(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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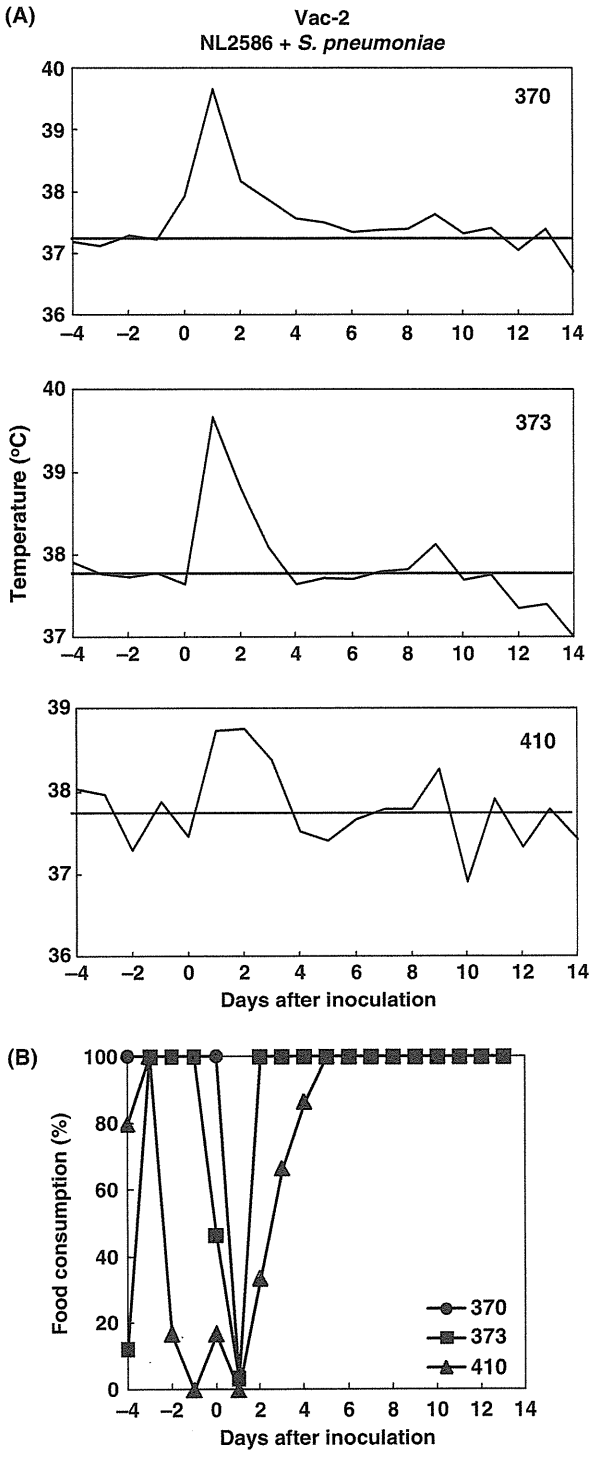

Fig. 5 Body temperatures and food consumption of vaccinated macaques after inoculation of H7N7 HPAIV (NL2586) with *Streptococcus pneumoniae*. Cynomolgus macaques (Nos 370, 373 and 410) were subcutaneously vaccinated twice. Seven weeks after the first vaccination (5 weeks after the second vaccination), 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

