inoculated with 10^6 PFU (500 μl) of S-OIVs or KUTK-4. On days 3 and 6 pi, 3 ferrets per group were euthanized for virologic and pathologic examinations. The virus titres in nasal washes and 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, Yamanashi, Japan), which were serologically negative by AniGen AIV Ab ELISA for currently circulating influenza viruses, were used in this study. Baseline body temperatures were measured once prior to infection. Four pigs per group were intranasally inoculated with $10^{6.2}$ PFU (1 ml) of viruses. Nasal swabs were collected daily. On day 3 pi, two pigs per group were euthanized and their tissues collected for examination. On day 14 pi, the remaining two pigs per group were euthanized for virologic and pathologic 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, Tokyo, Japan) 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.

Pathologic examination

Excised tissues of the nasal turbinates, trachea and/or lungs of euthanized 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 hematoxylin-and-eosin procedure, while 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., Tokyo, Japan).

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 PFU (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 naïve 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, Hercules, CA), whereas macaque lung homogenates were measured with the MILLIPLEX MAP Non-human Primate Cytokine/Chemokine Panel - Premixed 23-Plex (Millipore, Bedford, MA). 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 `Method Summary' section and one hour later administered the following antiviral compounds: (i) oseltamivir phosphate: 8 or 80 mg/kg/400 μl (divided into two oral administrations per day) for 5 days; (ii) zanamivir: 0.8 or 8 mg/kg/50 μl in one daily intranasal administration for 5 days; (iii) CS-8958: 0.7 mg/kg/50 μl in one intranasal administration; (iv) T-705: 60 or 300 mg/kg/400 μl (divided into two oral administrations per day) for 5 days; (v) or distilled water

orally (200 μl) and PBS intranasally (50 μl). Three mice per group were euthanized on days 3 or 6 pi 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. Based on the $TCID_{50}$ value, the 90% inhibitory concentration (IC_{90}) was calculated.

Neuraminidase inhibition assay—To assess the sensitivity of viruses to neuraminidase inhibitors (i.e., 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) ethanesulfonic acid containing calcium chloride, and incubated for 30 min at 37°C. Then, we added methylumbelliferyl-N-acetylneuraminic acid (Sigma, St Louis, MO) as a fluorescent substrate to this mixture. After incubation for one hour 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 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 to 112 years as of 2009, mean = 92.7 ± 15.0 years) or #2 (age range: 20 to 68 years as of 2009, mean = 48.2 ± 23.7 years), respectively. These sera were treated with receptor-destroying enzyme (DENKA SEIKEN CO., LTD, Tokyo, Japan) 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 30 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 hours 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, Carlsbad, CA), and examined with a confocal laser- scanning microscope (LSM510META; Carl Zeiss, Jena, Germany).

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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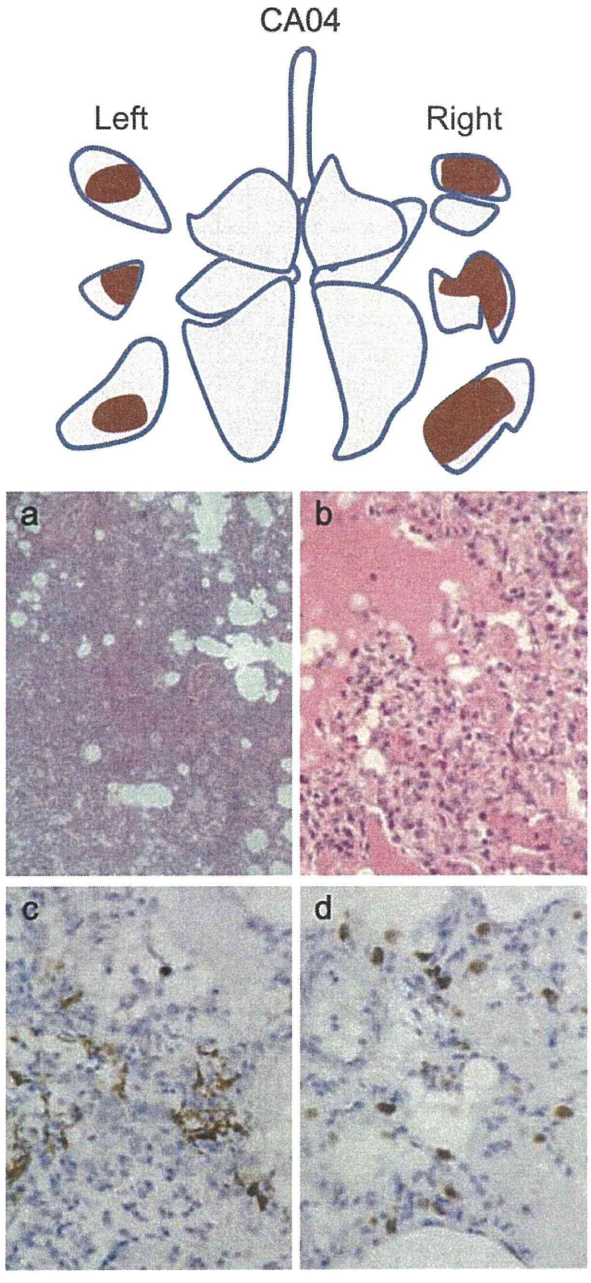

