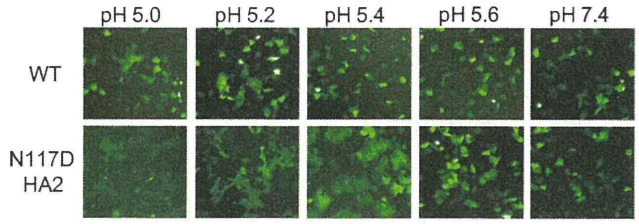

FIG 3 Syncytium formation of Vero cells expressing WT PR8 and HA2 N117D mutant HAs. Vero cells were cotransfected with WT HA or a mutant HA expression plasmid and with a GFP expression plasmid. After treatment with the indicated pH buffers, the cells were observed under a fluorescence microscope.

number of cells involved in cell-cell fusion was greater in these cells than in wild-type HA-transfected cells, even at pH 5.0 and 5.2. We also performed cell fusion assays in MDCK cells with wild-type and mutant HAs, and the results were very similar to those obtained in Vero cells (data not shown). These data indicate that the HA2 N117D mutation both augments membrane fusion and widens the optimal pH range for virus membrane fusion in Vero cells.

Comparison of endosomal pH between Vero and MDCK cells. Although wild-type PR8 virus growth is suboptimal in Vero cells, wild-type PR8 grew to a level comparable to that of HA2 N117D mutants in MDCK cells (Fig. 4A). The optimal pH for viral membrane fusion was higher for the HA2 N117D mutant than for the wild type (Fig. 3), allowing us to hypothesize that the endosomal pH of Vero cells is higher than that of MDCK cells. To test this hypothesis, we compared the endosomal pH between Vero and MDCK cells by introducing dextran-conjugated fluorescent dye as a marker and measuring the intracellular intensity (Fig. 4A). This assay is based on the principle that the fluorescence intensity of Oregon green 488 is sensitive to low pH whereas the intensity of Alexa Fluor 647 is not pH sensitive (25). Thus, pHs can be compared by measuring the intensity of each fluorescent dye and calculating the intensity ratio between Alexa Fluor 647 and Oregon green 488. Vero and MDCK cells were incubated with Alexa Fluor 647- and Oregon green 488-conjugated dextran for 15 min at 37°C. After incubation, the cells were washed and the intensities of Alexa Fluor 647 and Oregon green 488 were measured by using confocal microscopy. The Oregon green 488/Alexa Fluor 647 intensity ratio was appreciably higher in Vero cells than in MDCK cells (Fig. 4B), suggesting that the early endosomal pH value is higher in Vero cells than in MDCK cells.

DISCUSSION

Here, we described an approach to enhance the growth of influenza vaccine seed viruses in Vero cells, which are approved for use in human vaccine production. Influenza vaccine seed viruses that provide robust growth in cell culture are needed to ensure an adequate supply of influenza vaccines as either a supplement to or an alternative method for egg-based vaccine production. Our approach involved introducing a single amino acid mutation (N117D) into the HA2 subunit of HA, which was found in a Vero cell-adapted PR8 virus. The seasonal influenza vaccine seed-like viruses (6:2 reassortants with a PR8 backbone) tested in this study grew poorly in Vero cells. However, the introduction of this HA2 single mutation into these viruses produced mutants that grew to 100 to 1,000 times higher titers in Vero cells than wild-type viruses. This strategy for virus growth enhancement based on the HA2 mutation could thus be feasible for the production of growth-enhanced seasonal or pandemic vaccine seed viruses.

The amino acid at position 117 of HA2 is located on the stem region of HA. One concern is the possibility that changing the amino acid residue at this position may affect the antigenicity of inactivated vaccines, although the major antigenic sites of H1 HA (Ca, Cb, Sa, and Sb), against which most neutralization antibodies elicited by inactivated vaccines are raised, are located on the globular head region that surrounds the receptor binding site (3). Recent studies revealed that antibodies against the HA stem region confer universal protection from influenza virus infection (5, 9, 17, 39, 40). However, such neutralizing antibodies are rarely induced by conventional vaccinations (28). These findings suggest that the HA2 N117D mutation would not affect the antigenicity or efficacy of the vaccines.

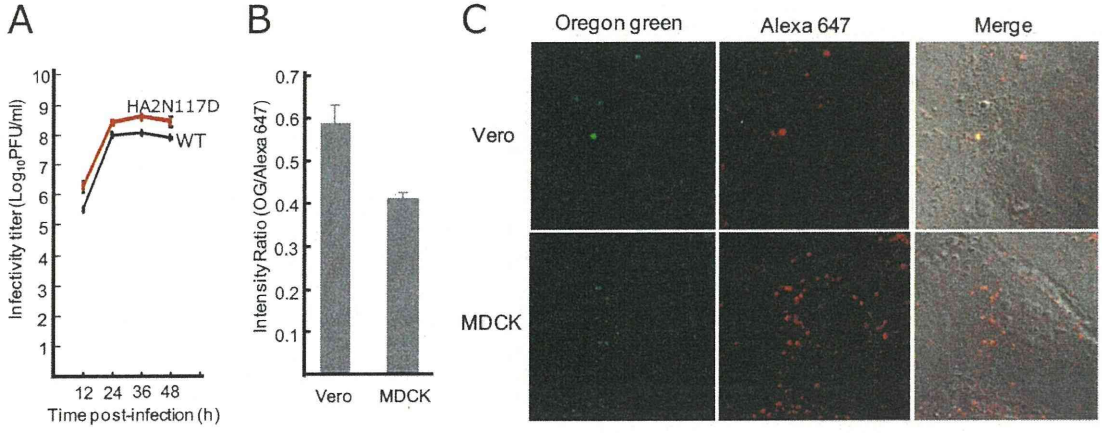

FIG 4 Comparison of endosomal pHs between Vero and MDCK cells. (A) Growth kinetics of WT PR8 and HA2 N117D mutant viruses in MDCK cells. The infectivity titers of WT and mutant viruses were determined following inoculation at an MOI of 0.01. The data are reported as mean titers with standard deviations for three independent experiments. (B) Oregon green (OG)-conjugated dextran (250 μg/ml) and Alexa 647-conjugated dextran (30 μg/ml) were internalized. After 15 min, fluorescence intensities were measured by using confocal microscopy and calculated as OG/Alexa 647 ratios. The data are reported as mean values with standard deviations obtained for five microscopic fields for each cell culture. (C) Representative microscopic images corresponding to panel B.

Here, we suggest that the growth enhancement was most likely due to broadening of the optimal pH range for virus membrane fusion mediated by HA2. The mutation site is located in the HA stalk region, close to the fusion peptide in the HA trimer (Fig. 2B). To accomplish virus membrane fusion, cleaved HA needs to be exposed to low pH (14). Previous reports demonstrate that the substitution of the neutral amino acid residue(s) in the HA stalk region for charged amino acids affects the optimal pH for virus membrane fusion (24, 32, 33, 43). Since the N117D substitution introduces a negative charge, it may change the electrostatic balance between the residue at position 117 and the fusion peptide, possibly resulting in fewer proton-dependent conformational changes in the HA molecule.

We revealed that the endosomal pH was higher in Vero cells than in MDCK cells 15 min after dextran intake. Based on this observation, we assume that this difference in endosomal pH affects virus growth in these cell lines. Indeed, cell-type-dependent endosomal pH kinetics are important for influenza virus infection (21, 22). In MDCK cells, influenza virus reaches the late endosome (pH 5.0) 10 min after endocytosis (21). On the other hand, in HeLa cells, which is a nonpermissive cell line for influenza virus infection (6), it takes 40 min for influenza virus to colocalize with a late endosome marker following endocytosis (36). However, highly pathogenic H5N1 virus, which requires a higher pH for optimal membrane fusion (pH 5.9) (32), grows in MDCK, Vero, and HeLa cells with comparable titers (44). Moreover, vesicular stomatitis virus (VSV) infectivity is much lower in MDCK cells than in Vero cells (11), possibly due to the higher optimal pH (pH 6.0) for membrane fusion of the VSV G protein (34). These facts imply that Vero cells may have a higher pH in the early endosome than MDCK cells. A precise assessment of endosomal pH changes is needed to better understand the mechanism of enhanced virus growth in Vero cells.

We used PR8 virus as a reassortant backbone virus in this study because it is attenuated in humans and is approved by the WHO for use as a genetic backbone for vaccine seed viruses (42). However, introducing the HA2 mutation identified here into any wild-type virus, including seasonal influenza viruses, could also enhance virus replication in Vero cells, providing alternative vaccine seed viruses for the production of inactivated influenza vaccines.

In conclusion, we propose a mutant virus possessing aspartic acid at HA2 position 117 as a seed virus for Vero cell-based influenza vaccine production. A virus with this single amino acid mutation can be produced easily by using reverse genetics. A cell culture-based vaccine strategy with this seed virus would allow the production of more doses of inactivated influenza vaccines in a timely, cost-effective manner, not only for seasonal, but also for pandemic vaccines.

