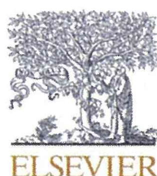
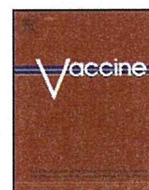
Vaccine 27 (2009) 7402–7408



Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



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

Yoshitaka Kashima[a,b,1], Mizuho Ikeda[a,1], Yasushi Itoh[a,*], Yoshihiro Sakoda[f], Tomoya Nagata[a,c], Taichiro Miyake[a,d], Kosuke Soda[f], Hiroichi Ozaki[h], Misako Nakayama[a], Hitomi Shibuya[f], Masatoshi Okamatsu[f], Hirohito Ishigaki[a], Hideaki Ishida[a], Toshihiro Sawai[a,e], Yoshihiro Kawaoka[i,j], Hiroshi Kida[f,g], Kazumasa Ogasawara[a]

[a] Department of Pathology, Shiga University of Medical Science, 485 Setatsukinowa, Otsu, Shiga 520-2192, Japan
[b] Department of Surgery, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[c] Department of Otorhinolaryngology-Head and Neck Surgery, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[d] Department of Ophthalmology, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[e] Department of Pediatrics, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[f] Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
[g] Center for Zoonosis Control, Hokkaido University, Sapporo 060-0818, Japan
[h] Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan
[i] Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[j] Department of Pathological Science, University of Wisconsin-Madison, Madison, WI 53706, USA

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form 4 August 2009
Accepted 24 August 2009
Available online 10 September 2009

Keywords:
Live vaccine
H5N1
Non-pathogenic influenza virus library

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.

© 2009 Elsevier Ltd. All rights reserved.

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.

* Corresponding author. Tel.: +81 77 548 2172; fax: +81 77 548 2423.
E-mail address: yasushii@belle.shiga-med.ac.jp (Y. Itoh).

[1] These authors contributed equally to this work.

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.08.089

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 (ASNENMET̲M for Vac-1, ASNENMEA̲M 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

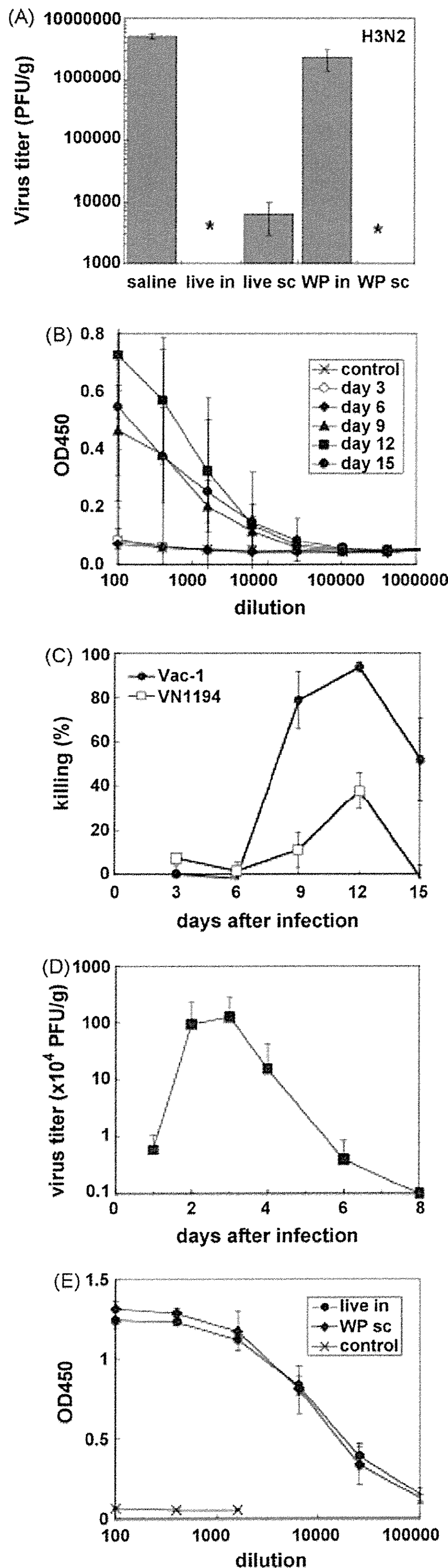


Fig. 1. Prevention of influenza virus replication by live vaccines and formalin-inactivated whole particle vaccines and immune responses induced by live Vac-1. (A) B6 mice were injected intranasally (in) or subcutaneously (sc) with 10^4 PFU of live influenza virus Aichi (live) or with whole particle 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 (=1×10^2 $TCID_{50}$/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 (log2)	Mouse	50% neutralization titer (log2)
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