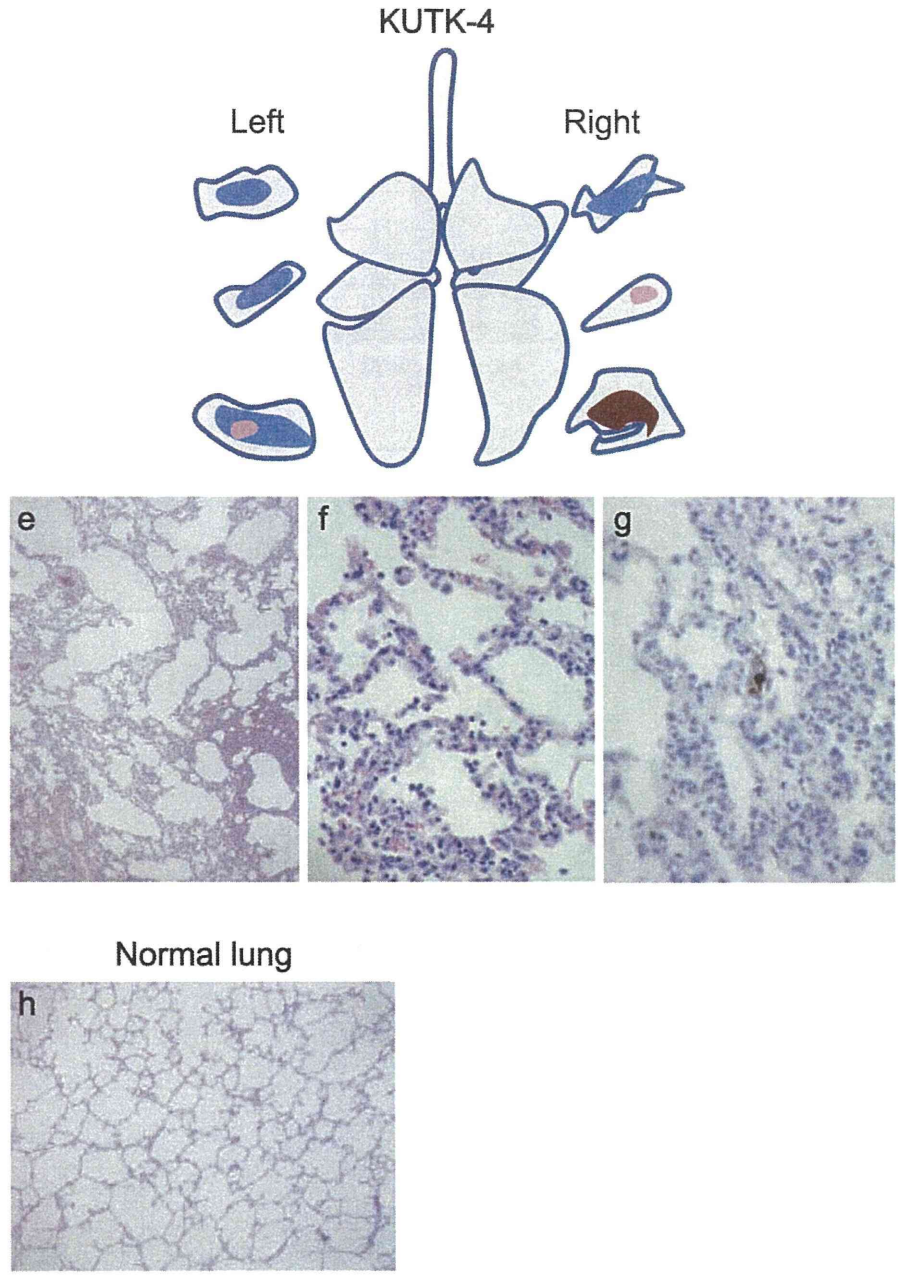

Figure 1. Pathologic examination of the lungs of infected cynomolgus macaques

Representative pathologic images of CA04- (macaque #1, **a-d**), KUTK-4- (macaque #7, **e-g**), and mock- (**h**) infected lungs on day 3 pi. 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.

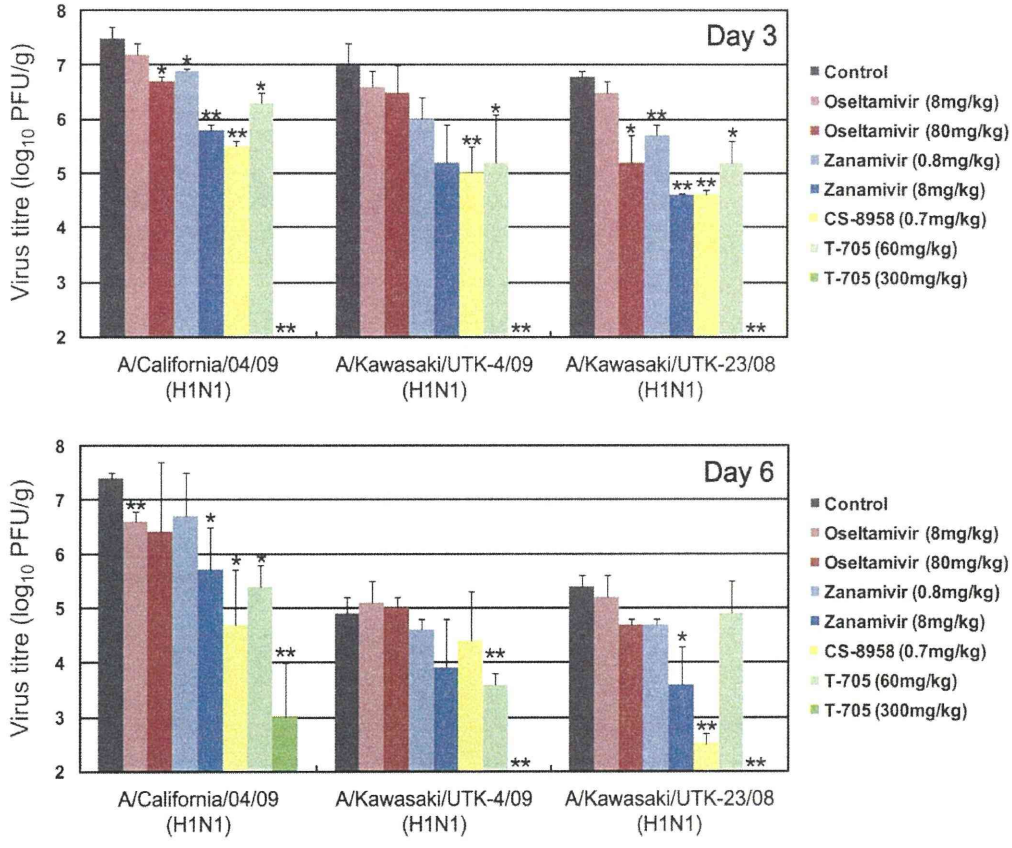

Figure 2. CA04 sensitivity to antiviral compounds in mice

Mice were intranasally inoculated with 10^4 PFU (50 μl) of CA04, KUTK-4, or A/Kawasaki/UTK-23/08 (H1N1). At 1 h pi, mice were administered oseltamivir phosphate, zanamivir, CS-8958, T-705, or distilled water and PBS (control). Three mice per group were euthanized on days 3 and 6 pi and the virus titres in lungs were determined by plaque assays in MDCK cells. *, $p<0.05$, **, $p<0.01$.