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-*

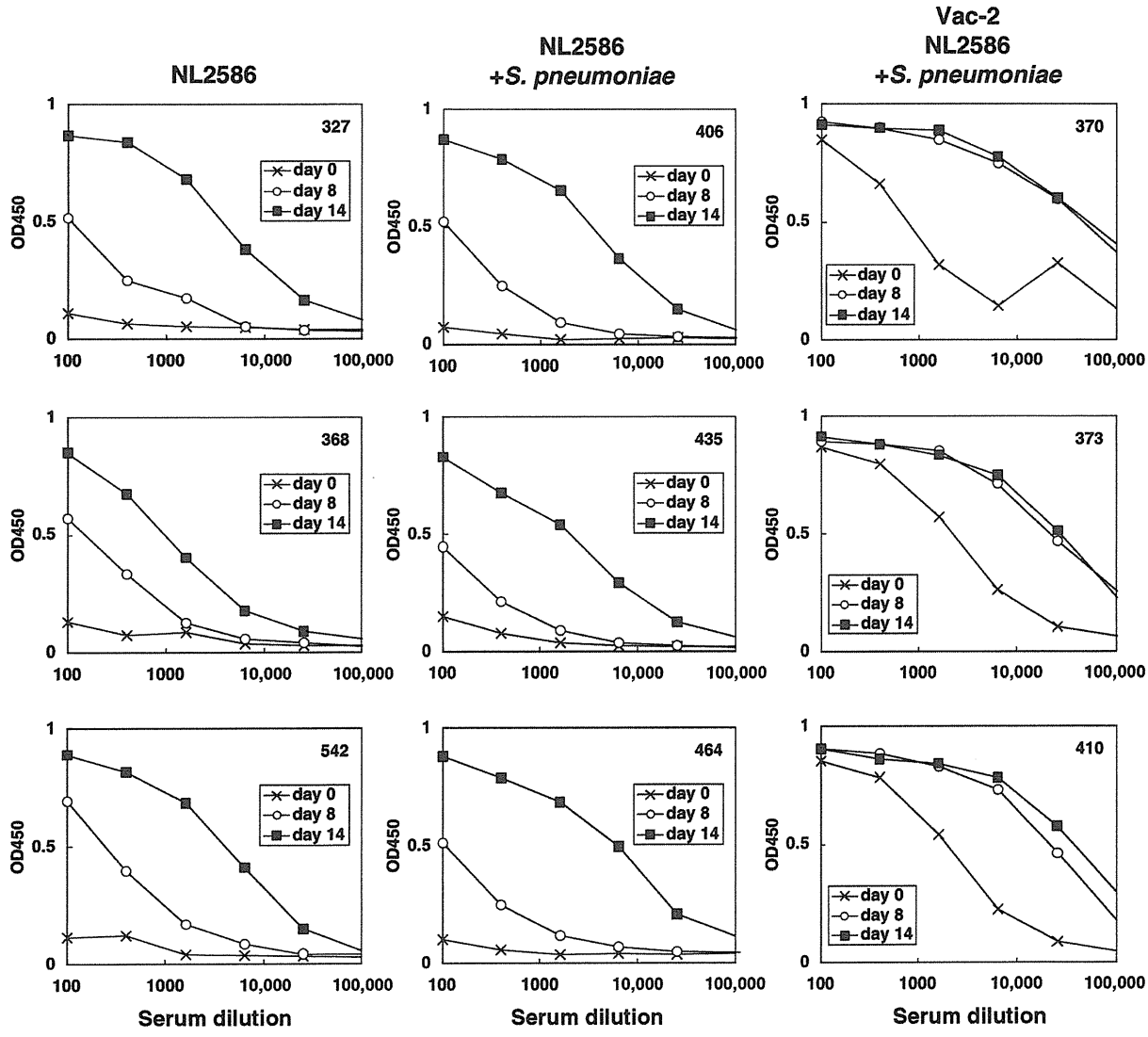

Fig. 6 Antibody responses specific for H7N7 vaccine antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation with NL2586 with or without *Streptococcus pneumoniae* (days 8 and 14). IgG antibodies specific for Vac-2 antigens in sera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

