-year-old cynomolgus macaques (Ina Research Inc.), five-to-eight-month-old male ferrets (Marshall Farms and Triple F Farms) and two-month-old female specific-pathogen-free miniature pigs (Nippon Institute for Biological Science) were used according to approved protocols for the care and use of animals. Detailed procedures are provided in Methods.

Antiviral sensitivity of viruses in mice. Five-week-old female BALB/c mice (Japan SLC Inc.; groups of six) were anaesthetized with sevoflurane and intranasally inoculated with 10^4 p.f.u. (volume, 50 μl) of CA04, KUTK-4, or A/Kawasaki/UTK-23/08 (H1N1). At 1 h after infection, mice were administered antiviral compounds as described in detail in Methods. Three mice per group were killed on days 3 or 6 after infection and the virus titres in lungs were determined by plaque assays in MDCK cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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LETTERS

In vitro and *in vivo* characterization of new swine-origin H1N1 influenza viruses

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Influenza A viruses cause recurrent outbreaks at local or global scale with potentially severe consequences for human health and the global economy. Recently, a new strain of influenza A virus was detected that causes disease in and transmits among humans, probably owing to little or no pre-existing immunity to the new strain. On 11 June 2009 the World Health Organization declared that the infections caused by the new strain had reached pandemic proportion. Characterized as an influenza A virus of the H1N1 subtype, the genomic segments of the new strain were most closely related to swine viruses[1]. Most human infections with swine-origin H1N1 influenza viruses (S-OIVs) seem to be mild; however, a substantial number of hospitalized individuals do not have underlying health issues, attesting to the pathogenic potential of S-OIVs. To achieve a better assessment of the risk posed by the new virus, we characterized one of the first US S-OIV isolates, A/California/04/09 (H1N1; hereafter referred to as CA04), as well as several other S-OIV isolates, *in vitro* and *in vivo*. In mice and ferrets, CA04 and other S-OIV isolates tested replicate more efficiently than a currently circulating human H1N1 virus. In addition, CA04 replicates efficiently in non-human primates, causes more severe pathological lesions in the lungs of infected mice, ferrets and non-human primates than a currently circulating human H1N1 virus, and transmits among ferrets. In specific-pathogen-free miniature pigs, CA04 replicates without clinical symptoms. The assessment of human sera from different age groups suggests that infection with human H1N1 viruses antigenically closely related to viruses circulating in 1918 confers neutralizing antibody activity to CA04. Finally, we show that CA04 is sensitive to approved and experimental antiviral drugs, suggesting that these compounds could function as a first line of defence against the recently declared S-OIV pandemic.

Sequence analyses of recently emerged swine-origin H1N1 viruses (S-OIVs) revealed the absence of markers associated with high pathogenicity in avian and/or mammalian species, such as a multibasic haemagglutinin (HA) cleavage site[2] or lysine at position 627 of the PB2 protein[3]. To characterize the new viruses *in vitro* and *in vivo*, we amplified the following S-OIVs in Madin–Darby canine kidney (MDCK) cells: A/California/04/09 (CA04), A/Wisconsin/WSLH049/09 (WSLH049), A/Wisconsin/WSLH34939/09 (WSLH34939), A/Netherlands/603/09 (Net603) and A/Osaka/164/09 (Osaka164). WSLH34939 was isolated from a patient who required hospitalization, whereas the remaining viruses were isolated from mild cases. These viruses represent the currently recognized neuraminidase (NA) variants among S-OIVs: CA04, NA-106V, NA-248N; Osaka164, NA-106I, NA-248N; WSLH049, NA-106I, NA-248D; WSLH34939, NA-106I, NA-248D; and Net603, NA-106V, NA-248N.

In MDCK cells and primary human airway epithelial cells, CA04 grew to titres comparable to those typically obtained for contemporary human H1N1 influenza viruses (Supplementary Fig. 1). Confocal, transmission electron and scanning electron microscopy revealed virions of remarkably filamentous shape (Supplementary Fig. 2), in marked contrast to the spherical shape observed with negatively stained virions (http://www.cdc.gov/h1n1flu/images.htm). The biological significance of the morphology of CA04 remains unknown.

To evaluate the pathogenicity of S-OIV in mammalian models, we conducted studies in mice, ferrets, non-human primates and pigs. BALB/c mice intranasally infected with a high dose ($>10^4$ plaque-forming units (p.f.u.)) of CA04 (Supplementary Fig. 3) experienced weight loss and those infected with the highest dose of this virus were humanely killed, in contrast to animals infected with a recent human H1N1 virus (A/Kawasaki/UTK-4/09, KUTK-4). The 50% mouse lethal dose (MLD_{50}) was $10^{5.8}$ p.f.u. for CA04 and $>10^{6.6}$ p.f.u. for

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METHODS

Viruses. A/California/04/09 (H1N1; CA04) was provided by the Centers for Disease Control (CDC). A/Wisconsin/WSLH049/09 (H1N1) was isolated from a patient with mild symptoms, whereas A/Wisconsin/WSLH34939/09 (H1N1) was isolated from a hospitalized patient. A/Netherlands/603/09 (H1N1) was isolated from a patient with mild symptoms and was provided by R. Fouchier. A/Osaka/164/09 (H1N1) was also isolated from a patient with mild symptoms.

The following influenza viruses served as controls: A/Kawasaki/UTK-4/09 (H1N1; KUTK-4; passaged twice in MDCK cells), an oseltamivir-resistant seasonal human virus; A/WSN/33 (H1N1; generated by reverse genetics and passaged twice in MDCK cells), a typical spherical influenza virus[19]; A/Kawasaki/UTK-23/08 (H1N1; passaged twice in MDCK cells), an oseltamivir-sensitive seasonal human virus; A/Victoria/3/75 (H3N2; passaged several times in eggs after it was obtained from the CDC), a human virus; A/swine/Hokkaido/2/81 (H1N1; passaged several times in eggs), a classical swine virus; and A/duck/Alberta/35/76 (H1N1; passaged several times in eggs), an avian virus. All experiments with S-OIV viruses were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Wisconsin-Madison, which are approved for such use by the CDC and the US Department of Agriculture, or in BSL3 containment laboratories at the University of Tokyo, the Shiga University of Medical Science, or the Hokkaido University, all of which are approved for such use by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Viral pathogenesis in mice. Six-week-old female BALB/c mice (Jackson Laboratory and Japan SLS Inc.) were used in this study. Baseline body weights were measured before infection. Three mice per group were anaesthetized with isoflurane and intranasally inoculated with 10^2, 10^3, 10^4, or 10^5 p.f.u. (50 μl) of CA04 and KUTK-4, or undiluted virus from virus stocks (CA04, $10^{6.5}$ p.f.u.; KUTK-4, $10^{6.6}$ p.f.u.). Body weight and survival were monitored daily for 14 days and mice with body weight loss of more than 25% of pre-infection values were killed. For virological and pathological examinations, 6 mice per group were intranasally infected with 10^5 p.f.u. of S-OIVs and KUTK-4 and 3 mice per group were killed on days 3 and 6 after infection. The virus titres in various organs were determined by plaque assays in MDCK cells.

Growth kinetics of virus in human airway epithelial (HAE) cells. Cultures of differentiated HAE cells were washed extensively with PBS to remove accumulated mucus and infected with virus at a multiplicity of infection (MOI) of 0.001 from the apical surface. The inoculum was removed after 1 h of incubation at 35 °C, and cells were further incubated at 35 °C. Samples were collected at 12, 24, 48, 72 and 96 h after infection from the apical surface. Apical harvesting was performed by adding 500 μl of medium to the apical surface, followed by incubation for 30 min at 35 °C, and removal of the medium from the apical surface. The titres of viruses released into the cell culture supernatant were determined by plaque assay in MDCK cells.

Experimental infection of cynomolgus macaques. Approximately three-to-four-year-old cynomolgus macaques (*Macaca fascicularis*) from the Philippines (obtained from Ina Research Inc.), weighing 2.1–3.0 kg and serologically negative by AniGen AIV antibody ELISA, which detects all influenza A virus subtypes (Animal Genetics Inc.), were used in this study. Baseline body weights were established by two or three measurements before infection. Under anaesthesia, telemetry probes (TA10CTA-D70, Data Sciences International) were implanted in the peritoneal cavities of animals to monitor body temperature. Six macaques per group were intramuscularly anaesthetized with ketamine (5 mg per kg) and xylazine (1 mg per kg) and inoculated with a suspension containing $10^{6.5}$ p.f.u. ml^{-1} of CA04 or KUTK-4 virus through a combination of intratracheal (4.5 ml), intranasal (0.5 ml per nostril), ocular (0.1 ml per eye) and oral (1 ml) routes (resulting in a total infectious dose of $10^{7.4}$ p.f.u.). Macaques were monitored every 15 min for changes in body temperature. On days 1, 3, 5 and 7 after infection, nasal and tracheal swabs and bronchial brush samples were collected. On days 3 and 7 after infection, 3 macaques per group were killed for virological and pathological examinations. The virus titres in various organs and swabs were determined by plaque assays in MDCK cells. Experiments were carried out in accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science, Shiga, Japan, and approved by the Shiga University of Medical Science Animal Experiment Committee and Biosafety Committee.

Experimental infection of ferrets. We used five-to-eight-month-old male ferrets (Marshall Farms and Triple F Farms), which were serologically negative by haemagglutination inhibition (HI) assay for currently circulating human influenza viruses. Baseline body temperatures and body weights were established by one or two measurements before infection. Six ferrets per group were intramuscularly anaesthetized with ketamine and xylazine (5 mg and 0.5 mg per kg of body weight, respectively) and intranasally inoculated with 10^6 p.f.u. (500 μl) of S-OIVs or KUTK-4. On days 3 and 6 after infection, 3 ferrets per group were killed for virological and pathological examinations. The virus titres in various organs were determined by plaque assays in MDCK cells.