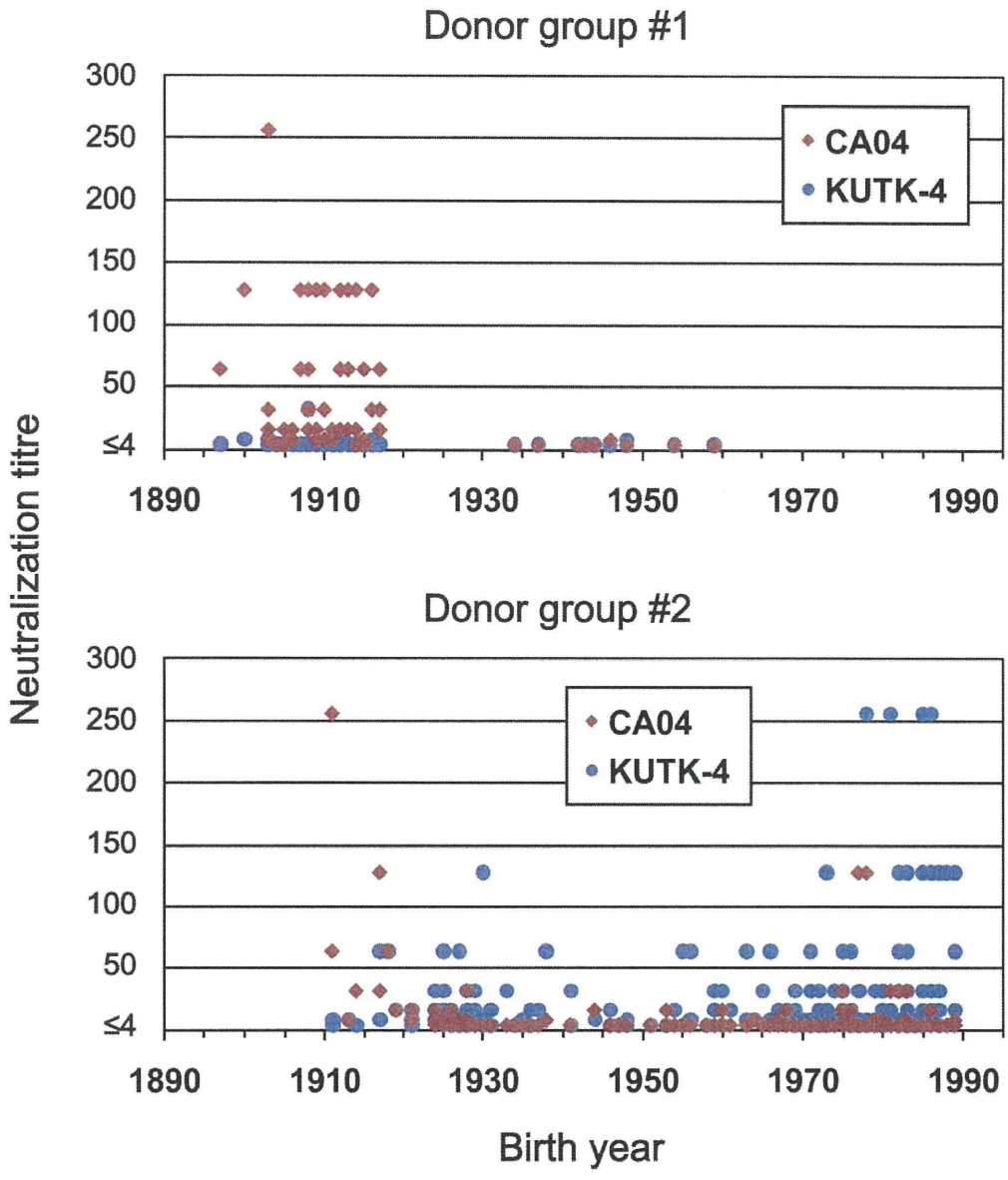

Figure 3. Neutralization activities in human sera against viruses

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. Since the sera of donor group #1 were collected in 1999, little neutralization activity was expected against KUTK-4, which was isolated in 2009.

Table 1

Virus titres in organs of infected cynomolgus macaques[a]

	A/California/04/09(H1N1)						A/Kawasaki/UTK-4/09 (H1N1)					
	day 3 pi			day 7 pi			day 3 pi			day 7 pi		
Animal ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
Nasal mucosa	4.7[b]	3.3	-[c]	-	-	-	-	-	-	-	-	-
Oro/nasopharynx	6.3	4.4	4.7	-	7.9	-	-	-	4.3	-	-	4.8
Tonsil	6.4	-	-	-	7.1	-	-	-	2.8	-	-	3.0
Trachea	5.9	2.0	5.6	-	-	-	2.0	4.1	-	3.7	-	5.4
Bronchus (right)	5.7	2.9	4.3	-	5.1	-	-	2.5	-	3.5	-	3.8
Bronchus (left)	5.9	-	6.1	-	5.1	-	-	-	-	3.3	-	5.1
Lung (upper right)	5.7	5.6	4.5	-	-	-	2.7	-	-	-	-	-
Lung (middle right)	5.6	6.4	6.9	-	-	-	2.3	2.6	2.5	-	-	-
Lung (lower right)	6.1	4.5	6.0	-	-	-	2.6	2.6	-	-	-	3.4
Lung (upper left)	4.7	4.3	6.4	-	-	-	-	-	-	-	-	-
Lung (middle left)	5.8	4.3	6.3	-	-	-	-	-	-	-	-	-
Lung (lower left)	6.7	4.5	6.6	-	-	-	-	-	-	-	-	2.3
Conjunctiva	3.6	-	-	-	-	-	-	-	-	-	-	-

[a] Cynomolgus macaques were inoculated with $10^{7.4}$ PFU of virus (6.7 ml) through multiple routes (see detailed procedure in Methods). Three macaques per group were euthanized on day 3 and 7 pi for virus titration. No virus was recovered from lymph nodes (chest), heart, spleen, kidneys, or liver of any animals.

