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

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

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

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

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

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

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

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ORIGINAL ARTICLE

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

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Abstract

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

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

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

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

Introduction

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

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

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

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

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

Materials and methods

Viruses

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

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

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Bacteria

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

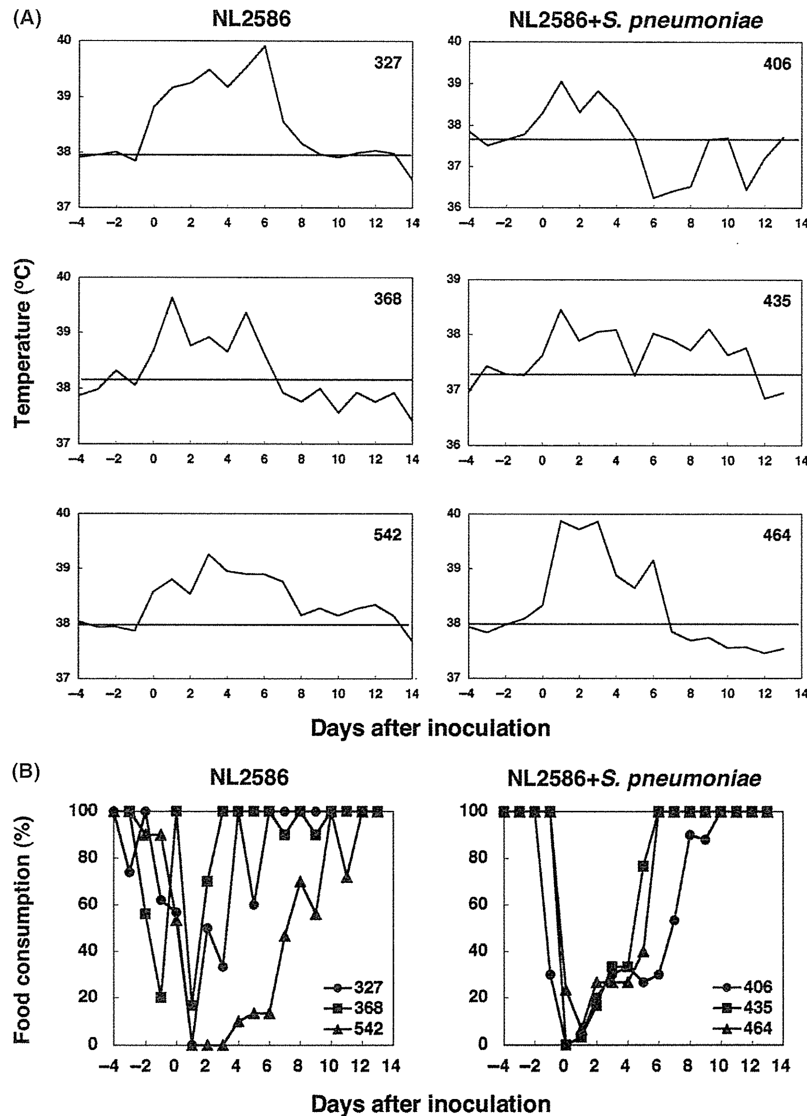

Fig. 1 Body temperatures and food consumption of macaques after inoculation of H7N7 HPAIV (NL2586) with or without *Streptococcus pneumoniae*. NL2586 (4×10^7 $TCID_{50}$) was inoculated onto conjunctivas and into nasal cavities and tracheas with *S. pneumoniae* (1.2×10^9 CFU) into tracheas of cynomolgus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