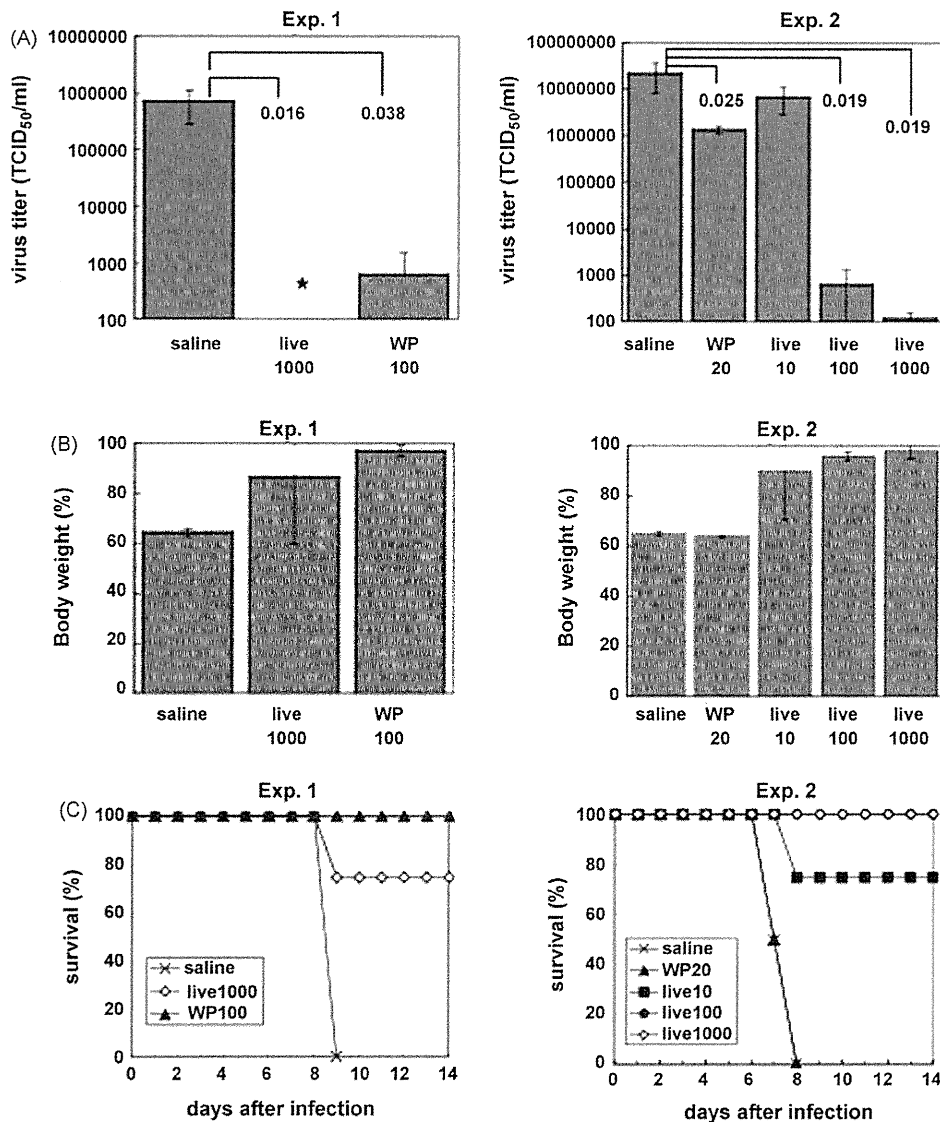


Fig. 2. Protection against H5N1 HPAIV in mice immunized with live vaccines or whole particle vaccines. B6 mice were intranasally inoculated with live Vac-1 (live, 10^3, 10^2, 10^1 PFU) or subcutaneously immunized with H5N1 whole particle vaccines (WP, 100 μg in Exp. 1 and 20 μg in Exp. 2) once. Saline 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.

Acknowledgements

This study was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT Japan. We thank Drs. Kunio Ishibashi, Hideaki Tsuchiya and Ryuzo Torii for maintenance of the biosafety level 3 facility.

References

[1] Gambotto A, Barratt-Boyes SM, de Jong MD, Neumann G, Kawaoka Y. Human infection with highly pathogenic H5N1 influenza virus. Lancet 2008;371(9622):1464–75.
[2] Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, et al. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet 2008;371(9622):1427–34.
[3] Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, et al. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 2004;363(9415):1099–103.
[4] Horimoto T, Kawaoka Y. Strategies for developing vaccines against H5N1 influenza A viruses. Trends Mol Med 2006;12(11):506–14.
[5] Johnson Jr PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. Comparison of long-term systemic and secretory antibody responses in children given live, attenuated, or inactivated influenza A vaccine. J Med Virol 1985;17(4):325–35.
[6] Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15(1):34–41.
[7] Rauh LW, Schmidt R. Measles Immunization with Killed Virus Vaccine. Serum antibody titers and experience with exposure to measles epidemic. Am J Dis Child 1965;109:232–7.
[8] Fulginiti VA, Eller JJ, Downie AW, Kempe CH. Altered reactivity to measles virus. Atypical measles in children previously immunized with inactivated measles virus vaccines. JAMA 1967;202(12):1075–80.
[9] Wareing MD, Tannock GA. Live attenuated vaccines against influenza; an historical review. Vaccine 2001;19(25–26):3320–30.
[10] Clements ML, Makhene MK, Karron RA, Murphy BR, Steinhoff MC, Subbarao K, et al. Effective immunization with live attenuated influenza A virus can be achieved in early infancy. Pediatric Care Center. J Infect Dis 1996;173(1):44–51.
[11] Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2004;53(RR-6):1–40.
[12] Jefferson TO, Rivetti D, Di Pietrantonj C, Rivetti A, Demicheli V. Vaccines for preventing influenza in healthy adults. Cochrane Database Syst Rev 2007;(2):CD001269.
[13] Kida H, Sakoda Y. Library of influenza virus strains for vaccine and diagnostic use against highly pathogenic avian influenza and human pandemics. Dev Biol (Basel) 2006;124:69–72.
[14] Sawai T, Itoh Y, Ozaki H, Isoda N, Okamoto K, Kashima Y, et al. Induction of cytotoxic T-lymphocyte and antibody responses against highly pathogenic avian influenza virus infection in mice by inoculation of apathogenic H5N1 influenza virus particles inactivated with formalin. Immunology 2008;124:155–65.
[15] Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin A, Stephenson I, et al. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 2006;24(44–46):6588–93.
[16] Clements ML, Betts RF, Tierney EL, Murphy BR. Resistance of adults to challenge with influenza A wild-type virus after receiving live or inactivated virus vaccine. J Clin Microbiol 1986;23(1):73–6.
[17] Powers DC, Sears SD, Murphy BR, Thumar B, Clements ML. Systemic and local antibody responses in elderly subjects given live or inactivated influenza A virus vaccines. J Clin Microbiol 1989;27(12):2666–71.
[18] Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004;59(1):1–15.
[19] Atmar RL, Keitel WA, Cate TR, Munoz FM, Ruben F, Couch RB. A dose-response evaluation of inactivated influenza vaccine given intranasally and intramuscularly to healthy young adults. Vaccine 2007;25(29):5367–73.
[20] Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. Vaccine 2002;20(25–26):3123–9.
[21] Soda K, Sakoda Y, Isoda N, Kajihara M, Haraguchi Y, Shibuya H, et al. Development of vaccine strains of H5 and H7 influenza viruses. Jpn J Vet Res 2008;55(2–3):93–8.
[22] Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004;350(12):1179–88.
[23] Nagata T, Toyota T, Ishigaki H, Ichihashi T, Kajino K, Kashima Y, et al. Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses. Vaccine 2007;25(26):4914–21.
[24] Kida H, Brown LE, Webster RG. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 1982;122(1):38–47.
[25] Ozaki H, Sugiura T, Sugita S, Imagawa H, Kida H. Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. Vet Microbiol 2001;82(2):111–9.
[26] Itoh Y, Ozaki H, Tsuchiya H, Okamoto K, Torii R, Sakoda Y, et al. A vaccine prepared from a non-pathogenic H5N1 avian influenza virus strain confers protective immunity against highly pathogenic avian influenza virus infection in cynomolgus macaques. Vaccine 2008;26(4):562–72.
[27] Lindstrom SE, Cox NJ, Klimov A. Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957-1972: evidence for genetic divergence and multiple reassortment events. Virology 2004;328(1):101–19.
[28] Ilyushina NA, Rudneva IA, Shilov AA, Klenk HD, Kaverin NV. Postreassortment changes in a model system: HA-NA adjustment in an H3N2 avian-human reassortant influenza virus. Arch Virol 2005;150(7):1327–38.
[29] Suguitan Jr AL, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, et al. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006;3(9):e360.
[30] Droebner K, Haasbach E, Fuchs C, Weinzierl AO, Stevanovic S, Buttner M, et al. Antibodies and $CD4^+$ T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain. Vaccine 2008;26(52):6965–74.
[31] Wright PF, Neumann G, Kawaoka Y. Orthomyxoviruses. In: Fields Virology. 5th ed. Philadelphia: Lippincott Williams & Wilkins, a Wolters Kluwer Business; 2007, 1727 pp [Chapter 48].
[32] Scholtissek C. Potential hazards associated with influenza virus vaccines. Dev Biol Stand 1995;84:55–8.
[33] Batten CA, Maan S, Shaw AE, Maan NS, Mertens PP. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. Virus Res 2008;137(1):56–63.
[34] Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, et al. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 2005;280(7):5571–80.
[35] Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, et al. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. Immunity 2007;27(2):240–52.
[36] Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 2007;179(7):4711–20.