[b] Virus titre (mean $\log_{10}$ PFU/g).

[c] -, virus not detected (detection limit: 2 $\log_{10}$ PFU/g).

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Intranasal administration of a live non-pathogenic avian H5N1 influenza virus from a virus library confers protective immunity against H5N1 highly pathogenic avian influenza virus infection in mice: Comparison of formulations and administration routes of vaccines

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ABSTRACT

Outbreaks of highly pathogenic avian influenza viruses (HPAIVs) would cause disasters worldwide. Various strategies against HPAIVs are required to control damage. It is thought that the use of non-pathogenic avian influenza viruses as live vaccines will be effective in an emergency, even though there might be some adverse effects, because small amounts of live vaccines will confer immunity to protect against HPAIV infection. Therefore, live vaccines have the advantage of being able to be distributed worldwide soon after an outbreak. In the present study, we found that intranasal administration of a live H5N1 subtype non-pathogenic virus induced antibody and cytotoxic T lymphocyte responses and protected mice against H5N1 HPAIV infection. In addition, it was found that a small amount (100 PFU) of the live vaccine was as effective as 100 μg (approximately 10^{10-11} PFU of virus particles) of the inactivated whole particle vaccine in mice. Consequently, the use of live virus vaccines might be one strategy for preventing pandemics of HPAIVs in an emergency.

1. Introduction

Highly pathogenic avian influenza viruses (HPAIVs) have raised the concern that a pandemic will cause enormous damage worldwide after HPAIVs acquire the ability for human-to-human transmission because most humans do not possess immunity against these viruses [1,2]. It would probably take several months to prepare and distribute inactivated vaccines against HPAIVs [3,4]. However, since small amounts of non-pathogenic live vaccines against HPAIVs should be effective for protection against virus infection, it would be possible to produce live vaccines soon after an outbreak of HPAIVs and prevent a pandemic. In the present study, we examined whether nasal inoculation with a small amount of a live vaccine could prevent H5N1 HPAIV replication in mouse lungs.

Live vaccines are generally more effective than inactivated vaccines in protection against virus infection [5,6]. In the case of measles virus vaccines, it has been shown that inactivated virus did not work as a vaccine to induce protective immunity [7,8]. Both live and inactivated vaccines are available for seasonal influenza

Abbreviations: CPE, cytopathic effects; CTL, cytotoxic T lymphocyte; CFSE, carboxyfluorescein diacetate succinimidyl ester; CMTMR, (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine); HPAIV, highly pathogenic avian influenza virus; LD_{50}, 50% lethal dose; NP, nucleoprotein; OVA, ovalbumin; PFU, plaque-forming unit; $TCID_{50}$, 50% tissue infectious dose.

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virus infection [9]. However, live vaccines against influenza viruses cannot be used for children younger than 2 years of age, persons over 50 years of age, and immunocompromised patients because of side effects [10–12]. It is also possible that live vaccines will acquire pathogenicity by mutation in vaccinated hosts. In contrast, although inactivated vaccines do not have these risks, inoculation is required every year because of their weak potency for eliciting immunological memory [5].

We have established a virus library that contains 144 strains of non-pathogenic avian influenza virus with combinations between 16 hemagglutinins (HA) and 9 neuraminidases (NA) [13]. We previously reported that whole virus particles inactivated by formalin (whole particle vaccines) induced antibody and cytotoxic T lymphocyte (CTL) responses more vigorously than did ether-split vaccines and that whole particle vaccines conferred more effective protection against H3N2 and H5N1 viruses than did ether-split vaccines [14]. Since viruses in the library are non-pathogenic in chickens, we have postulated that these viruses are also non-pathogenic in mice and probably in humans without an attenuation process and gene recombination [15].

In the present study, we compared antigen-specific responses induced by a live vaccine against H5N1 HPAIV with those induced by a whole particle vaccine, since immunological rationales for differences between two types of vaccines have not been revealed [16–19]. We also examined the effects of different routes of inoculation with the live vaccine. We found that intranasal administration of the live vaccine induced antibody and CTL responses more effectively than did subcutaneous administration and conferred protection against infection, whereas subcutaneous immunization with the whole particle vaccines was more effective than intranasal immunization in protective efficacy. In addition, quantitative analysis revealed that a small amount (100 PFU) of the live vaccine was as effective as 100 μg (approximately 10^{10-11} PFU of virus particles) of the whole particle vaccine in mice. Therefore, live viruses from the non-pathogenic avian influenza virus library might be vaccine candidates for worldwide distribution soon after outbreaks of new subtypes of influenza viruses, including HPAIVs.

2. Materials and methods

2.1. Influenza viruses and vaccines

The influenza A virus A/Aichi/2/1968 (H3N2) (Aichi) is a low pathogenic influenza virus [20]. A genetic reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1, formally described as A/R(duck/Mongolia/54/01-duck/Mongolia/47/01)/2004 (H5N1), National Center for Biotechnology Information taxonomy database ID: 376899), was generated by mixed infection with A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [14]. PB2, PB1, PA, HA, NP, and M genes of Vac-1 were derived from the H5N2 virus, and NA and NS genes were derived from the H7N1 virus [21]. The influenza virus A/Vietnam/1194/2004 (H5N1) (VN1194) is a highly pathogenic strain [22]. The percent sequence similarities between Vac-1 and VN1194 are 92% in HA and 90% in NA at the amino acid level. Live viruses used for inoculation were prepared from the culture supernatant of infected Madin-Darby canine kidney (MDCK) cells. For preparation of whole particle vaccines, viruses were propagated in the allantoic cavities of 10-day-old embryonated hen's eggs at 35 °C for 36–48 h. Then the viruses were purified by ultracentrifugation (112,500 × *g* for 90 min) of allantoic fluid through a 10–50% sucrose density gradient. Formalin-inactivated vaccines were prepared with 0.1% formalin at 4 °C for a week. The purified fixed viruses were then suspended in PBS. Inactivation of the viruses was confirmed by the absence of detectable hemagglutination after one passage of the treated viruses in 10-day-old embryonated hen eggs [14]. 1.4×10^{11} 50% egg infectious dose (EID_{50}) of purified Vac-1 was used to prepare 100 μg of whole particle vaccine of Vac-1. The amount of whole particle vaccines was indicated as that of entire protein including HA and the other viral proteins. The whole particle vaccine of H5N1 virus used in the present study contained 15, 170 HA units of HA antigen in 100 μg vaccine. The nucleotide sequences of HA, NA and NP of Vac-1 grown in embryonated eggs and MDCK cells were analyzed and amino acid sequences were compared in Table 1. There were three nucleotide replacements accompanying amino acid changes between Vac-1 grown in MDCK cells and that grown in embryonated eggs, whereas there was no difference in NA and NP genes between these two viruses.

