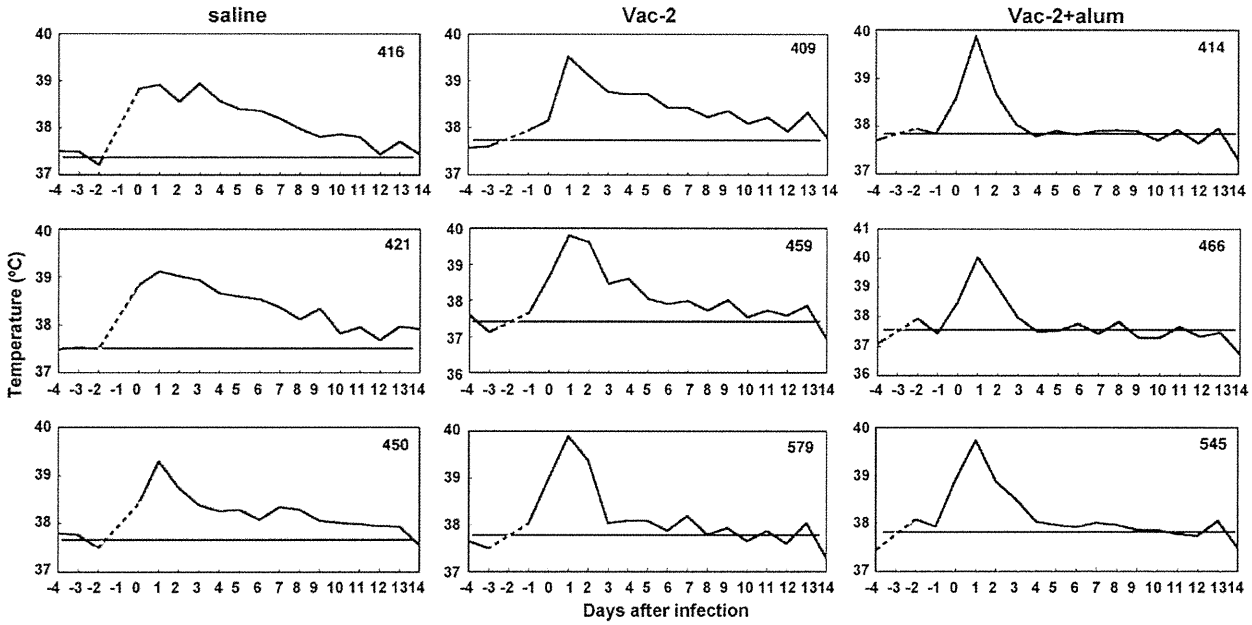


Fig. 5. Body temperatures of macaques after inoculation of H7N7 HPAIV (NL2586). Cynomolgus macaques were subcutaneously immunized with Vac-2 alone (middle column) or with Vac-2 and alum (right column). On day 0, 10 weeks after the second vaccination, NL2586 was inoculated onto conjunctivas and into nasal 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.