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

Animals

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

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

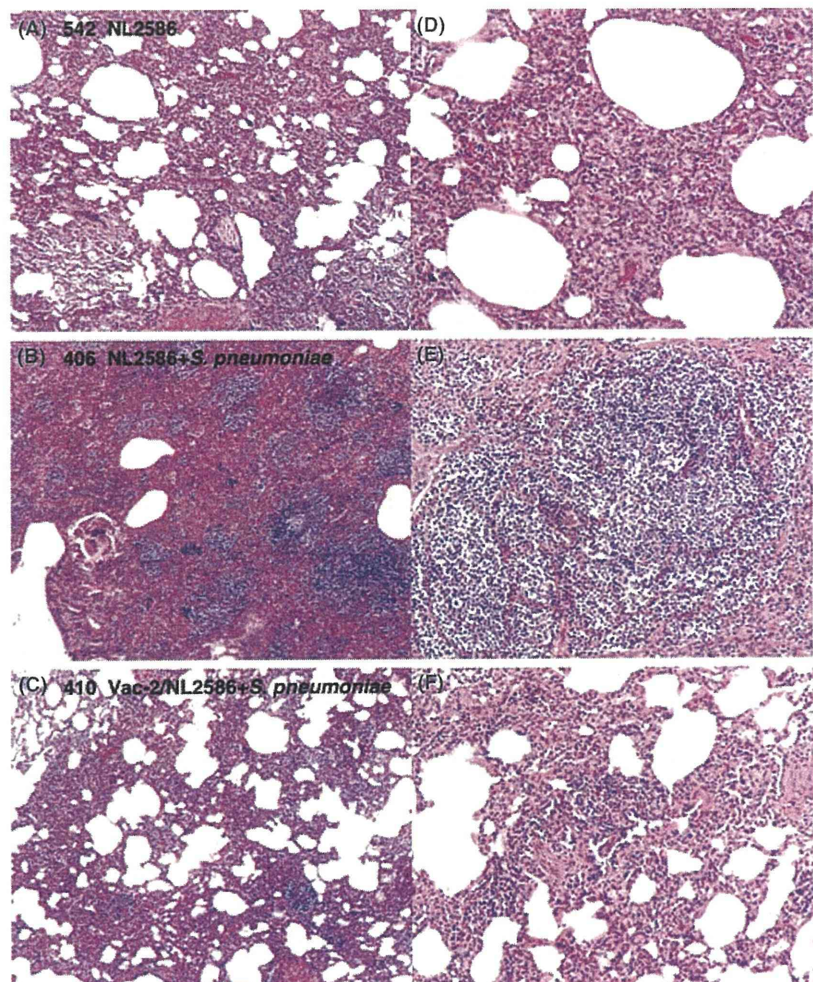

Fig. 2 Lung histology of cynomolgus macaques inoculated with NL2586 with or without *Streptococcus pneumoniae*. Cynomolgus macaques were subcutaneously vaccinated with inactivated whole particles of Vac-2 twice. Seven weeks after the first vaccination (5 weeks after the second vaccination), the macaques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

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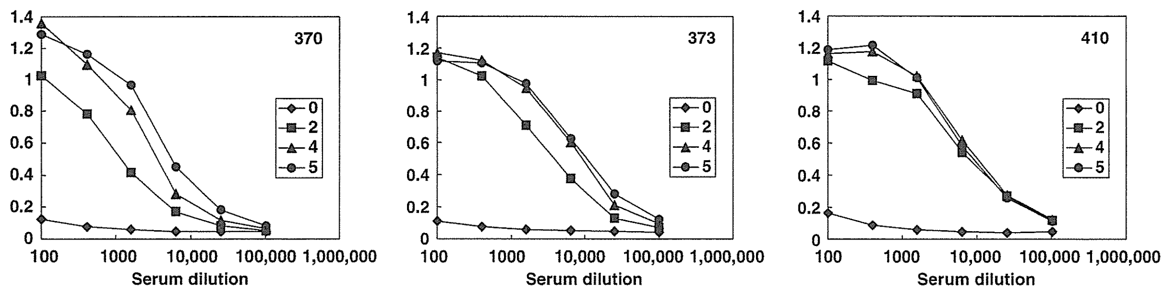

Fig. 3 Antibody responses specific for H7N7 vaccine antigens in cynomolgus macaques immunized with whole virus particle vaccines. Cynomolgus macaques were subcutaneously inoculated with whole virus particle vaccines of Vac-2 (1 mg/dose) with alum twice with a 2-week interval between injections. Sera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

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

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

Histological examination

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

Enzyme-linked immunosorbent assays

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

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

Virus neutralization assay

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

Results

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

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

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Table 1 *Streptococcus pneumoniae* titers in bronchial swabs

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

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

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

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

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

Table 2 *Streptococcus pneumoniae* titers in lungs at autopsy

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

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

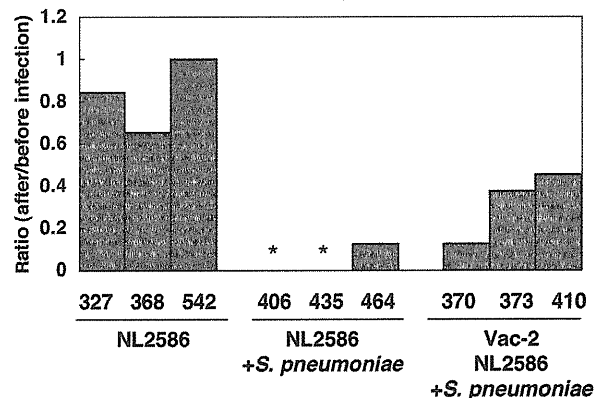

Fig. 4 IL-10 secretions in nasal swab samples. The levels of IL-10 in the samples were analyzed by ELISA. Nasal swabs were collected on day 0 before inoculation and day 8 after inoculation with NL2586 with or without *Streptococcus pneumoniae* as described in Table 3. The levels on day 8 were compared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

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(pneumonia) was observed in lungs of macaques infected with both NL2586 and *S. pneumoniae* (Fig. 2B,E). It was likely that acute-phase inflammation with neutrophils against *S. pneumoniae* disappeared following lymphocyte infiltration (i.e. tissue-repairing phase) 2 weeks after the simultaneous infection.

The above findings indicated that simultaneous infection resulted in histopathological pneumonitis and pneumonia but did not result in enhanced morbidity in the macaques. Thus, we histologically examined the efficacy of a vaccine, Vac-2, against H7N7 HPAIV for improving the bacterial pneumonia. Macaques were subcutaneously immunized twice with inactivated Vac-2, and subsequent increase in vaccine antigen-specific IgG in sera was confirmed (Fig. 3). Five weeks after the second vaccination, NL2586 and *S. pneumoniae* were inoculated into the macaques. Fourteen days after the challenge, lung tissue was examined at autopsy. The lungs of vaccinated macaques showed slight pneumonitis, but only weak pneumonia was seen in the lungs even after infection with both NL2586 and *S. pneumoniae* (Fig. 2C,F). Thus, we concluded that the vaccine against H7N7 HPAIV prevented severe bacterial pneumonia.