Table 1
Comparison of amino acids of HA in H5N1 viruses used in this study.

Virus	Amino acid positions in HA		
	282	371	492
Vac-1 live vaccine	E	K	A
Vac-1 whole particle vaccine	K	E	E

Amino acid positions are determined based on the positions of H5 protein of Vac-1. The sequences of NA and NP genes were identical between live vaccine and whole particle vaccine of Vac-1. The position 282 in HA1 is located outside of a sialic acid binding site. The positions 371 and 492 in HA2 are located near the virion lipid membrane. VN1194 possesses identical amino acids on these three positions as Vac-1 used for whole particle vaccines.

2.2. Immunization

C57BL/6 mice (B6) (6–10 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Aichi or Vac-1 was subcutaneously (in 100 μl PBS) or intranasally (in 15 μl PBS) inoculated into mice. VN1194 (3×10^4 50% tissue infectious dose ($TCID_{50}$) = 100 × 50% lethal dose (LD_{50}) in 30 μl PBS) was intranasally inoculated into mice. All experiments were performed with the approval of the Shiga University of Medical Science.

2.3. In vivo CTL assay

Spleen cells (2×10^7 cells/ml) from naïve B6 mice were incubated with 0.5 μM ovalbumin (OVA) OVA257-264 peptide (SIINFEKL) or influenza virus nucleoprotein (NP) NP366-374 peptide (ASNENMETM for Vac-1, ASNENMEAM for VN1194) for 2 h at 37 °C. After washing twice with PBS, the recovered cells (2×10^7 cells/ml) were labeled with different concentrations of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE/CFSE) (0.25 μM or 2.5 μM, Molecular Probes, Eugene, OR) or (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (CMTMR) (5 μM, Molecular Probes) at room temperature for 10 min. Labeling was stopped with one half volume of fetal calf serum followed by two additional washes. Five million cells carrying each peptide were mixed and injected intravenously into immunized mice. At 14 h after injection, the spleens were harvested to prepare single cell suspensions. CFSE/CMTMR-positive cells were analyzed by a flow cytometer with exclusion of dead cells by ethidium monoazide bromide (Molecular Probes) staining. Cells from infected mice were treated with PBS containing 4% paraformaldehyde before analysis [14,23].

2.4. Virus titration

MDCK cells were cultured in Eagle's MEM supplemented with 10% FCS, L-glutamine, and antibiotics. Diluted suspensions of lung

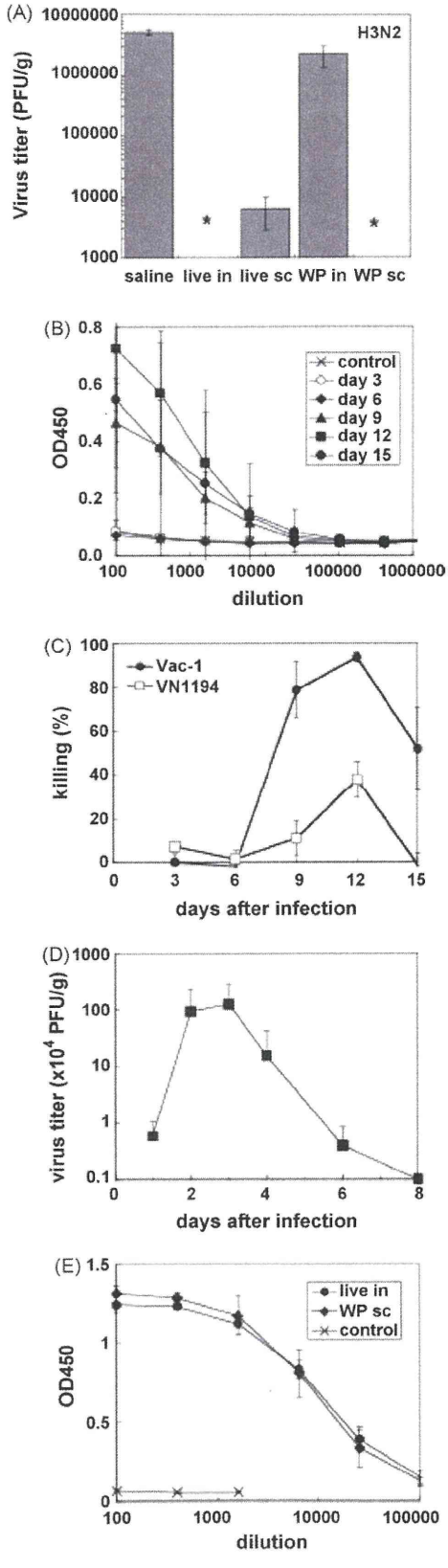

Fig. 1. Prevention of influenza virus replication by live vaccines and formalin-inactivated whole particle vaccines and immune responses induced by live Vac-1. (A) B6 mice were injected intranasally (in) or subcutaneously (sc) with 10^4 PFU of live influenza virus Aichi (live) or with whole particle vaccines (20 μg) (WP). Twenty-nine days after the vaccination, mice were intranasally challenged with 10^4 PFU of Aichi. Five days after infection, viral titers in the lungs were examined