[37] Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, Fukutomi A, et al. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem 1992;267(19):13573–9.
[38] Scheibe M, Bethge C, Witt M, Hummel T. Intranasal administration of drugs. Arch Otolaryngol Head Neck Surg 2008;134(6):643–6.
[39] Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG. Regulation of airway tight junctions by proinflammatory cytokines. Mol Biol Cell 2002;13(9):3218–34.
[40] Youakim A, Ahdieh M. Interferon-gamma decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. Am J Physiol 1999;276(5 Pt 1):G1279–88.
[41] Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441(7089):101–5.
[42] Han JH, Akira S, Calame K, Beutler B, Selsing E, Imanishi-Kari T. Class switch recombination and somatic hypermutation in early mouse B cells are mediated by B cell and Toll-like receptors. Immunity 2007;27(1):64–75.

JOURNAL OF VIROLOGY, Feb. 2009, p. 1572–1578 Vol. 83, No. 4
0022-538X/09/$08.00+0 doi:10.1128/JVI.01879-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

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

Rashid Manzoor,[1] Yoshihiro Sakoda,[1] Naoki Nomura,[1] Yoshimi Tsuda,[1] Hiroichi Ozaki,[2] Masatoshi Okamatsu,[1] and Hiroshi Kida[1,3]*

Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan[1]; Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan[2]; and Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan[3]

Received 7 September 2008/Accepted 24 November 2008

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

* Corresponding author. Mailing address: Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, hokkaido university, Kita 18 Nishi 9, Kita-Ku, Sapporo 060-0818, Japan. Phone: (81) 11-706-5207. Fax: (81) 11-706-5273. E-mail: kida@vetmed.hokudai.ac.jp.

▽ Published ahead of print on 3 December 2008.

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.

ACKNOWLEDGMENTS

We thank Erich Hoffmann, St. Jude Children's Research Hospital, for kindly providing pHW2000. We also thank Richard Webby, St. Jude Children's Research Hospital, for kindly providing pHW72-

EGFP. We also thank T. Umemura, Graduate School of Veterinary Medicine, Hokkaido University, for excellent technical and editorial assistance.

The present work was supported by the Program of Founding Research Centers for Emerging and Re-Emerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