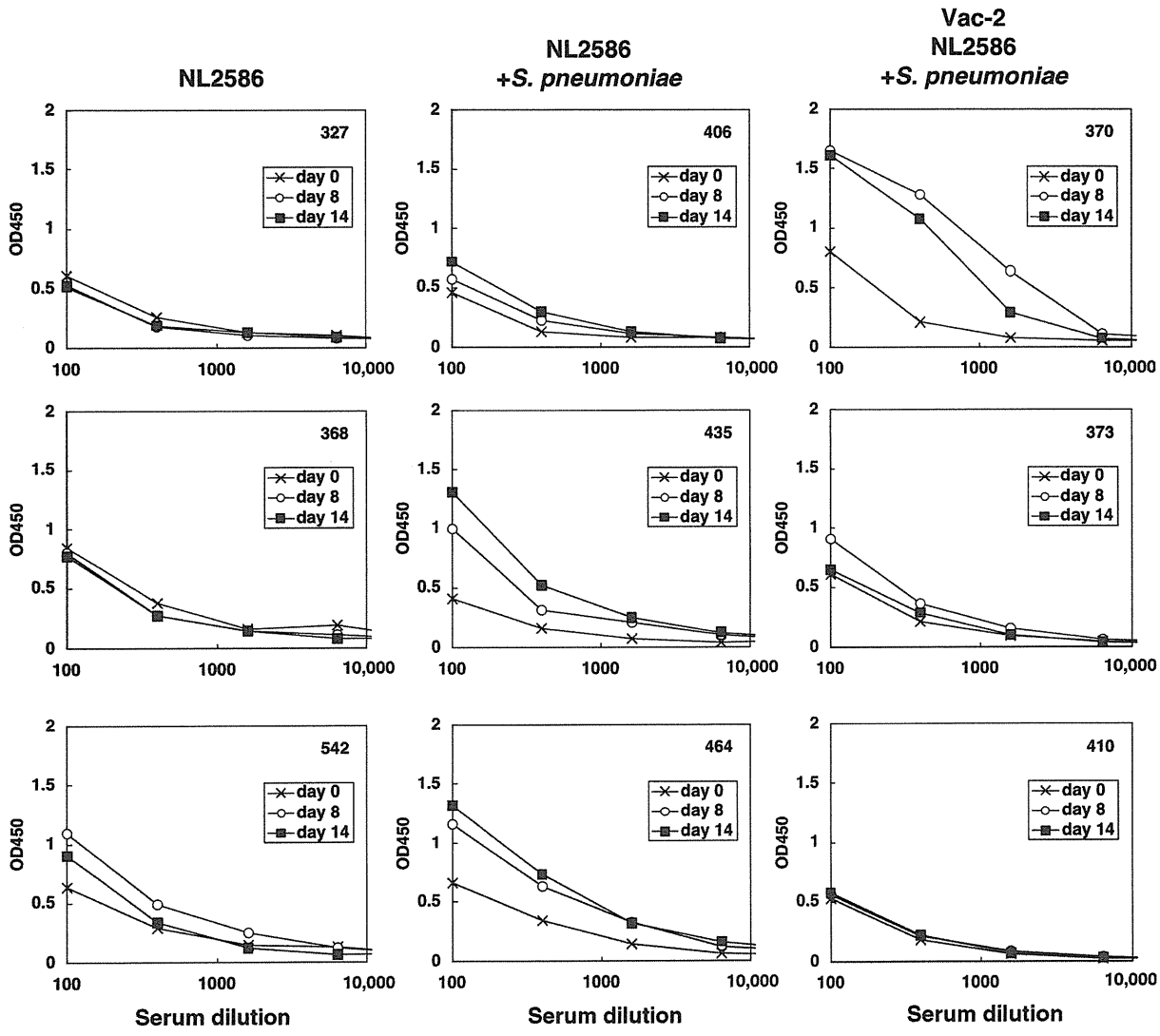

Fig. 7 Antibody responses specific for *Streptococcus pneumoniae* antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation of NL2586 with or without *S. pneumoniae* (days 8 and 14). IgG antibodies specific for *S. pneumonia* antigens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

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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 (Oligoadenylate 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			

MSM-*Mx*, and B6.MSM-*Oas* mice were injected intraperitoneally with 200 μg of the synthetic double-stranded RNA (dsRNA) analog, poly (I:C) (GE Healthcare, UAS). Animals were sacrificed at 24 hr after the injection and the spleens were dissected. Total RNAs were isolated from the spleens using TRIzol reagent (Invitrogen, USA), and cDNAs were generated with oligo (dT) primers using ReverTra Ace (TOYOBO, Japan). PCR was performed using the following primers: ACGATGGATTCTGTGAATAATCTGT (nt 211–235) and TCTAGATAGCCTGGTTAATCG GAGAATTT (nt 2,095–2,121) for *Mx1*; GGCTG CAGAGGTATTAGCTGGACCT (nt 36–60) and CAGGAGGATGGCAATATCCAAGACA (nt 1202–1226) for *Oas1b*; and TGATGGTGGGAAT GGGTCAG (nt 207–226) and GAAGGCTG GAAAAGAGCCTC (nt 854–873) for mouse *Actb* (NM_007393). PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. Sequences of the *Oas1b* PCR products were determined with an ABI Prism 377 DNA Sequencer and an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Experimental infection of mice with influenza virus and West Nile virus: To assess the lethality of viruses in the congenic mice, 8-week-old male mice were infected intranasally with 30 μl inoculum containing 10^2–10^4 × 50% of lethal dose (LD_{50}) of a highly pathogenic avian influenza strain, A/whooper swan/Mongolia/3/2005 (H5N1), diluted with phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin G and 100 μg streptomycin under anesthesia with 50 mg/kg sodium pentobarbital (Somnopentyl, Schering-Plough Animal Health U. S. A). The LD_{50} value was calibrated using 8-week-old B6 mice. After infection, mice were monitored daily for 21 days. For West Nile virus infection, 6-week-old male mice were infected intraperitoneally with 200 μl inoculum containing 1 or 10 plaque forming units (PFUs) of West Nile virus 6LP strain diluted with PBS under anesthesia with diethyl ether inhalation. After infection, mice were followed up for 4 weeks.