Experimental infection of miniature pigs. Two-month-old female specific-pathogen-free miniature pigs (Nippon Institute for Biological Science), which were serologically negative by AniGen AIV antibody ELISA for currently circulating influenza viruses, were used in this study. Baseline body temperatures were measured once before infection. Four pigs per group were intranasally inoculated with $10^{6.2}$ p.f.u. (1 ml) of viruses. Nasal swabs were collected daily. On day 3 after infection, two pigs per group were killed and their tissues collected for examination. On day 14 after infection, the remaining two pigs per group were killed for virological and pathological examinations. Virus titres in various organs and swabs were determined by plaque assays in MDCK cells. The miniature pigs used in this study were housed in self-contained isolator units (Tokiwa Kagaku) at a BSL3 facility and experiments were conducted in accordance with guidelines established by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Pathological examination. Excised tissues of the nasal turbinates, trachea and/or lungs of killed mice, macaques, ferrets and pigs were preserved in 10% phosphate-buffered formalin. Tissues were then processed for paraffin embedding and cut into 5-μm-thick sections. One section from each tissue sample was stained using a standard haematoxylin-and-eosin procedure, whereas another one was processed for immunohistological staining with an anti-influenza virus rabbit antibody (R309; prepared in our laboratory) that reacts comparably with CA04 and KUTK-4. Specific antigen–antibody reactions were visualized by 3,3′-diaminobenzidine tetrahydrochloride staining using a Dako EnVision system (Dako Co. Ltd).

Ferret transmission study. For transmission studies in ferrets, animals were housed in adjacent transmission cages that prevent direct and indirect contact between animals but allow spread of influenza virus through the air. Three or two 5-to-8-month-old ferrets were intranasally inoculated with 10^6 p.f.u. (500 μl) of CA04, KUTK-4, A/Victoria/3/75 (H3N2) or A/duck/Alberta/35/76 (H1N1) (inoculated ferrets). One day after infection, three or two naive ferrets were each placed in a cage adjacent to an inoculated ferret (contact ferrets). All ferrets were monitored daily for changes in body temperature and weight, and the presence of clinical signs. To assess viral replication in the upper respiratory tract, viral titres were determined in nasal washes collected from virus-inoculated and contact ferrets on day 1 after inoculation or co-housing, respectively, and then every other day (up to 9 days).

Cytokine and chemokine measurement. For cytokine and chemokine measurement, homogenates of mouse lungs were processed with the Bio-Plex Mouse Cytokine 23-Plex and 9-Plex panels (Bio-Rad Laboratories), whereas macaque lung homogenates were measured with the MILLIPLEX MAP Non-human Primate Cytokine/Chemokine Panel–Premixed 23-Plex (Millipore). Array analysis was performed by Bio-Plex Protein Array system (Bio-Rad Laboratories).

Antiviral sensitivity of viruses in mice. To test the antiviral sensitivity of viruses in mice, animals were infected as described in the Methods Summary section and 1 h later administered the following antiviral compounds: (1) oseltamivir phosphate: 8 or 80 mg per kg per 400 μl (divided into two oral administrations per day) for 5 days; (2) zanamivir: 0.8 or 8 mg per kg per 50 μl in one daily intranasal administration for 5 days; (3) CS-8958: 0.7 mg per kg per 50 μl in one intranasal administration; (4) T-705: 60 or 300 mg per kg per 400 μl (divided into two oral administrations per day) for 5 days; (5) or distilled water orally (200 μl) and PBS intranasally (50 μl). Three mice per group were killed on day 3 or 6 after infection and the virus titres in lungs were determined by plaque assays in MDCK cells.

Sensitivity to antiviral compounds in tissue culture. MDCK cells overexpressing the β-galactoside α2,6-sialyltransferase I gene (or, for studies with T-705, regular MDCK cells) were infected with CA04, KUTK-4, or A/Kawasaki/UTK-23/08 (H1N1) at a multiplicity of infection of 0.001. After incubation for 1 h at 37 °C, growth medium containing various concentrations of oseltamivir carboxylate (the active form of oseltamivir), zanamivir, R-125489 (the active form of CS-8958), or T-705 was added to the cells. Twenty-four hours later, the culture supernatants were harvested and the 50% tissue-culture infectious dose ($TCID_{50}$) in MDCK cells determined. On the basis of the $TCID_{50}$ value, the 90% inhibitory concentration (IC_{90}) was calculated.

Neuraminidase inhibition assay. To assess the sensitivity of viruses to neuraminidase inhibitors (that is, oseltamivir, zanamivir and CS-8958), neuraminidase inhibition assays were performed as described previously[20]. Briefly, diluted viruses were mixed with various concentrations of oseltamivir carboxylate, zanamivir, or R-125489 in 2-(*N*-morpholino)ethanesulphonic acid containing calcium chloride, and incubated for 30 min at 37 °C. Then, we added methylumbelliferyl-*N*-acetylneuraminic acid (Sigma) as a fluorescent substrate to this mixture. After incubation for 1 h at 37 °C, sodium hydroxide in 80% ethanol was added to the mixture to stop the reaction. The fluorescence of the solution was measured at an

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excitation wavelength of 360 nm and an emission wavelength of 465 nm and the 50% inhibitory concentration (IC_{50}) was calculated.

Neutralization assay with human sera. Human sera were collected in 1999 or 2009 from donor group 1 (age range: 50–112 years as of 2009, mean = 92.7 ± 15.0 years) or 2 (age range: 20–68 years as of 2009, mean = 48.2 ± 23.7 years), respectively. These sera were treated with receptor-destroying enzyme (DENKA SEIKEN CO.) to remove inhibitors of influenza virus replication. One hundred $TCID_{50}$ (50% tissue culture infectious dose) of CA04 and KUTK-4 were pre-incubated with twofold serial dilutions of treated sera, incubated for 60 min on MDCK cells, which were then observed for cytopathic effects to determine the neutralizing activity of the test sera. Our research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo (approval numbers: 21-6-0428 for donor group 1; 21-7-0529 for donor group 2).

Immunofluorescence microscopy. MDCK cells were infected with CA04, KUTK-4, or WSN and fixed with 4% paraformaldehyde 16–24 h later. Infected cells were incubated with the following primary antibodies: mouse anti-HA (7B1b), anti-HA (IVC102), or mouse anti-HA (WS3-54) antibody against CA04, KUTK-4 or WSN, respectively. Cells were then incubated with Alexa Fluor 488 goat anti-mouse immunoglobulin G (Invitrogen), and examined with a confocal laser-scanning microscope (LSM510META; Carl Zeiss).

Electron microscopy. MDCK cells were infected with CA04, KUTK-4 or WSN at a multiplicity of infection of 10. At 16–24 h after infection, cells were processed for ultrathin section electron microscopy and scanning electron microscopy as described previously[19,21].

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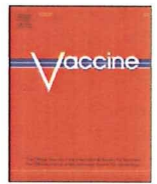

Short communication

Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus

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ABSTRACT

An influenza vaccine was prepared from inactivated whole particles of the non-pathogenic strain A/duck/Hokkaido/Vac-1/04 (H5N1) virus using an oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). The vaccine was injected intramuscularly into five 4-week-old chickens, and 138 weeks after vaccination, they were challenged intranasally with 100 times 50% chicken lethal dose of the highly pathogenic avian influenza (HPAI) virus A/chicken/Yamaguchi/7/04 (H5N1). All 5 chickens survived without exhibiting clinical signs of influenza, although 2 days post-challenge, 3 vaccinated chickens shed limited titres of viruses in laryngopharyngeal swabs.

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1. Introduction

Influenza A viruses are divided into H1–H16 and N1–N9 subtypes on the basis of antigenic specificity of two glycoproteins [1,2]. Each of the subtypes has been isolated from migrating waterfowls, which play a role in the spread of the influenza A viruses [3,4]. During repeated passage through a chicken population, the viruses acquire transmissibility and pathogenicity against chickens, resulting in highly pathogenic avian influenza (HPAI) outbreaks in domestic poultry [5–9].

HPAI caused by H5 and H7 subtype viruses have occurred in many parts of the world, and such outbreaks have resulted in huge economic losses in poultry industries. A recent outbreak of H5N1 virus infection emerged in South Asia and spread through Eurasia and Africa. In addition, direct transmission of H5N1 viruses from birds to humans with high mortality occurred. Since 2003, more than 400 human cases with 60% mortality have been reported as of 11 May 2009 [10].

The standard measure undertaken for the control of HPAI in poultry is stamping out. Vaccination is allowed as an optional-tool to decrease the amount of viruses shed from infected chickens when standard measure cannot enough to control the outbreak in the field [11]. Although commercial vaccines prepared from viruses of the North American lineage are available, they may be less effective for the control of HPAI outbreaks caused by infection by viruses of the Eurasian lineage [12]. Thus, a vaccine prepared from a Eurasian lineage virus may provide better protection against Asian HPAI virus infections [13–16].

We have developed an H5N1 reassortant virus of the Eurasian lineage that is non-pathogenic for chickens and chicken embryos, and exhibits good growth in embryonated chicken eggs [12]. Subsequently, we prepared test vaccines using this reassortant virus, and confirmed the optimal antigen concentration and its protective potency against a currently prevalent Eurasian lineage HPAI virus [17]. We demonstrated that the test vaccine is able to induce protective immunity against HPAI virus starting 8 days post-vaccination and that chickens challenged with a HPAI virus, A/whooperswan/Mongolia/3/2005 (H5N1) strain did not show clinical signs of HPAI [14]. In the present study, we examined whether the vaccine induces long lasting immunity (138 weeks) by challenging vaccinated chickens with HPAI virus.

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2. Materials and methods

2.1. Viruses

The A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04) virus belonging to the Eurasian lineage of a non-pathogenic avian influenza (AI) virus, generated as a reassortant virus between A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1), was used for vaccine preparation [12].

A/chicken/Yamaguchi/7/04 (H5N1) (Ck/Yamaguchi/04) virus, isolated by the National Institute of Animal Health (Ibaraki, Japan) from a dead chicken during the HPAI outbreak in 2004 in Japan, was used as the challenge virus [18,19].

To prepare virus suspensions, the Dk/Vac-1/04 and Ck/Yamaguchi/04 viruses were inoculated into the allantoic cavity of embryonated chicken eggs and incubated at 34 °C for 48 h and 35 °C for 24 h, respectively.

2.2. Vaccine preparation

A virus suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4 °C. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs.