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REFERENCES

1. Barrett PN, et al. 2011. Efficacy, safety, and immunogenicity of a Vero-cell-culture-derived trivalent influenza vaccine: a multicentre, double-blind, randomised, placebo-controlled trial. Lancet 377:751–759.
2. Barrett PN, Mundt W, Kistner O, Howard MK. 2009. Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines. Expert Rev. Vaccines 8:607–618.
3. Brownlee GG, Fodor E. 2001. The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. Philos. Trans. R. Soc. Lond. B Biol. Sci. 356:1871–1876.
4. Clark TW, et al. 2009. Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N. Engl. J. Med. 361:2424–2435.
5. Corti D, et al. 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest. 120:1663–1673.
6. De BK, Nayak DP. 1980. Defective interfering influenza viruses and host cells: establishment and maintenance of persistent influenza virus infection in MDBK and HeLa cells. J. Virol. 36:847–859.
7. Ehrlich HJ, et al. 2009. A cell culture (Vero)-derived H5N1 whole-virus vaccine induces cross-reactive memory responses. J. Infect. Dis. 200: 1113–1118.
8. Ehrlich HJ, et al. 2008. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N. Engl. J. Med. 358:2573–2584.
9. Ekiert DC, et al. 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324:246–251.
10. Genzel Y, Fischer M, Reichl U. 2006. Serum-free influenza virus production avoiding washing steps and medium exchange in large-scale microcarrier culture. Vaccine 24:3261–3272.
11. Green RF, Meiss HK, Rodriguez-Boulan E. 1981. Glycosylation does not determine segregation of viral envelope proteins in the plasma membrane of epithelial cells. J. Cell Biol. 89:230–239.
12. Gupta RK, and Nguyen-Van-Tam JS. 2006. Oseltamivir resistance in influenza A (H5N1) infection. N. Engl. J. Med. 354:1423–1424.
13. Halperin SA, et al. 2002. Safety and immunogenicity of a trivalent, inactivated, mammalian cell culture-derived influenza vaccine in healthy adults, seniors, and children. Vaccine 20:1240–1247.
14. Harrison SC. 2008. Viral membrane fusion. Nat. Struct. Mol. Biol. 15: 690–698.
15. Horimoto T, et al. 2007. Enhanced growth of seed viruses for H5N1 influenza vaccines. Virology 366:23–27.
16. James JM, et al. 1998. Safe administration of influenza vaccine to patients with egg allergy. J. Pediatr. 133:624–628.
17. Kashyap AK, et al. 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc. Natl. Acad. Sci. U. S. A. 105:5986–5991.
18. Katz JM, Webster RG. 1992. Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. J. Gen. Virol. 73:1159–1165.
19. Keitel WA, et al. 2009. Safety and immunogenicity of inactivated, Vero cell culture-derived whole virus influenza A/H5N1 vaccine given alone or with aluminum hydroxide adjuvant in healthy adults. Vaccine 27: 6642–6648.
20. Kobasa D, et al. 2004. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 431:703–707.
21. Lakadamyali M, Rust MJ, Babcock HP, Zhuang X. 2003. Visualizing infection of individual influenza viruses. Proc. Natl. Acad. Sci. U. S. A. 100:9280–9285.
22. Lakadamyali M, Rust MJ, Zhuang X. 2004. Endocytosis of influenza viruses. Microbes Infect. 6:929–936.
23. Le QM, et al. 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437:1108.
24. Lin YP, et al. 1997. Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion. Virology 233:402–410.
25. Marchetti A, Lelong E, Cosson P. 2009. A measure of endosomal pH by flow cytometry in Dictyostelium. BMC Res. Notes 2:7.
26. Montagnon BJ. 1989. Polio and rabies vaccines produced in continuous cell lines: a reality for Vero cell line. Dev. Biol. Stand. 70:27–47.
27. Murakami S, et al. 2008. Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production. J. Virol. 82:1605–1609.
28. Nabel GJ, Fauci AS. 2010. Induction of unnatural immunity: prospects

for a broadly protective universal influenza vaccine. Nat. Med. 16: 1389–1391.
29. Neumann G, et al. 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. 96:9345–9350.
30. Octaviani CP, Ozawa M, Yamada S, Goto H, Kawaoka Y. 2010. High level of genetic compatibility between swine-origin H1N1 and highly pathogenic avian H5N1 influenza viruses. J. Virol. 84:10918–10922.
31. Ozawa M, Goto H, Horimoto T, Kawaoka Y. 2007. An adenovirus vector-mediated reverse genetics system for influenza A virus generation. J. Virol. 81:9556–9559.
32. Reed ML, et al. 2010. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks. J. Virol. 84:1527–1535.
33. Reed ML, et al. 2009. Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein. J. Virol. 83:3568–3580.
34. Roche S, Bressanelli S, Rey FA, Gaudin Y. 2006. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 313:187–191.
35. Rybak SL, Murphy RF. 1998. Primary cell cultures from murine kidney and heart differ in endosomal pH. J. Cell Physiol. 176:216–222.
36. Sieczkarski SB, Whittaker GR. 2003. Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. Traffic 4:333–343.
37. Smith KA, Colvin CJ, Weber PS, Spatz SJ, Coussens PM. 2008. High titer growth of human and avian influenza viruses in an immortalized chick embryo cell line without the need for exogenous proteases. Vaccine 26:3778–3782.
38. Sugawara K, et al. 2002. Development of Vero cell-derived inactivated Japanese encephalitis vaccine. Biologicals 30:303–314.
39. Sui J, et al. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. 16:265–273.
40. Wang TT, et al. 2010. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc. Natl. Acad. Sci. U. S. A. 107:18979–18984.
41. WHO. 1995. Cell culture as a substrate for the production of influenza vaccines: memorandum from a WHO meeting. Bull. World Health Organ. 73:431–435.
42. Wood JM, Robertson JS. 2004. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat. Rev. Microbiol. 2:842–847.
43. Xu R, Wilson IA. 2011. Structural characterization of an early fusion intermediate of influenza virus hemagglutinin. J. Virol. 85:5172–5182.
44. Yang W, Qu S, Liu Q, Zheng C. 2009. Avian influenza virus A/chicken/Hubei/489/2004 (H5N1) induces caspase-dependent apoptosis in a cell-specific manner. Mol. Cell Biochem. 332:233–241.

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Subcutaneous inoculation of a whole virus particle vaccine prepared from a non-pathogenic virus library induces protective immunity against H7N7 highly pathogenic avian influenza virus in cynomolgus macaques

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ABSTRACT

Development of H7N7 highly pathogenic avian influenza virus (HPAIV) vaccines is an urgent issue since human cases of infection with this subtype virus have been reported and most humans have no immunity against H7N7 viruses. We made an H7N7 vaccine combining components from an influenza virus library of non-pathogenic type A influenza viruses. Antibody and T cell recall responses specific against the vaccine strain were elicited by subcutaneous inoculation with the whole virus particle vaccine with or without alum as an adjuvant in cynomolgus macaques. No significant difference was observed in magnitude of antibody responses between vaccination with alum and vaccination without alum, though vaccination with alum induced longer recall responses of $CD8^+$ T cells than did vaccination without alum. After challenge with a subtype of H7N7 HPAIV, the virus was detected in nasal swabs of unvaccinated macaques for 8 days but only for 1 day in the animals vaccinated either with or without alum, although the macaques vaccinated with alum showed elevated body temperature more briefly after infection. These findings demonstrated that this H7N7 HPAIV strain is pathogenic to macaques and that the vaccine conferred protective immunity to macaques against H7N7 HPAIV infection.

1. Introduction

H7 subtype influenza viruses fall into two geographically distinct lineages, Eurasian and North American [1]. The Eurasian lineage contains highly pathogenic avian influenza viruses (HPAIVs) that possess multibasic amino acids in hemagglutinin (HA) cleaved by not only trypsin-like enzymes but also other ubiquitous proteases, resulting in high mortality in poultry [2]. Of these, outbreaks of H7N7 HPAIV infection caused 89 human cases with conjunctivitis in the Netherlands in 2003, including one fatal case. More than 50% of the infected people had serum antibodies after recovery that reacted with H7N7 virus [3,4]. The recent increase in findings of H7 avian influenza viruses from outbreaks in poultry and humans has raised concerns about the possibility of additional zoonotic transmissions of influenza viruses from poultry to humans, which would pose a public health threat [5,6]. In addition, H7 influenza viruses from the North American lineage have acquired sialic acid-binding properties that closely resemble those in human influenza viruses, and are transmissible in the ferret model [7]. These findings suggest that H7 HPAIVs may become transmissible in humans. Since most people have no immunity against H7 influenza viruses, development of vaccines against H7 subtype viruses is an urgent issue [8–10].

In a similar study on H5 vaccination, we previously demonstrated that formalin-inactivated whole virus particle vaccines induced protective immune responses including antibody and cytotoxic T lymphocyte (CTL) responses in mice and that the whole virus particle vaccines conferred protective immunity against H5N1 HPAIV challenge in cynomolgus macaques [11,12]. Although the unvaccinated cynomolgus macaques infected with H5N1 HPAIV

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did not die as did ferrets and mice [13–16], H5N1 HPAIV was detected for 5 days after challenge with H5N1 HPAIV, a duration sufficient to assess the effects of vaccines. Indeed, H5N1 HPAIV was recovered from the vaccinated macaques only for 1 day after challenge with the virus. Hence, we believe that prevention of virus replication in animals is a more reliable indicator of vaccine efficacy than is prevention of death. This is especially true when using mice, since most laboratory mice possess a defective version of the *Mx* gene related to prevention of virus replication in humans and it may be difficult to determine the degree of vaccination effect when using survival rates alone [17]. The macaque model allowed us better to analyze pathogenicity of HPAIV and the effects of vaccines for preclinical studies.

In this H7N7 study, we made a non-pathogenic H7N7 virus vaccine using two strains from a virus library of 144 different combinations of 16 HA and 9 neuraminidase (NA) subtypes of non-pathogenic viruses [18]. One parental strain, A/duck/Mongolia/736/2002 (H7N7), is non-pathogenic in chickens, but its growth potential in embryonated eggs is insufficient for vaccine manufacture. Therefore, we selected A/duck/Hokkaido/49/1998 (H9N2), which has better growth potential in embryonated eggs and is likewise not pathogenic in chickens, as a partner for the genetic reassortment to obtain a vaccine strain [10,19]. The product was grown and inactivated to make a whole virus particle vaccine as described previously [10,19]. At first, we examined immune responses induced by subcutaneous inoculation with the inactivated whole virus particle vaccine. Next, we examined the pathogenicity of the H7N7 HPAIV in cynomolgus macaques to establish a non-human primate model for assessing vaccine efficacy and subsequent protection against an H7N7 HPAIV by vaccination.

We also examined the effects of an adjuvant, alum (aluminum hydroxide), in combination with the whole virus particle vaccine. Alum has been used in vaccines for humans [20–22] and is considered to enhance Th2 type responses [23]. Indeed, it was recently demonstrated that alum activated the Nalp3 inflammasome pathway, resulting in production of pro-inflammatory cytokines IL-1β and IL-18 and that Nalp3-deficient mice failed to mount a significant antibody response to an antigen administered with alum [24–26]. In contrast to the results of some studies, we found that alum with the H7N7 whole virus particle vaccine induced not only antigen-specific antibody responses but also $CD8^+$ T cell responses in cynomolgus macaques. Furthermore, the vaccine with alum conferred $CD8^+$ T cell recall responses for a longer period than without alum, although alum did not affect the period of virus replication in the nasal cavity, trachea, or bronchus.

2. Materials and methods

2.1. Viruses

For a vaccine, we used the influenza virus A/duck/Hokkaido/Vac-2/2004 (H7N7) (Vac-2, National Center for Biotechnology Information (NCBI) taxonomy database ID: 390987); this is a genetic reassortant generated by coinfection with A/duck/Mongolia/736/2002 (H7N7) and A/duck/Hokkaido/49/1998 (H9N2) in chicken embryos. The PB2, PB1, PA, HA, NA, and the NS genes of Vac-2 were derived from the H7N7 virus, and NP and M genes were derived from the H9N2 virus [10].