1. **Almond, J. W.** 1977. A single gene determines the host range of influenza virus. Nature **270**:617–618.
2. **Bai, G. R., Y. Sakoda, A. S. Mweene, N. Kishida, T. Yamada, H. Minakawa, and H. Kida.** 2005. Evaluation of the Espline influenza A&B-N kit for the diagnosis of avian and swine influenza. Microbiol. Immunol. **49**:1063–1067.
3. **Beare, A. S., and R. G. Webster.** 1991. Replication of avian influenza viruses in humans. Arch. Virol. **119**:37–42.
4. **Castrucci, M. R., I. Donatelli, L. Sidoli, G. Barigazzi, Y. Kawaoka, and R. G. Webster.** 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology **193**:503–506.
5. **Choi, Y. K., T. D. Nguyen, H. Ozaki, R. J. Webby, P. Puthavathana, C. Buranathal, A. Chaisingh, P. Auewarakul, N. T. Hanh, S. K. Ma, P. Y. Hui, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J. Virol. **79**:10821–10825.
6. **Claas, E. C., Y. Kawaoka, J. C. de Jong, N. Masurel, and R. G. Webster.** 1994. Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology **204**:453–457.
7. **Clements, M. L., E. K. Subbarao, L. F. Fries, R. A. Karron, W. T. London, and B. R. Murphy.** 1992. Use of single-gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of wild-type human influenza A virus for squirrel monkeys and adult human volunteers. J. Clin. Microbiol. **30**:655–662.
8. **Deng, T., O. G. Engelhardt, B. Thomas, A. V. Akoulitchev, G. G. Brownlee, and E. Fodor.** 2006. Role of Ran binding protein 5 in nuclear import and assembly of the influenza virus RNA polymerase complex. J. Virol. **80**: 11911–11919.
9. **Gabriel, G., B. Dauber, T. Wolff, O. Planz, H. D. Klenk, and J. Stech.** 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. USA **102**:18590–18595.
10. **Gabriel, G., A. Herwig, and H. D. Klenk.** 2008. Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathog. **4**:e11.
11. **Guan, Y., K. F. Shortridge, S. Krauss, P. H. Li, Y. Kawaoka, and R. G. Webster.** 1996. Emergence of avian H1N1 influenza viruses in pigs in China. J. Virol. **70**:8041–8046.
12. **Hatta, M., P. Halfmann, K. Wells, and Y. Kawaoka.** 2002. Human influenza a viral genes responsible for the restriction of its replication in duck intestine. Virology **295**:250–255.
13. **Hinshaw, V. S., R. G. Webster, C. W. Naeve, and B. R. Murphy.** 1983. Altered tissue tropism of human-avian reassortant influenza viruses. Virology **128**:260–263.
14. **Hoffmann, E., S. Krauss, D. Perez, R. Webby, and R. G. Webster.** 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine **20**:3165–3170.
15. **Hoffmann, E., G. Neumann, G. Hobom, R. G. Webster, and Y. Kawaoka.** 2000. "Ambisense" approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. Virology **267**:310–317.
16. **Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA **97**:6108–6113.
17. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146**:2275–2289.
18. **Honda, A., K. Mizumoto, and A. Ishihama.** 1999. Two separate sequences of PB2 subunit constitute the RNA cap-binding site of influenza virus RNA polymerase. Genes Cells **4**:475–485.
19. **Isoda, N., Y. Sakoda, N. Kishida, G. R. Bai, K. Matsuda, T. Umemura, and H. Kida.** 2006. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch. Virol. **151**:1267–1279.
20. **Ito, T., J. N. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster, and Y. Kawaoka.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. **72**:7367–7373.
21. **Jorba, N., S. Juarez, E. Torreira, P. Gastaminza, N. Zamarreno, J. P. Albar, and J. Ortin.** 2008. Analysis of the interaction of influenza virus polymerase complex with human cell factors. Proteomics **8**:2077–2088.
22. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. **63**:4603–4608.
23. **Keawcharoen, J., K. Oraveerakul, T. Kuiken, R. A. Fouchier, A. Amonsin, S. Payungporn, S. Noppornpanth, S. Wattanodorn, A. Theamboonlers, R. Tantilertcharoen, R. Pattanarangsan, N. Arya, P. Ratanakorn, D. M. Osterhaus, and Y. Poovorawan.** 2004. Avian influenza H5N1 in tigers and leopards. Emerg. Infect. Dis. **10**:2189–2191.
24. **Kida, H., L. E. Brown, and R. G. Webster.** 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology **122**:38–47.
25. **Kida, H., T. Ito, J. Yasuda, Y. Shimizu, C. Itakura, K. F. Shortridge, Y. Kawaoka, and R. G. Webster.** 1994. Potential for transmission of avian influenza viruses to pigs. J. Gen. Virol. **75**:2183–2188.
26. **Kida, H., K. F. Shortridge, and R. G. Webster.** 1988. Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. Virology **162**: 160–166.
27. **Labadie, K., E. Dos Santos Afonso, M. A. Rameix-Welti, S. van der Werf, and N. Naffakh.** 2007. Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology **362**:271–282.
28. **Landolt, G. A., A. I. Karasin, M. M. Schutten, and C. W. Olsen.** 2006. Restricted infectivity of a human-lineage H3N2 influenza A virus in pigs is hemagglutinin and neuraminidase gene dependent. J. Clin. Microbiol. **44**: 297–301.
29. **Li, Z., H. Chen, P. Jiao, G. Deng, G. Tian, Y. Li, E. Hoffmann, R. G. Webster, Y. Matsuoka, and K. Yu.** 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J. Virol. **79**:12058–12064.
30. **Manzoor, R., Y. Sakoda, S. Sakabe, T. Mochizuki, Y. Namba, Y. Tsuda, and H. Kida.** 2008. Development of a pen-site test kit for the rapid diagnosis of H7 highly pathogenic avian influenza. J. Vet. Med. Sci. **70**:557–562.
31. **Mase, M., N. Tanimura, T. Imada, M. Okamatsu, K. Tsukamoto, and S. Yamaguchi.** 2006. Recent H5N1 avian influenza A virus increases rapidly in virulence to mice after a single passage in mice. J. Gen. Virol. **87**:3655–3659.
32. **Mase, M., K. Tsukamoto, T. Imada, K. Imai, N. Tanimura, K. Nakamura, Y. Yamamoto, T. Hitomi, T. Kira, T. Nakai, M. Kiso, T. Horimoto, Y. Kawaoka, and S. Yamaguchi.** 2005. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. Virology **332**: 167–176.
33. **Momose, F., C. F. Basler, R. E. O'Neill, A. Iwamatsu, P. Palese, and K. Nagata.** 2001. Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. J. Virol. **75**:1899–1908.
34. **Momose, F., H. Handa, and K. Nagata.** 1996. Identification of host factors that regulate the influenza virus RNA polymerase activity. Biochimie **78**: 1103–1108.
35. **Murphy, B. R., D. L. Sly, E. L. Tierney, N. T. Hosier, J. G. Massicot, W. T. London, R. M. Chanock, R. G. Webster, and V. S. Hinshaw.** 1982. Reassortant virus derived from avian and human influenza A viruses is attenuated and immunogenic in monkeys. Science **218**:1330–1332.
36. **Myers, K. P., C. W. Olsen, and G. C. Gray.** 2007. Cases of swine influenza in humans: a review of the literature. Clin. Infect. Dis. **44**:1084–1088.
37. **Naito, T., F. Momose, A. Kawaguchi, and K. Nagata.** 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J. Virol. **81**:1339–1349.
38. **Ohtsu, Y., Y. Honda, Y. Sakata, H. Kato, and T. Toyoda.** 2002. Fine mapping of the subunit binding sites of influenza virus RNA polymerase. Microbiol. Immunol. **46**:167–175.
39. **Peiris, J. S., Y. Guan, D. Markwell, P. Ghose, R. G. Webster, and K. F. Shortridge.** 2001. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J. Virol. **75**:9679–9686.
40. **Poole, E., D. Elton, L. Medcalf, and P. Digard.** 2004. Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology **321**:120–133.
41. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493.
42. **Saito, T., H. Suzuki, K. Maeda, K. Inai, N. Takemae, Y. Uchida, and H. Tsunemitsu.** 2008. Molecular characterization of an H1N2 swine influenza virus isolated in Miyazaki, Japan, in 2006. J. Vet. Med. Sci. **70**:423–427.
43. **Salomon, R., J. Franks, E. A. Govorkova, N. A. Ilyushina, H. L. Yen, D. J. Hulse-Post, J. Humberd, M. Trichet, J. E. Rehg, R. J. Webby, R. G. Webster, and E. Hoffmann.** 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J. Exp. Med. **203**:689–697.
44. **Scholtissek, C., H. Burger, O. Kistner, and K. F. Shortridge.** 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology **147**:287–294.
45. **Scholtissek, C., W. Rohde, V. Von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87**:13–20.
46. **Sever, J. L.** 1962. Application of a microtechnique to viral serological investigations. J. Immunol. **88**:320–329.
47. **Shapiro, G. I., and R. M. Krug.** 1988. Influenza virus RNA replication in

vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J. Virol. **62:**2285–2290.

48. **Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster.** 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology **252:**331–342.
49. **Snyder, M. H., A. J. Buckler-White, W. T. London, E. L. Tierney, and B. R. Murphy.** 1987. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. J. Virol. **61:**2857–2863.
50. **Songserm, T., A. Amonsin, R. Jam-on, N. Sae-Heng, N. Pariyothorn, S. Payungporn, A. Theamboonlers, S. Chutinimitkul, R. Thanawongnuwech, and Y. Poovorawan.** 2006. Fatal avian influenza A H5N1 in a dog. Emerg. Infect. Dis. **12:**1744–1747.
51. **Songserm, T., A. Amonsin, R. Jam-on, N. Sae-Heng, N. Meemak, N. Pariyothorn, S. Payungporn, A. Theamboonlers, and Y. Poovorawan.** 2006. Avian influenza H5N1 in naturally infected domestic cat. Emerg. Infect. Dis. **12:** 681–683.
52. **Subbarao, E. K., W. London, and B. R. Murphy.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. **67:**1761–1764.
53. **Tian, S. F., A. J. Buckler-White, W. T. London, L. J. Reck, R. M. Chanock, and B. R. Murphy.** 1985. Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/Mallard/NY/78 virus and its reassortants in squirrel monkey respiratory tract. J. Virol. **53:**771–775.
54. **Van Reeth, K.** 2007. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet. Res. **38:**243–260.
55. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.
56. **Webster, R. G., and W. G. Laver.** 1972. The origin of pandemic influenza. Bull. W. H. O. **47:**449–452.
57. **Yao, Y., L. J. Mingay, J. W. McCauley, and W. S. Barclay.** 2001. Sequences in influenza A virus PB2 protein that determine productive infection for an avian influenza virus in mouse and human cell lines. J. Virol. **75:**5410–5415.
58. **Yasuda, J., K. F. Shortridge, Y. Shimizu, and H. Kida.** 1991. Molecular evidence for a role of domestic ducks in the introduction of avian H3 influenza viruses to pigs in southern China, where the A/Hong Kong/68 (H3N2) strain emerged. J. Gen. Virol. **72:**2007–2010.