The inactivated Dk/Vac-1/04 virus suspension was diluted with phosphate-buffered saline (PBS) to appropriate concentrations based on hemagglutination (HA) titres. A 2.5 volume of viral suspension with HA titre of 1:256 was mixed with a 7.5 volume of oil adjuvant containing 3.9% anhydromannitol-octadecenoate-ether (AMOE) and sufficient light mineral oil to comprise the remaining volume. This mixture was homogenized using an ultra-homomixer (PRIMIX Co. Ltd.) to produce a water-in-oil type of adjuvant test vaccine. The virus concentration in the test vaccine was 640 HA units per dose [17].

2.3. Animals and serum sampling

Specific pathogen-free white leghorn chickens were obtained from Kyoto Biken Laboratories, Inc., Kyoto, Japan. Ten 4-week-old chickens were vaccinated intramuscularly in the lower thigh with 0.5 mL of the test vaccine, and 4 other 4-week-old chickens were used as non-vaccinated controls. The two groups of chickens were reared separately for 138 weeks after the vaccination. Five vaccinated chickens, however, kept separately, died due to air conditioning accident of rearing facility 44 weeks after vaccination. Thus we used the remaining 5 vaccinated chickens for the experiment. For the Ck/Yamaguchi/04 challenge test, the chickens were transported to a bio-safety level 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All procedures were performed according to the animal experiment guidelines of Hokkaido University. A blood sample was obtained from each chicken every week after vaccination for 5 weeks, and then at 11–14-week intervals until 138 weeks. In addition, blood samples were obtained from all surviving chickens 2 weeks after the Ck/Yamaguchi/04 challenge.

2.4. Hemagglutination-inhibition (HI) test

The HI test was performed according to the Japanese Standards for Veterinary Biological Products. Briefly, 1 volume of each serum sample was mixed with 3 volumes of 10% chicken red blood cells (RBCs) and incubated overnight at 4 °C. The mixtures were centrifuged at 1000 × g for 5 min and the supernatants collected as four-fold diluted sera. One-hundred microliters of each supernatant were dispensed into wells in the first lane of a plastic V-bottomed microtitration plate. Fifty microliters of PBS was dispensed into all other wells, after which 50 μL of two-fold serial dilutions of the supernatants were added to the PBS-containing wells. The Dk/Vac-1/04 and Ck/Yamaguchi/04 virus suspensions were inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4 °C and 0.1% for 7 days at 4 °C, respectively, followed by dilution of the antigen with PBS to adjust the HA titre to 1:8. Fifty microliters of each HA antigen was then dispensed into all wells of the plates and they were incubated for 30 min at room temperature. Finally, 100 μL of 0.5% chicken RBCs was dispensed into all previously prepared wells and the plates were incubated again for 60 min at room temperature. The HI antibody titres were expressed as the highest dilution of the serum sample that showed complete inhibition of hemagglutination.

2.5. Protection test of vaccinated chickens against HPAI virus challenge

All chickens were challenged intranasally with 100 times 50% chicken lethal dose (i.e. $10^{5.5}$ times 50% egg infectious dose) of Ck/Yamaguchi/04 at 138 weeks after the Dk/Vac-1/04 vaccination. Clinical signs, such as lethargy, loss of appetite and nervous symptoms, were monitored for 14 days post-challenge (p.c.). To detect virus shedding, laryngopharyngeal and cloacal swabs were individually collected from all surviving chickens on days 2 and 4 p.c. Laryngopharyngeal and cloacal swabs were also collected individually at the time of death or at euthanasia on day 14 p.c. Swabs were individually suspended in 1.0 mL of minimal essential medium. A 0.1-mL aliquot of each suspension was then inoculated into the allantoic cavity of embryonated chicken eggs. The infectivity titres of the swabs were calculated using the method of Reed and Muench [20], and expressed as 50% egg infectious dose per millilitre (EID_{50}/mL).

3. Results

The serum HI antibodies against Dk/Vac-1/04 reached a maximum geometric mean (GM) titre of 1:2048 at 4 weeks after vaccination, and then gradually decreased until reaching a GM titre level of 1:111 at 138 weeks after vaccination (Table 1). The serum HI titres of 5 vaccinated chickens that died due to an accident were basically the same as those of the 5 chickens in the present results at every points of time until the time of the accident (44 weeks after vaccination, data not shown).

During the challenge test, all chickens in the vaccinated group survived for 14 days p.c. without showing any clinical signs of HPAI (Table 2). In contrast, all chickens in the non-vaccinated group showed typical HPAI symptoms 1–2 days p.c. and died within 3 days p.c.

A marked secondary antibody response was observed 14 days p.c. in serum HI titres of chickens vaccinated with Dk/Vac-1/04 and challenged with Ck/Yamaguchi/04 viruses (Table 3). In addition, $10^{1.3}$–$10^{1.7}$ EID_{50}/mL of challenge virus were recovered from the laryngopharyngeal swabs from 3 of the 5 vaccinated chicken 2 days p.c. but there was no virus recovery at 4 and 14 days p.c. However, $10^{4.5}$–$10^{7.5}$ EID_{50}/mL of the challenge virus were recovered from laryngopharyngeal and cloacal swabs of the non-vaccinated chickens.

These findings demonstrated that all of the chickens vaccinated with Dk/Vac-1/04 survived the Ck/Yamaguchi/04 challenge without exhibiting any clinical signs. Three of those chickens shed small amounts of Ck/Yamaguchi/04 virus in their laryngopharyngeal swabs, only on day 2 p.c.

Table 1
HI antibody titres against Dk/Vac-1/04 in chickens during the 138 weeks following Dk/Vac-1/04 vaccination.

Group	Chicken No.	HI antibody titres and weeks after vaccination															
		1	2	3	4	5	16	28	40	52	64	76	88	100	112	124	138
Vaccinated	2	<4	128	2048	4096	2048	1024	512	512	512	256	256	256	256	256	256	256
	3	<4	256	1024	2048	2048	2048	1024	1024	1024	512	512	512	512	256	256	128
	6	<4	128	512	1024	1024	1024	256	256	256	128	128	128	128	64	64	64
	7	<4	128	512	2048	2048	256	128	128	128	128	128	128	128	64	64	64
	8	<4	512	1024	2048	2048	1024	512	512	512	512	512	512	256	128	128	128
GM[a]		<4	194	891	2048	1783	891	388	388	388	256	256	256	223	128	128	111
Non-vaccinated	9	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	10	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	11	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	12	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
GM[a]		<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

[a] GM: geometric mean.

Table 2
Clinical signs of influenza in vaccinated chickens after challenge with the HPAI virus Ck/Yamaguchi/04.

Group	Chicken No.	Serum HI antibody titre[a] and virus strain		Clinical signs on days following HPAI challenge													
		Dk/Vac-1/04	Ck/Yamaguchi/04	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccinated	2	256	256	–[b]	–	–	–	–	–	–	–	–	–	–	–	–	–
	3	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	64	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	7	64	64	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	8	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-vaccinated	9	<4	<4	–	+[b]	D[b]											
	10	<4	<4	+	D												
	11	<4	<4	–	D												
	12	<4	<4	–	+	D											

[a] HI antibody titre at the time of challenge.
[b] '–' no abnormal signs; '+': typical clinical signs (lethargy, loss of appetite and nervous symptoms); D: death.

Table 3
Antibody response and virus isolation from laryngopharyngeal and cloacal swabs of chickens after challenge with the Ck/Yamaguchi/04 virus.

Group	Chicken no.	Serum HI antibody titre and virus strain				Virus titres[a] on the following days after challenge							
		Dk/Vac-1/04		Ck/Yamaguchi/04		2		3		4		14	
		Pre[b]	Post[b]	Pre	Post	L[c]	C[c]	L	C	L	C	L	C
Vaccinated	2	256	2048	256	2048	–[d]	–	NT[d]	NT	–	–	–	–
	3	128	8192	128	8192	1.7	–	NT	NT	–	–	–	–
	6	64	512	128	512	–	–	NT	NT	–	–	–	–
	7	64	2048	64	2048	1.5	–	NT	NT	–	–	–	–
	8	128	512	128	256	1.3	–	NT	NT	–	–	–	–
Non-vaccinated	9	<4	NT	<4	NT	6.8	5.5	6.0	4.5	NT	NT	NT	NT
	10	<4	NT	<4	NT	4.5	5.5	NT	NT	NT	NT	NT	NT
	11	<4	NT	<4	NT	6.5	4.8	NT	NT	NT	NT	NT	NT
	12	<4	NT	<4	NT	7.5	5.5	4.5	4.8	NT	NT	NT	NT

[a] Virus titre expressed as $\log_{10}$ EID_{50}/mL.
[b] Pre: at the time of challenge; Post: 14 days post-challenge.
[c] L: laryngopharynx; C: cloacal.
[d] '–': Indicates a virus recovery titre lower than 0.5 $\log_{10}$ EID_{50}/mL; NT: not tested.

4. Discussion

In the present study, we demonstrated that an avian influenza test vaccine produced using an oil adjuvant containing AMOE as a surfactant induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination. Oda *et al.* reported that the surfactant contained in our oil adjuvant plays a key role in stimulation of an antibody response [21]. They also described that oligosaccharide oleate ester is the most important element in the adjuvant activity of AMOE. It is, thus, considered that AMOE is a potent adjuvant equivalent to QS-21 saponin or alum adjuvants in mice.

Other elements of AMOE may play an important role in the creation of stable water-in-oil emulsions of light mineral oil. Such emulsion stability may improve and prolong the adjuvant activity of AMOE by enhancing the potency of the vaccine as a foreign substance and by capturing the antigen at the injection site. Recently, Hikida *et al.* reported that phospholipase C-gamma 2 is essential for formation and maintenance of memory B cells [22]. It is possible that the long-lasting immunological memory and the marked

antibody production following the HPAI virus challenge in the present vaccinated chickens may have been regulated through similar immunological mechanisms.

Both Dk/Vac-1/04 and Ck/Yamaguchi/04 were viruses of the Eurasian lineage, and both showed similar antigenic cross reactivity in the HI test. This suggests that the potency of a vaccine prepared from a virus strain belonging to the same lineage as the outbreak virus may be higher than that of a vaccine prepared from a virus strain belonging to a heterologous lineage strain [12,13].