Bacterial growth in the bronchi and lungs of cynomolgus macaques infected with NL2586

We also examined bacterial growth in the bronchi and lungs of macaques simultaneously infected with NL2586 and *S. pneumoniae*. Bacterial colonies were detected in the bronchi of macaques 435 and 464 until day 4 after inoculation (Table 1), whereas bacterial colonies were observed in the lungs of all three macaques on day 14 (Table 2). On the other hand, bacterial colonies were decreased in the lungs of vaccinated macaques compared with those in the non-vaccinated macaques, although the reduction was not significant ($P = 0.47$) (Table 2). In addition, no bacterial colony was detected in the bronchi of vaccinated macaques, whereas a few bacterial colonies were detected in the bronchi of non-vaccinated macaques (Table 1). These findings are compatible with the histological severity.

We analyzed IL-10 production after the infection as it has been reported that IL-10 controlled lung inflammation during influenza virus infection [23, 28]. IL-10 production in nasal swab samples from macaques infected with NL2586 alone was not altered on day 8 after the infection comparing with that before the infection, whereas IL-10 production after the inoculation with NL2586 and *S. pneumoniae* was significantly decreased in macaques with and without vaccination ($P = 0.002$ in NL2586 alone vs. NL2586 + *S. pneumoniae*, $P = 0.02$ in NL2586 alone and Vac-2 + NL2586 + *S. pneumoniae*; Fig. 4). However, vaccinated macaques infected with HPAIV and *S. pneumoniae* showed intermediate IL-10 reduction because of low bacterial growth in the vaccinated macaques (Tables 1 and 2). These findings suggest that *S. pneumoniae* infection suppress IL-10 production in macaques and that low production of IL-10 may enhance inflammation in the lung of unvaccinated macaques as observed in histological results (Fig. 2).

Virus replication in swab samples from cynomolgus macaques after challenge with NL2586 or both NL2586 and Streptococcus pneumoniae

We examined virus replication in nasal swab samples from cynomolgus macaques after challenge with

Table 3 Virus recovery from nasal swabs of macaques inoculated with NL2586

		Virus titer [log 10 ($TCID_{50}$/ml)]									
Inoculation	Animal[1] (dpi)	1	2	3	4	5	6	7	8	10	12
NL2586	327	3.50	3.67	3.00	5.33	3.50	2.67	<	<	<	<
	368	2.67	2.33	2.00	2.23	<	<*	<	<	<	<
	542	<*	<	<*	1.67	1.67	2.50	<	<	<	<
NL2586	406	4.23	2.00	2.50	<*	<	<*	<*	<	<	<
+*S. pneumoniae*	435	3.50	3.00	2.50	2.00	2.50	3.00	<*	<*	<	<
	464	3.33	1.50	2.67	2.50	<*	<*	<	<	<	<
Vac-2	370	2.33	2.33	<	<	<	<	<	<	<	<
NL2586	373	<*	<	<	<	<	<	<	<	<	<
+*S. pneumoniae*	410	<	<	<	<	<	<	<	<	<	<

dpi, days post infection.
The symbol '<' indicates that the virus titer was less than the detection limit (<10 $TCID_{50}$/ml).
The symbol '<*' indicates that one cytopathic effect-positive well was observed in quadruplicate culture of undiluted sample solution.
[1]Each macaque was inoculated with NL2586 (4×10^7 $TCID_{50}$) and/or *S. pneumoniae* (1.2×10^9 CFU) on day 0.

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Fig. 5 Body temperatures and food consumption of vaccinated macaques after inoculation of H7N7 HPAIV (NL2586) with *Streptococcus pneumoniae*. Cynomolgus macaques (Nos 370, 373 and 410) were subcutaneously vaccinated twice. Seven weeks after the first vaccination (5 weeks after the second vaccination), NL2586 (4×10^7 $TCID_{50}$) was inoculated onto conjunctivas and into nasal cavities and tracheas with *S. pneumoniae* (1.2×10^9 CFU) into tracheas. (A) Body temperatures were monitored by telemetry transmitters implanted in the peritoneal cavities. Average temperatures of the highest and lowest temperatures on one day are time-dependently shown. Lines drawn horizontally indicate the average temperature levels at pre-infection. (B) Appetite was reflected by the amount of food consumed, which was calculated from the numbers of residual and fed pellets.

NL2586 or both NL2586 and *S. pneumoniae*. The virus was detected in nasal swab samples from the macaques until day 6 after inoculation with NL2586 (average 6 days; Table 3). Similarly, after simultaneous infection with NL2586 and *S. pneumoniae*, the virus was detected in nasal swab samples from the macaques until days 6–8 after the challenge (average 7 days, $P = 0.16$ vs. NL2586 alone). On the other hand, in swab samples from the vaccinated macaques, the virus was detected until day 2 after simultaneous challenge with NL2586 and *S. pneumoniae* (average 1 day, $P = 0.002$ vs. without vaccination). Therefore, it was clearly shown that pre-inoculation with whole particles of Vac-2 decreased H7N7 HPAIV replication in the respiratory tract after simultaneous infection with H7N7 HPAIV and *S. pneumoniae*. In addition, the average weight of vaccinated macaques on day 14 after challenge was 92% of the weight on day 0. Therefore, loss of weight was significantly reduced by vaccination ($P = 0.025$, vs. 88% in unvaccinated macaques as stated above). Furthermore, the average duration of abnormal body temperature after inoculation with NL2586 and *S. pneumoniae* was 2.67 days in vaccinated macaques (Fig. 5A) but 10.67 days in unvaccinated macaques (Fig. 1A) ($P = 0.01$). The average duration of appetite loss was 2.67 days in vaccinated macaques (Fig. 5B) but 7.33 days in unvaccinated macaques (Fig. 1B), although the difference was not statistically significant ($P = 0.06$). Thus, prevention of viral replication by the vaccine would decrease bacterial growth, severity of bacterial pneumonia and morbidity of infection.