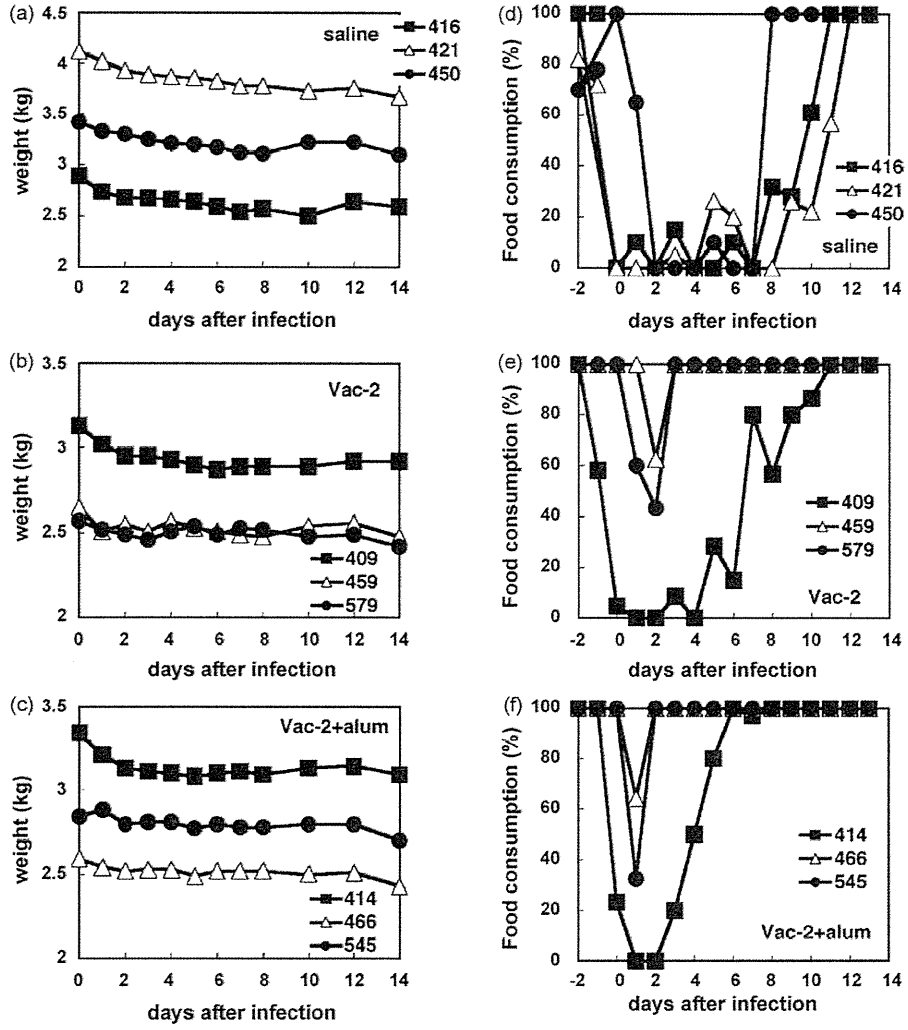


Fig. 6. Body weight and food consumption of cynomolgus macaques inoculated with NL2586. Body weight (a–c) and appetite (d–f) was measured on the indicated days. The average duration of appetite loss was 10, 4.67, and 2.67 days in macaques inoculated with saline, Vac-2 alone, and Vac-2 with alum, respectively. *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

This study was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT Japan. We thank Dr. Ilaria Capua for providing NL2586, Dr. Akira Yokoe for his help in autopsy preparation, Drs. Kunio Ishibashi, Norio Okahara, and Takahiro Nakagawa for animal care, and Drs. Piers Vigers and Reiko Aiura for careful reading of the manuscript.

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.

Published in final edited form as:
Nature. 2009 August 20; 460(7258): 1021–1025. doi:10.1038/nature08260.

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

Yasushi Itoh[1,*], **Kyoko Shinya**[2,*], **Maki Kiso**[3,*], **Tokiko Watanabe**[4,*], **Yoshihiro Sakoda**[5,*], **Masato Hatta**[4,*], **Yukiko Muramoto**[3,*], **Daisuke Tamura**[3], **Yuko Sakai-Tagawa**[3], **Takeshi Noda**[6], **Saori Sakabe**[3], **Masaki Imai**[4], **Yasuko Hatta**[4], **Shinji Watanabe**[4], **Chengjun Li**[4], **Shinya Yamada**[3], **Ken Fujii**[3], **Shin Murakami**[3], **Hirotaka Imai**[3], **Satoshi Kakugawa**[3], **Mutsumi Ito**[3], **Ryo Takano**[3], **Kiyoko Iwatsuki-Horimoto**[3], **Masayuki Shimojima**[3], **Taisuke Horimoto**[3], **Hideo Goto**[3], **Kei Takahashi**[3], **Akiko Makino**[2], **Hirohito Ishigaki**[1], **Misako Nakayama**[1], **Masatoshi Okamatsu**[5], **Kazuo Takahashi**[7], **David Warshauer**[8], **Peter A. Shult**[8], **Reiko Saito**[9], **Hiroshi Suzuki**[9], **Yousuke Furuta**[10], **Makoto Yamashita**[11], **Keiko Mitamura**[12], **Kunio Nakano**[12], **Morio Nakamura**[12], **Rebecca Brockman-Schneider**[13], **Hiroshi Mitamura**[14], **Masahiko Yamazaki**[15], **Norio Sugaya**[16], **M. Suresh**[4], **Makoto Ozawa**[4], **Gabriele Neumann**[4], **James Gern**[12], **Hiroshi Kida**[5], **Kazumasa Ogasawara**[1], and **Yoshihiro Kawaoka**[2,3,4,6,17,18]