To assure the safety of vaccines in humans, ether-split influenza vaccines are widely adopted for human use. We chose formalin-inactivated whole virus particles as an antigen in order to maintain efficacy while reducing the cost of the vaccine. Recently, Hagenaars *et al.* reported that whole inactivated virus particles, containing all viral components, produced the best results in potency tests as compared to split, subunit and virosomes presented through intramuscular and intranasal routes [23]. Their report supports our approach of using whole virus particles for vaccine development. Furthermore, it is anticipated that a whole virus particle vaccine should be effective against H5 viruses, even on an antigenically drifted virus [14]. Thus, whole virus particles appear to be one of the best candidates for chicken vaccine development.

The present results show that the test vaccine constructed using an apparently optimum adjuvant composition and an appropriate lineage strain of whole inactivated virus particles induces long-lasting protective immunity in chickens.

It is possible that potency of the test vaccine may be different for SPF from conventional lines of chicken. Accordingly, studies to compare the immunological response to vaccination in SPF and conventional lines of chicken are under way.

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PB2 Protein of a Highly Pathogenic Avian Influenza Virus Strain A/chicken/Yamaguchi/7/2004 (H5N1) Determines Its Replication Potential in Pigs[▽]

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It has been shown that not all but most of the avian influenza viruses replicate in the upper respiratory tract of pigs (H. Kida et al., J. Gen. Virol. 75:2183–2188, 1994). It was shown that A/chicken/Yamaguchi/7/2004 (H5N1) [Ck/Yamaguchi/04 (H5N1)] did not replicate in pigs (N. Isoda et al., Arch. Virol. 151:1267–1279, 2006). In the present study, the genetic basis for this host range restriction was determined using reassortant viruses generated between Ck/Yamaguchi/04 (H5N1) and A/swine/Hokkaido/2/1981 (H1N1) [Sw/Hokkaido/81 (H1N1)]. Two in vivo-generated single-gene reassortant virus clones of the H5N1 subtype (virus clones 1 and 2), whose PB2 gene was of Sw/Hokkaido/81 (H1N1) origin and whose remaining seven genes were of Ck/Yamaguchi/04 (H5N1) origin, were recovered from the experimentally infected pigs. The replicative potential of virus clones 1 and 2 was further confirmed by using reassortant virus (rg-Ck-Sw/PB2) generated by reverse genetics. Interestingly, the PB2 gene of Ck/Yamaguchi/04 (H5N1) did not restrict the replication of Sw/Hokkaido/81 (H1N1), as determined by using reassortant virus rg-Sw-Ck/PB2. The rg-Sw-Ck/PB2 virus replicated to moderate levels and for a shorter duration than parental Sw/Hokkaido/81 (H1N1). Sequencing of two isolates recovered from the pigs inoculated with rg-Sw-Ck/PB2 revealed either the D256G or the E627K amino acid substitution in the PB2 proteins of the isolates. The D256G and E627K mutations enhanced viral polymerase activity in the mammalian cells, correlating with replication of virus in pigs. These results indicate that the PB2 protein restricts the growth of Ck/Yamaguchi/04 (H5N1) in pigs.

Influenza A viruses have been isolated from a variety of species, including humans, birds, pigs, horses, minks, seals, whales, cats, dogs, and tigers (23, 50, 51, 55). Indeed, influenza A viruses exhibit a restricted host range with efficient replication in their natural hosts and poor or no replication in other host species (3, 12, 13, 35); however, influenza viruses may cross this species barrier. Interspecies transmission of human, swine, and avian influenza viruses has been documented on several occasions (4, 6, 36, 54). The causative viruses of both the 1957 (Asian) and the 1968 (Hong Kong) pandemics were reassortant viruses which acquired the polymerase basic protein 1 (PB1), hemagglutinin (HA), and neuraminidase (NA) genes and the PB1 and HA genes, respectively, from avian influenza viruses (22, 26, 45, 56, 58). The role of pigs in the generation of new influenza viruses is well documented (25). It was shown that the H3 HA gene of the Hong Kong pandemic strain A/Hong Kong/1968 (H3N2) was of a migratory duck origin and was acquired as a result of reassortment with the precedent human H2N2 influenza virus in pigs (26, 58). Furthermore, avian-human reassortant viruses were isolated from Italian pigs (4), and those isolated from children in The Netherlands in 1993 were found to be avian-human reassortants circulating in pigs in Europe (6). These findings indicate that pigs can support the growth of both avian and human influenza viruses and are therefore termed “mixing vessels” (44). Nevertheless, not all influenza viruses replicate in pigs, as demonstrated by Kida et al. (25) in a study of the replication potential of 38 different H1 to H13 subtypes of avian influenza viruses.

The molecular bases for influenza virus host-range restriction and adaptation to a new host species are poorly understood. The first host range barrier is offered at the cell surface where receptor-mediated entry into cells starts (20). After cell entry, a second level of host range barrier is offered where the interaction between viral and cellular proteins takes place. In addition to surface glycoproteins, influenza virus internal proteins also harbor determinants for host range and virulence (7, 29, 53). Among these internal proteins, PB2 is a well-documented component of the viral polymerase complex required for virus replication. The PB2 protein has been shown to be involved in host range restriction and pathogenicity (1, 52).

In late December 2003, there was an influenza outbreak in a layer chicken farm in Yamaguchi Prefecture, Japan. The causative agent was identified as the highly pathogenic avian influenza virus A/chicken/Yamaguchi/7/2004 (H5N1) [Ck/Yamaguchi/04 (H5N1)] (32). This virus was shown to be highly pathogenic to chickens, quails, budgerigars, and ducklings and less virulent for mice, while miniature pigs were resistant to infection with the virus (19). This virus offers a good subject with which to study the mechanism underlying interspecies

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transmission to a new host. The classical swine influenza viruses or avian-human reassortant viruses have been reported to be circulating in pigs in Europe and Asia (4, 6, 11). These viruses can contribute genes to viruses like Ck/Yamaguchi/04 (H5N1) and enable them to replicate in new host species, thereby facilitating the interspecies transmission. Therefore, the present study was conducted to address the molecular basis of restricted replication of Ck/Yamaguchi/04 (H5N1) in pigs by using classical swine influenza virus, A/swine/Hokkaido/2/1981 (H1N1) [Sw/Hokkaido/81 (H1N1)].

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (Nissui, Japan) supplemented with 5% calf serum. Human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum. Both cell lines were maintained at 37°C in a 5% CO_2 atmosphere. Two viruses (designated parent viruses) were used in this study. Sw/Hokkaido/81 (H1N1), a classical swine influenza virus (42), was obtained from the virus repository of our laboratory, while Ck/Yamaguchi/04 (H5N1), a highly pathogenic avian influenza virus, was provided by the National Institute of Animal Health, Ibaraki, Japan (32).

All viruses in the present study were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C. Before the infectious allantoic fluid was harvested, the eggs were chilled at 4°C overnight, and the harvested allantoic fluid was stored at −80°C until use.

Experimental infection of pigs. Three- to four-week-old, crossbred (Landrace × Duroc × Yorkshire) specific pathogen-free pigs, free of antibodies against influenza A viruses (Takikawa swine station, Hokkaido, Japan), were housed in the biosafety level 3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The serum antibody titers against influenza A viruses were determined by enzyme-linked immunosorbent assay (24).

The pigs were inoculated intranasally with 500 μl of infectious allantoic fluid containing $10^{7.0}$ to $10^{7.5}$ 50% egg infectious doses (EID_{50}) of viruses, except for Ck/Yamaguchi/04 (H5N1). The Ck/Yamaguchi/04 (H5N1) strain was inoculated intranasally with 500 μl of infectious allantoic fluid containing $10^{8.4}$ EID_{50} of virus. The nasal swabs were collected either for 7 days postinoculation (p.i.) from pigs inoculated with infectious allantoic fluid prepared from coinoculated eggs or for 10 days p.i. from pigs inoculated with other viruses used in this study. The nasal swabs were collected in 1 ml of virus transport medium (30). Preinoculation blood samples and blood sampled at 14 days p.i. for serum were collected, and antibody titers were determined using enzyme-linked immunosorbent assay (24). The infectivity titers of the different viruses in the nasal swabs of pigs were calculated in embryonated chicken eggs by the 50% end-point method (41) and were expressed as EID_{50}/ml of swab.

All animal experiments were conducted in accordance with the guidelines of the institutional animal care and use committee of Hokkaido University, Japan.

In vivo selection of H5N1 reassortant viruses, generated between Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) capable of replication in pigs. The virus inoculum containing reassortant viruses was produced by coinoculation of 10-day-old embryonated chicken eggs with 100 μl of inoculum containing Ck/Yamaguchi/04 (H5N1) ($10^{7.4}$ EID_{50}/50 μl) and Sw/Hokkaido/81 (H1N1) ($10^{3.0}$ EID_{50}/50 μl) viruses. The harvested infectious allantoic fluid was used as the inoculum for pigs, to select the H5N1 reassortant viruses capable of replication in the pigs. The inoculum contained parental H5N1, H1N1, and reassortant viruses. The nasal swabs were collected for 7 days p.i. and were used for selecting virus clones by plaque cloning.

Virus clones were selected from nasal swabs by plaque cloning on MDCK cells as described by Kida et al. (25). Individual virus clones were selected and propagated in 10-day-old embryonated chicken eggs at 35°C. The eggs were chilled at 4°C overnight, and allantoic fluid was harvested. The HA subtype of virus clones was determined by hemagglutination inhibition assay (46).

Evaluation of replicative potential of H5N1 subtype virus clones recovered from pigs. Eleven H5N1 subtype virus clones were isolated by plaque cloning. All gene segments of these virus clones were amplified and partially sequenced. It was found that two virus clones were single-gene reassortants, while the gene constellation of the remaining nine virus clones was like that of parental Ck/Yamaguchi/04 (H5N1) virus. For the determination of their replicative potential, two single-gene reassortant virus clones and two virus clones of the Ck/Yamaguchi/04 (H5N1)-like gene constellation were reinoculated into pigs. Nasal swabs were collected for 10 days p.i., and infectivity titers were measured as described above.