Immune responses against HPAIV affected by mixed infection with Streptococcus pneumoniae

We examined immune responses against H7N7 virus and *S. pneumoniae* after infection to see whether mixed infection altered responses against the pathogens. As it was technically difficult to prepare purified antigen of NL2586 for ELISA, we used Vac-2 antigen to examine antibody responses against H7N7 virus. Serum IgG

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responses specific for Vac-2 antigen in the unvaccinated macaques were elevated on day 8 after infection with NL2586 alone (Fig. 6, left panels) and the levels on day 14 were higher than those on day 8. IgG responses specific for the Vac-2 in sera from the unvaccinated macaques infected with NL2586 and *S. pneumoniae* were comparable to those from the macaques infected with NL2586 alone (Fig. 6, middle panels). Recall IgG responses in the vaccinated macaques were observed on day 8 after the infection as the antibody responses on day 8 had rapidly increased to the similar level on day 14 (Fig. 6, right panels). Infection with *S. pneumoniae* did not affect IgG responses against H7N7 virus. This finding was also seen in a neutralization assay against NL2586 (Table 4): no difference in neutralization activities of sera between macaques infected with NL2586 alone and macaques infected with NL2586 and *S. pneumoniae* was observed.

Next, we examined IgG responses against *S. pneumoniae* (Fig. 7). Two of the three unvaccinated macaques (Nos 435 and 464) showed IgG specific responses against *S. pneumoniae* after inoculation with NL2586 and *S. pneumoniae*, and one macaque (No. 406) showed very weak IgG responses against *S. pneumo-*

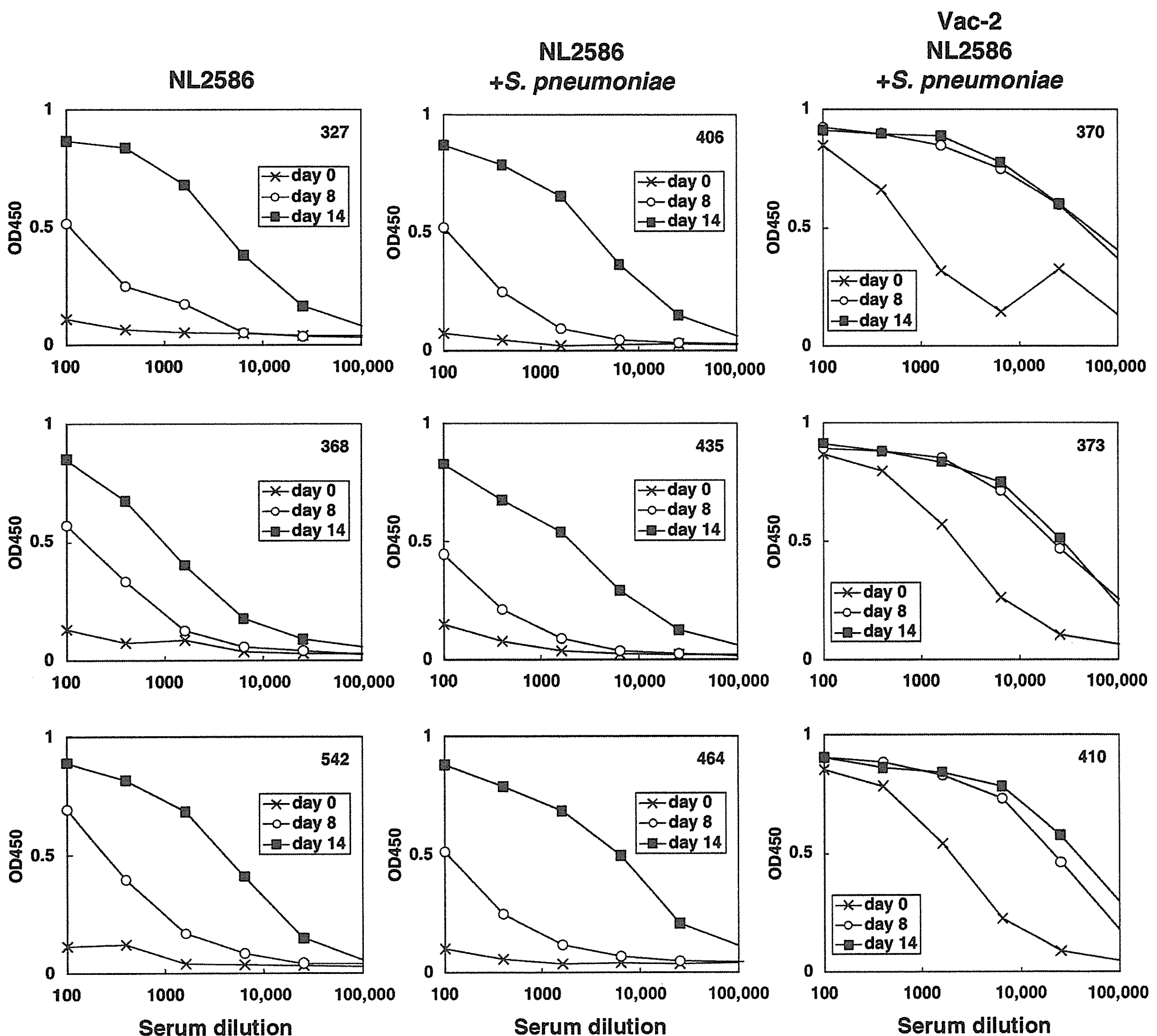

Fig. 6 Antibody responses specific for H7N7 vaccine antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation with NL2586 with or without *Streptococcus pneumoniae* (days 8 and 14). IgG antibodies specific for Vac-2 antigens in sera were analyzed at indicated dilutions as described in Fig. 2.