tissue homogenates (10%, w/v) were added onto confluent MDCK cell monolayers in 6-well plates for plaque-forming assay and incubated at room temperature for 1 h for adsorption. The suspension was removed and cells were covered with MEM containing 1% agar and 5 μg/ml trypsin (Difco Laboratories, Detroit, MI). After incubation at 35 °C for 2 days, the plaque number was counted. The limit of detection in this assay was 0.5×10^3 PFU/g lung tissue [14]. In some experiments, diluted homogenates of lungs were inoculated onto MDCK cell monolayers in 96-well plates with quadruplicate culture. After incubation at 35 °C for 3 days, virus titer ($TCID_{50}$/ml) was determined according to cytopathic effects (CPE). The limit of detection in this assay was 1×10^3 $TCID_{50}$/g ($=1 \times 10^2$ $TCID_{50}$/ml). Statistical significance was calculated by the two-tailed Student's *t*-test.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed by a previously reported method with modification [24]. Briefly, 96-well plates were coated with 50 μl of purified Vac-1 (20 μg/ml) disrupted with 0.05 M Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl [25]. After washing three times with PBS containing 0.05% Tween20, PBS with 3% BSA was added for blocking. Serially diluted samples were incubated overnight in the coated plates. After washing five times, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) (1/2000 × 50 μl) was used. Horseradish peroxidase activity was assessed by 3,3′,5,5′-tetramethyl benzidine substrate (100 μl). The reaction was stopped by 1 M HCl (100 μl). Optical density was measured at 450 nm.

2.6. Virus neutralization assay

Sera from immunized mice were pretreated with receptor destroying enzyme (RDEII, Denka Seiken, Tokyo, Japan). Serially diluted sera were mixed with 50 $TCID_{50}$ of VN1194 for 1 h. The mixture was then added onto an MDCK cell monolayer in 96-well plates. After incubation for 1 h, medium was added and cells were cultured for 3 days. Neutralization titers were determined by calculating the concentration of serum showing 50% inhibition of CPE.

3. Results

3.1. Non-pathogenic avian influenza virus as an H5N1 live vaccine candidate

Firstly, in order to compare vaccination routes for live and whole particle vaccines of influenza virus, B6 mice were intranasally or subcutaneously inoculated with the live vaccine or the whole particle vaccine of H3N2 influenza virus and then challenged with an H3N2 influenza virus Aichi 4 weeks after the vaccination. Five days after the challenge infection, plaque-forming units in the lungs were counted (Fig. 1A). Intranasal administration of the live vaccine completely inhibited viral replication, whereas subcutaneous inoculation reduced viral replication significantly but less effectively than did intranasal inoculation. The virus was not detected in the lungs of mice subcutaneously immunized with the whole particle vaccine, whereas intranasal inoculation with the whole particle vaccine slightly reduced viral replication but not as effective as subcutaneous inoculation. Reduction of viral replication was concordant with the level of antibody responses (data not shown). In conclusion, the optimal routes of vaccination were intranasal inoculation for live vaccines and subcutaneous injection for whole particle vaccines.

There were two H5N1 strains, Vac-1 and A/duck/Hokkaido/Vac-3/2007 (H5N1) (Vac-3), in a virus library containing 144 subtypes

of non-pathogenic influenza viruses [21]. Both strains possessed efficient growth potential in embryonated eggs and their mean death times of chicken embryos were longer than 48 h, indicating usefulness of two strains for vaccine preparation. However, Vac-3 showed pathogenicity in chickens compared with Vac-1 when Vac-3 was inoculated intracerebrally into chickens [21]. Therefore, we selected Vac-1 as a live vaccine candidate in the present study. In previous studies, we showed that the whole particle vaccine of Vac-1 was effective for protection against H5N1 HPAIV infection in mice and cynomolgus macaques [13,14,26]. To examine whether Vac-1 worked as a live vaccine against H5N1 HPAIV in mammalians, live Vac-1 was intranasally inoculated into B6 mice. Levels of serum IgG antibodies specific for viral antigens of Vac-1 were increased 9 days after the inoculation (Fig. 1B). Furthermore, these antibodies recognized the H5N1 HPAIV strain VN1194. Sera from mice inoculated with Vac-1 showed neutralization activity against VN1194 at the lowest dilution (Table 2). Similarly, intranasal administration with Vac-1 elicited CTL responses specific for NP366-374 (i.e., ASNENMETM) derived from a sequence of Vac-1 and NP366-374 (i.e., ASNENMEAM) derived from a sequence of VN1194 (Fig. 1C). Nonetheless, CTL responses specific for ASNENMEAM were smaller than those for ASNENMETM on either day 9 or day 12 when the killing activity against the immunogen reached a maximal response.

We next examined replication capacity of Vac-1 in mice (Fig. 1D). After intranasal inoculation of Vac-1 (10^3 PFU), virus replication reached a maximal level (10^6 PFU/g) in the lungs on day 3, and the virus titer was clearly decreased on day 6. Finally, no virus was observed in the lungs on day 8. These results indicated that Vac-1 fully replicated in mice as did H3 subtype viruses that were adapted to humans [27,28], although Vac-1 was generated from viruses in fecal samples of migratory ducks. In addition, no mice died or showed significant symptoms including piloerection and malaise for 15 days after infection (data not shown). Intranasal inoculation with live Vac-1 (10^3 PFU) induced a comparable level of antigen-specific IgG as subcutaneous inoculation with inactivated whole particles of Vac-1 (100 μg) (Fig. 1E). In mice, Vac-1 replicated and provoked immune responses without significant adverse symptoms. Although the results seen in mice would not completely be true of humans, Vac-1 seemed to be a candidate of live vaccines against H5N1 HPAIV in humans without further attenuation processes.

3.2. Comparison of the protective effects of live vaccine and whole particle vaccine against H5N1 HPAIV

We compared the protective effects against the H5N1 HPAIV strain VN1194 of the live vaccine inoculated intranasally and the whole particle vaccine inoculated subcutaneously. Four weeks after the vaccination, the mice were intranasally challenged with VN1194 using a dose 100-time higher than the mean lethal dose ($100 \times LD_{50}$). Five days after the challenge with VN1194, viral replication in lungs was determined (Fig. 2A). The virus was not detected in lungs of mice vaccinated with 10^3 PFU of live Vac-1, while the virus was slightly detected but the replication was considerably inhibited in mice immunized with 100 μg of the whole particle vaccine compared with that in mice inoculated with saline (Exp. 1). Subsequently, we intranasally inoculated mice with serially diluted live Vac-1 (Exp. 2). The virus titer was considerably reduced in the lungs of mice vaccinated with 100 PFU of live Vac-1, but reduction in virus titer was not seen in the lungs of mice immunized with 10 PFU of live Vac-1. On the other hand, subcutaneous inoculation with 20 μg of the whole particle vaccine slightly but significantly reduced virus titers in the lungs compared with those in control mice inoculated with saline ($P = 0.025$). In conclusion, 100 PFU of the live vaccine would be as effective as 100 μg of the whole particle vaccine in mice. These findings suggest that even a small amount of the live vaccine is effective in preventing virus replication in lungs compared with the inactivated whole particle vaccine.