[1]Department of Pathology, Shiga University of Medical Science, Ohtsu, Shiga 520-2192, Japan

[2]Department of Microbiology and Infectious Diseases, Kobe University, Hyogo 650-0017, Japan

[3]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

[4]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI 53711, USA

[5]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[6]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

[7]Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka 537-0025 Japan

[8]Wisconsin State Laboratory of Hygiene, Madison, WI 53706, USA

[9]Department of Public Health, Niigata University, Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan

[10]Toyama Chemical Co., Ltd., Toyama 930-8508, Japan

[11]Daiichi Sankyo Co Ltd, Shinagawa, Tokyo 140-8710, Japan

*These authors contributed equally to this work'

Author Contributions Y.I., K.S., M.K., T.W., Y.S., M.H., Y.M., D.T., Y.S.T., T.N., M.I., S.W., K.I.H., T.H., N.S., H.K., K.O., and Y.K. designed the experiments; Y.I., K.S., M.K., T.W., Y.S., M.H., D.T., Y.S.T., T.N., S.S., M.I., Y.H., S.W., C.L., S.Y., K.F., S.M., H.I., S.K., M.I., R.T., K.I.H., M.S., T.H., K.T., A.M., H.I., M.N., M.O., K.T., D.W., P.A.S., R.S., H.S., Y.F., M.Y., K.M., K.N., M.N., R.B.S., J.G., H.M., and M.Y. performed the experiments; Y.I., K.S., M.K., T.W., Y.S., M.H., Y.M., Y.S.T., T.N., M.I., S.W., C.L., S.Y., K.I.H., T.H., H.G., M.S., M.O., G.N., H.K., K.O., and Y.K. analyzed data; Y.I., K.S., M.K., T.W., Y.S., M.H., Y.M., Y.S.T., T.N., M.I., K.I.H., M.S., M.O., G.N., K.O., and Y.K. wrote the manuscript.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

[12]Eiju General Hospital, Tokyo 110-8654, Japan

[13]School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53792, USA

[14]Department of Internal Medicine, Mitamura Clinic, Shizuoka 413-0103, Japan

[15]Department of Pediatrics, Zama Children's Clinic, Kanagawa 228-0023, Japan

[16]Keiyu Hospital, Kanagawa 220-0012, Japan

[17]Creative Research Initiative, Sousei, Hokkaido University, Sapporo 060-0818, Japan

[18]ERATO Infection-Induced Host Responses Project, Saitama 332-0012, Japan

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.

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

inoculated with 10^6 PFU (500 μl) of S-OIVs or KUTK-4. On days 3 and 6 pi, 3 ferrets per group were euthanized for virologic and pathologic examinations. The virus titres in nasal washes and various organs were determined by plaque assays in MDCK cells.

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

Pathologic examination

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

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

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

Antiviral sensitivity of viruses in mice

To test the antiviral sensitivity of viruses in mice, animals were infected as described in the `Method Summary' section and one hour later administered the following antiviral compounds: (i) oseltamivir phosphate: 8 or 80 mg/kg/400 μl (divided into two oral administrations per day) for 5 days; (ii) zanamivir: 0.8 or 8 mg/kg/50 μl in one daily intranasal administration for 5 days; (iii) CS-8958: 0.7 mg/kg/50 μl in one intranasal administration; (iv) T-705: 60 or 300 mg/kg/400 μl (divided into two oral administrations per day) for 5 days; (v) or distilled water

orally (200 μl) and PBS intranasally (50 μl). Three mice per group were euthanized on days 3 or 6 pi and the virus titres in lungs were determined by plaque assays in MDCK cells.