J Med Primatol doi:10.1111/j.1600-0684.2009.00395.x

ORIGINAL ARTICLE

Amelioration of pneumonia with *Streptococcus pneumoniae* infection by inoculation with a vaccine against highly pathogenic avian influenza virus in a non-human primate mixed infection model

Taichiro Miyake[1,2], Kosuke Soda[3], Yasushi Itoh[1], Yoshihiro Sakoda[3], Hirohito Ishigaki[1], Tomoya Nagata[1,4], Hideaki Ishida[1], Misako Nakayama[1], Hiroichi Ozaki[5], Hideaki Tsuchiya[6], Ryuzo Torii[6], Hiroshi Kida[3,7] & Kazumasa Ogasawara[1]

1 Department of Pathology, Shiga University of Medical Science, Otsu, Shiga, Japan
2 Department of Ophthalmology, Shiga University of Medical Science, Otsu, Shiga, Japan
3 Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
4 Department of Otorhinolaryngology, Head and Neck Surgery, Shiga University of Medical Science, Otsu, Shiga, Japan
5 Faculty of Agriculture, Tottori University, Tottori, Japan
6 Research Center for Animal Life Science, Shiga University of Medical Science, Otsu, Shiga, Japan
7 Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

Keywords
cynomolgus macaque – H7N7 – superinfection

Correspondence
Yasushi Itoh, Department of Pathology, Shiga University of Medical Science, 485 Setatsukinowa, Otsu, Shiga 520-2192, Japan.
Tel.: +81 77 548 2172;
fax: +81 77 548 2423;
e-mail: yasushii@belle.shiga-med.ac.jp

Accepted October 9, 2009.

Abstract

Background Highly pathogenic avian influenza virus (HPAIV) infection has a high mortality rate in humans. Secondary bacterial pneumonia with HPAIV infection has not been reported in human patients, whereas seasonal influenza viruses sometimes enhance bacterial pneumonia, resulting in substantial morbidity and mortality. Therefore, if HPAIV infection were accompanied by bacterial infection, an increase in mortality would be expected. We examined whether a vaccine against HPAIV prevents severe morbidity caused by mixed infection with HPAIV and bacteria.

Methods H7N7 subtype of HPAIV and *Streptococcus pneumoniae* were inoculated into cynomolgus macaques with or without vaccination of inactivated whole virus particles.

Results Vaccination against H7N7 HPAIV decreased morbidity caused by HPAIV and pneumonia caused by *S. pneumoniae*. Bacterial replication in lungs was decreased by vaccination against HPAIV, although the reduction in bacterial colonies was not significant.

Conclusions Vaccination against HPAIV reduces pneumonia caused by bacterial superinfection and may improve prognosis of HPAIV-infected patients.

Introduction

Influenza virus and *Streptococcus pneumoniae* are the two pathogens that cause the majority of respiratory infections in humans. Influenza virus infection results in pneumonitis in which lymphocytes infiltrate into lung interstices, whereas bacteria induce pneumonia in which neutrophils infiltrate into lung alveoli. Although influenza virus infection alone results in pneumonitis, secondary bacterial pneumonia may be a major cause of substantial morbidity and mortality during typical influenza pandemics, including the major pandemic of 1918–1919 [2, 21]. Although bacterial pneumonia has not been reported in patients infected with H5N1 or H7N7 highly pathogenic avian influenza virus (HPAIV) [5, 26], the possibility of mortality being increased by

© 2009 John Wiley & Sons A/S

mixed infection with HPAIV and bacteria has not been ruled out. Thus, it seems reasonable to assume that the prevention of influenza virus replication will improve bacterial pneumonia in case of mixed infection, resulting in decreased morbidity and mortality; however, this assumption has not been examined, at least in macaque models. In this study, as HPAIV A/chicken/Netherlands/2586/2003 (H7N7) (NL2586) was more pathogenic, i.e. duration of high fever and loss of appetite, in cynomolgus macaques than A/Vietnam/1194/2004 (H5N1) [8] (Itoh, Y., *et al.*, unpublished data), we examined the efficacy of inoculation with a vaccine against H7N7 HPAIV for ameliorating bacterial pneumonia in a non-human primate model with mixed infection.

In recent years, results of epidemiological and animal model studies have demonstrated that initial respiratory tract infection alters immunity to a second unrelated pathogen, even long after the resolution of the first pathogen and in the absence of cross-reactive immunity [15, 29]. Several factors have been proposed to be involved in this altered immunity, including suppression of neutrophil function [1, 4, 13] and induction of inhibitory interleukin (IL)-10 [27, 28]. In another study, it was shown that interferon-γ produced by T cells in the lung after viral infection inhibits alveolar macrophage-mediated microbial clearance and, consequently, leads to enhanced susceptibility to secondary bacterial infection [24].

We previously demonstrated that whole virus particle vaccines inactivated by formalin induced protective immune responses, including antibody and cytotoxic T lymphocyte responses, against HPAIV in mice [18]. We selected vaccine strains of non-pathogenic H5N1 and H7N7 viruses, A/duck/Hokkaido/Vac-1/2004 (Vac-1) and A/duck/Hokkaido/Vac-2/2004 (Vac-2) respectively, from a virus library containing 144 different combinations of 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of non-pathogenic viruses [10] and produced whole virus particle vaccines as described previously [17, 20]. Then, we examined immune responses induced by subcutaneous inoculation with the whole virus particle vaccine and protective efficiency against H5N1 and H7N7 HPAIVs in non-human primate models [8] (Itoh, Y., *et al.*, unpublished data). The whole virus particle vaccines ameliorated morbidity including high fever and appetite loss in cynomolgus macaques. HPAIV was detected in samples from unvaccinated macaques for 5–7 days after challenge with HPAIV, whereas HPAIV was recovered from samples of the vaccinated macaques only for 1–2 days after challenge.

In the present study, vaccination with Vac-2 against H7N7 HPAIV decreased histopathological pneumonia caused by *S. pneumoniae* in macaques simultaneously infected with H7N7 HPAIV and *S. pneumoniae*. Bacterial growth in the lung was diminished by vaccination with Vac-2, although the reduction was not significant. Therefore, development of vaccines against influenza virus might be crucial for preventing high rates of morbidity and mortality in pandemics.

Materials and methods

Viruses

Non-pathogenic influenza virus A/duck/Hokkaido/Vac-2/2004 (H7N7) (Vac-2, National Center for Biotechnology Information taxonomy database ID: 390987) is a genetic reassortant generated by co-infection with A/duck/Mongolia/736/2002 (H7N7) and A/duck/Hokkaido/49/1998 (H9N2) in chicken embryos. PB2, PB1, PA, HA, NA and NS genes of Vac-2 were derived from the H7N7 virus, and NP and M genes were derived from the H9N2 virus [17]. HPAIV A/chicken/Netherlands/2586/2003 (H7N7) (NL2586, National Center for Biotechnology Information taxonomy database ID: 533037) was provided by Dr Ilaria Capua [L'Office International des Épizooties (OIE), Food and Agriculture Organization of the United Nations (FAO), and National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Italy]. The percent sequence similarity between Vac-2 and NL2586 was 97% in HA and 98% in NA at the amino acid level. The viruses were propagated in the allantoic cavities of 10-day-old embryonated hen's eggs at 35°C for 48 hours. For an inactivated vaccine, the infectious allantoic fluids were concentrated and purified by high-speed centrifugation through a 10–50% sucrose density gradient (112,500 *g* for 90 minutes) and then treated with 0.1% formalin at 4°C for 1 week. The purified viruses were then suspended in PBS. Inactivation of the viruses was confirmed by the absence of detectable hemagglutination following inoculation of the materials into 10-day-old embryonated hen's eggs after one passage [20]. The amount of whole particle vaccines was indicated as that of entire protein including HA and the other viral proteins. The vaccine used in this study contained 42,667 HA units of HA antigen in 1 mg vaccine.