For our test (challenge) pathogen, an HPAIV, A/chicken/Netherlands/2586/2003 (H7N7) (NL2586, NCBI taxonomy database ID: 533037), was provided by Dr. I. 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) [10,27]. The amino acid sequence identities between Vac-2 and NL2586 were 97% in HA and 98% in NA.

Both the Vac-2 viruses for vaccine and the NL2586 for challenge to macaques were propagated in allantoic cavities of 10-day-old embryonated hen's eggs at 35 °C for 48 h. To prepare an inactivated vaccine, the Vac-2 infected allantoic fluids were concentrated and purified by high-speed centrifugation (112,500 × *g* for 90 min) through a 10–50% sucrose density gradient and then treated in 0.1% formalin at 4 °C for 1 week [10]. The amount of whole particle vaccines was indicated as that of entire protein including HA and the other viral proteins. The vaccine used in the present study contained 42,667 HA units in 1 mg vaccine.

In order to assess virus replication, serial dilutions of swabs and whole blood samples were inoculated onto confluent Madin–Darby canine kidney (MDCK) cells as described previously [11]. Cytopathic effects were then examined under a microscope 72 h later.

2.2. Animals

Five- to seven-year-old cynomolgus macaques (*Macaca fascicularis*) were used with permission of the Shiga University of Medical Science Animal Experiment Committee and Biosafety Committee. Cynomolgus macaques in the present study were healthy young adults old enough for pregnancy. In the text and figures, individual macaques are distinguished by identification numbers. The absence of H7N7-specific antibodies in their sera was confirmed before experiments using antigen-specific enzyme-linked immunosorbent assays (ELISA). Under anesthesia 2 weeks before virus inoculation, a telemetry probe (TA10CTA-D70, Data Sciences International, St. Paul, MN) was implanted in the peritoneal cavity of each macaque to monitor body temperature. The macaques used in this study were free from B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica* [11].

The vaccine (1 mg/dose) was inoculated subcutaneously into macaques using syringes with and without alum (500 μl, Superfos Biosector, Vaerloese, Denmark) twice with a 2-week interval between injections. Saline instead of the vaccine was inoculated into macaques as unvaccinated controls. The macaques were challenged with NL2586 (2×10^7 $TCID_{50}$) on the conjunctiva (1×10^6 $TCID_{50}$/50 μl for each eye) and into nasal cavities (9×10^6 $TCID_{50}$/450 μl for each nasal cavity) with pipettes 10 weeks after the second vaccination. 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 (TE8201, Eiken Chemical, Ltd., Tokyo, Japan) were used to collect fluid samples in nasal cavities and tracheas, and the sticks were subsequently immersed in 1 ml of PBS containing 0.1% BSA and antibiotics. A bronchoscope (MEV-2560, Machida Endoscope Co., Ltd., Tokyo, Japan) and brushes (BC-203D-2006, Olympus, Tokyo, Japan) were used to collect bronchial samples [28]. The brushes were quickly immersed in 1 ml of PBS containing BSA and antibiotics.

2.3. Antibody assays

The antibody titers of serum and swab samples against Vac-2 antigens were determined using ELISA [29]. For ELISA, 96-well plates were coated with 50 μl of purified Vac-2 (20 μg/ml) disrupted with 0.05 M Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl. Serially diluted samples were incubated overnight in the coated plates. After washing five times, horseradish peroxidase-conjugated anti-monkey IgG antibodies (MP Biomedicals, Inc./Cappel, Aurora, OH) (1:1000 × 50 μl) or anti-monkey IgA antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) (1:4000 × 50 μl) were added and incubated for 1 h at room temperature. Horseradish peroxidase activity was assessed using 3,3′,5,5′-tetramethyl benzidine substrate (100 μl). The reac-

tion was stopped by the addition of 1 M hydrogen chloride (100 μl). Optical density was measured at 450 nm.

2.4. 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 h. Diluted samples were mixed with 50 $TCID_{50}$ of viruses for 1 h. 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 and 5 μg/ml trypsin. 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.

2.5. Hemagglutination-inhibition (HI) test

Serum samples were pretreated as described in Section 2.4. Serially diluted sera were mixed with 8 HA units of Vac-2 or NL2586 virus antigen for 1 h at room temperature. The mixture was then incubated with chicken red blood cells. After 30-min incubation, the HI titers were determined.

2.6. Cell proliferation

Lymphocytes were purified from peripheral blood of the macaques using a density gradient (Wako Pure Chemical Industries Ltd., Osaka, Japan). After washing, $CD8^+$ cells were isolated using CD8 microbeads for non-human primates and magnetic cell sorting (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by separation of $CD4^+$ cells using CD4 microbeads. The purity of the isolated $CD4^+$ and $CD8^+$ population was confirmed after flow cytometry to be more than 90%. The cells remaining after removal of $CD4^+$ and $CD8^+$ cells were used as antigen-presenting cells (APC) after irradiation at 30 Gy. $CD4^+$ or $CD8^+$ T cells (1×10^5/well) and APC (0.5×10^5/well) were cultured with inactivated whole particle Vac-2 antigens in 96-well U-bottom plates for 72 h. [^{3}H]-Thymidine (0.5 μCi/well) was added for the last 16 h of culture. Incorporation of [^{3}H]-Thymidine was measured.

3. Results

3.1. Antibody responses after inoculation of the inactivated whole virus particle vaccine prepared from a non-highly pathogenic H7N7 strain in the virus library

The vaccine was given twice to each macaque, and during a 10-week period between the second vaccination and challenge with H7N7 HPAIV we examined antibody and T lymphocyte responses in the vaccinated and non-vaccinated macaques on schedule as described in Fig. 1. The macaques showed no systemic symptoms after vaccination. No skin reaction at the site of injection was observed after inoculation with whole particle vaccines alone, though subcutaneous nodules without skin erosion or ulceration were observed at the site of injection in macaques vaccinated with alum.

Two weeks after the first vaccination and immediately before the second vaccination, serum IgG specific against Vac-2 antigen was detected in all vaccinated macaques (data not shown), and serum levels of IgG antibodies specific against the H7N7 vaccine antigens reached a maximum in all of the macaques inoculated with inactivated Vac-2 with or without alum by 4 weeks after the second vaccination (Fig. 2a). Antigen-specific IgG antibodies were detected in nasal and tracheal swabs from all the vaccinated macaques except in the nasal swabs of #466 and in the tracheal swabs of #459 (Fig. 2c and e). Antigen-specific IgA was detected in sera from two macaques vaccinated without alum (#459 and #579) and in the nasal and tracheal swabs from one macaque vaccinated with inactivated Vac-2 with alum (#545) (Fig. 2b, d, and f).

Table 1
Hemagglutination-inhibition activities of serum samples after vaccination.

Vaccination	Animal	Antigen							
		Vac-2				NL2586			
		Week							
		4	6	8	10	4	6	8	10
Vac-2	409	256	128	64	64	<4	<4	<4	<4
	459	64	32	16	16	<4	<4	<4	<4
	579	256	128	64	32	<4	<4	<4	<4
Vac-2 + alum	414	256	128	32	16	<4	<4	<4	<4
	466	256	128	64	32	<4	<4	<4	<4
	545	128	64	32	32	<4	<4	<4	<4

Week: weeks after the first vaccination ('week 4' means 2 weeks after the second vaccination). Detection limit is 1:4. The difference between Vac-2 alone and Vac-2 with alum is not significant ($P > 0.05$).

In order to compare the duration of antibody responses between macaques vaccinated with alum and without alum, the serum HI activities were examined periodically (Table 1). HI activity against Vac-2 was observed in sera from macaques vaccinated with Vac-2 alone and Vac-2 with alum 2 weeks after the second vaccination, whereas no HI activity against the highly pathogenic strain NL2586 was ever detected. HI activity against Vac-2 in sera gradually declined after 4 weeks. However, the sera from vaccinated macaques 8 weeks after the second vaccination showed activity in neutralizing infectivity of Vac-2 virus and less potent but significant neutralization activity against the highly pathogenic strain NL2586, compared with those from unvaccinated macaques (Table 2). It is known that HI tests do not accurately detect neutralizing antibodies, especially when avian influenza viruses are used as HA antigens [29–31]. Therefore, the results of neutralization activity suggest induction of antibodies against NL2586 by vaccination with the whole particles of inactivated Vac-2. Furthermore, addition of alum did not show significant enhancement of the HI activity and neutralization activities.

IgG antibody responses specific for Vac-2 antigens in the nasal swabs were examined 10 weeks after the second vaccination (Fig. 3). Both the macaques vaccinated with alum and those vac-

Table 2
Neutralization of Vac-2 and NL2586 with sera obtained after the second vaccination.

Vaccination	Animal	50% neutralization titer (log 2)	
		Virus	
		Vac-2	NL2586
Saline	416	<2	<2
	421	<2	<2
	450	<2	<2
Vac-2	409	8.00	4.50
	459	7.23	2.33
	579	7.67	3.50
Vac-2 + alum	414	8.23	2.50
	466	9.18	3.00
	545	7.83	4.50

Cynomolgus macaques were immunized with Vac-2 as described in Fig. 2. Sera were collected 8 weeks after the second vaccination (10 weeks after the first vaccination). The averages of 50% neutralization titers against Vac-2 were 7.67 and 8.53 in sera from macaques vaccinated with Vac-2 alone and macaques vaccinated with Vac-2 with alum, respectively. P values with Student's t-test are 0.003 (saline vs. Vac-2 alone) and 0.03 (saline vs. Vac-2 with alum) when 50% neutralization titers below 2 are calculated as 2.