Furthermore, we examined body weight and survival of the vaccinated mice after challenge with VN1194. All control mice lost weight, whereas the vaccinated mice did not show loss of weight except for one mouse that was vaccinated with 10^3 PFU of live Vac-1 and died on day 9 in Exp. 1 (Fig. 2B). All of the control mice died on day 9 in Exp. 1 and by day 8 in Exp. 2. Similarly, inoculation with 20 μg of the whole particle vaccine did not prevent loss of body weight and death in Exp. 2 (Fig. 2B and C). One of the four mice vaccinated with 10 PFU of live Vac-1 in Exp. 2 died on day 8, but the other mice vaccinated with live Vac-1 survived until the end of the experiment (Fig. 2C). Regardless of one death seen in mice inoculated with 10^3 PFU of live Vac-1, which might be due to an experimental variation, inoculation of more than 100 PFU of live Vac-1 could prevent H5N1 HPAIV replication with no mortality in mice. Therefore, inoculation with 100 PFU of live Vac-1 would be as effective as inoculation with 100 μg of Vac-1 whole particle vaccine.

Table 2
Neutralization of an HPAIV with sera from mice vaccinated with live Vac-1.

Exp. 1		Exp. 2	
Mouse	50% neutralization titer (log 2)	Mouse	50% neutralization titer (log 2)
1	<2	1	<2
2	2.0	2	2.5
3	2.0	3	<2
4	<2	4	<2[a]

B6 mice were intranasally inoculated with Vac-1 (10^3 PFU) as described in the legend to Fig. 2. Sera were collected from four mice 15 days after inoculation in each experiment. Diluted sera were incubated with HPAIV VN1194. 50% neutralization titers of sera from individual mice are shown.

[a] One CPE-negative well was observed in quadruplicate cultures of the lowest dilution (4×).

4. Discussion

We propose the use of non-pathogenic avian influenza viruses as live vaccines to cope with outbreaks of infections with newly emerging influenza viruses, including HPAIV, because live vaccines can be prepared and distributed worldwide soon after an outbreak. In the present study, we investigated the effects of different administration routes and the efficacy of a live vaccine against H5N1 subtype HPAIV in mice. Intranasal inoculation with a small amount of the live vaccine elicited antibody and CTL responses and therefore inhibited H5N1 HPAIV infection more effectively than did subcutaneous administration. These findings suggest that the use of live virus vaccines is one prophylactic method for preventing infection with not only seasonal influenza virus but also HPAIV, though further fundamental investigation is necessary [29,30].

One advantage of live vaccines is that a low dose of the vaccines is sufficient for protection against viral infection. Indeed, a small amount (100 PFU) of live Vac-1 prevented replication of H5N1 HPAIV in mouse lungs. Although it is difficult to directly compare the amounts of virus contents between live vaccines and whole particle vaccines, 100 μg of whole particle vaccine seem to be approximately 10^{10-11} PFU of the virus [31]. Therefore, live vaccines could be easily prepared for many people in a short period, and the use of live vaccines would be one strategy for preventing pandemics caused by emerging influenza viruses, including HPAIVs. However, live vaccines still have the possibility of genetic reassortment between live vaccines (e.g., H5N1) and seasonal influenza viruses (e.g., H3N2 and H1N1) [32,33]. Given that, live vaccines should be administrated in the early phase of outbreaks of newly