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Table 4 Neutralization activity of NL2586 with sera obtained after challenge with NL2586 and *Streptococcus pneumoniae*

Inoculation	Animal	50% neutralization titer (log 2)
NL2586	327	6.67
	368	5.50
	542	5.33
NL2586	406	5.17
+*S. pneumoniae*	435	4.00
	464	5.67
Vac-2	370	4.67
NL2586	373	5.83
+*S. pneumoniae*	410	6.23

Serum samples were collected 14 days after challenge infection with NL2586 with or without *S. pneumoniae*. The averages of 50% neutralization titers against NL2586 were 5.83, 4.95 and 5.58 in sera from macaques infected with NL2586 alone, macaques infected with NL2586 and *S. pneumoniae*, and macaques infected with NL2586 and *S. pneumoniae* after vaccination respectively. *P*-values with Student's *t*-test are >0.05 (NL2586 vs. NL2586 + *S. pneumoniae*, NL2586 vs. Vac-2 + NL286 + *S. pneumoniae*, and NL2586 + *S. pneumoniae* vs. Vac-2 + *S. pneumoniae*).

niae (Fig. 7, middle panels). A vaccinated macaque 370 showed higher increase in IgG specific for *S. pneumoniae* on day 8 than in that on day 14. This seems that immunological memory against *S. pneumoniae* has been present due to previous infection (Fig. 7, right panels). In two other vaccinated macaques (Nos 373 and 410), very weak or almost no IgG response was detected in sera. These findings suggest that viral replication may enhance IgG responses against *S. pneumoniae* in naïve animals, although IgG detected on day 14 was not sufficient to inhibit bacterial growth in the lungs (Table 2). Alternatively, as bacterial colonies in the vaccinated macaques were fewer than those in the unvaccinated macaques (Tables 1 and 2), IgG responses against *S. pneumoniae* might not be significant in the vaccinated macaques (Nos 373 and 410).

Discussion

Seasonal influenza virus infection occasionally induces secondary bacterial infection that results in substantial morbidity and mortality. Suppression of host immunity, including neutrophil function and macrophage-mediated microbial clearance [1, 4, 13, 24], and induction of inhibitory IL-10 [27, 28] are thought to be involved in the increased morbidity and mortality. It has not been determined whether mixed infection with HPAIV and bacteria causes more severe morbidity and higher mortality than infection with HPAIV alone, although co-infection with HPAIV and bacteria has not been detected in patients [5, 26]. Thus, in order to prevent severe morbidity and mortality in humans in future pandemics, it should be determined whether HPAIV infection with bacterial infection causes more severe morbidity than does HPAIV infection alone and whether regulation of HPAIV replication ameliorates bacterial pneumonia in experimental mixed infection using a macaque model as a preclinical study.

Some studies have shown that preceding influenza virus infection enhanced bacterial pneumonia with severe morbidity [6, 9, 11, 14]. Influenza viruses induce lung epithelial apoptosis via macrophage activation, resulting in the loss of defense capacity against bacteria in lung epithelia and the establishment of appropriate environment for bacterial growth [7]. Nonetheless, simultaneous infection with H7N7 HPAIV and *S. pneumoniae* in macaques did not induce severe pneumonia including diffuse alveolar damage at autopsy; only modest pneumonia immediately following lymphocyte infiltration was observed. Histology of the lungs of macaques simultaneously challenged with H7N7 HPAIV and *S. pneumoniae* indicated that the pneumonia seemed to be in a repairing phase with inflammatory cells infiltrating the alveoli being mainly lymphocytes with a few neutrophils, although pneumonia caused by *S. pneumoniae* occurred in addition to pneumonitis caused by H7N7 HPAIV, and pneumonia with neutrophils was not significant in lungs of macaques inoculated with NL2586 alone. As lung epithelial apoptosis via macrophage activation by influenza viruses seems to be responsible for severe pneumonia in co-infection, simultaneous infection but not preceding influenza viral infection might induce modest pneumonia. Alternatively, it is possible that pathogenicity of *S. pneumoniae* in macaques was low or *S. pneumoniae* growth might be regulated in some degree by host responses as *S. pneumoniae* might have been inoculated before desensitization of TLR by influenza virus infection in the simultaneous inoculation with HPAIV and *S. pneumonia* [4]. Further study should be required to reveal whether *S. pneumoniae* infection after HPAIV infection causes severe morbidity and mortality in a macaque model.