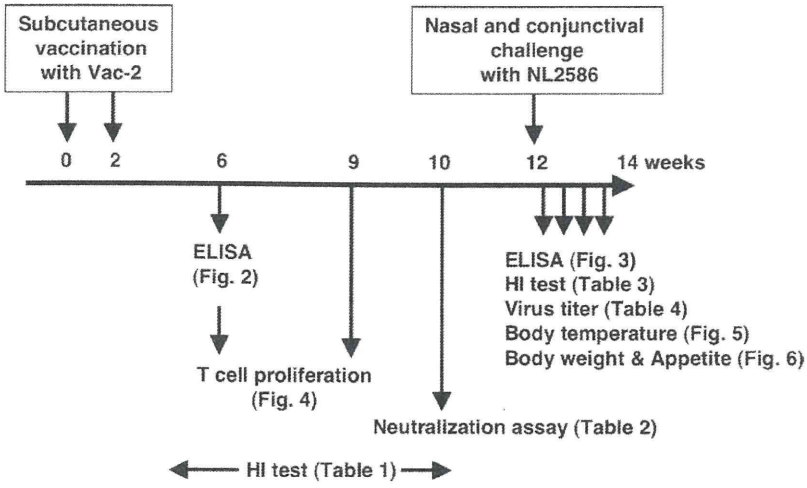

Fig. 1. Experimental schedule of the H7N7 vaccination and challenge in cynomolgus macaques. Cynomolgus macaques were subcutaneously immunized with whole virus particle vaccines of Vac-2 (1 mg/dose) without alum or with alum twice with a 2-week interval between injections. Blood and swab samples were collected after vaccination to examine antibody responses. Peripheral blood cells were used for proliferation assay in the 6th and 9th weeks. Neutralization assay was performed using sera collected in the 10th week. Ten weeks after the second vaccination, macaques were inoculated with HPAIV NL2586 onto conjunctivas and into nasal cavities. Swab samples were collected after inoculation with virus for virus titration. Body temperature, weight, and food consumption were recorded.

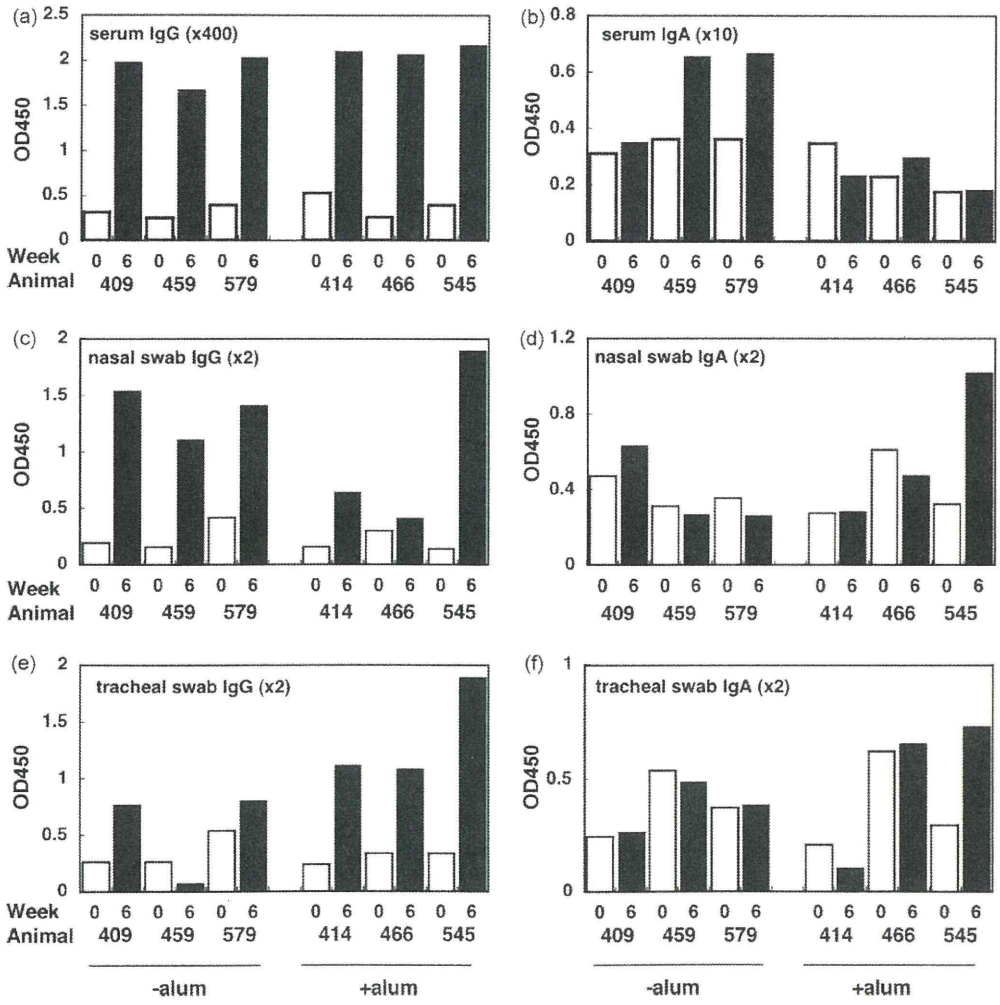

Fig. 2. Antibody responses specific against H7N7 vaccine antigens in cynomolgus macaques immunized with whole virus particle vaccines. Cynomolgus macaques were subcutaneously immunized with whole virus particle vaccines of Vac-2 without alum (#409, #459, and #579) or with alum (#414, #466, and #545). Four weeks after the second vaccination, sera and swabs were collected. IgG (a, c, and e) and IgA (b, d, and f) antibodies specific for Vac-2 antigens in sera (a and b), nasal swabs (c and d), and tracheal swabs (e and f) were analyzed at indicated dilutions using ELISA. Optical densities at 450 nm are shown. Open and filled bars indicate antibodies against Vac-2 antigens in pre-vaccinated samples and in samples 6 weeks after the first vaccination, respectively.

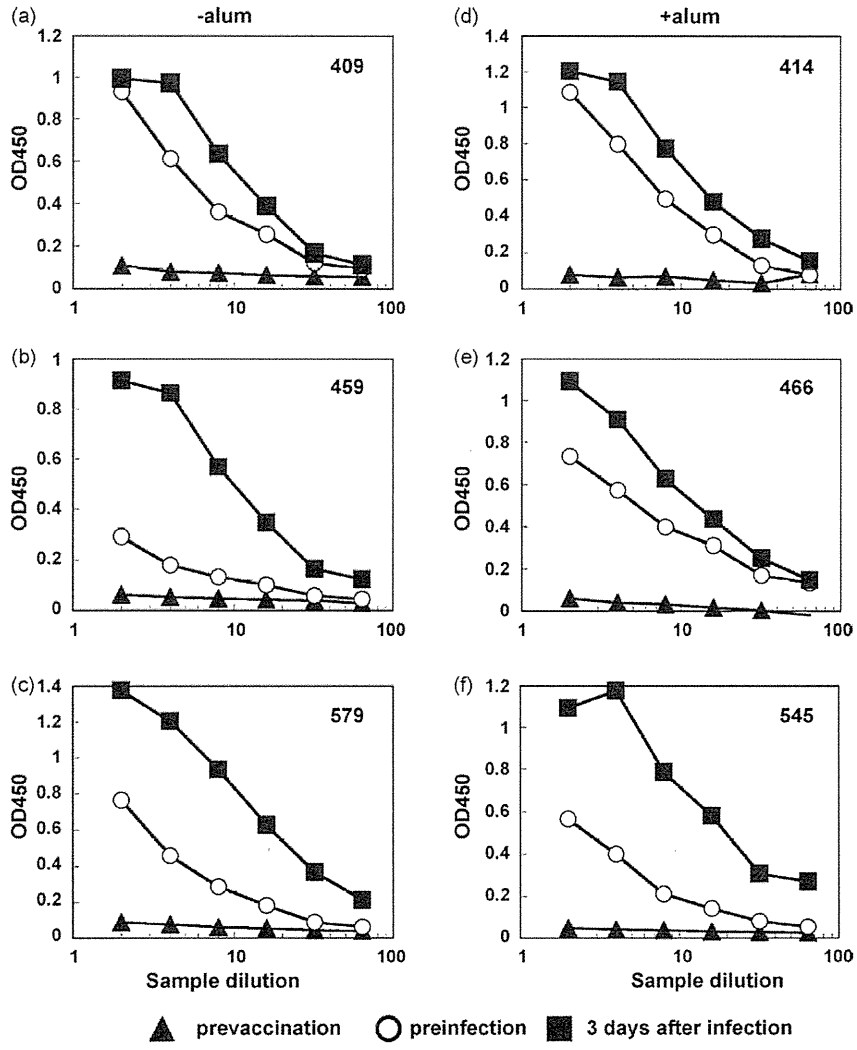

Fig. 3. Antibody responses specific against H7N7 vaccine antigens in cynomolgus macaques 10 weeks after vaccination and 3 days after the HPAIV challenge. Cynomolgus macaques were subcutaneously immunized with Vac-2 without alum (a–c) or with alum (d–f) as described in the legend of Fig. 2. Ten weeks after the second vaccination (open circle) and 3 days after the virus challenge (closed square), nasal swabs were collected. IgG antibodies specific against Vac-2 antigens were examined as described in Fig. 2. Closed triangles indicate antibodies against Vac-2 antigens in pre-vaccinated samples.

cinated without alum maintained high levels of IgG in nasal swabs, although one of the three macaques vaccinated without alum (#459) showed only a slight increase in IgG (Fig. 3b). In addition, recall responses of the antibodies detected 3 days after the virus challenge in the macaques vaccinated with alum were the same as those in the macaques vaccinated without alum (Fig. 3). Similarly, the levels of serum IgG antibodies specific for the Vac-2 antigens in all of the vaccinated macaques without alum at 10 weeks after the second vaccination were almost the same as the levels in the macaques vaccinated with alum (data not shown). Thus, alum did not affect the duration or intensity of antibody responses elicited by the vaccination.

3.2. T lymphocyte responses induced by whole virus particle vaccines prepared from a non-pathogenic H7N7 strain, Vac-2

T lymphocyte responses to Vac-2 antigens were assessed in peripheral blood of the macaques. $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood of the vaccinated animals were purified and cultured with the Vac-2 antigens (Fig. 4). Both $CD4^+$ and $CD8^+$ T lymphocytes obtained from three macaques inoculated with inactivated Vac-2 with alum 4 weeks after the second vaccination showed antigen-specific proliferation (Fig. 4a and b). $CD4^+$ and $CD8^+$ T lymphocytes obtained from two of the three macaques vaccinated without alum also showed antigen-specific proliferation. Proliferative responses of $CD4^+$ T lymphocytes obtained from the vaccinated macaques were seen 7 weeks after the second vaccination (Fig. 4c), whereas proliferative responses of $CD8^+$ T lymphocytes were elicited in cultures from the macaques vaccinated with alum but not from the macaques vaccinated without alum. Therefore, vaccination with alum evoked $CD8^+$ T lymphocyte recall responses for a longer period after vaccination than did vaccination without alum.