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

Neuraminidase inhibition assay—To assess the sensitivity of viruses to neuraminidase inhibitors (i.e., oseltamivir, zanamivir, and CS-8958), neuraminidase inhibition assays were performed as described previously[20]. Briefly, diluted viruses were mixed with various concentrations of oseltamivir carboxylate, zanamivir, or R-125489 in 2-(N- morpholino) ethanesulfonic acid containing calcium chloride, and incubated for 30 min at 37°C. Then, we added methylumbelliferyl-N-acetylneuraminic acid (Sigma, St Louis, MO) as a fluorescent substrate to this mixture. After incubation for one hour at 37°C, sodium hydroxide in 80% ethanol was added to the mixture to stop the reaction. The fluorescence of the solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm and the 50% inhibitory concentration (IC_{50}) was calculated.

Neutralization assay with human sera—Human sera were collected in 1999 or 2009 from donor group #1 (age range: 50 to 112 years as of 2009, mean = 92.7 ± 15.0 years) or #2 (age range: 20 to 68 years as of 2009, mean = 48.2 ± 23.7 years), respectively. These sera were treated with receptor-destroying enzyme (DENKA SEIKEN CO., LTD, Tokyo, Japan) to remove inhibitors of influenza virus replication. One hundred $TCID_{50}$ (50% tissue culture infectious dose) of CA04 and KUTK-4 were pre-incubated with twofold serial dilutions of treated sera, incubated for 30 min on MDCK cells, which were then observed for cytopathic effects to determine the neutralizing activity of the test sera. Our research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo (approval numbers; 21-6-0428 for donor group #1, 21-7-0529 for donor group #2).