Results

Generation of Mx1 and Oas1b congenic mice

Congenic strains were generated using a marker-assisted speed congenic strategy as reported previously[17]. To generate congenic mice in which the *Mx* or *Oas* locus from MSM mice was introduced into the genetic background of B6 mice, the B6 and MSM alleles of *Mx1* and *Oas1b* were determined to select candidate mice. In the *Mx1* locus of B6 mice, a large deletion from exon 9 to 11 was reported previously[2,33]. Therefore, primers were designed for amplifying the region from intron 10 to exon 11 to distinguish the B6 allele from the MSM allele as reported previously[2]. As shown in Fig. 1A, these primers can detect MSM allele. On the other hand, since a single nucleotide polymorphism has been found in the *Hin*fI site of the *Oas1b* gene in the MSM allele (Fig. 1B), genotyping was performed by digestion of the PCR products of the *Oas1b* gene with *Hin*fI (Fig. 1C). The 'best' male mice, those carrying the most homozygous B6 alleles in 134 microsatellite markers with heterozygosity in the *Mx1* or *Oas1b* gene, were selected for breeding next generation. Backcrossing was performed six and seven times for exchanging to the B6 genetic background in the B6.MSM-*Oas* and B6.MSM-*Mx* mice, respectively. Finally, heterozygous sibling pairs were mated and homozygous mice were selected. To estimate the length of the chromosomal regions derived from MSM mice, the genotype and position of microsatellite markers surrounding the *Mx* or *Oas* locus were confirmed (Fig. 2). As shown in Fig. 2A, the genotypes of *D16Mit71* and *D16Mit106* in the B6.MSM-*Mx* mice were homozygous MSM (M/M), whereas that of *D16Mit20* was homozygous B6 (B/B), suggesting that the region between *D16Mit71* and *D16Mit106* was derived from MSM and recombination occurred at two points between *D16Mit20*