3.3. Pathogenicity of H7N7 HPAIV in cynomolgus macaques and protective effects of the inactivated whole virus particle vaccine

HPAIV NL2586 was inoculated on conjunctivas and in nasal cavities of macaques. Body temperature was expressed by calculating the average of the highest and lowest temperatures on 1 day, and the body temperature after the virus challenge was compared with that before the virus challenge. Higher body temperature than that before the challenge was observed for 11–13 days after inocula-

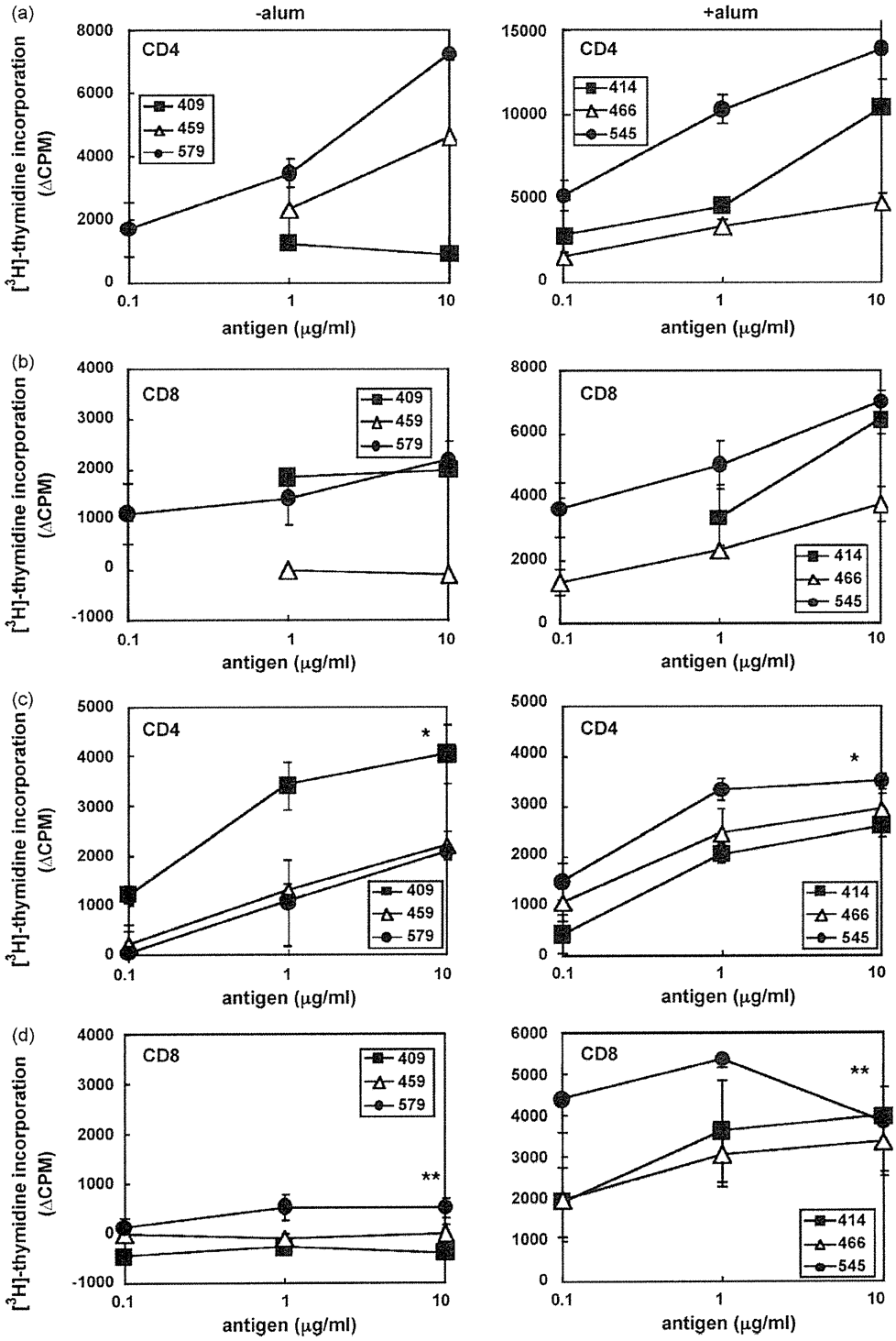

Fig. 4. CD4⁺ and CD8⁺ T lymphocyte proliferative responses specific for Vac-2 antigens. Cynomolgus macaques were subcutaneously immunized with Vac-2 without alum (left column) or with alum (right column) as described in the legend to Fig. 2. CD4⁺ (a and c) and CD8⁺ (b and d) lymphocytes were isolated from peripheral blood of the vaccinated macaques 4 weeks (a and b) and 7 weeks after the second vaccination (c and d). The differences of average proliferative responses of CD4⁺ T cells are not significant between Vac-2 alone and Vac-2 with alum in (c) (*$P > 0.05$). The differences of average proliferative responses of CD8⁺ T cells are significant between Vac-2 alone and Vac-2 with alum in (d) (**$P < 0.05$). T lymphocytes from macaques inoculated with saline did not show any proliferative responses against Vac-2 antigens (data not shown).

tion in the unvaccinated macaques (#416, #421, #450) (Fig. 5, left panels), while raised body temperature in the macaques vaccinated with inactivated Vac-2 alone was observed until day 7 (#579), day 9 (#459) and day 13 (#409) (Fig. 5, middle panels). Although the inactivated Vac-2 alone slightly decreased the duration of raised body temperature, inoculation of Vac-2 with alum considerably reduced the duration of raised body temperature to 4 days (Fig. 5, right panels). Therefore, these results indicate that vaccination with inactivated Vac-2, especially with alum, accelerated the recovery of body temperature.

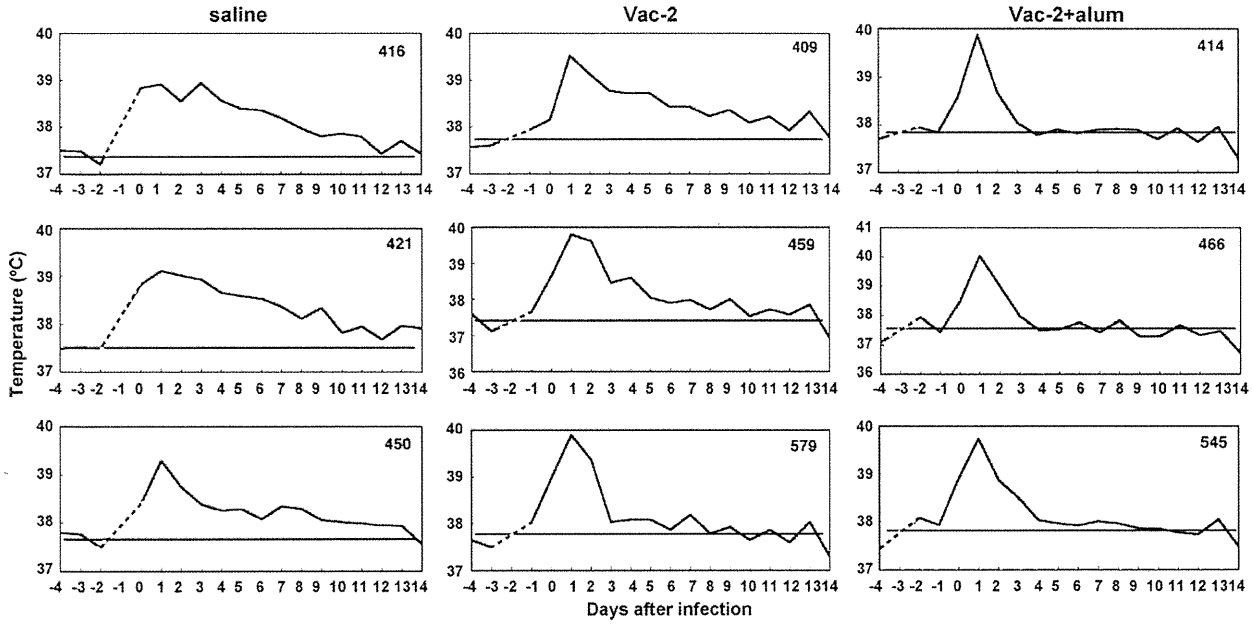

Fig. 5. Body temperatures of macaques after inoculation of H7N7 HPAIV (NL2586). Cynomolgus macaques were subcutaneously immunized with Vac-2 alone (middle column) or with Vac-2 and alum (right column). On day 0, 10 weeks after the second vaccination, NL2586 was inoculated onto conjunctivas and into nasal cavities of vaccinated macaques. Cynomolgus macaques injected with saline were used as unvaccinated controls (left column). Lines drawn horizontally indicate the average temperature levels at pre-infection. Temperature data were not plotted on day −1 in saline control, on day −2 in macaques inoculated with Vac-2 alone, and on day −3 in macaques inoculated with Vac-2 and alum since temperature data were not recorded because of power failure (dotted lines). The average duration of high temperature was 12.3, 9.67, and 3.33 days in macaques inoculated with saline, Vac-2 alone, and Vac-2 with alum, respectively. *P* values calculated with Student's *t*-test are 0.2 (saline vs. Vac-2 alone), 0.0003 (saline vs. Vac-2 with alum), and 0.02 (Vac-2 alone vs. Vac-2 with alum).

All three control unvaccinated macaques lost weight and appetite for 7–11 days after NL2586 challenge (Fig. 6a and d). Two vaccinated macaques (#409 and #414) lost weight after the virus challenge (Fig. 6b and c). The average percent weights after challenge (weight on day 14/weight on day 0) were 89.8%, 93.7% and 93.8% in macaques inoculated with saline, Vac-2, and Vac-2 with alum, respectively. There were significant differences in relative weight-losses between macaques inoculated with saline and macaques inoculated with Vac-2 without alum ($P = 0.002$) and between macaques inoculated with saline and macaques inoculated with Vac-2 with alum ($P = 0.01$). Loss of appetite was observed in all animals after challenge, but the duration of appetite loss in the vaccinated macaques was much shorter than that in the control macaques without vaccination (Fig. 6d–f). The raised body temperature probably contributed to the loss appetite and weight. No conjunctivitis was observed in any of the macaques. In addition, the macaques showed no clinical signs including sneezing and coughing.