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

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

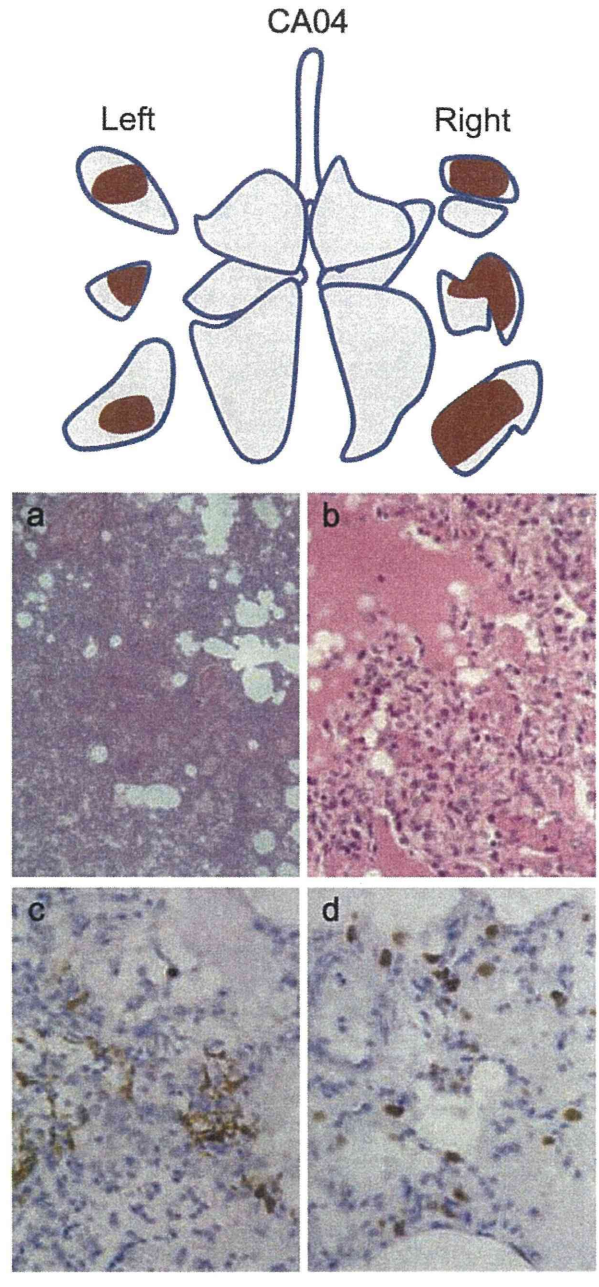
Acknowledgements

We thank the Centers for Disease Control (CDC) for A/California/04/09 virus and Ron Fouchier (Erasmus University, Rotterdam, The Netherlands) for A/Netherlands/603/09 virus. We thank Krisna Wells for editing the manuscript, Martha McGregor, Rebecca Moritz, Anthony Hanson, Hideaki Ishida, Hideaki Tsuchiya, Ryuzo Torii, Naoki Yamamoto, Kosuke Soda, Naoki Nomura, and Hiromi Yoshida for excellent technical assistance. We also thank Takashi Umemura, Yuji Sunden, and Tomohisa Tanaka for pathological analyses of virus-infected pigs. This work was supported by National Institute of Allergy and Infectious Diseases Public Health Service research grants; by an NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C), by Grant-in-Aid for Specially Promoted Research, by a contract research fund for the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, and by grants-in-aid from the Ministry of Health and by ERATO (Japan Science and Technology Agency). G.N. is named as co-inventor on several patents about influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals. Y.K. is named as inventor/co-inventor on several patents about influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals.

Reference List

1. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. N. Engl. J Med. 2009
2. Kawaoka Y, Webster RG. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A 1988;85:324–8. [PubMed: 2829180]
3. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular Basis for High Virulence of Hong Kong H5N1 Influenza A Viruses. Science 2001;293:1840–1842. [PubMed: 11546875]
4. Iwakura Y, Nakae S, Saijo S, Ishigame H. The roles of IL-17A in inflammatory immune responses and host defense against pathogens. Immunol Rev 2008;226:57–79. [PubMed: 19161416]
5. Hamada H, et al. Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. J Immunol 2009;182:3469–3481. [PubMed: 19265125]
6. Baskin CR, et al. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. Proc. Natl. Acad. Sci U. S. A 2009;106:3455–3460. [PubMed: 19218453]
7. Rimmelzwaan GF, et al. Pathogenesis of influenza A (H5N1) virus infection in a primate model. J Virol 2001;75:6687–91. [PubMed: 11413336]
8. Kobasa D, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;445:319–323. [PubMed: 17230189]
9. Maines TR, et al. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc. Natl. Acad. Sci U. S. A 2006;103:12121–12126. [PubMed: 16880383]
10. Yamashita M, et al. CS-8958, a prodrug of the new neuraminidase inhibitor R-125489, shows long-acting anti-influenza virus activity. Antimicrob. Agents Chemother 2009;53:186–192. [PubMed: 18955520]
11. Furuta Y, et al. In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob. Agents Chemother 2002;46:977–981. [PubMed: 11897578]
12. Hayden FG, et al. Inhaled zanamivir for the prevention of influenza in families. Zanamivir Family Study Group. N. Engl. J Med 2000;343:1282–1289. [PubMed: 11058672]
13. Nakajima K, Desselberger U, Palese P. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. Nature 1978;274:334–339. [PubMed: 672956]
14. Peiris JS, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617–619. [PubMed: 14987888]
15. Wright, PF.; Neumann, G.; Kawaoka, Y. Fields Virology. Knipe, DM., et al., editors. Wolters Kluwer, Lippincott Willams & Wilkins; Philadelphia: 2007. p. 1691-1740.
16. Moscona A. Global transmission of oseltamivir-resistant influenza. N. Engl. J Med 2009;360:953–956. [PubMed: 19258250]
17. Hatakeyama S, et al. Enhanced expression of an alpha2,6-linked sialic acid on MDCK cells improves isolation of human influenza viruses and evaluation of their sensitivity to a neuraminidase inhibitor. J Clin Microbiol 2005;43:4139–4146. [PubMed: 16081961]