For virus titration, serial dilutions of swabs and whole blood samples were inoculated onto confluent Madin–Darby canine kidney (MDCK) cells. The MDCK cells were then cultured in MEM including 0.1% BSA. Cytopathic effects were examined with a microscope 72 hours later, and mean tissue infectious dose ($TCID_{50}$)/ml was calculated [8].

© 2009 John Wiley & Sons A/S

Bacteria

Streptococcus pneumoniae was obtained from Dr Takayuki Ezaki (Gifu University, GTC261, NCTC7465). The bacteria were stored at −80°C in 10% (w/v) skimmed milk. For preparation of the animal inoculation, 10-μl aliquots of bacteria were removed from frozen stock and inoculated into 40 ml of brain–heart infusion broth (Becton, Dickinson and Company, Sparks, MD, USA) and then incubated at 37°C for 17 hours. Bacteria were collected by centrifugation at 700 *g* for 30 minutes and resuspended in 10 ml saline. A 1-ml aliquot of the saline suspension was used for inoculation into each animal, and the remainder was used for quantification and colony counting by serial dilution [16, 19].

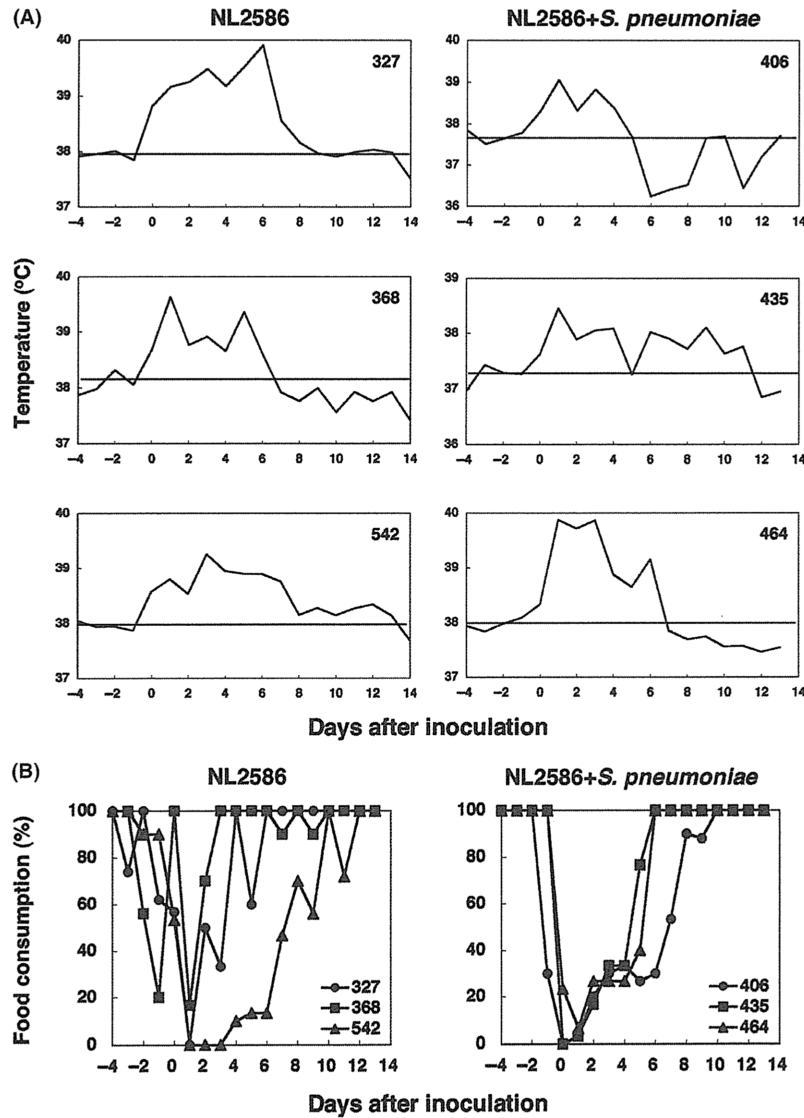


Fig. 1 Body temperatures and food consumption of macaques after inoculation of H7N7 HPAIV (NL2586) with or without *Streptococcus pneumoniae*. NL2586 (4×10^7 $TCID_{50}$) was inoculated onto conjunctivas and into nasal cavities and tracheas with *S. pneumoniae* (1.2×10^9 CFU) into tracheas of cynomolgus macaques (Nos 406, 435 and 464). The other macaques (Nos 327, 368 and 542) were inoculated with NL2586 (4×10^7 $TCID_{50}$) onto conjunctivas and into nasal cavities and tracheas. (A) Body temperatures were monitored by telemetry transmitters implanted in the peritoneal cavities. Average temperatures of the highest and lowest temperatures on one day are time-dependently shown. Lines drawn horizontally indicate the average temperature levels at pre-infection. (B) Appetite was reflected by the amount of food consumed, which was calculated from the numbers of residual and fed pellets.

© 2009 John Wiley & Sons A/S

Swab samples were collected as described in the following. To count the colony number, swab samples were serially diluted. Diluted fluid was cultured on blood agar plates with 5% sheep blood (Eiken Chemical Co. Ltd, Tokyo, Japan) at 37°C for 21 hours. *Streptococcus pneumoniae* colonies were identified by their hemolytic activity and counted. *Streptococcus pneumoniae* colonies characteristically produce a zone of alpha hemolysis (incomplete, green) on blood agar [16].

Animals

Five- to seven-year-old cynomolgus macaques (*Macaca fascicularis*) from Vietnam were used with permission of the Shiga University of Medical Science Animal Experiment Committee and Biosafety Committee and in accordance with Guidelines for the Husbandry and Management of Laboratory Animals of Research Center for Animal Life Science at Shiga University of Medical Science. In the text and figures, individual macaques are distinguished by identification numbers. The absence of H7N7-specific antibody in the sera was confirmed before experiments using antigen-specific enzyme-linked immunosorbent assays (ELISA). Under anesthesia 2 weeks before virus inoculation, telemetry probes (TA10CTA-D70; Data Sciences International, St Paul, MN, USA) to monitor body temperature were implanted in the macaques' peritoneal cavities. The macaques used in this study did not carry B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp. or *Entamoeba histolytica* [8].