Generation of viruses by reverse genetics. Eight genes from each of the Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) viruses were cloned to produce viruses by reverse genetics (rg) as described by Hoffmann et al. (14). In brief, the RNA of viruses was extracted using TRI reagent LS (Sigma). The cDNAs were amplified by reverse transcription of viral RNA, using Uni 12 primer (5′-AGC AAA AGC AGG-3′). Full-length genes of Ck/Yamaguchi/04 and Sw/Hokkaido/81 were amplified by using gene-specific universal primer sets (17). The amplified genes were then sequenced using a GenomeLab DTCS Quick Start kit (Beckman Coulter) according to the manufacturer's instructions and analyzed with a CEQ 2000XL sequencer (Beckman Coulter). The amplified genes were first cloned into the pCR 2.1 TOPO cloning vector (Invitrogen) and then into the pHW2000 expression vector (kindly provided by E. Hoffmann, St. Jude Children's Research Hospital), except for the PA, HA, and NA genes of Ck/Yamaguchi/04 (H5N1) and all eight genes of Sw/Hokkaido/81 (H1N1), which were directly cloned into the pHW2000 expression vector. Genes cloned into pCR 2.1 TOPO or pHW2000 were sequenced, and only those clones with sequences identical to the consensus sequence were selected. Ligation of the genes into the pHW2000 expression vector was carried out using a DNA ligation kit (version 2.1; Takara, Japan) according to the manufacturer's instructions.

For generating viruses by reverse genetics using eight plasmids, MDCK and 293T cells were used as described previously (16). The rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) viruses were inoculated into pigs to compare their potential to replicate in pigs with that of parental viruses. The rg-Ck-Sw/PB2 virus [the PB2 gene from Sw/Hokkaido/81 (H1N1) and seven genes from Ck/Yamaguchi/04 (H5N1)] was inoculated into pigs to evaluate the replicative behavior of virus clones 1 and 2 in pigs. The rg-Sw-Ck/PB2 virus [the PB2 gene from Ck/Yamaguchi/04 (H5N1) and seven genes from Sw/Hokkaido/81 (H1N1)] was inoculated into pigs to study the host range-restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) on seven genes of Sw/Hokkaido/81 (H1N1) in pigs.

Site-directed mutagenesis. The E627K and D256G mutations were introduced into the PB2 gene of Ck/Yamaguchi/04 (H5N1) cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations and the absence of unwanted mutations were confirmed by sequencing the full length of the cloned PB2 genes.

Luciferase assay. The luciferase assay was conducted as described by Salomon et al. (43). The luciferase reporter plasmid (pHW72-Luc) was constructed by replacing the open reading frame of the enhanced green fluorescent protein (EGFP) in the pHW72-EGFP plasmid (kindly provided by R. Webby, St. Jude Children's Research Hospital) with the luciferase gene (15). Sixty percent-confluent 293T cell monolayers (in 12-well tissue culture plates; Nunclone) were transfected with 2 μg of pHW72-Luc, 1 μg of pHW2000-PB2, 1 μg of pHW2000-PB1, 1 μg of pHW2000-PA, and 2 μg of pHW2000-NP, using Trans-IT-293 (Mirus) according to the manufacturer's instructions. After 24 h of transfection, cell extracts were prepared in 250 μl of passive lysis buffer, and luciferase levels were assayed with a dual-luciferase assay system (Promega) using a Lumat LB 9507 (Berthold, Germany) instrument. The results were recorded from two independent experiments, and each experiment was run in triplicate.

RESULTS

Selection of in vivo-generated reassortant viruses capable of replication in pigs. The nasal swabs, collected from pigs intranasally administered inoculum prepared from embryonated chicken eggs coinoculated with Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1), were used for picking virus clones by plaque cloning. A total of 119 virus plaque clones were picked from nasal swabs collected from days 1 to 3 p.i. (Table 1). Of the total, 11 virus clones were of the H5N1 subtype. The H5N1 subtype virus clones were plaque purified on MDCK cells. Partial genome sequencing of these virus clones revealed that two of these (virus clones 1 and 2) were single-gene reassortants deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) virus and the remaining seven genes from Ck/Yamaguchi/04 (H5N1). The remaining 9 virus clones (clones 3 to 11) derived all of their genes from Ck/Yamaguchi/04 (H5N1) (Table 2).

TABLE 1. Recovery and subtyping of virus clones from nasal swab samples

Day postinoculation	Total no. of plaques picked	No. of plaques of HA subtype:	
		H5	H1
1	61	11	50
2	38	–[a]	38
3	20	–	20

[a] –, No H5 subtype virus clone was recovered.

Virus clones recovered by in vivo selection in pigs are capable of replication in pigs. Virus clones 1 and 2 [the PB2 gene of Sw/Hokkaido/81 (H1N1) and the remaining seven genes of Ck/Yamaguchi/04 (H5N1) origin] and virus clones 3 and 4 [all eight genes of Ck/Yamaguchi/04 (H5N1) origin] were reinoculated into pigs to assess the virus clones' replicative potential (Table 2). Virus clones 1 and 2 were recovered from nasal swabs, and virus shedding occurred from days 1 to 5 p.i., while virus clones 3 and 4 were not recovered from nasal swabs. The sera collected 14 days p.i. from pigs inoculated with virus clones 1 and 2 showed seroconversion, while sera from pigs inoculated with virus clones 3 and 4 did not show any seroconversion (Table 2). This finding suggested that the PB2 gene of Sw/Hokkaido/81 (H1N1) conferred replicative potential to the reassortant virus possessing the seven genes of Ck/Yamaguchi/04 (H5N1) origin.

The replication potential of rg-Ck-Sw/PB2 is similar to that of virus clones 1 and 2. To confirm the importance of the PB2 gene of Sw/Hokkaido/81 (H1N1) for the replicative potential in pigs, reassortant viruses were produced by reverse genetics. The replicative potential of rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) was similar to that of the parental viruses (Table 3) (2, 19). A single-gene reassortant virus (rg-Ck-Sw/PB2) possessing a gene constellation like that of virus clones 1 and 2 (Table 2) was generated by reverse genetics and inoculated intranasally into the pigs. The rg-Ck-Sw/PB2 was shed for 2 to 3 days, as were the parent virus clones 1 and 2 (Table 3). This finding further supported the results obtained by reinoculation of in vivo-selected reassortant virus clones into pigs.

Amino acid substitutions found in the PB2 protein of rg-Sw-Ck/PB2 after a single passage in pigs. If the PB2 gene of Ck/Yamaguchi/04 (H5N1) restricted viral replication in pigs, it should also restrict the replication of Sw/Hokkaido/81 (H1N1) in pigs. Therefore, rg-Sw-Ck/PB2, possessing seven genes from Sw/Hokkaido/81 (H1N1) and the PB2 gene from Ck/Yamaguchi/04 (H5N1), was inoculated intranasally into two pigs (Table 3, pigs 7 and 8). Interestingly, rg-Sw-Ck/PB2 virus was first recovered on day 3 p.i. from nasal swabs, in contrast to rg-Sw/Hokkaido/81 (H1N1), which was recovered on day 1 p.i. During the first 3 days (days 3 to 5 p.i.) of rg-Sw-Ck/PB2 virus shedding, virus titers were 2 to 4 logs lower than those of rg-Sw/Hokkaido/81 (H1N1) (Table 3). Moreover, the duration of rg-Sw-Ck/PB2 virus shedding was 3 to 4 days shorter than that of rg-Sw/Hokkaido/81 (H1N1). The full-length genes of virus isolates (Table 4, Pig 7-day 3 and Pig 8-day 3) recovered from pigs on day 3 p.i., as well as from rg-Sw-Ck/PB2 (inoculum), were sequenced and compared. Predicted amino acid sequences of all genes, except for the PB2 gene, were identical to those of rg-Sw-Ck/PB2 (inoculum) (Table 4). The PB2 proteins of both isolates, Pig 7-day 3 and Pig 8-day 3, had amino acid substitutions of glutamic acid to lysine at position 627 (E627K) and glycine to aspartic acid at position 256 (D256G), respectively.

To reconfirm the replicative potential of recovered virus, isolates Pig 7-day 3 and Pig 8-day 3 were inoculated into the pigs. The viruses were recovered from the nasal swabs from day 1 p.i., in contrast to rg-Sw-Ck/PB2 which was recovered on day 3 p.i. (Table 3). This finding suggested that the E627K and D256G mutations must have played important roles in host adaptation.

The D256G and E627K amino acid substitutions enhance polymerase activity. In order to assess the polymerase activity, a luciferase reporter gene construct was used. The polymerase activity of Sw/PB2-PB1-PA-NP was approximately twice that of Ck/PB2-PB1-PA-NP. However, there was a considerable increase in the polymerase activity of ribonucleoprotein (RNP) expressed by the Sw/PB2-Ck/PB1-PA-NP polymerase complex, achieved by replacing the PB2 gene of Ck/Yamaguchi/04 (H5N1) with that of Sw/Hokkaido/81 (H1N1) (Table 5). This finding correlates with the replication of in vivo-isolated virus clones 1 and 2 or in vitro-generated rg-Ck-Sw/PB2 virus in pigs. Interestingly, the RNP expressed by Ck/PB2-Sw/PB1-PA-NP, produced by replacing the PB2 gene of Sw/Hokkaido/81 (H1N1) with that of Ck/Yamaguchi/04

TABLE 2. Genome segment origin and susceptibility of pigs to H5N1 subtype virus clones recovered from nasal swabs of inoculated pigs

Virus clone(s)	Genome segment origin[a]								Susceptibility of pigs to virus clone[b]	
	PB2	PB1	PA	HA	NP	NA	M	ND	Replication	Antibody response
1	Sw	Ck	Ck	Ck	Ck	Ck	Ck	Ck	+[c]	+
2	Sw	Ck	Ck	Ck	Ck	Ck	Ck	Ck	+[d]	+
3	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	–	–
4	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	–	–
5–11[e]	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	ND	ND

[a] Genome segment origins were determined by partial sequencing. Sw, swine; Ck, chicken.
[b] Inoculum contained 10^7 to $10^{7.5}$ EID_{50}/500 μl of viruses. ND, not determined.
[c] Virus was recovered from days 2 to 5 p.i., and titers ranged from $10^{1.5}$ to $10^{3.8}$ EID_{50}/ml.
[d] Virus was recovered from days 1 to 3 p.i., and titers ranged from $10^{1.5}$ to $10^{2.8}$ EID_{50}/ml.
[e] Susceptibility of pigs to virus clones 5 to 11 was not determined.