18. Jakiela B, Brockman-Schneider R, Amineva S, Lee WM, Gern JE. Basal cells of differentiated bronchial epithelium are more susceptible to rhinovirus infection. Am J Respir. Cell Mol. Biol 2008;38:517–523. [PubMed: 18063839]
19. Noda T, et al. Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature 2006;439:490–492. [PubMed: 16437116]
20. Kiso M, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004;364:759–765. [PubMed: 15337401]
21. Neumann G, et al. Ebola virus VP40 late domains are not essential for viral replication in cell culture. J Virol 2005;79:10300–10307. [PubMed: 16051823]



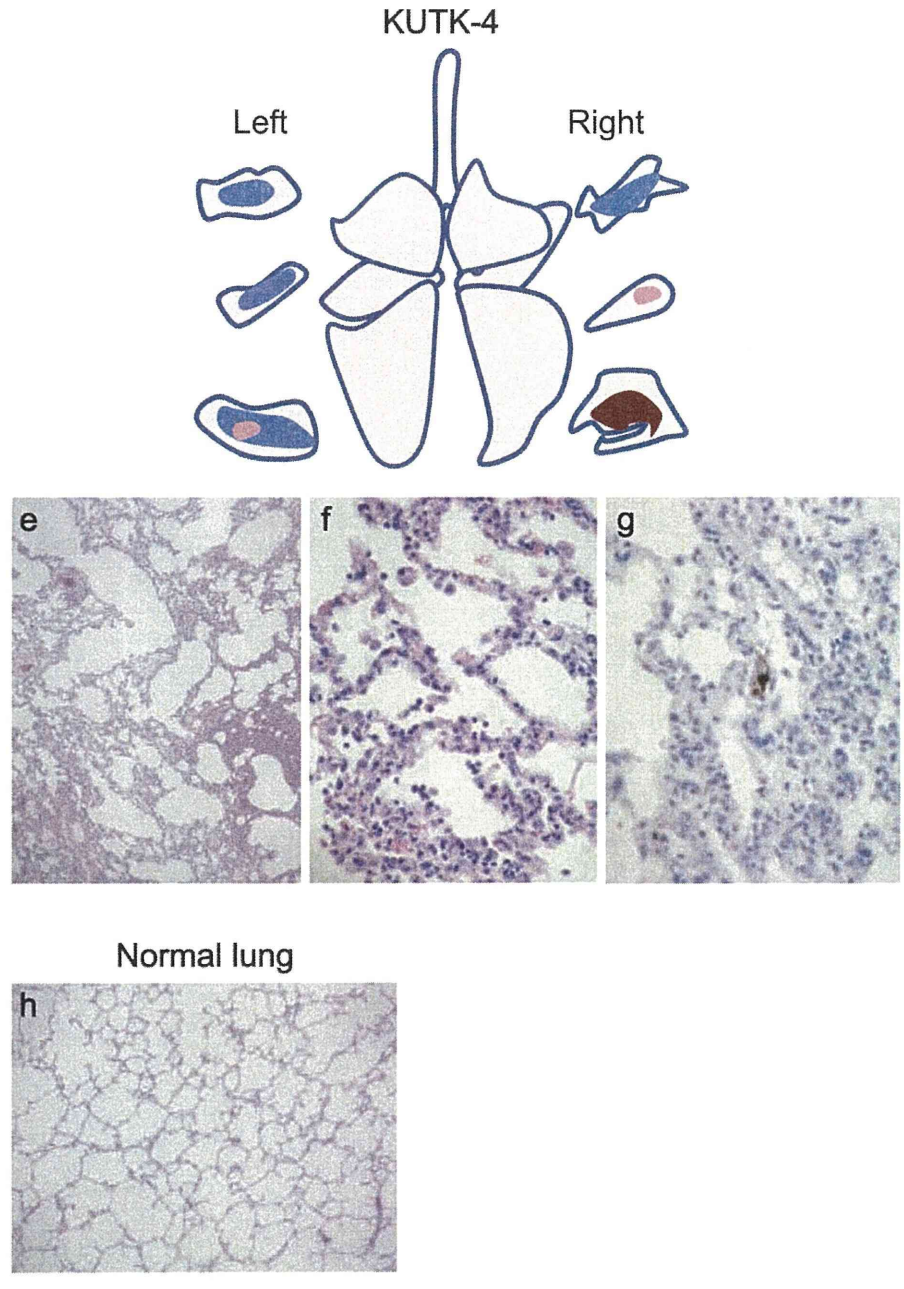


Figure 1. Pathologic examination of the lungs of infected cynomolgus macaques

Representative pathologic images of CA04- (macaque #1, **a-d**), KUTK-4- (macaque #7, **e-g**), and mock- (**h**) infected lungs on day 3 pi. One or two sections per lung lobe were examined; representative findings are shown to depict the distribution of lesions in the sections (shown as cross sections placed next to illustrations of each lung lobe), with or without viral antigen, as follows: brown, severe lung lesion containing moderate to many viral antigen-positive cells; pink, mild lung lesions containing a few viral antigen-positive cells; blue, lung lesions with alveolar wall thickening, with remaining air spaces unaffected.