The vaccines (1 mg/dose) were inoculated subcutaneously with syringes with alum (500 μl; Superfos Biosector, Vaerloese, Denmark) twice with a 2-week interval between injections. Saline (500 μl) was injected into control animals. Five weeks after the second vaccination, NL2586 (4×10^7 $TCID_{50}$) was inoculated on conjunctivas (1×10^6 $TCID_{50}$/50 μl for each eye) and

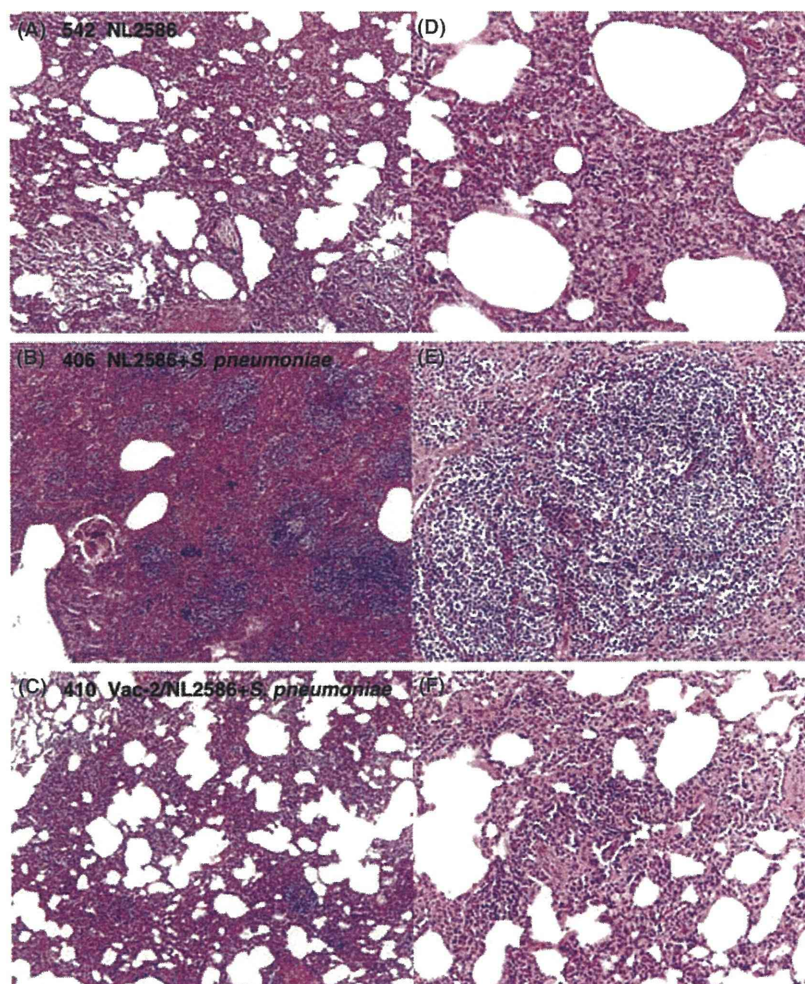


Fig. 2 Lung histology of cynomolgus macaques inoculated with NL2586 with or without *Streptococcus pneumoniae*. Cynomolgus macaques were subcutaneously vaccinated with inactivated whole particles of Vac-2 twice. Seven weeks after the first vaccination (5 weeks after the second vaccination), the macaques were inoculated with pathogens. After autopsy (i.e. 14 days after the challenge), the lungs were fixed in 10% formalin. Hematoxylin and eosin (H&E) staining was conducted as described in Materials and methods. The figures (A–C) show the low power magnification and (D–F) high power magnification. (A, D) An unvaccinated macaque (No. 542) inoculated with NL2586 alone; (B, E) an unvaccinated macaque (No. 406) inoculated with NL2586 and *S. pneumoniae*; (C, F) a vaccinated macaque (No. 410) inoculated with NL2586 and *S. pneumoniae*.

© 2009 John Wiley & Sons A/S

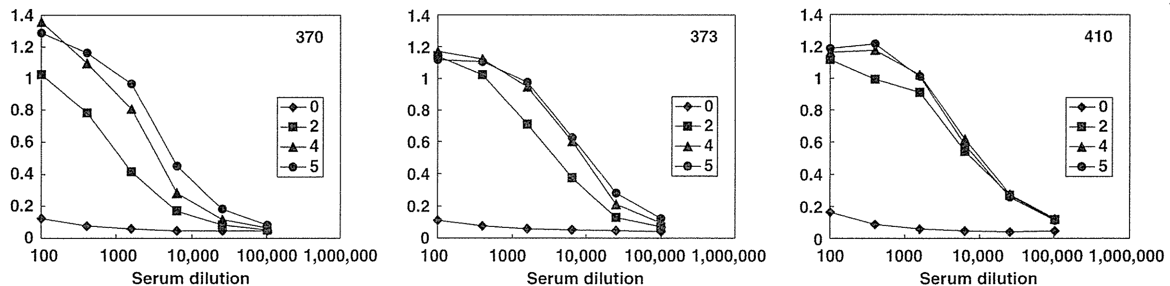


Fig. 3 Antibody responses specific for H7N7 vaccine antigens in cynomolgus macaques immunized with whole virus particle vaccines. Cynomolgus macaques were subcutaneously inoculated with whole virus particle vaccines of Vac-2 (1 mg/dose) with alum twice with a 2-week interval between injections. Sera were collected before (0 week) and after vaccination (2, 4 and 5 weeks after the first vaccination). IgG antibodies specific for Vac-2 antigens in sera were analyzed at indicated dilutions using ELISA. Optical densities at 450 nm are shown.

into nasal cavities (9×10^6 $TCID_{50}$/450 μl for each nasal cavity) with pipettes and into tracheas (2×10^7 $TCID_{50}$/1 ml) with catheters. *Streptococcus pneumoniae* (1.2×10^9 CFU/1 ml) was inoculated into tracheas with catheters. Experiments using NL2586 were performed in the biosafety level 3 facility of the Research Center for Animal Life Science, Shiga University of Medical Science.

Under anesthesia, two cotton sticks were used to collect each swab; subsequently, the sticks were immersed in 1 ml of PBS containing 0.1% BSA. A bronchoscope (MEV-2560; Machida Endoscope Co., Ltd, Tokyo, Japan) and brushes (BC-203D-2006; Olympus, Tokyo, Japan) were used to collect samples of bronchi [25]. The brushes were immersed in 1 ml of PBS containing BSA.

Histological examination

After autopsy, the lungs were fixed in 10% formalin for at least 1 week. Hematoxylin and eosin staining was conducted as previously described [8].