TABLE 3. Virus titers in nasal swabs of pigs inoculated with viruses produced by reverse genetics

Virus	Pig	Virus titer (log10 EID_{50}/ml) at p.i. day[a]										Antibody response
		1	2	3	4	5	6	7	8	9	10	
rg-Ck/Yamaguchi/04 (H5N1)	1	—	—	—	—	—	—	—	ND	ND	ND	–
	2	—	—	—	—	—	—	—	ND	ND	ND	–
rg- Sw/Hokkaido/81 (H1N1)	3	5.8	≥6.5	6.3	5.5	3.8	4.8	—	1.8	1.8	1.3	+
	4	5.3	≥6.5	6.3	5.8	2.5	3.3	—	—	3.5	2.3	+
rg-Ck-Sw/PB2	5	3.5	3.8	1.3	—	—	—	—	ND	ND	ND	+
	6	—	1.5	1.3	—	—	ND	ND	ND	ND	ND	+
rg-Sw-Ck/PB2	7	—	—	3.8[b]	5.8	4.3	4.8	2.5	2.8	1.8	—	+
	8	—	—	1.3[b]	3.5	2.3	4.8	3.5	2.8	—	—	+
Pig 7-day 3	9	3.8	4.8	5.8	4.5	2.8	2.8	—	—	—	ND	+
	10	2.5	2.5	2.8	2.8	2.8	3.3	1.3	—	—	ND	+
Pig 8-day 3	11	4.3	≥4.5	5.5	5.5	3.8	2.3	—	—	—	ND	+
	12	2.8	4.8	5.3	4.8	4.5	3.3	—	—	—	ND	+

[a] ND, not determined; —, virus titer determined to be <1.5 log_{10} EID_{50}/ml.
[b] Virus isolates were sequenced and results are shown in Table 4.

(H5N1), showed lower polymerase activity than that of Sw/PB2-PB1-PA-NP (Table 5); conversely, Ck/PB2$_{D256G}$-Sw/PB1-PA-NP and Ck/PB2$_{E627K}$-Sw/PB1-PA-NP showed 3 to 15 times higher polymerase activity than that shown by Ck/PB2-Sw/PB1-PA-NP. These findings also correlate with the replicative behavior of virus isolates Pig 7-day 3 and Pig 8-day 3, which were isolated on day 3 p.i. from pigs inoculated with rg-Sw-Ck/PB2. After reinoculation into pigs, both virus isolates were isolated on day 1 p.i. The effect of the D256G and E627K amino acid substitutions on polymerase activity was further evaluated by using RNP expressed by homologous Ck/PB2$_{D256G}$PB1-PA-NP and Ck/PB2$_{E627K}$PB1-PA-NP polymerase complexes. There was a 43 to 175 times increase in the polymerase activity of RNP expressed by Ck/PB2$_{D256G}$PB1-PA-NP and Ck/PB2$_{E627K}$PB1-PA-NP compared to that of Ck/PB2-PB1-PA-NP and a 12 to 14 times increase compared to that of Ck/PB2$_{D256G}$-Sw/PB1-PA-NP and Ck/PB2$_{E627K}$-Sw/PB1-PA-NP, respectively. These findings suggest that the D256G and E627K amino acid substitutions in the PB2 protein of Ck/Yamaguchi/04 (H5N1) counteracted the suppressive effects of the naïve PB2 protein of Ck/Yamaguchi/04 (H5N1).

DISCUSSION

It has been shown that avian and human H5N1 viruses isolated in 1997 (48) and 2004 (5) replicated to moderate levels in the upper respiratory tracts of experimentally infected pigs. There is also evidence of cocirculation of avian and human influenza viruses in pigs in China (39). Therefore, it is reasonable to think that pigs can provide opportunity for the reassortment and subsequent emergence of new reassortant influenza viruses.

Kida et al. (25) inoculated pigs with A/duck/Hokkaido/8/1980 (H3N8) (nonreplicating strain) and Sw/Hokkaido/81 (H1N1) (replicating strain). They recovered both the viruses possessing the parental gene constellation and the reassortant viruses. The recovered H3N8 subtype viruses, after reinoculation, did not replicate in the pigs, while reassortant viruses replicated. Similarly, in the present study, viruses with the

TABLE 4. Comparison of amino acid sequences of PB2 gene products of isolates recovered from pigs inoculated with rg-Sw-Ck/PB2

Virus isolate	PB2 amino acid at:	
	Position 256	Position 627
rg-Sw-Ck/PB2 (inoculum)	D	E
Pig 7-day 3	D	K
Pig 8-day 3	G	E
rg-Sw/Hokkaido/81 (H1N1)	D	K

TABLE 5. Viral polymerase activity correlates with the virus replication potential in pigs[a]

Genome segment origin[b]				Luciferase activity ± SD (10^4 RLU)
PB2	PB1	PA	NP	
Ck	Ck	Ck	Ck	38.3 ± 2.2
Sw	Sw	Sw	Sw	63.3 ± 2.3
Sw	Ck	Ck	Ck	234.2 ± 5.4
Ck	Sw	Sw	Sw	38.4 ± 1.2
Ck_{E627K}	Sw	Sw	Sw	579.7 ± 78.5
Ck_{D256G}	Sw	Sw	Sw	120.3 ± 9.0
Ck_{E627K}	Ck	Ck	Ck	6696.8 ± 233.0
Ck_{D256G}	Ck	Ck	Ck	1654.1 ± 146.5
Control[c]				2.2 ± 0.7

[a] Polymerase activity was assayed by a viral untranslated region-driven luciferase reporter gene. 293T cells were transfected with the pHW2000 plasmid containing the cloned PB2, PB1, PA, and NP genes of Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1). After 24 h of transfection, luciferase activity (in relative light units [RLU]) was assayed in cell lysates. Results are the means ± standard deviations (SD) of two independent experiments, and each experiment was conducted in triplicate ($n = 6$).
[b] Sw (swine) and Ck (chicken) indicate that the respective genes are derived from Sw/Hokkaido/81 (H1N1) or Ck/Yamaguchi/04 (H5N1), respectively.
[c] 293T cells were transfected with pHW2000 and pHW-Luc as the negative control.

parental gene constellation were recovered, and two of these virus clones (virus clones 3 and 4) were found to be identical to the parental virus and rg-Ck/Yamaguchi/04 (H5N1) in that all three had the capability to replicate in pigs; therefore, the isolation of entire H5N1 virus clones could be due to concurrent infection of cells lining the upper respiratory tract of the inoculated pigs, with different reassortant viruses present in the inoculum which might have provided all eight gene segments of Ck/Yamaguchi/04 (H5N1).

The role of the PB2 protein in determining the host range has been studied extensively using squirrel monkeys (7), mice (9, 29), and mammalian cells (57). In the present study, we found that the PB2 gene of Ck/Yamaguchi/04 (H5N1) restricted its replication in pigs, since its replacement by the PB2 gene of Sw/Hokkaido/81 (H1N1) enabled it to replicate in the pigs, as observed for naturally selected virus clones 1 and 2 and rg-Ck-Sw/PB2 virus. Kida et al. (25) isolated triple-gene reassortants deriving the NP, NA, and M or NP, NA, and NS genes from the replicating strain Sw/Hokkaido/81 (H1N1) and the remaining five genes from the nonreplicating strain A/duck/Hokkaido/8/1980 (H3N8). In the present study, single-gene reassortant virus clones deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) were isolated. It could be due to differences in the gene constellations of nonreplicating influenza virus strains bearing different host range determinants (28, 49), as used by Kida et al. (25) and in the present study.

The restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) virus was evaluated by studying the replication of rg-Sw-Ck/PB2 virus in pigs. Interestingly, the viruses were recovered on day 3 p.i. and replicated to moderate levels for a shorter duration than rg-Sw/Hokkaido/81 (H1N1) (Table 3). These findings indicate that during the first 2 days p.i., the virus might have undergone adaptive changes. This assumption was supported by examining the predicted amino acid sequences of the two virus isolates, Pig 7-day 3 and Pig 8-day 3, whose PB2 proteins had E627K and D256G amino acid substitutions, respectively. Amino acid substitution at position 256 in the PB2 protein has not been reported previously, while amino acid substitution at position 627 has been reported to be a host range determinant. Li et al. (29) inoculated mice with two duck isolates of contrasting pathogenicity for mice. They found that more than 50% of the virus isolates recovered from mouse lungs had E627K substitutions in the PB2 protein. Similarly, viruses recovered from mice inoculated with Ck/Yamaguchi/04 (H5N1) had the E627K substitution in the PB2 protein (31); therefore, these studies suggested that the presence of E or K at position 627 is host dependent and is an indicator of avian-to-mammalian adaptation. The finding that the Pig 7-day 3 and Pig 8-day 3 isolates were isolated from pigs on day 1 p.i. and previous findings suggest that the E627K and D256G substitutions enabled the Pig 7-day 3 and Pig 8-day 3 isolates to replicate in pigs like that of parental or rg-Sw/Hokkaido/81 (H1N1) virus.

The in vivo replicative behavior of virus clones 1 and 2 or of virus isolates Pig 7-day 3 and Pig 8-day 3 was further supported by the luciferase assay. The E627K amino acid substitution has been shown to increase the polymerase activity (9), while the D256G amino acid substitution found in the present study has not been reported previously. The findings suggest that replication of virus clones 1 and 2 or virus isolates Pig 7-day 3 and Pig 8-day 3 in pigs may be due to enhancement of viral polymerase activity in the epithelial cells lining the upper respiratory tract of pigs.