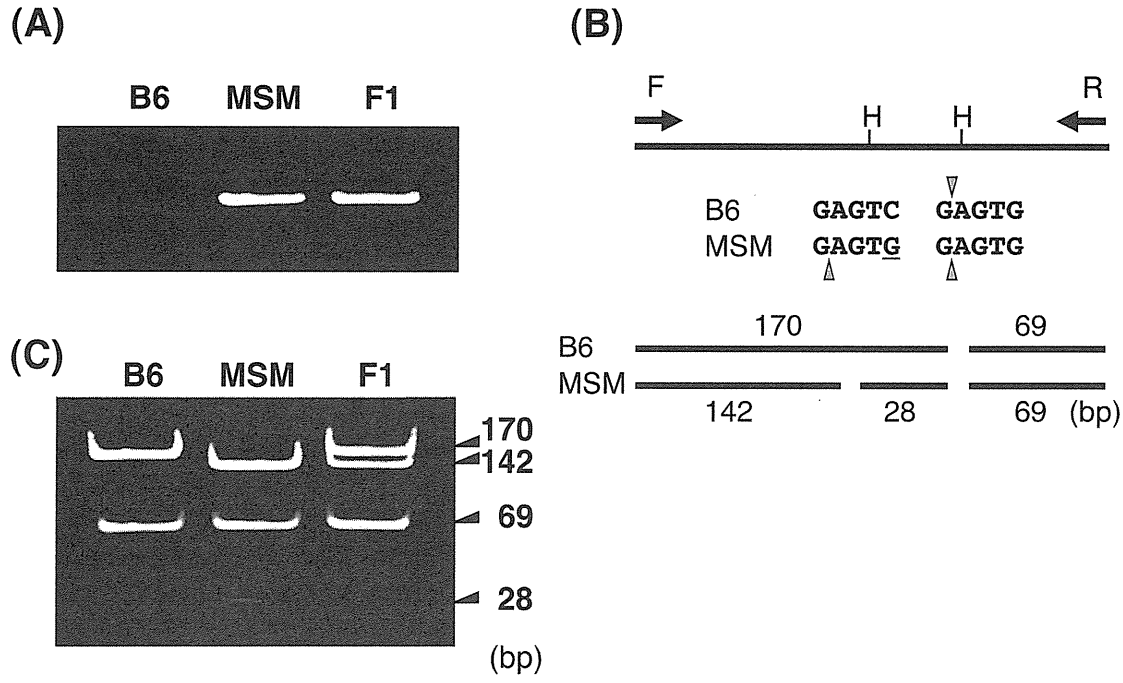

Fig. 1. Detection of the B6 and MSM alleles of the *Mx1* and *Oas1b* genes. (A) The results of PCR amplification of the Mx1 gene. The alleles of MSM but not of B6 mice show the PCR product. (B) Schematic diagram of the *Oas1b* gene in B6 and MSM mice. The arrows show PCR primers used for *Oas1b* genotyping. The lower diagram shows the expected results of *Hin*fI digestion after PCR amplification. H; *Hin*fI site. (C) The result of genotyping of the *Oas1b* gene. The 28-bp and 142-bp bands are derived from the MSM allele.

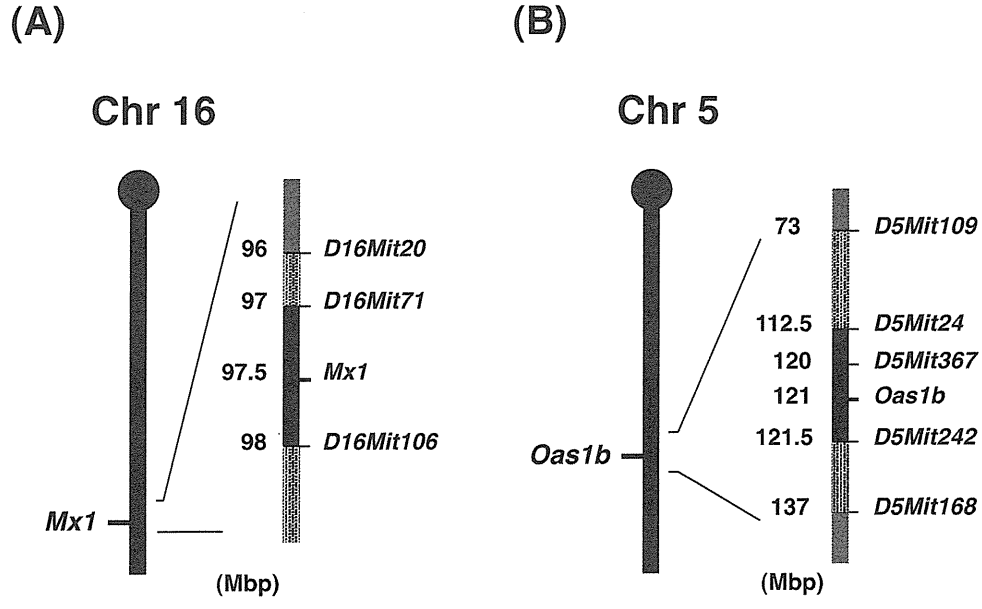

Fig. 2. Schematic diagrams of the genomic structure surrounding *Mx1* and *Oas1b* genes in the B6.MSM-*Mx* (A) and B6.MSM-*Oas* (B) congenic strains, respectively. Black and gray bars represent the MSM-derived and B6-derived genomes, respectively. Dotted bars represent recombined regions between the MSM and B6 genomes. The numbers to the left of the bars represent physical locations based on the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview).

and *D16Mit71,* and between *D16Mit106* and telomere. On the other hand, the genotypes of *D5Mit24*, *D5Mit367*, and *D5Mit242* in B6.MSM-*Oas* mice were M/M, whereas those of *D5Mit109* and *D5Mit168* were B/B (Fig. 2B), suggesting that the region between *D5Mit24* and *D5Mit242* was derived from MSM mice and recombination occurred at two points between *D5Mit109* and *D5Mit24*, and between *D5Mit242* and *D5Mit168*.