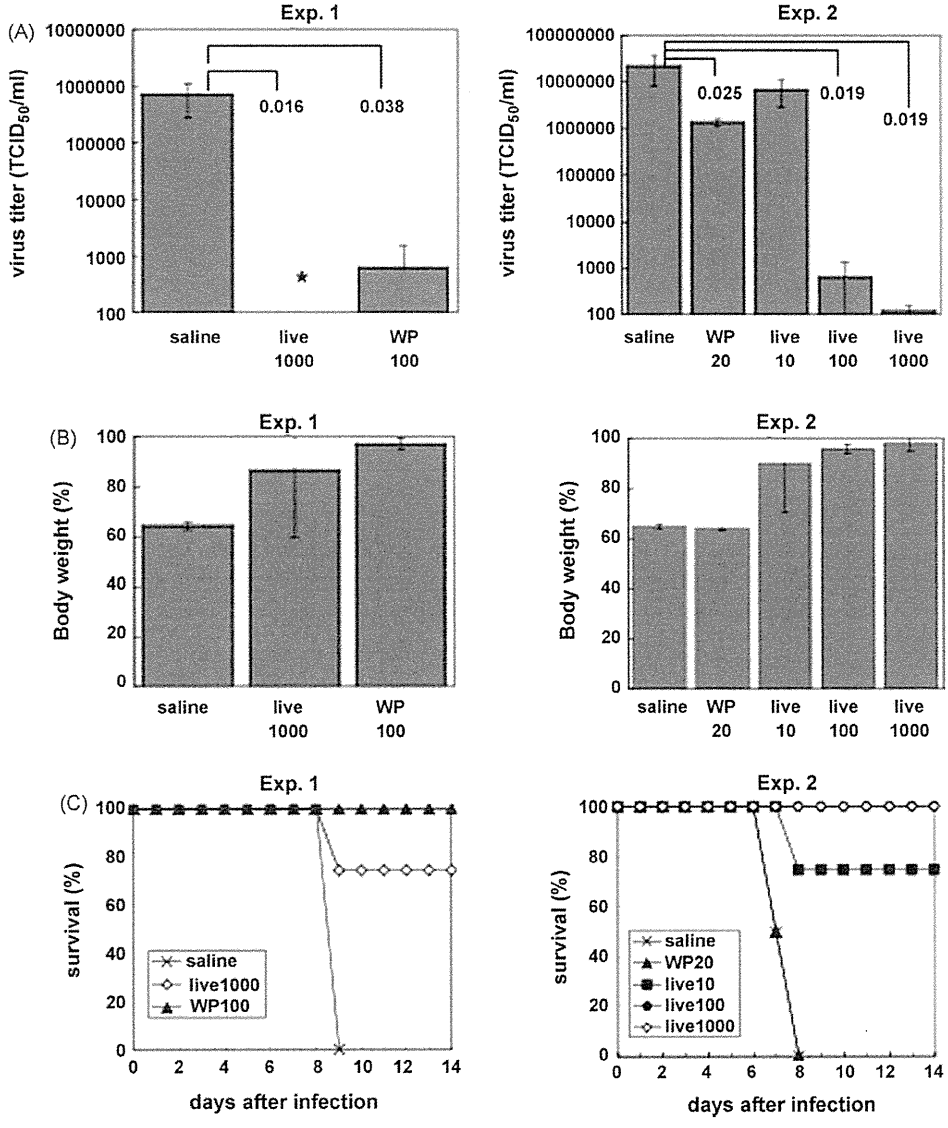

Fig. 2. Protection against H5N1 HPAIV in mice immunized with live vaccines or whole particle vaccines. B6 mice were intranasally inoculated with live Vac-1 (live, 10^3, 10^2, 10^1 PFU) or subcutaneously immunized with H5N1 whole particle vaccines (WP, 100 μg in Exp. 1 and 20 μg in Exp. 2) once. Saline was used for control mice. Four weeks after vaccination, all mice were challenged intranasally with 100 LD_{50} of VN1194 in 30 μl PBS. Results from two independent experiments are shown (left panels, Exp. 1; right panels, Exp. 2). Three or four mice were used in each group. (A) Lungs were collected 5 days after challenge with VN1194. Viral titers in the lungs are indicated as $TCID_{50}$/ml. Virus titers were under the detection limit in the lungs of mice immunized with 10^3 PFU of live Vac-1 (live1000) (*: $< 1 \times 10^2$ $TCID_{50}$/ml) and two of three mice immunized with whole particle vaccines in Exp. 1. In Exp. 2, virus titers were under the detection limit in the lungs of one and three of four mice immunized with 10^2 and 10^3 PFU of live Vac-1 (live100 and live1000), respectively. *P* values were calculated by Student's *t*-test with virus titers below 10^2 $TCID_{50}$/ml being calculated as 100 ($P = 0.016$ for live1000 vs. saline, $P = 0.038$ for WP100 vs. saline in Exp. 1; $P = 0.025$ for WP20 vs. saline, $P = 0.019$ for live100 vs. saline, $P = 0.019$ for live1000 vs. saline in Exp. 2). (B) Body weight was measured on day 9 in Exp. 1 and on day 7 in Exp. 2 after inoculation of VN1194. Body weight was calculated as the percentage of body weight on day 0. Averages and standard deviations of four mice in each group are shown. (C) Protective effects of vaccines are shown as the percent survival of vaccinated mice. In Exp. 2, mice inoculated with saline and whole particle vaccines showed identical survival curves and all mice vaccinated with 10^2 and 10^3 PFU of live Vac-1 survived for 14 days after inoculation with VN1194.

emerging strains to fill a deficit of immunity against them before inactivated vaccines will be prepared.

The present study on the inactivated whole particle vaccine suggested the limitation of an effective dose for induction of protective immunity against HPAIV. As shown in Fig. 1, 20 μg of whole particle vaccines were effective for protection of H3N2 virus infection because antigenicity between vaccine strain and challenge strain was identical. In addition, vaccination with 20 μg of H5N1 subtype whole particle vaccines reduced virus titers after challenge with VN1194, which of HA showed 92% similarity to HA of Vac-1 (Fig. 2). However, the reduction was not sufficient for improvement of the survival rate in mice vaccinated with 20 μg of H5N1 inactivated whole particle vaccines. Therefore, a dose of inactivated vaccines might be also important to induce sufficient crossreactive responses to protect lethal infection though antigenic similarity between vaccine strains and possible pandemic strains is crucial in vaccine development. In other words, a small amount of live vaccines might induce crossreactive responses to challenged strains more effectively than did inactivated vaccines.

Intranasal inoculation with a live vaccine was more effective for induction of immune responses and protection against influenza virus infection than was subcutaneous inoculation. On the other

hand, subcutaneous inoculation with an inactivated whole particle vaccine was more effective than intranasal inoculation. These findings indicated that viral replication in respiratory epithelial cells was required for live vaccines to work effectively, and type I interferon produced by the infected epithelial cells might be crucial for antigen presentation of mucosal antigen-presenting cells to activate T cells [34–36]. Subcutaneous inoculation of live virus might not induce amplification of the virus since subcutaneous dendritic cells might not possess trypsin-like proteases [37]. In addition, since antigen delivery by mucosal inoculation with pipettes was less effective than that by subcutaneous injection as observed in vaccination with inactivated whole virus particles, virus replication in respiratory epithelial cells would overcome the insufficient delivery of mucosal vaccines in quantity of antigen [38]. Furthermore, since it has been reported that proinflammatory cytokines alter permeability of airway tight junctions [39], live vaccines might induce production of proinflammatory cytokines and decrease barrier function of the airway mucosa [40], resulting in effective absorption and presentation of the antigen.

It is generally considered that live vaccines yield immunological memory cells [5]. As to the capacity of memory cell induction, we have not yet compared live vaccines with inactivated whole particle vaccines. There is a possibility that live vaccines generate signals through toll-like receptors (TLR) and retinoic acid-inducible gene (RIG)-I to induce memory cells effectively [6,41,42]. In the near future, we will examine the capacity of the live vaccine to yield memory cells against a virus challenge in mice.

In this study we could not exactly examine adverse effects of live Vac-1 because it was difficult to monitor fever and appetite loss in mice. In addition, it might be difficult to estimate the optimal amount of the live vaccine for human application based on the experiments with mice. Therefore, in macaques we are going to examine the adverse effects of live Vac-1 by monitoring body temperature and appetite [26], and further analyze correlation between dose and efficacy of live vaccines as a pre-clinical stage to use them for humans in an emergency.

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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.