Vaccination against H7N7 HPAIV decreased morbidity caused by H7N7 HPAIV and *S. pneumoniae* in the macaques. Bacterial growth in the lungs was decreased by vaccination against H7N7 HPAIV, although the reduction in bacterial colonies was not statistically significant. Similarly, mitigation of the pneumonia was histologically observed in the lungs of vaccinated macaques. It is likely that the aforementioned lung epithelial apoptosis caused by influenza viruses is somehow involved in the enhancement

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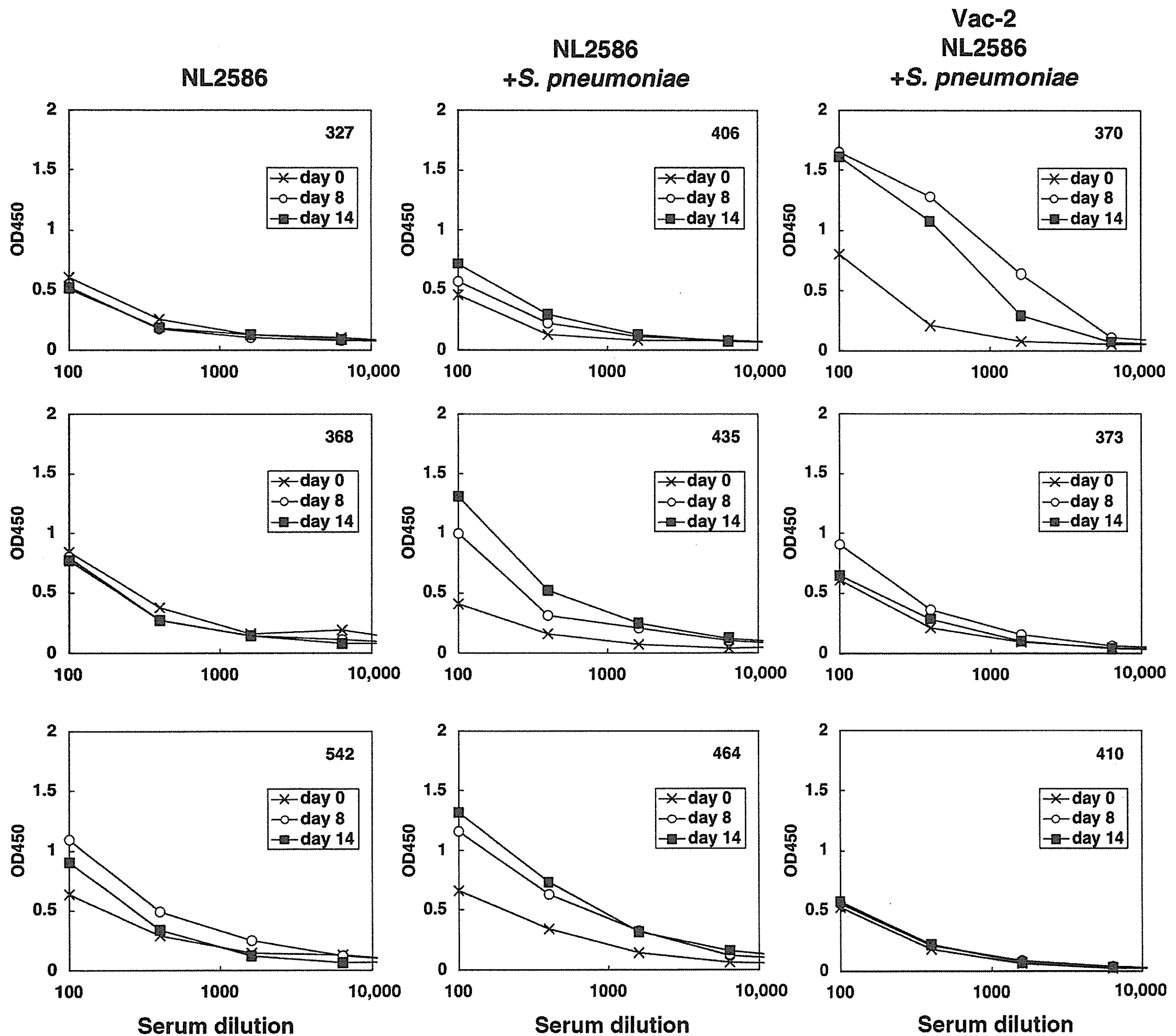

Fig. 7 Antibody responses specific for *Streptococcus pneumoniae* antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation of NL2586 with or without *S. pneumoniae* (days 8 and 14). IgG antibodies specific for *S. pneumonia* antigens in sera were analyzed at indicated dilutions as described in Fig. 2.

of bacterial pneumonia and that H7N7 HPAIV and *S. pneumoniae* interact with each other directly and indirectly in the development of pneumonia and pneumonitis.

In general, IL-10 expression is upregulated in influenza virus and *S. pneumoniae* infection of mice and IL-10 inhibits severe inflammatory responses in the lungs [12, 27, 28]. However, in the present study, *S. pneumoniae* infection suppressed IL-10 production, supporting histological findings and efficacy of antiviral vaccination. Furthermore, the reduction of IL-10 is assumed to finally induce cytokine storm after mixed infection with HPAIV and *S. pneumoniae* [3]. This assumption should be evidenced in near future.

Vaccination against HPAIV decreased the severity of pneumonia caused by bacterial superinfection, and prognosis of HPAIV-infected patients might be improved. Therefore, it might be crucial to prepare vaccines against future pandemic strains in order to prevent severe bacterial pneumonia as observed in the H1N1 pandemic in 1918 [10].