We then examined antibody recall responses against Vac-2 and responses against NL2586 using HI test. HI activity against Vac-2 but not against NL2586 was observed in sera from vaccinated macaques 10 weeks after the second vaccination (Table 3). On the other hand, HI activity against Vac-2 was not detected in sera from the unvaccinated macaques before the virus challenge. HI activity against Vac-2 prominently increased and reacted with NL2586 in sera from all of the vaccinated macaques 7 days after the challenge with NL2586, whereas HI activity against Vac-2 (but not against NL2586) in sera from the unvaccinated macaques was observed on day 14. These findings indicate that the vaccinated macaques showed antibody recall responses against Vac-2 containing responses against common epitopes between Vac-2 and NL 2586 more promptly than did the unvaccinated macaques. In addition, the latter finding induced a speculation that perhaps some *in vivo* mutation after infection elicited antibodies specific for the mutated NL2586 that weakly reacted with the original NL2586 [32].

Finally, we examined the virus titers in the swab samples with and without vaccination after challenge with NL2586. The virus was detected in the nasal, tracheal and bronchial swabs of the unvaccinated macaques on days 1–8 after inoculation of NL2586 (Table 4). On the other hand, in the macaques vaccinated either with or without alum, the virus was detected on day 1 and a very small amount of the virus was detected on day 3. Therefore, it was clear that inoculation with the inactivated whole virus particles of Vac-2 derived from the non-pathogenic virus library accelerated elimination of HPAIV in the upper and lower respiratory tracts.

Table 3
Hemagglutination-inhibition activities of serum samples after infection with NL2586.

Vaccination	Animal	Antigen					
		Vac-2			NL2586		
		dpi					
		0	7	14	0	7	14
Saline	416	<4	<4	512	<4	<4	<4
	421	<4	<4	8	128	<4	<4
	450	<4	<4	128	<4	<4	<4
Vac-2	409	64	4096	4096**	<4	64	16
	459	16	2048	4096**	<4	32	32
	579	32	8192	8192**	<4	128	256
Vac-2 +alum	414	16*	1024	1024	<4	32	32
	466	32*	4096	8192	<4	256	256
	545	32*	1024	1024	<4	32	32

dpi: days post-infection. Sera were collected on indicated days after infection with NL2586. Day 0 means 10 weeks after the second vaccination and before infection with NL2586. Detection limit is 1:4. *P* values with Student's *t*-test are less than 0.05 in comparison between saline and Vac-2 with alum on day 0 (*) and in comparison between saline and Vac-2 alone on day 14 (**) against Vac-2 antigen when HI titers below 4 are calculated as 4.

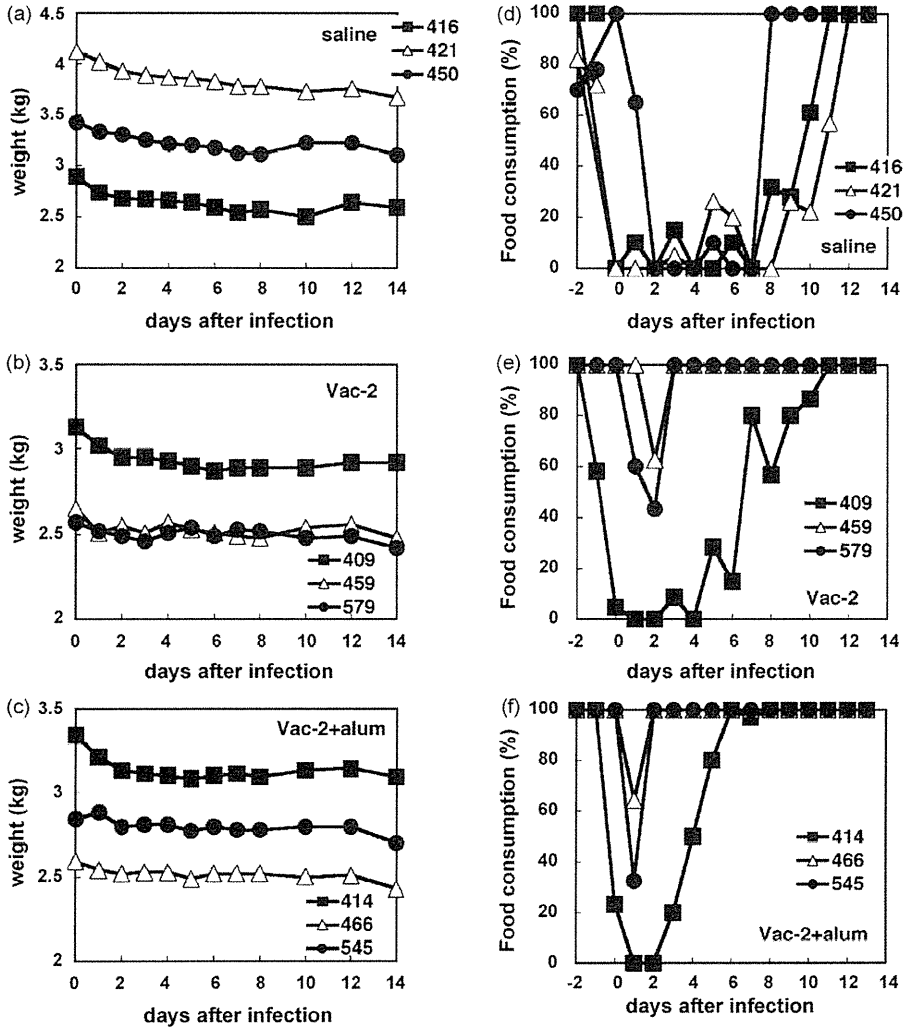

Fig. 6. Body weight and food consumption of cynomolgus macaques inoculated with NL2586. Body weight (a–c) and appetite (d–f) was measured on the indicated days. The average duration of appetite loss was 10, 4.67, and 2.67 days in macaques inoculated with saline, Vac-2 alone, and Vac-2 with alum, respectively. *P* values calculated with Student's *t*-test are 0.2 (saline vs. Vac-2 alone) and 0.03 (saline vs. Vac-2 with alum). (a and d) Macaques without vaccination, (b and e) macaques immunized with Vac-2 alone, (c and f) macaques immunized with Vac-2 and alum.

Table 4
Virus recovery from macaques inoculated with NL2586.

Vaccination	Animal	Virus titer (log 10 ($TCID_{50}$/ml))														
		Nasal swab					Tracheal swab					Bronchial swab				
		dpi														
		1	3	5	7	8	1	3	5	7	8	1	3	5	7	8
Saline	416	3.00	2.50	3.33	4.33	<	3.67	<	1.50	2.50	<	<*	<*	1.50	2.50	<
	421	3.50	2.67	2.00	2.50	<	3.23	<*	1.50	<	<*	2.00	<*	<*	2.67	<
	450	2.67	1.67	2.00	2.67	<*	3.00	<	2.00	<*	<	3.50	<	<	<*	<
Vac-2	409	3.51	<	<	<	<	<*	<	<	<	<	3.00	<	<	<	<
	459	2.50	<	<	<	<	3.23	<	<	<	<	<*	<	<	<	<
	579	2.50	<	<	<	<	1.67	<	<	<	<	<	<	<	<	<
Vac-2 + alum	414	3.00	<*	<	<	<	1.50	<	<	<	<	<*	<	<	<	<
	466	2.50	<*	<	<	<	2.00	<	<	<	<	<*	<	<	<	<
	545	2.33	<	<	<	<	<	<	<	<	<	<	<	<	<	<

dpi: days post-infection. Each macaque was inoculated with NL2586 (2×10^7 $TCID_{50}$) on day 0. The symbol '<' indicates that the virus titer was less than the detection limit (<10 $TCID_{50}$/ml). The symbol '<*' indicates that the one cytopathic effect-positive well was observed in quadruplicate culture of undiluted samples. No virus was detected in swab samples collected on days 10 and 12. *P* values are below 0.05 (saline vs. Vac-2 and saline vs. Vac-2 with alum) in nasal swabs on days 3, 5, and 7 and in tracheal swabs on day 5 when virus titers below 10 $TCID_{50}$/ml are calculated as 10.

4. Discussion

For an initial test of H7 vaccine potential, we made a vaccine from two non-pathogenic strains, with H7 structure very similar to that of a highly pathogenic H7 strain. Secondly, we also found that an H7N7 HPAIV, NL2586, replicated in the upper and lower respiratory tract of cynomolgus macaques and resulted in severe symptoms, including high body temperature and appetite loss. Then, we found that subcutaneous inoculation with inactivated whole virus particle of Vac-2 elicited neutralizing antibody responses and prompt antibody recall responses specific against NL2586, and we found that the vaccine worked for reduction of virus replication and prevention of symptoms in cynomolgus macaques.

To our knowledge, this is the first report demonstrating pathogenicity of H7N7 HPAIVs in cynomolgus macaques. Although the cynomolgus macaques infected with H7N7 HPAIVs did not die as ferrets and mice did [2,7], the challenge viruses were recovered from unvaccinated macaques until 8 days after the challenge. Indeed, H7N7 HPAIV was recovered from the vaccinated macaques for only 1 day after challenge with the virus. In addition, the NL2586 strain used in the present study had more than 99% identity in amino acid sequence of PB2, PB1, PB1-F2, PA, HA, NP, NA, M1 and NS1 compared with A/Netherlands/219/2003 (H7N7) (NCBI taxonomy database ID: 251298) isolated from a human. Therefore, the macaque model allowed us to test the pathogenicity of H7N7 HPAIV and efficacy of vaccines as a model for preclinical studies.

The H7N7 HPAIV, NL2586, appears to be more pathogenic than the H5N1 HPAIV in cynomolgus macaques, since intranasal inoculation with the HPAIV A/Vietnam/1194/2004 (H5N1) (VN1194) induced fever in cynomolgus macaques for 2 days, but the macaques did not show loss of appetite [11]. In contrast, in the present study, all of the unvaccinated macaques lost their appetite and showed raised body temperature for more than 12 days. Therefore we believe that NL2586 is more pathogenic than VN1194 at least in cynomolgus macaques.

The increase of IgG antibodies in nasal swabs from vaccinated macaques after the virus challenge, which indicates the recall responses of antibodies, seems to be critical for preventing viral replication (Fig. 3), and indeed, antibodies recalled after the virus challenge showed HI activity against the challenge virus (Table 3). In general, IgA in mucosal tissue is thought to play an important role in protection against influenza virus infection [33]. However, IgA responses in nasal and tracheal swabs were not significant after subcutaneous inoculation of the Vac-2 vaccine with or without alum, but rather we observed an increase of IgG in the swabs (Fig. 2). Similarly, in our previous study, intranasal inoculation of the vaccine against H5N1 HPAIV showed no advantage compared with subcutaneous inoculation in protection against H5N1 HPAIV in cynomolgus macaques [11]. Therefore, mucosal vaccines for increasing IgA in the mucosal tissues may not be essential for protection against influenza virus infection, at least in the macaque model.