Enzyme-linked immunosorbent assays

The antibody titers of serum samples against Vac-2 antigens were determined using ELISA. Then 96-well plates were coated with 50 μl of purified Vac-2 (20 μg/ml) [8]. For analysis of antibody responses against *S. pneumoniae*, *S. pneumoniae* was cultured as described previously. Thereafter, *S. pneumoniae* was suspended in PBS and inactivated by heat (100°C, 10 minutes). ELISA plates were coated with 50 μl of inactivated *S. pneumoniae* (2×10^5 CFU) [22]. Serially diluted samples were incubated overnight in the coated plates. After washing five times, horseradish peroxidase-conjugated anti-monkey IgG antibody (MP Biomedicals, Inc./Cappel, Aurora, OH, USA) (1:1000 × 50 μl) was added and incubated for 1 hour at room temperature. Horseradish peroxidase activity was assessed using 3, 3′, 5, 5′-tetramethyl benzidine substrate (100 μl). The reaction was stopped by the addition of 1 M hydrogen chloride (100 μl). Optical density was measured at 450 nm.

For detection of IL-10, a monkey IL-10 ELISA kit was used as according to a manufacturer's instruction (Bender MedSystems GmbH, Vienna, Austria). IL-10 secretion was expressed as relative changes comparing with IL-10 production after infection and before infection.

Virus neutralization assay

The serum samples were pretreated with receptor destroying enzyme (RDEII; Denka Seiken, Tokyo, Japan) at 37°C overnight and then inactivated at 56°C for 1 hour. Diluted samples were mixed with 50 $TCID_{50}$ of NL2586 for 1 hour. Then the mixture was added onto an MDCK monolayer. After 1-h incubation, the suspension was removed, and the cells were cultured in MEM containing 0.1% BSA. After incubation at 35°C for 3 days, the number of wells with cytopathic effects was counted in quadruplicate culture. Neutralization titers were expressed as the dilution in which cytopathic effects were observed in 50% of the wells.

Results

Pathogenicity of simultaneous infection with H7N7 HPAIV and Streptococcus pneumoniae in cynomolgus macaques and efficacy of ameliorating pneumonia with Streptococcus pneumoniae by a vaccine against H7N7 HPAIV

H7N7 HPAIV NL2586 (4×10^7 $TCID_{50}$) or both NL2586 and *S. pneumoniae* were inoculated on conjunctivas and in nasal cavities and tracheas of cynomolgus

J Med Primatol **39** (2010) 58–70
© 2009 John Wiley & Sons A/S

Table 1 *Streptococcus pneumoniae* titers in bronchial swabs

		Number of colonies (×10^2 CFU/ml)								
	Animal (dpi)	0	1	2	3	4	5	6	7	8
Without Vac-2	406	0	0	0	0	0	0	0	0	0
	435	0	1	33	2	0	0	0	0	0
	464	0	0	0	0	1	0	0	0	0
With Vac-2	370	0	0	0	0	0	0	0	0	0
	373	0	0	0	0	0	0	0	0	0
	410	0	0	0	0	0	0	0	0	0

Macaques were subcutaneously vaccinated with Vac-2 twice. Five weeks after the second vaccination, the macaques were inoculated with NL2586 and *S. pneumoniae*. Bronchial swabs were collected with a bronchoscope on the indicated days. dpi, days post inoculation with NL2586 and *S. pneumoniae*.

macaques. The body temperature was time-dependently expressed by an average of highest and lowest temperatures on 1 day, and the body temperature after the virus challenge was compared with that before the virus challenge. After NL2586 inoculation, higher body temperature than that before the challenge was observed for 6–13 days in the macaques (Nos 327, 368, 542; Fig. 1A, left panels). After simultaneous inoculation with NL2586 and *S. pneumoniae*, high body temperature was observed until day 4 (No. 406, followed by unstable low temperature until day 12), day 6 (No. 464) and day 11 (No. 435) (Fig. 1A, right panels).

After NL2586 challenge, loss of appetite was observed in two macaques for 2–5 days and in one macaque for 10 days, and after challenge with both NL2586 and *S. pneumoniae*, loss of appetite was observed in two macaques for 6 days and in one macaque for 10 days (Fig. 1B). We also compared weight loss among macaques challenged with the pathogens. All of the macaques had lost weight on day 14 after the virus challenge; the average weight ratio (after/before the challenge) in macaques challenged with NL2586 alone was 90%, whereas that in macaques challenged with NL2586 and *S. pneumoniae* was 88%. These were not significantly different and coincide with the findings for appetite loss (P = 0.33).

Next, we performed histological examination of the lungs 14 days after the challenge. The lungs of macaques infected with NL2586 alone indicated interstitial lymphocyte infiltration in thick alveolar walls (pneumonitis) (Fig. 2A,D), whereas pneumonitis and alveolar lymphocyte infiltration with a few neutrophils

Table 2 *Streptococcus pneumoniae* titers in lungs at autopsy

	Number of colonies (×10^2 CFU/ml)					
	Without Vac-2			With Vac-2		
Lung lobe	406	435	464	370	373	410
Upper right	0	1	5	0	4	1
Middle right	0	13	3	1	1	3
Lower right	0	0	6	4	1	2
Upper left	1	0	5	0	0	2
Middle left	0	2	1	1	1	1
Lower left	0	1	1	0	0	2
Total	1	17	21	6	7	11

Lung tissues were collected at autopsy (14 days after inoculation with NL2586 and *S. pneumoniae*). Tissues were homogenized and suspended in PBS to be adjusted to 10% (w/v) solution.

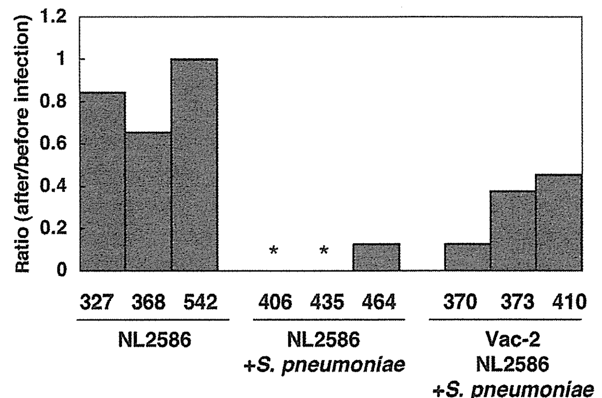


Fig. 4 IL-10 secretions in nasal swab samples. The levels of IL-10 in the samples were analyzed by ELISA. Nasal swabs were collected on day 0 before inoculation and day 8 after inoculation with NL2586 with or without *Streptococcus pneumoniae* as described in Table 3. The levels on day 8 were compared with the day 0 baseline to determine the relative changes in each macaque. Average fold-changes of IL-10 (day 8/day 0) are 0.83, 0.04 and 0.32 in macaques inoculated with NL2586 alone, unvaccinated macaques inoculated with NL2586 and *S. pneumoniae*, and vaccinated macaques inoculated with NL2586 and *S. pneumoniae* respectively, when the concentrations below the detection limit were calculated as 0 unit/ml (*). The differences of IL-10 relative levels are significant in NL2586 alone vs. NL2586 and *S. pneumoniae* (P = 0.002) and in NL2586 alone vs. Vac-2 + NL2586 and *S. pneumoniae* (P = 0.02).

© 2009 John Wiley & Sons A/S