Acknowledgments

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FULL PAPER

Generation of congenic mouse strains by introducing the virus-resistant genes, *Mx1* and *Oas1b*, of feral mouse-derived inbred strain MSM/Ms into the common strain C57BL/6J

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Abstract

Mx1 (Myxovirus resistance protein) and Oas1b (Oligoadenylate synthetase-1), induced by type 1 interferon (IFN), play a role in early antiviral innate immunity by inhibiting the replication of viruses. In mice, Mx1 and Oas1b confer resistance to the infection of orthomyxoviruses including influenza viruses and flaviviruses including West Nile viruses, respectively. Laboratory mice have been used to study the mechanisms of the pathogenesis of these virus infections; however, it is possible that they are not a suitable model system to study these viruses, since most of the inbred laboratory mouse strains lack both genes. It has been reported that feral mouse-derived inbred strains show resistance to the infection of these viruses due to the presence of intact both genes. In this study, we generated congenic strains in which the *Mx* or *Oas* locus of the MSM/Ms (MSM) mouce was introduced to the most widely used mouse strain, C57BL/6J (B6). B6.MSM-*Mx* mice showed resistance to the infection of influenza virus but not of West Nile virus. On the other hand, B6.MSM-*Oas* mice showed resistance to the infection of West Nile virus but not of influenza virus. Our results indicate that Mx1 and Oas1b show highly antiviral specificity in mice possessing the same genetic background. Therefore, these congenic mice are useful for not only infection study but also investigation of host defense mechanism to these viruses.

Key words: congenic mouse, flavivirus, *Mx1, Oas1b,* orthomyxovirus,

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Introduction

Type 1 interferons (IFNs), IFN α/β, are produced and secreted from virus-infected cells and cause the surrounding cells to induce a number of cellular proteins, including the Mx (Myxovirus resistance protein) and Oas (Oligo-adenylate synthetase)[26]. These IFN-inducible proteins play important roles in the host's innate defense by inhibiting viral replication. Mx proteins can be found in a wide variety of organisms including mammals, birds, fish, and even invertebrate species[4,24,32,34,38] and belong to the dynamin superfamily of high molecular weight GTPases[22,23]. In mice, two *Mx* genes, *Mx1* and *Mx2*, have been identified to locate in the *Mx* locus on Chromosome (Chr) 16. *Mx1* has been identified as a gene encoding an anti-viral protein to orthomyxoviruses including influenza viruses[28,33]. Oas family proteins are also highly conserved among many species[19,27,40]. In virus-infected cells, Oas proteins are activated by the binding of viral double-stranded RNAs and are known to synthesize 2'-5' oligoadenylate (2-5A). The 2-5A subsequently binds to and activates latent ribonuclease RNase L, resulting in the degradation of viral RNAs and the enhancement of IFN signaling[9,16,28,41]. In mice, the *Oas* locus locates on Chr 5 and is composed of the *Oas* gene cluster, consisting of *Oas1a-h*, *Oas2*, and *Oas3*. Among these genes, *Oas1b* has been identified as a flavivirus-resistant gene[12,18,19,26,27].

Most strains of laboratory mice such as C57BL/6J (B6), BALB/c, and DBA/2, lost the functional *Mx1*, *Mx2*, and *Oas1b* genes during selective breeding[2,18,26,33]. Therefore, laboratory mice are susceptible to both viruses, whereas feral mouse-derived inbred strains are known to possess intact *Mx1*, *Mx2*, and *Oas1b* genes[11,18,26,29,36]. Because of its widespread use in medical and basic science, the B6 strain was selected as the reference strain for the mouse genome sequencing. Additionally, B6 mice are also commonly used in the production of both transgenic and knockout mice as well as many mutagenesis projects[20]. Susceptibility to viruses has been believed to be associated with a genetic background in humans and other animals. This genetic effect is often complex and difficult to identify, since it is further modified by environmental factors. Laboratory mice such as B6 afford a useful alternative for the study of host defenses against infections, because variations among strains allow the identification of the genes associated with resistance or susceptibility to virus infection. However, as B6 mice lack the important *Mx1* and *Oas1b* genes, they are not, perhaps, the most suitable model system for the study of both orthomyxo- and flaviviruses. Therefore, we established the congenic strains, B6.MSM-*Mx* and B6.MSM-*Oas* that carry the *Mx* and *Oas* locus, respectively, from the Japanese feral mouse-derived inbred strain, MSM/Ms. These congenic mice were found to be more resistant to lethal challenge with the two highly pathogenic viruses than were the original B6 mice. These congenic mice provide a useful model for the study of not only the antiviral function of Mx1 and Oas1b, but also the infectious mechanism of these viruses in humans and other animals.

Materials and Methods

Generation of congenic strains: The laboratory mouse strain, B6 was purchased from Charles River Japan (Tokyo, Japan) and the feral mouse-derived inbred strain MSM/Ms was provided by Prof. T. Shiroishi, National Institute of Genetics, Japan. *Mx* and *Oas* congenic mice were generated using the speed congenic method[17]. Animal breeding rooms were maintained at 22 ± 4°C and 50 ± 20% relative humidity with a 12-hr light-dark cycle. Research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Veterinary Medicine, Hokkaido University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University.