We investigated the effect of alum in combination with whole virus particle vaccines in the present study. We found that the addition of alum enabled maintenance of the recall responses of $CD8^+$ T cells against Vac-2 antigens in cynomolgus macaques (Fig. 4). Similarly, CTL responses against simian human immunodeficiency virus (SHIV) and human papilloma virus (HPV) antigens have been reported in non-human primates immunized with vaccines and alum [34,35]. However, it is generally considered that alum stimulates Th2 responses and enhances antibody production but not CTL responses [36,37]. Indeed, we confirmed that the whole virus particle vaccine of H3N2 and H5N1 virus with alum did not elicit CTL responses in mice (unpublished observations). This suggests some different mechanisms in cynomolgus macaques and mice for activating dendritic cells and for priming CTL through the pathogen-associated molecular pattern (PAMP) sensory system stimulated by alum. Recently, Nalp3 has been reported to recognize uric acid produced by inoculation of alum in mice and human cells [24–26]. However, it is still not clear that CTL responses are not elicited by alum in humans [22,38,39], because involvement of Nalp3 has only been shown *in vitro*. Further studies are needed to reveal activation of the Nalp3 inflammasome in humans and non-human primates [40].

In the present study, we established a non-human primate model for assessing vaccine efficacy against H7N7 virus. We found that the vaccine, Vac-2 effectively reduced replication of H7N7 HPAIV in the upper and lower respiratory tracts. Vac-2 is thus one of the candidates for vaccines against H7 HPAIVs. Together with the previous report in which an H5N1 vaccine seed in the virus library protected against H5N1 HPAIV replication [11], this indicates that the virus library would be a highly useful resource to provide early access to crossprotective vaccine seeds in the onset of a pandemic.

Acknowledgments

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References

[1] Belser JA, Lu X, Maines TR, Smith C, Li Y, Donis RO, et al. Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. J Virol 2007;81(20):11139–47.
[2] Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K. Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. J Virol 2007;81(19):10558–66.
[3] Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101(5):1356–61.
[4] van Beest Holle MDR, Meijer A, Koopmans M, de Jager CM, van de Kamp EEHM, Wilbrink B, et al. Human-to-human transmission of avian influenza A/H7N7, the Netherlands, 2003. Eurosurveiillance 2005;10(10–12):264–8.
[5] Hirst M, Astell CR, Griffith M, Coughlin SM, Moksa M, Zeng T, et al. Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg Infect Dis 2004;10(12):2192–5.
[6] Puzelli S, Di Trani L, Fabiani C, Campitelli L, De Marco MA, Capua I, et al. Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. J Infect Dis 2005;192(8):1318–22.
[7] Belser JA, Blixt O, Chen LM, Pappas C, Maines TR, Van Hoeven N, et al. Contemporary North American influenza H7 viruses possess human receptor specificity: implications for virus transmissibility. Proc Natl Acad Sci USA 2008;105(21):7558–63.
[8] de Wit E, Munster VJ, Spronken MI, Bestebroer TM, Baas C, Beyer WE, et al. Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. J Virol 2005;79(19):12401–7.
[9] Jadhao SJ, Achenbach J, Swayne DE, Donis R, Cox N, Matsuoka Y. Development of Eurasian H7N7/PR8 high growth reassortant virus for clinical evaluation as an inactivated pandemic influenza vaccine. Vaccine 2008;26(14):1742–50.
[10] Sakabe S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, et al. A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens. Vaccine 2008;26(17):2127–34.
[11] 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.
[12] 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(2):155–65.
[13] Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, et al. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73(4):3184–9.

[14] Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol 1999;73(7):5903–11.
[15] Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol 2002;76(9):4420–9.
[16] Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, et al. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol 2005;79(4):2191–8.
[17] Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol Cell Biol 1988;8(10):4518–23.
[18] 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.
[19] 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.
[20] Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM. Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clin Exp Immunol 1985;61(1):143–51.
[21] Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants—a balance between toxicity and adjuvanticity. Vaccine 1993;11(3):293–306.
[22] Lindblad EB. Aluminium compounds for use in vaccines. Immunol Cell Biol 2004;82(5):497–505.
[23] Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 2008;205(4):869–82.
[24] Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008;453(7198):1122–6.
[25] Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 2008;9(8):847–56.
[26] Li H, Willingham SB, Ting JP, Re F. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J Immunol 2008;181(1):17–21.
[27] Manzoor R, Sakoda Y, Sakabe S, Mochizuki T, Namba Y, Tsuda Y, et al. Development of a pen-site test kit for the rapid diagnosis of H7 highly pathogenic avian influenza. J Vet Med Sci 2008;70(6):557–62.
[28] Thomas MJ, Flanary LR, Brown BA, Katze MG, Baskin CR. Use of human nasal cannulas during bronchoscopy procedures as a simple method for maintaining adequate oxygen saturation in pigtailed macaques (*Macaca nemestrina*). J Am Assoc Lab Anim Sci 2006;45(4):44–8.
[29] 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.
[30] Yoden S, Kida H, Yanagawa R. An avian influenza virus of which infectivity is neutralized by antisera lacking hemagglutination-inhibition activity. Arch Virol 1982;74(2–3):205–10.
[31] Kida H, Yoden S, Kuwabara M, Yanagawa R. Interference with a conformational change in the haemagglutinin molecule of influenza virus by antibodies as a possible neutralization mechanism. Vaccine 1985;3:219–22.
[32] Hoffmann E, Lipatov AS, Webby RJ, Govorkova EA, Webster RG. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. Proc Natl Acad Sci USA 2005;102(36):12915–20.
[33] Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol 1986;24(1):157–60.
[34] Notka F, Stahl-Hennig C, Dittmer U, Wolf H, Wagner R. Construction and characterization of recombinant VLPs and Semliki-forest virus live vectors for comparative evaluation in the SHIV monkey model. Biol Chem 1999;380(3):341–52.
[35] Palker TJ, Monteiro JM, Martin MM, Kakareka C, Smith JF, Cook JC, et al. Antibody, cytokine and cytotoxic T lymphocyte responses in chimpanzees immunized with human papillomavirus virus-like particles. Vaccine 2001;19(27):3733–43.
[36] Speidel K, Osen W, Faath S, Hilgert I, Obst R, Braspenning J, et al. Priming of cytotoxic T lymphocytes by five heat-aggregated antigens in vivo: conditions, efficiency, and relation to antibody responses. Eur J Immunol 1997;27(9):2391–9.
[37] Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 2008;26(19):2350–9.
[38] Perales MA, Schwartz DH, Fabry JA, Lieberman J. A vaccinia-gp160-based vaccine but not a gp160 protein vaccine elicits anti-gp160 cytotoxic T lymphocytes in some HIV-1 seronegative vaccinees. J Acquir Immune Defic Syndr Hum Retrovirol 1995;10(1):27–35.
[39] Benson EM, Clarkson J, Law M, Marshall P, Kelleher AD, Smith DE, et al. Therapeutic vaccination with p24-VLP and zidovudine augments HIV-specific cytotoxic T lymphocyte activity in asymptomatic HIV-infected individuals. AIDS Res Hum Retroviruses 1999;15(2):105–13.
[40] Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 2006;440(7081):237–41.

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

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Abstract

Influenza A viruses cause recurrent outbreaks of local or global scale with potentially severe consequences for human health and the global economy. Recently, a new strain of influenza A virus was detected that causes disease in and transmits among humans, probably owing to little or no pre-existing immunity to the new strain. On June 11, 2009, the WHO declared that the infections caused by the new strain had reached pandemic proportion. Characterized as an influenza A virus of the H1N1 subtype, the genomic segments of the new strain were most closely related to swine viruses[1]. Most human infections with swine-origin H1N1 influenza viruses (S-OIVs) appear to be mild; however, more than 50% of hospitalized individuals do not have underlying health issues, attesting to the pathogenic potential of S-OIVs. To better assess the risk posed by the new virus, we characterized one of the first US S-OIV isolates, A/California/04/09 (H1N1; CA04), as well as several other S-OIV isolates, *in vitro* and *in vivo*. In mice and ferrets, CA04 and other S-OIV isolates tested replicate more efficiently than a currently circulating human H1N1 virus. In addition, CA04 replicates efficiently in nonhuman primates, causes more severe pathologic lesions in the lungs of infected mice, ferrets, and nonhuman primates than a currently circulating human H1N1 virus, and transmits among ferrets. In specific-pathogen free miniature pigs, CA04 replicates without clinical symptoms. The assessment of human sera from different age groups suggests that infection with human H1N1 viruses antigenically closely related to viruses circulating in 1918 confers neutralizing antibody activity to CA04. Finally, we show that CA04 is sensitive to approved and experimental antiviral drugs, suggesting these compounds as a first line of defence against the recently declared S-OIV pandemic.

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

In MDCK cells and primary human airway epithelial cells, CA04 grew to titres comparable to those typically obtained for contemporary human H1N1 influenza viruses (Supplementary Fig. S1). Confocal, transmission electron, and scanning electron microscopy revealed virions of remarkably filamentous shape (Supplementary Fig. S2), in marked contrast to the spherical shape observed with negatively stained virions (http://www.cdc.gov/h1n1flu/images.htm). The biological significance of the morphology of CA04 remains unknown.

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To evaluate the pathogenicity of S-OIV in mammalian models, we conducted studies in mice, ferrets, nonhuman primates, and pigs. BALB/c mice intranasally infected with a high dose ($>10^4$ plaque-forming units [PFU]) of CA04 (Supplementary Fig. S3) experienced weight loss and those infected with the highest dose of this virus were humanely euthanized, in contrast to animals infected with a recent human H1N1 virus (A/Kawasaki/UTK-4/09, KUTK-4). The 50% mouse lethal dose (MLD_{50}) was $10^{5.8}$ plaque-forming units (PFU) for CA04 and $>10^{6.6}$ PFU for KUTK-4. For the additional S-OIV isolates tested, the MLD_{50} values were $>10^{6.4}$ PFU for Osaka164, $>10^{6.6}$ PFU for WSLH049, $10^{4.5}$ PFU for WSLH34939, and $>10^{5.8}$ PFU for Net603.