Next we confirmed by RT-PCR that *Mx* or *Oas1b* mRNA was transcribed from both MSM alleles in congenic mice. After induction of IFN using poly (I:C) injection, expression of the *Mx1* gene in the spleens of B6.MSM-*Mx*, MSM, and B6 mice was analyzed. Intact *Mx1* could be distinguished from the mutant type due to the large deletion in the *Mx1* gene of the B6 mouse (Fig. 3A). In addition, no *Mx2* gene expression was detected in the spleens of B6.MSM-*Mx* and MSM mice (Fig. 3A). On the other hand, since *Oas1b* genes expressed from the B6 and MSM mice could not be distinguished by the length of RT-PCR products (Fig. 3B), we determined the origin of the *Oas1b* gene by DNA sequencing. A nonsense mutation was observed in the B6 *Oas1b* cDNA, whereas the sequence of the *Oas1b* cDNA in the B6.MSM-*Oas* mice was identical to that of MSM mice (data not shown). Interestingly, both genes are basally expressed in B6 background and are more induced by dsRNA stimulation than those of original MSM mice (Fig. 3). This result is in agreement with previous report showing that MSM strain is hyporesponsive to poly (I:C) due to a mutation in toll-like receptor 3 activated by dsRNA[35]. Thus, we have confirmed that these congenic strains are able to express the intact *Mx1* and *Oas1b* genes by the stimulation of dsRNA.

Experimental infection of congenic strain with orthomyxovirus and flavivirus

We performed viral injection to confirm whether these congenic mice were resistant to the infection of orthomyxovirus and flavivirus. A highly pathogenic avian influenza virus and West Nile virus were selected as the representative orthomyxo- and flaviviruses, respectively. When

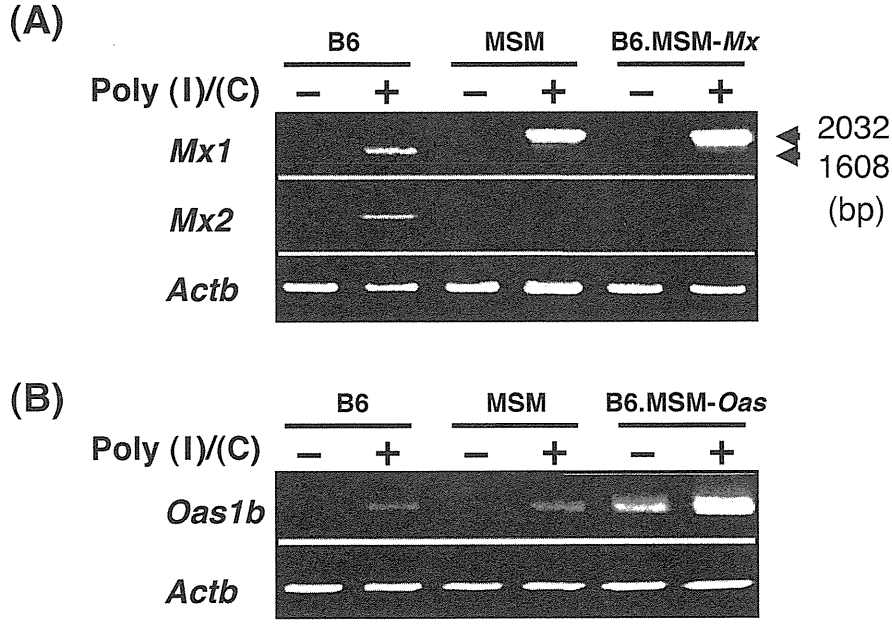

Fig. 3. Expression of intact *Mx1* and *Oas1b* genes in congenic mice. (A) Expression of *Mx1* and *Mx2* genes in the spleen tissues of B6, MSM, and B6.MSM-*Mx* mice in response to poly (I)/(C). (B) Expression of *Oas1b* gene in the spleen tissues of B6, MSM, and B6.MSM-*Oas* mice in response to poly (I)/(C).