The PB2, PB1, and PA proteins make up the viral RNA polymerase complex. The presence of overlapping PB1 and NP functional regions on the PB2 protein has suggested their role in switching the transcriptase to replicase activity (40, 47). The D256G substitution is located in the functional domain of the PB2 protein. This region has been shown to be related to a cap binding function (18, 40), interaction with NP protein (40), and interaction with PB1 protein (38). Similarly, the E627K substitution is located in the C-terminal region of the PB2 protein, which interacts with both the PB1 and NP proteins (40). Labadie et al. (27) suggested that the presence of K at position 627 in the PB2 protein helps to stabilize the PB2-NP interaction in human cells through an unknown host cellular factor, while K at this position impairs this interaction in avian cells. Many host cell proteins have been shown to interact with different subunits of influenza virus polymerase complex, and some of these were involved either in translocation of viral RNPs such as importin α (10), Ran binding protein 5 (8), or heat shock protein 90 (37) or in regulation of polymerase activity (33, 34). Recently, Jorba et al. (21) identified many influenza virus polymerase-interacting nuclear and cytosolic proteins involved in transcription, modification, and translocation. Those findings suggest that interaction of polymerase components with each other to carry out transcription or replication involves host cellular factors; thus, adaptive changes to host cellular factors might play an important role in host range determination.

The role of the D256G and E627K amino acid substitutions in the adaptation of influenza viruses to new hosts is reflected by a significant increase in the polymerase activity of both homologous and heterologous polymerase complexes (Table 5). This result indicates that the D256G and E627K amino acid substitutions might be critical changes to control polymerase activities independently, not only for the reassortant virus rg-Sw-Ck/PB2 but also for the original Ck/Yamaguchi/04 (H5N1). It was interesting to find that out of 3,146 predicted amino acid sequences of the PB2 gene obtained from GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html), only one isolate, A/swine/Wisconsin/1/1967 (H1N1), had the D256G amino acid substitution, while one swine and one duck isolate had the D256R and D256I amino acid substitutions, respectively. This finding indicates that D256G might not be a common mutation in the process of virus evolution. In any case, it is speculated that the mutations D256G and E627K might have appeared as a result of the interaction of the PB2 protein of Ck/Yamaguchi/04 (H5N1) with pig cellular proteins, resulting in enhanced replication of virus isolates Pig 7-day 3 and Pig 8-day 3 in pigs.

In light of earlier and present findings, it is reasonable to conclude that the PB2 protein of Ck/Yamaguchi/04 (H5N1) determined its host range. However, the molecular events which lead to the appearance of D256G and E627K substitutions have yet to be elucidated.

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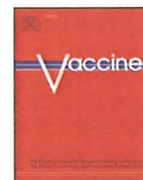

PolyI:polyC$_{12}$U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants

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ABSTRACT

The highly pathogenic avian H5N1 influenza virus has the potential to incite a global pandemic. Therefore, there is an urgent need to develop effective vaccines against these viruses. Because it is difficult to predict which strain of influenza will cause a pandemic, it is advantageous to develop vaccines that will confer cross-protective immunity against variants of the influenza virus. Recently, we reported that the Toll-like receptor 3 agonist, polyI:polyC$_{12}$U (Ampligen®), has been proven to be safe in a Phase III human trial, and is an effective mucosal adjuvant for intranasal H5N1 influenza vaccination. Intranasal administration of an Ampligen® adjuvanted pre-pandemic H5N1 vaccine (NIBRG14), which was derived from the A/Vietnam/1194/2004 strain, resulted in the secretion of vaccine-specific IgA and IgG in nasal mucosa and serum, respectively, and protected mice against homologous A/Vietnam/1194/2004 and heterologous A/Hong Kong/483/97 and A/Indonesia/6/2005 viral challenge.

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1. Introduction

There are presently a number of pre-pandemic H5N1 vaccines in existence, which were derived from currently circulating strains of the virus. However, the continual mutation of H5N1 renders them of limited use. Therefore, it is of crucial importance to develop an influenza vaccine that confers cross-protective immunity not only against the homologous influenza virus but also against the variants that arise from mutation of the virus.

Inactivated vaccines against the influenza virus have been administered parenterally to induce viral-specific serum IgGs that are highly protective against homologous virus infection. However, they are much less effective against heterologous virus infection [1]. By contrast, a number of studies have shown that the mucosal immunity acquired through natural infection, which is mainly mediated by the secreted form of IgA (sIgA) in the respiratory tract, is more effective and cross-protective against heterologous virus infections than the systemic immunity induced by parenteral vaccination [1–3]. It is believed that sIgA is more cross-protective against heterologous influenza compared with IgG due to its divalency (higher avidity) and location [1]. In this regard, induction of virus-specific sIgA in the respiratory tract has a great advantage in conferring protection against an unpredictable pandemic of highly pathogenic avian influenza viruses.

We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C) is a promising and effective intranasal adjuvant for influenza virus vaccine. Poly(I:C) interacts with Toll-like receptor 3 (TLR3), which plays a key role in the innate immune system and activates immune cell responses. Intranasal administration with split influenza vaccine in combination with poly(I:C) increased both the mucosal and systemic humoral immune response, resulting in complete protection against homologous and heterologous influenza viruses in mice [4]. Although poly(I:C) is a potent mucosal adjuvant that induces type I interferons (IFNs) and has the potential to bridge the gap between innate and adaptive immunity [5], it has been associated with serious adverse events during clinical trials [6].

PolyI:polyC$_{12}$U (Ampligen®), a dsRNA compound that is similar to poly(I:C), degrades easily *in vivo* due to the existence of mismatched residues in the nucleotide. It has a good safety profile based on clinical trials, including a recently conducted double-blind, placebo-controlled Phase III clinical trial [7]. To date, more than 75,000 doses of Ampligen have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. We examined the cross-protective effect of intranasal vaccine given in combination with Ampligen in mice. We demonstrated that co-administration of the vaccine with Ampligen elicited cross-protective immunity against heterologous A/Hong Kong/483/97 and A/Indonesia/6/2005 viruses.

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2. Materials and methods

The strains of H5N1 viruses used in this study were A/Hong Kong/483/97, A/Vietnam/1194/2004, and A/Indonesia/6/2005 [8]. The A/Hong Kong/483/97 virus was prepared in Madin–Darby canine kidney (MDCK) cells without any special step for mouse adaptation. The A/Vietnam/1194/2004 and A/Indonesia/6/2005 viruses were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 °C. The formalin-inactivated whole virus vaccine (NIBRG14) was prepared from a recombinant avirulent avian virus that contains modified hemagglutinin (HA) and neuraminidase from the highly pathogenic avian influenza strain A/Vietnam/1194/2004 and other viral proteins from the influenza strain A/PuertoRico/8/34 (A/PR8, H1N1) [9]. The trivalent-inactivated influenza vaccine (split-product HA vaccines) prepared for the 2005–2006 season, including A/NewCaledonia/20/99 (H1N1), A/NewYork/55/2004 (H3N2), and B/Shanghai/361/2002, was purchased from Kitasato Institute (Saitama, Japan). PolyI:polyC$_{12}$U (Ampligen®) was kindly provided by Hemispherx Biopharma (Philadelphia, PA).

BALB/c mice were anaesthetized with diethyl ether and immunized 2 or 3 times, either intranasally or subcutaneously, with 1 μg of NIBRG14 [10] or trivalent split-product virus vaccines [11] with or without adjuvant at 3-week intervals. Each mouse was anaesthetized and infected by intranasal administration of 4 μl of PBS containing virus suspension with 1000 PFU of H5N1 virus into each nostril (2 μl/nostril) at 2 weeks after final vaccination. The immune response elicited after vaccination was examined 2 weeks after the final vaccination using a number of immunological assays (ELISA, hemagglutination inhibition (HI), and virus neutralization (VN) assays) [10,11]. The protective efficacy of the vaccines was examined by assessing viral titer in the nasal wash and monitoring survival rate of mice after the challenge. All animal experiments were performed in accordance with the Guides for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of NIID. Infection with H5N1 virus was performed under Biosafety Level 3 containment and was approved by NIID.

3. Results and discussion

3.1. Antibody responses in mice immunized intranasally or subcutaneously with NIBRG14 vaccine and Ampligen

To determine the efficacy of Ampligen as a mucosal adjuvant for H5N1 vaccines, the antibody response to NIBRG14 was examined. Mice were immunized twice by intranasal or subcutaneous administration of NIBRG14, with or without Ampligen, and their antibody response was measured by ELISA. In nasal washes, higher levels of anti-NIBRG14 IgA Ab were observed in animals immunized intranasally with 1 μg of NIBRG14 and 10 μg of Ampligen (Fig. 1A). A small IgA response was elicited by intranasal administration of NIBRG14 without adjuvant, and no IgA response was evident in any of the mice which received a subcutaneous vaccination of NIBRG14 with or without Ampligen. Neutralizing activity against homologous A/Vietnam/1194/2004 virus was detected in the sera from mice immunized either intranasally or subcutaneously, with or without adjuvant. However, no neutralizing activity against heterologous A/Hong Kong/483/97 or A/Indonesia/6/2005 viruses was detected in the sera from any immunized group, suggesting that serum IgG antibodies are insufficient to neutralize heterologous virus and IgA antibodies at the mucosal surface might be more important than serum IgG antibodies for the protection against heterologous viruses. However, nor was neutralizing activity detected in the nasal wash from any group against both homologous and heterologous viruses. We suspect that, due to the dilution by PBS when the nasal wash samples were collected, the concentration of vaccine-specific IgA in our samples was much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal wash may not have been detectable.