On day 3 post infection (pi) of mice, similar titres were detected in nasal turbinates of mice infected with 10^5 PFU of S-OIVs or KUTK-4 (Supplementary Table 1); however, S-OIVs replicated more efficiently in the lungs of infected animals, which may account for the prominent bronchitis and alveolitis with viral antigen on day 3 postinfection with CA04 (Supplementary Fig. S4a–b). On day 6 pi, virus titres followed a similar trend and the lungs of CA04-infected mice showed a bronchoaveolitis with viral antigen, although signs of regeneration were apparent (Supplementary Fig. S4c). We detected viral antigen-positive bronchial epithelial, but not alveolar, cells on day 3 pi of mice infected with KUTK-4 (Supplementary Fig. S4e). By day 6, infection in KUTK-4-inoculated mice had progressed to a bronchitis and peribronchitis; however, viral antigen was rarely detected in these lesions (Supplementary Fig. S4f).

There were marked differences in the induction of pro-inflammatory cytokines in the lungs of mice infected with CA04 versus KUTK-4 (Supplementary Fig. S5a–c). Infection with KUTK-4 resulted in limited induction of pro-inflammatory cytokines/chemokines in the lungs, in striking contrast to infection with CA04. Increased production of IL-10 (Supplementary Fig. S5a) in lungs of CA04-infected mice at day 6 pi likely reflects a host response to dampen over-exuberant pulmonary inflammation and promote tissue repair. Infection with CA04 led to strong induction of both IFNγ and IL-4 in the lungs. The selective induction of the T_H2 cytokine IL-5 in CA04- but not in KUTK-4-infected mice on day 6 pi is noteworthy (Supplementary Fig. S5b), but further studies are needed to understand the relevance of this finding to viral control. IL-17 has been reported to play a role in protection against lethal influenza and also in eliciting inflammatory responses[4,5]. However, the enhanced viral replication and lung pathology observed in CA04-infected mice was not linked to dysregulated IL-17 production.

Cynomolgus macaques (*Macaca fascicularis*) have been used to study highly pathogenic avian H5N1 viruses[6,7] and the 1918 pandemic virus[8]. Infection of cynomolgus macaques with CA04 (see 'Methods' section for detailed procedures) resulted in a more prominent increase in body temperature than infection with KUTK-4 (Supplementary Fig. S6). This difference might originate from the observed differences in virus titres (Table 1 and Supplementary Table 2). No remarkable difference in body weight loss was found between the two groups (data not shown). CA04 replicated efficiently in the lungs and other respiratory organs of infected animals, similar to highly pathogenic influenza viruses[6,8] (Table 1). By contrast, conventional human influenza viruses are typically limited in their replicative ability in the lungs of infected primates[6,8] (Table 1), although a seasonal H1N1 virus was isolated from one animal on day 7 pi. Pathologic examination revealed that CA04 caused more severe lung lesions than did KUTK-4 (Fig. 1 and Supplementary Fig. S7). On day 3 pi with CA04, alveolar spaces were occupied by edematous exudate and inflammatory infiltrates (Fig. 1a); severe thickening of alveolar walls was also observed (Fig. 1b). Viral antigen-positive cells were distributed in the inflammatory lesions, and many of these cells were elongated with thin cytoplasm and hemming around the alveolar wall, indicating type I pneumocytes (Fig. 1c). In addition to type I pneumocytes, CA04 viral antigens were also detected in considerable numbers of cuboidal, cytokeratin-positive cells, hence identified as type II pneumocytes (Fig. 1d, and Supplementary

Fig. S8), as has been reported for highly pathogenic avian H5N1 influenza viruses[6]. Upon infection with KUTK-4, large sections of infected lungs showed thickening of the alveolar wall on day 3 pi (Fig. 1e). Although the infiltration of inflammatory cells was prominent at the alveolar wall (Fig. 1f), viral antigens were sparse and detected in type I (but not type II) pneumocytes (Fig. 1g). By contrast, the lungs of noninfected animals show clear alveolar spaces (Fig. 1h).

On day 7 pi, lung pathology remained more severe for CA04- than for KUTK-4-infected lungs (Supplementary Fig. S7), although regenerative changes were seen for CA04. Nonetheless, considerable numbers of antigen-positive cells were still detectable (Supplementary Fig. S7c). Collectively, these findings demonstrate that CA04 causes more severe lung lesions in nonhuman primates than does a contemporary human influenza virus.

Induction of pro-inflammatory cytokines/chemokines in the lungs of CA04-infected macaques was variable at day 3 pi (Supplementary Fig. S9). However, consistent with persisting lung pathology and inflammation on day 7 pi, the levels of MCP-1, MIP-1α, IL-6, and IL-18 were markedly higher in the lungs of 2 of 3 CA04-infected macaques.

Ferrets are widely accepted as a suitable small-animal model for influenza virus pathogenicity and transmissibility studies. Infection of ferrets with S-OIVs or KUTK-4 did not cause marked changes in body temperature or weight in any group (data not shown). Although all test viruses were detected in nasal turbinates at similar titres on day 3 pi (Supplementary Table 3), S-OIVs replicated to higher titres in trachea and lungs.

Pathological examination detected similar levels of viral antigen in the nasal mucosa of both CA04- and KUTK-4-infected ferrets (Supplementary Fig. S10a and e). However, the lungs of CA04-infected ferrets showed more severe bronchopneumonia with prominent viral antigen expression in the peribronchial glands and a few alveolar cells (Supplementary Fig. S10b-d) on day 3 pi. By contrast, most of the lung appeared normal following infection with KUTK-4 (Supplementary Fig. S10f and g). Thus, in all three mammalian models tested, CA04 appeared to be more pathogenic than a contemporary human H1N1 virus, KUTK-4.

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

Genetic analysis suggests that S-OIV originated in pigs[1]. However, there were no confirmed influenza virus outbreaks in Central American pigs prior to the reported S-OIV infections in humans. To assess S-OIV replication in pigs, we inoculated specific-pathogen free miniature pigs, which are easier to manage, with CA04 or a classical swine influenza virus (A/swine/Hokkaido/2/81, H1N1). No signs of disease were observed (data not shown), although both viruses replicated efficiently in the respiratory organs of these animals (Supplementary Tables 5 and 6). Slightly higher titres of CA04 were detected in lungs on day 3 pi, which is supported by pathological findings that show more apparent bronchitis and bronchiolitis in pigs infected with CA04 (Supplementary Fig. S11). The asymptomatic infection of CA04, despite efficient virus replication, might explain the lack of reports of S-OIV outbreaks in pigs prior to virus transmission to humans.

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

A recent report suggested that 33% of individuals over 60 years of age had neutralizing antibodies to CA04 (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5819a1.htm:MMWR), likely due to previous exposure to antigenically similar H1N1 viruses. In fact, both the human H1N1 viruses that circulated until 1957 and the classical swine virus HA gene of S-OIVs are descendants of the 1918 pandemic virus, possibly explaining their antigenic relatedness. In 1977, H1N1 viruses re-emerged that were genetically and antigenically very closely related to viruses circulating in the 1950s[13] and should thus have elicited neutralizing antibodies to CA04 among younger age groups; however, this does not appear to be the case, according to the above described report. To resolve this puzzling finding, we assessed the neutralizing activities of sera collected from a broad range of age groups against CA04 and KUTK-4. We used two sets of donor sera, collected in 1999 from residents and workers in a nursing home (donor set #1), and in April of 2009 from workers and patients in a hospital (donor set #2). High neutralizing activity against KUTK-4 was detected for many sera in donor set #2 (Fig. 3), but not for sera in donor set #1, likely because these sera were collected prior to the emergence of the current human H1N1 viruses. Interestingly, with few exceptions, no appreciable neutralizing antibodies against CA04 were found for individuals born after 1920; however, many of those born before 1918 had high neutralizing antibody titres (individual neutralizing antibody titres are shown in Supplementary Table 9). These data indicate that infection with the 1918 pandemic virus or closely related human H1N1 viruses, but not infection with antigenically divergent human H1N1 viruses circulating in the 1920s to 1950s, and again since 1977, elicited neutralizing antibodies to S-OIVs.

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

Methods Summary

Viruses and cells

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

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

Animals

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

Antiviral sensitivity of viruses in mice

Five-week-old female BALB/c mice (Japan SLC Inc., Shizuoka, Japan; groups of six) were anesthetized with sevoflurane and intranasally inoculated with 10^4 plaque-forming units (volume: 50 μl) of CA04, KUTK-4, or A/Kawasaki/UTK-23/08 (H1N1). At 1 h pi, mice were administered antiviral compounds as described in detail in the `Methods' section. Three mice per group were euthanized on days 3 or 6 pi and the virus titres in lungs were determined by plaque assays in MDCK cells.

Methods

Viruses

A/California/04/09 (H1N1; CA04) was kindly provided by the Centers for Disease Control (CDC). A/Wisconsin/WSLH049/09 (H1N1) was isolated from a patient with mild symptoms, while A/Wisconsin/WSLH34939/09 (H1N1) was isolated from a hospitalized patient. A/Netherlands/603/09 (H1N1) was isolated from a patient with mild symptoms and was kindly provided by Ron Fouchier (Erasmus University, Rotterdam, The Netherlands). A/Osaka/164/09 (H1N1) was also isolated from a patient with mild symptoms.

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

of Agriculture, or in BSL3 containment laboratories at the University of Tokyo (Tokyo, Japan), the Shiga University of Medical Science (Shiga, Japan), or the Hokkaido University (Sapporo, Japan), all of which are approved for such use by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Viral pathogenesis in mice—Six-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. Baseline body weights were measured prior to infection. Three mice per group were anesthetized with isoflurane and intranasally inoculated with 10^2, 10^3, 10^4, or 10^5 PFU (50 μl) of CA04 and KUTK-4, or undiluted virus from virus stocks (CA04; $10^{6.5}$ PFU, KUTK-4; $10^{6.6}$ PFU). Body weight and survival were monitored daily for 14 days and mice with body weight loss of more than 25% of pre-infection values were euthanized. For virologic and pathologic examinations, 6 mice per group were intranasally infected with 10^5 PFU of S-OIVs and KUTK-4 and 3 mice per group were euthanized on days 3 and 6 pi. The virus titres in various organs were determined by plaque assays in MDCK cells.

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

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

Experimental infection of ferrets—We used five-to-eight-month-old male ferrets (Marshall Farms, Wolcott, NY and Triple F Farms, Sayre, PA), which were serologically negative by hemagglutination inhibition (HI) assay for currently circulating human influenza viruses. Baseline body temperatures and body weights were established by one or two measurements prior to infection. Six ferrets per group were intramuscularly anesthetized with ketamine and xylazine (5 mg and 0.5 mg per kg of body weight, respectively) and intranasally