infected with a 10^2 LD_{50} dose of influenza A virus, all B6 and B6.MSM-*Oas* mice died within 14 days after infection, whereas 5 of 7 (71%) B6.MSM-*Mx* mice survived. When B6.MSM-*Mx* mice were infected with 2 higher doses, 10^3 LD_{50} and 10^4 LD_{50}, 6 of 7 (86%) and 7 of 7 (100%) B6.MSM-*Mx* mice survived, respectively (Fig. 4A). On the other hand, all B6.MSM-*Oas* mice infected with 1 and 10 PFUs of West Nile virus survived, whereas most of B6 and B6.MSM-*Mx* mice died within 14 days after infection. The survival rate in each experiment was as follows; B6 mice infected with 1 PFU, B6 with 10 PFU, B6.MSM-*Mx* with 1 PFU, and B6.MSM-*Mx* with 10 PFU were 20% (3 out of 15), 7% (1 out of 14), 10% (1 out of 10), and 30% (3 out of 10), respectively (Fig. 4B). These results indicate that Mx1 and Oas1b have specific anti-virus activity against influenza- and West Nile viruses, respectively, in mice with the same B6 genetic background.

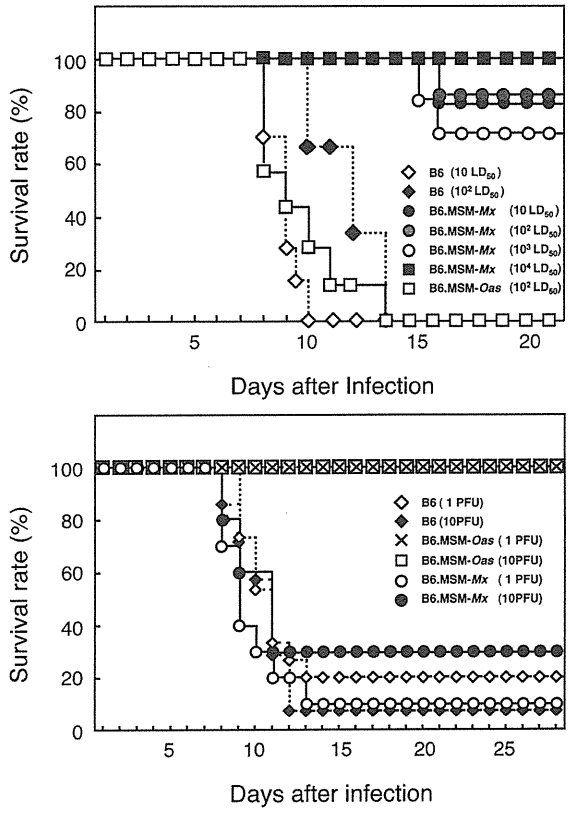

Fig. 4. Experimental infection with orthomyxovirus and flavivirus using congenic mouse strains. Survival rates of B6 mice are shown by dotted lines in each experiment. (A) Black and white diamonds represent B6 mice infected with 10 LD_{50} and 10^2 LD_{50} doses of influenza virus, respectively, whereas white squares represent B6.MSM-*Oas* mice infected with 10^2 LD_{50}. B6.MSM-*Mx* mice infected with 10 LD_{50}, 10^2 LD_{50}, 10^3 LD_{50}, and 10^4 LD_{50} inoculum doses are shown by black circles, white circles, gray circles, and black squares, respectively. (B) Survival rates of B6 (diamonds) and B6.MSM-*Mx* (circles) mice after infection with 1 PFU (white symbols) and 10 PFU (black symbols) of West Nile virus, respectively. Crosses and white squares represent B6.MSM-*Oas* mice infected with 1 PFU and 10 PFU, respectively.