3.2. Intranasal vaccination with NIBRG14 and Ampligen protects mice against highly pathogenic avian influenza virus infection

We next examined the protective effect of intranasal vaccination with NIBRG14 in combination with Ampligen against homologous and heterologous H5N1 viruses. Mice were immunized, either intranasally or subcutaneously, with 1 μg of NIBRG14 and 10 μg of Ampligen, and then challenged by infection with homologous A/Vietnam/1194/2004, heterologous A/Hong Kong/483/97 or heterologous A/Indonesia/6/2005 viruses. All of the mice immunized intranasally with combined vaccine and Ampligen completely cleared the viruses in their nasal cavity (Table 1). By contrast, significantly higher levels of virus in nasal wash samples were detected in mice immunized subcutaneously with vaccine and Ampligen. All of the mice in both groups survived following homologous A/Vietnam/1194/2004 viral challenge (Table 1). In the heterologous viral challenge experiment, the virus titer in the nasal wash of the intranasal vaccination group was significantly lower than that of the subcutaneous vaccination group following infection with the A/Hong Kong/483/97 or A/Indonesia/6/2005 virus. Consequently, though intranasally immunized mice survived lethal infection with A/Hong Kong/483/97 or A/Indonesia/6/2005 viruses, the 100% of A/Hong Kong/483/97 and 60% of A/Indonesia/6/2005 infected mice

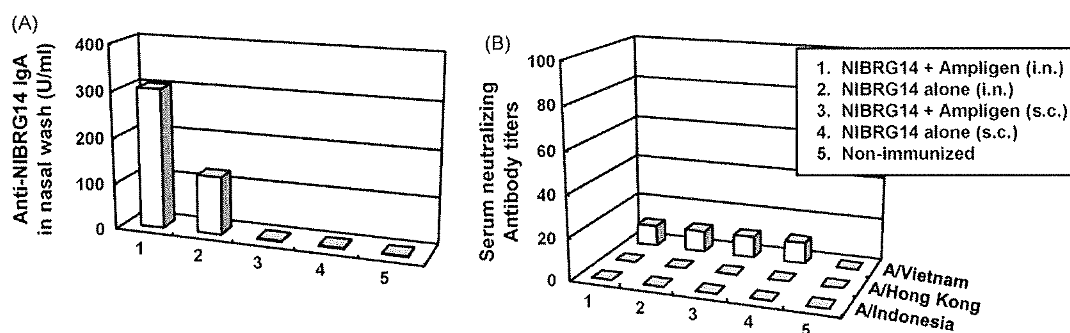

Fig. 1. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally or subcutaneously with vaccine alone, or in combination with Ampligen. Nasal washes and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA (A). The serum collected at 2 weeks after the booster was analyzed for the presence of neutralizing antibodies against homologous or heterologous influenza virus (B). Inhibition of the virus was assessed by the additional reduction in infectivity beyond the background of naive mice. Sample was run in duplicate, and data are presented per group, where the ability to inhibit 100% of infection at the indicated dilution is shown.

Table 1
H5N1 virus titers in nasal washes and survival rates after challenge with homologous and heterologous viruses.

Vaccination (route)	Challenge virus					
	A/Vietnam/1194/2004		A/Hong Kong/483/97		A/indonesia/6/2005	
	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)
NIBRG14 + Ampligen (i.n.)	0	100	9 ± 2	80	63 ± 24	100
NIBRG14 + Ampligen (s.c.)	112 ± 72	100	10415 ± 4649	0	1393 ± 534	40
-	1110 ± 494	0	2765 ± 2065	0	27600 ± 3355	20

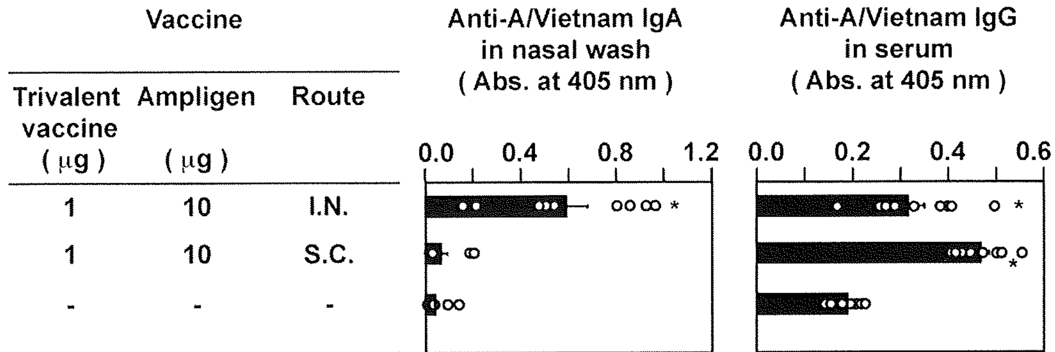

Fig. 2. Cross-reactive IgA and IgG antibodies to A/Vietnam/1194/04. The mice were initially immunized with 1 μg of trivalent-inactivated vaccines with Ampligen through intranasal or subcutaneous route. Immunization was repeated at 3 and 5 weeks after the initial immunization. The nasal washes and serum samples were collected 2 weeks after the final immunization. The concentrations of IgA and IgG antibodies titer to A/Vietnam/1194/04 were measured by ELISA. Bars represent the means ± S.E. of 1:5 diluted samples (nasal washes) or 1:200 diluted samples (sera) and open circles indicate individual animals.

immunized subcutaneously with vaccine and Ampligen succumbed to death (Table 1). These data indicate that intranasal vaccination with combined H5N1 vaccine and Ampligen is more effective than subcutaneous vaccination in protection against homologous and heterologous H5N1 influenza virus challenge [10].

3.3. *Induction of cross-reactive antibodies to H5N1 virus by intranasal vaccination with seasonal influenza vaccine and Ampligen*

We next characterized the cross-reactive antibody response to A/Vietnam/1194/2004 (H5N1) virus in mice immunized, either intranasally or subcutaneously, with 1 μg of seasonal influenza vaccine and 10 μg of Ampligen. Compared to that in the subcutaneously immunized mice, the concentration of IgA antibodies against A/Vietnam/1194/2004 in nasal wash samples was significantly increased in mice inoculated intranasally with the trivalent vaccine and Ampligen (Fig. 2). The concentration of IgG antibodies against A/Vietnam/1194/2004 in serum was also significantly increased in mice inoculated either intranasally or subcutaneously with the trivalent vaccine and Ampligen combination (Fig. 2). HI titers with regard to heterologous A/Vietnam/1194/2004, A/Hong Kong/483/97, and A/Indonesia/6/2005 virus were also examined *in vitro* using serum and nasal samples from the same group of mice. However, these samples did not show any appreciable cross-neutralizing activity against the H5N1 virus strains. The inability to detect any neutralizing activity in the nasal wash samples was, again, likely due to the dilution of antibodies by PBS when the nasal wash samples were collected.

3.4. *Cross-protection against different H5N1 influenza virus strains by intranasal inoculation with seasonal influenza vaccine and Ampligen*

We next examined whether the combination of the seasonal influenza vaccine and Ampligen could confer cross-protection against heterologous H5N1 influenza viruses, including the A/Vietnam/1194/2004, A/Hong Kong/483/97 and A/Indonesia/6/2005 strains (Table 2). We immunized mice, either intranasally or subcutaneously, with 1 μg of seasonal influenza vaccine and 10 μg of Ampligen, then monitored viral titer and survival of mice after intranasal challenge with a lethal dose of A/Vietnam/1194/2004, A/Hong Kong/483/97 or A/Indonesia/6/2005 virus. Mice that had been inoculated intranasally or subcutaneously with a combination of trivalent virus and Ampligen showed a significant reduction in A/Vietnam/1194/2004 virus titer, compared with non-inoculated mice. Furthermore, 50% of the intranasally inoculated mice survived, whereas all of the subcutaneously inoculated mice had succumbed to death by 14 days post-infection with A/Vietnam/1194/2004 virus (Table 2). In challenges with 1000 PFU of A/Hong Kong/483/97 virus, mice that had been inoculated with both the trivalent vaccine and Ampligen showed a 25% reduction in virus titer, compared with non-inoculated mice

Table 2
Cross-protective effect of inoculation with seasonal influenza vaccine and Ampligen against H5N1 influenza viruses.

Vaccination (route)	Challenge virus					
	A/Vietnam/1194/2004		A/Hong Kong/483/97		A/Indonesia/6/2005	
	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)
Trivalent vaccine + Ampligen (i.n.)	435 ± 231	50	103 ± 94	100	20500 ± 4843	100
Trivalent vaccine + Ampligen (s.c.)	726 ± 281	0	138 ± 54	70	12400 ± 1198	20
-	4505 ± 1113	0	484 ± 195	60	45200 ± 5492	20

(Table 2). At 14 days after challenge with A/Hong Kong/483/97, all of the intranasally inoculated mice were still alive, whereas 30% of the subcutaneously inoculated mice ($n = 10$) and 40% of the non-inoculated mice ($n = 10$) had died. Finally, in challenges with A/Indonesia/6/2005 virus, mice that had been inoculated intranasally or subcutaneously with the trivalent vaccine and Ampligen combination showed a significant reduction in virus titer compared with non-inoculated mice. At 14 days after challenge with A/Indonesia/6/2005 virus, all of the intranasally inoculated mice were still alive, whereas 80% of the subcutaneously inoculated mice had died (Table 2). Taken together, these results indicate that intranasal inoculation with the trivalent vaccine combined with Ampligen is more effective against infection with heterologous H5N1 influenza virus than subcutaneous vaccination.

4. Concluding remarks

To develop an effective influenza vaccine, it is beneficial to mimic the process of natural infection that bridges the innate and adaptive immune systems [12]. In the present study, we showed that polyI:polyC_{12}U (Ampligen®) has mucosal adjuvant activity when co-administered intranasally with formalin-inactivated H5N1 influenza whole-virion vaccine or the trivalent-inactivated influenza vaccine licensed in Japan for the 2005–2006 season. It increased both the mucosal and systemic humoral responses, and protected mice against homologous and heterologous highly pathogenic H5N1 avian influenza viruses [10,11]. Because TLR3, the receptor that is activated in response to Ampligen [13], is localized to the endosomal compartment in cells, concomitant administration of Ampligen and liposomes may be more effective than Ampligen alone. In fact, chitin microparticles, as a carrier for poly(I:C), enhanced antibody responses and provided protection against lethal H5N1 influenza virus challenge when administered in conjunction with poly(I:C) [14]. We have also observed that co-administration of H5N1 vaccine with Ampligen as a mucosal adjuvant elicited high levels of vaccine-specific IgA titer in saliva and IgG titer in the serum in Cynomolgus macaques (Ichinohe et al. unpublished data). Plans to test the efficacy of the adjuvant-combined intranasal influenza vaccine in human clinical trials will be underway in Japan as early as 2010. Finally, it would be of great benefit to develop biocompatible materials that will enhance the adhesion and uptake of vaccines in the nasal cavity and the respiratory tract. This could significantly enhance the efficacy of inoculation in humans, since the relative extent of the nasal cavity differs from that in mice, and most of the vaccine is ingested when it is intranasally administered to humans.

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