Genotyping was performed by polymerase chain reaction (PCR) with tail DNA. The forward and reverse primers for the *Mx1* gene were designed based on the sequence of the MSM genome corresponding to the deleted-region of the B6 genome; GTGACCTTTGAACCTGCTTCCT (intron 10) and GCAGACTCTTCCAGGGCTTTGA (exon 11) as described previously[2]. PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. The forward and reverse primers for the *Oas1b* (accession number: NM_001083925) were, GCTCAAGGGCAGGT CAGAC (nt 15-33 of exon 3) and TCAAAC TTCACCTCCTTCAGC (nt 231-251 of exon 3), respectively. PCR products were digested with 2 U *Hin*fI for 1 hr, followed by electrophoresis in 8% acrylamide gels and visualization by ethidium bromide staining. Microsatellite markers used for genotyping are listed in Table 1.

Expression of intact Mx1 and Oas1b genes in congenic mice: Eight-week-old female B6, B6.

Table 1. List of microsatellite markers used for the marker-assisted speed congenic methods

Microsatellite Markers	Chr	cM	Microsatellite Markers	Chr	cM	Microsatellite Markers	Chr	cM	Microsatellite Markers	Chr	cM	Microsatellite Markers	Chr	cM
D1Mit316	1	7.9	*D4Mit111*	4	21.9	*D8Mit94*	8	13	*D12Mit63*	12	19	*D16Mit71*	16	70.7
D1Mit58	1	8.3	*D4Mit80*	4	33.7	*D8Mit339*	8	23	*D12Mit114*	12	29	*D16Mit106*	16	71.5
D1Mit123	1	21	*D4Mit166*	4	44.5	*D8Mit7*	8	32	*D12Mit229*	12	41	*D17Mit198*	17	16
D1Mit18	1	29.7	*D4Mit52*	4	54.9	*D8Mit33*	8	45	*D12Mit277*	12	50	*D17Mit49*	17	23.2
D1Miit251	1	38.1	*D4Mit13*	4	71	*D8Mit213*	8	54	*D13Mit116*	13	10	*D17Mit251*	17	31
D1Mit415	1	52	*D4Mit42*	4	81	*D8Mit200*	8	58	*D13Mit159*	13	47	*D17Mit89*	17	36
D1Mit30	1	70	*D5Mit180*	5	10	*D8Mit56*	8	73	*D13Mit226*	13	59	*D17Mit93*	17	44.5
D1Mit14	1	81.6	*D5Mit176*	5	18.2	*D9Mit2*	9	17	*D13Mit260*	13	65	*D17Mit221*	17	56.7
D1Mit145	1	89	*D5Mit109*	5	34	*D9Mit328*	9	23	*D13Mit77*	13	73	*D18Mit132*	18	11
D1Mit403	1	100	*D5Mit24*	5	60	*D9Mit49*	9	35	*D14Mit49*	14	3	*D18Mit177*	18	20
D1Mit20	1	106	*D5Mit367*	5	65	*D9Mit133*	9	43	*D14Mit45*	14	12.5	*D18Mit51*	18	37
D2Mit312	2	1	*D5Mit242*	5	66	*D9Mit76*	9	49	*D14Mit268*	14	19	*D18Mit184*	18	41
D2Mit294	2	15	*D5Mit168*	5	78	*D9Mit18*	9	71	*D14Mit37*	14	27.5	*D18Mit186*	18	45
D2Mit433	2	31.7	*D6Mit166*	6	0.6	*D10Mit248*	10	7	*D14Mit115*	14	40	*D18Mit106*	18	50
D2Mit37	2	45	*D6Mit74*	6	20.5	*D10Mit124*	10	15	*D14MIt196*	14	47	*D18Mit4*	18	57
D2Mit101	2	52.5	*D6Mit188*	6	32.5	*D10Mit3*	10	21	*D15Mit10*	15	9.9	*D19Mit69*	19	6
D2Mit255	2	69	*D6Mit104*	6	45.5	*D10Mit221*	10	31	*D15Mit111*	15	17.8	*D19Mit80*	19	22
D2Mit343	2	84.2	*D6Mit194*	6	61.5	*D10Mit69*	10	52	*D15Mit156*	15	39.1	*D19Mit13*	19	33
D2Mit229	2	99	*D7Mit76*	7	3.4	*D10Mit180*	10	64	*D15Mit71*	15	46.7	*D19Mit10*	19	47
D3Mit164	3	2.4	*D7Mit117*	7	11	*D11Mit62*	11	1.5	*D15Mit79*	15	66.2	*D19Mit84*	19	53
D3Mit305	3	11.2	*D7Mit26*	7	23	*D11Mit53*	11	16	*D16Mit165*	16	10.3	*DXMit166*	X	15.5
D3Mit333	3	22	*D7Mit84*	7	28.4	*D11Mit4*	11	37	*D16Mit212*	16	27.3	*DXMit25*	X	27.8
D3Mit241	3	33	*D7Mit173*	7	43	*D11Mit35*	11	##	*D16Mit147*	16	28.2	*DXMit16*	X	37
D3Mit12	3	49.2	*D7Mit321*	7	48.5	*D11Nds7*	11	62	*D16Mit42*	16	33	*DXMit19*	X	43.2
D3Mit14	3	64.1	*D7Mit222*	7	52.6	*D11Mit61*	11	70	*D16Mit140*	16	42.8	*DXMit130*	X	55
D3Mit129	3	84.9	*D7Mit66*	7	57.5	*D11Mit48*	11	77	*D16Mit70*	16	57	*DXMit10*	X	63.2
D4Mit149	4	0	*D7Mit105*	7	63.5	*D12Mit270*	12	13	*D16Mit20*	16	69.5			