Discussion

Orthomyxovirus and flavivirus are considered to be important viruses from both the medical and sanitary position. Influenza A, B, and C viruses, classified as orthomyxoviruses, cause the epidemic respiratory disease known as 'flu' in humans that spreads worldwide. Last century, pandemic influenza emerged several times. Further, a new emerging pandemic influenza virus is much concerned[13,15,21]. On the other hand, 20–30 members of the flavivirus family are known to be involved in human diseases. Some of these viruses cause severe diseases such as fatal encephalitis and haemorrhagic fevers in humans[10,31]. Last century, yellow fever virus, Dengue virus, Japanese encephalitis virus, tick-borne encephalitis virus, and West Nile virus caused large outbreaks worldwide[10]. Taken together, infectious diseases associated with orthomyxoviruses and flaviviruses are the subject of much important study. In mice, both Mx1 and Mx2 proteins have been identified, and Mx1 localizes in the nucleus and inhibits virus polymerase activity[5,14,22,25,39]. Therefore, mice carrying intact Mx1 show resistance to orthomyxoviruses such as influenzavirus[1,33]. B6.MSM-*Mx* and B6 mice showed notably different survival rates after a challenge with a highly pathogenic avian influenza virus. Although it remains to be determined whether Mx in all vertebrates possesses anti-viral

activity against orthomyxoviruses, anti-viral activity of the Mx protein has been shown in rodents, human, and other animals, suggesting that laboratory mice lacking these genes may not reflect the normal infectious conditions in humans and animals[39]. In addition, these data indicate that studies performed using mice lacking Mx would lead to incorrect evaluations of viral virulence, effects of vaccine, drug and therapy and so on. MSM mice do not express Mx2, and B6.MSM-*Mx* mice do not recover Mx2 expression, suggesting that the absence of Mx2 expression in MSM mice is not due to the MSM genetic background but due to the presence of a putative cis-acting element in the Mx locus. Mouse Mx2 protein localizes in the cytoplasm in the same manner as the Mx proteins in humans and some animals[2,25,39]. Mouse Mx2 can inhibit the replication of negative-stranded RNA viruses that replicate in the cytoplasm such as vesicular stomatitis virus and hanta virus, but not those that replicate in the nucleus such as influenza virus. On the other hand, Mx proteins in humans and some animals localize in cytoplasm and can inhibit influenza virus replication[2,25,39]. The reason for the differential anti-viral activity between mouse Mx2 and Mx proteins of other species is unknown.

Oas genes are induced by type 1 IFN, and synthesize 2-5 A, and consequently RNA degradation, by activating latent RNase L[9,28,41]. In mice, a gene cluster has been identified on Chr 5 that is comprised of ten *Oas* family genes[19,27]. Although mouse *Oas1b* is one of these genes, Oas1b protein is not regarded as a typical Oas protein, because it lacks enzymatic activity[7]. Although mice carrying intact *Oas1b* show resistant in flavivirus infection, the mechanism by which Oas1b confers resistance to the infection of flaviviruses on mice remains unclear[18,26,30]. In recent reports, the *Oas1b* gene of wild-derived mice was shown to confer differential resistance to the infection of flaviviruses due to the polymorphisms[8,29,38]. In our study, B6.MSM-*Mx* but not B6.MSM-*Oas* mice showed resistance to the infection of influenza virus. On the other hand, B6.MSM-*Oas* but not B6.MSM-*Mx* mice showed resistance to the infection of West Nile virus, suggesting that the Oas1b protein of MSM mice possesses anti-West Nile virus characteristics but murine Mx1 protein does not. In addition, these results indicate that Mx1 and Oas1b specifically inhibit influenza virus and West Nile virus replications, respectively.

As there is only limited information on the mechanisms of the pathogenesis of virus infection and the role of host innate immune response in humans, animal models are necessary to identify relationship between virus and host defense in infectious diseases. Mice are frequently used as an animal model to study the viral virulence and vaccine efficacy before using other larger animals[3,6]. However, standard laboratory mice do not possess certain key components of the innate immune system that mediates protection against the infection of these viruses. It has been reported that the proinflammatory cytokines, such as interleukin 1, interleukin 6, and macrophage inflammatory protein-1, are significantly increased in highly pathogenic influenza-infected mice; however, the course of the disease and the extent of virus replication and spread in these knockout mice were not different from those observed in wild-type mice[36]. On the other hand, IFN α/β receptor-deficient mice are highly susceptible to pathogenic influenza virus[37]. These results suggest a role for IFN signaling, including Mx1, which is essential for protection of the host in the early stages of infection in mice. Thus, the congenic mice generated in this study are useful for the further investigation of orthomyxovirus and flavivirus infectious diseases, particularly, the precise mechanism and timing of the interplay between components of pro- and anti-inflammatory signaling pathways, and may allow the eventual identification of an effective target on these viruses.

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