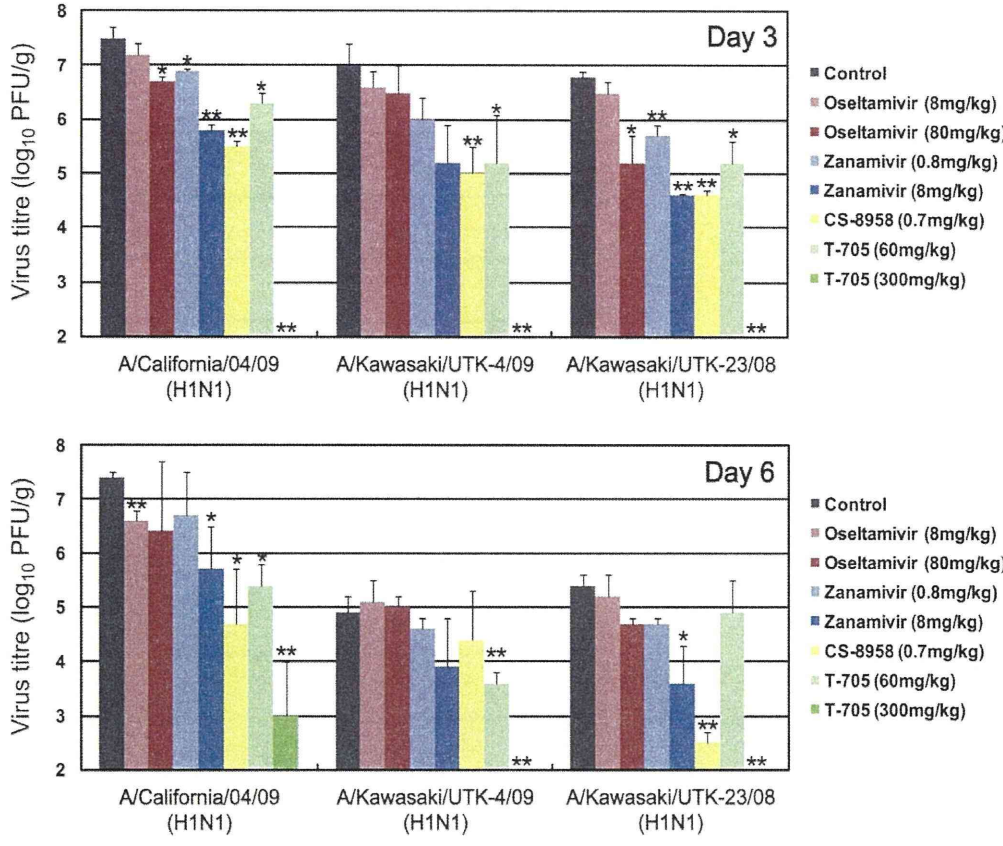


Figure 2. CA04 sensitivity to antiviral compounds in mice

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

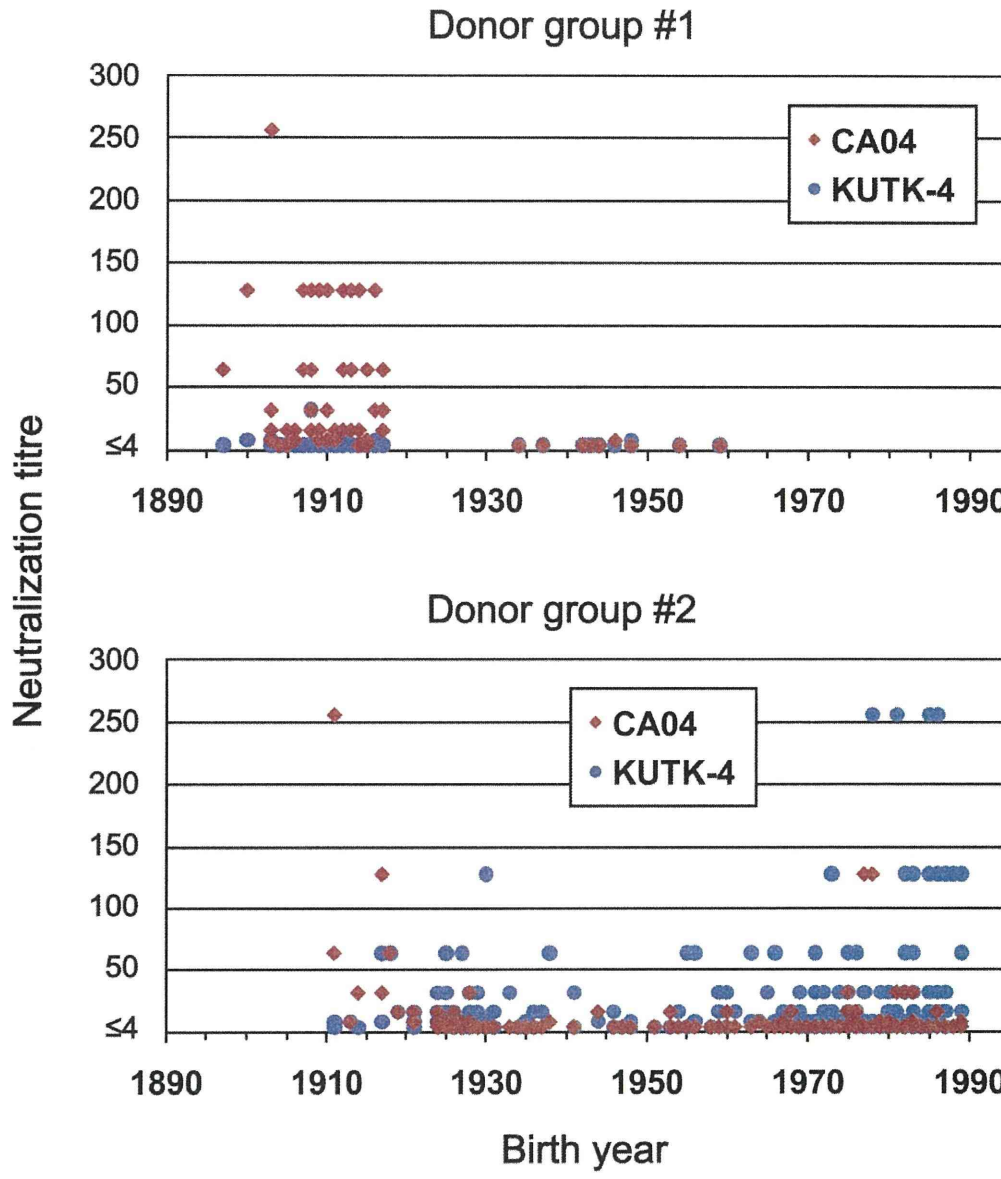


Figure 3. Neutralization activities in human sera against viruses

Human sera of donor groups #1 (collected in 1999) and #2 (collected in April and May of 2009) were subjected to neutralization assays with CA04 and KUTK-4. Since the sera of donor group #1 were collected in 1999, little neutralization activity was expected against KUTK-4, which was isolated in 2009.

Table 1

Virus titres in organs of infected cynomolgus macaques[a]

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

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

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

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