Virulence Determinants of Pandemic A(H1N1)2009 Influenza Virus in a Mouse Model

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A novel swine-origin H1N1 influenza virus [A(H1N1)pdm09 virus] caused the 2009 influenza pandemic. Most patients exhibited mild symptoms similar to seasonal influenza, but some experienced severe clinical signs and, in the worst cases, died. Such differences in symptoms are generally associated with preexisting medical conditions, but recent reports indicate the possible involvement of viral factors in clinical severity. To better understand the mechanism of pathogenicity of the A(H1N1)pdm09 virus, here, we compared five viruses that are genetically similar but were isolated from patients with either severe or mild symptoms. In a mouse model, A/Norway/3487/2009 (Norway3487) virus exhibited greater pathogenicity than did A/Osaka/164/2009 (Osaka164) virus. By exploiting reassortant viruses between these two viruses, we found that viruses possessing the hemagglutinin (HA) gene of Norway3487 in the genetic background of Osaka164 were more pathogenic in mice than other reassortant viruses, indicating a role for HA in the high virulence of Norway3487 virus. Intriguingly, a virus possessing HA, NA, and NS derived from Norway3487 exhibited greater pathogenicity in mice in concert with PB2 and PB1 derived from Osaka164 than did the parental Norway3487 virus. These findings demonstrate that reassortment between A(H1N1)pdm09 viruses can lead to increased pathogenicity and highlight the need for continued surveillance of A(H1N1)pdm09 viruses.

The swine-origin H1N1 influenza A virus [A(H1N1)pdm09], which emerged in Mexico and the United States in the spring of 2009, spread throughout 200 countries and caused the first human pandemic of the 21st century (1–3). In most cases, patients exposed to A(H1N1)pdm09 experienced mild infections. However, A(H1N1)pdm09 did cause severe disease in high-risk groups, including pregnant women, young children, older adults, and those with chronic lung disease, obesity, or other comorbidities (4–9). Many severe cases were also reported sporadically in individuals who did not have any of these risk factors and were otherwise healthy (6). However, it is not known whether these variations in disease outcome originated from differences in the pathogenicity of the A(H1N1)pdm09 viruses.

The virulence and host range of influenza A virus are determined by both host and viral factors. Two amino acids at positions 627 and 701 in PB2 are important for the adaptation of avian influenza virus to mammalian hosts (10–12). In the case of A(H1N1)pdm09 virus, whose PB2 protein originated from an avian-like Eurasian swine virus, a basic amino acid at position 591 of PB2, which is a newly identified mammalian-adapting amino acid, has been shown to compensate for the lack of amino acid substitutions at positions 627 and 701 of PB2 (13, 14). Moreover, we and others recently found that an amino acid change of aspartic acid to glycine at position 222 (H1 numbering) in hemagglutinin (HA), which altered receptor-binding specificity (15–19, 34), confers high virulence to A(H1N1)pdm09 viruses (19–21). Here, we asked whether A(H1N1)pdm09 virus possesses other as yet unknown molecular determinants of pathogenicity. To answer this question, we compared the virulence levels of five A(H1N1)pdm09 viruses isolated from patients with severe or mild symptoms. We then generated a series of reassortant viruses be-

tween two A(H1N1)pdm09 viruses that differ in their pathogenicities in mice and attempted to determine the viral factors responsible for the high pathogenicity.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) with 5% newborn calf serum. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Cells were maintained at 37°C in 5% CO₂. MDCK cells were used for growth kinetics assays, and 293T cells were used for plasmid-based reverse genetics, as described below.

Viruses. In this study, we used the following five A(H1N1)pdm09 viruses: A/California/04/2009 (Cal04), A/Norway/3568/2009 (Norway3568), A/Norway/3487/2009 (Norway3487), A/Osaka/164/2009 (Osaka164), and A/Lviv/N6/2009 (LvivN6). These viruses were isolated from patients who had no underlying risk factors for severe influenza; unfortunately, detailed medical records, including vaccination history, treatment, and medical support after the onset of influenza in these patients, were unavailable. Cal04, Norway3568, and Osaka164 were isolated from patients who exhibited mild influenza symptoms and recovered, whereas Norway3487 and LvivN6 were isolated from patients who experienced severe symptoms and died. All of these viruses were generated by reverse genetics (22).

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Plasmid-based reverse genetics. All viruses used in the study were generated by plasmid-based reverse genetics, as described previously (22). Briefly, viral RNA was extracted from the supernatants of virus-infected MDCK cells by using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany). Extracted RNA was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA) and the universal primers specific for influenza A virus genes to generate cDNA. The resulting products were PCR amplified by using KOD Fx DNA polymerase (Toyobo, Osaka, Japan) with specific primers for each virus gene and cloned into a plasmid under the control of the human RNA polymerase I (Pol I) promoter and the mouse RNA Pol I terminator (Pol I plasmids). Mutations in the PB2 genes of Norway3487 virus were generated by PCR amplification of the respective RNA polymerase I PB2 construct with primers possessing the desired mutations. Primer sequences are available upon request. All constructs were sequenced to ensure the absence of unwanted mutations.

Growth kinetics assays. Triplicate wells of confluent MDCK cells were infected with viruses at a multiplicity of infection (MOI) of 0.0001 with MEM containing 0.3% bovine serum albumin (BSA) and 1 μ g/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin and incubated at 37°C. Supernatants were harvested at a given number of hours postinfection (hpi). Virus titers were determined by plaque assays with MDCK cells.

Mouse experiments. Six-week-old female BALB/c mice weighing 15 to 20 g (Japan SLC, Inc., Shizuoka, Japan) were used in this study. Baseline body weights were measured prior to infection. Four mice per group were anesthetized with isoflurane and then intranasally inoculated with 10^4 , 10^5 , and 10^6 PFU (50 μ l) of virus. Body weight and survival were monitored daily for 14 days postinfection (dpi). The percentage of body weight change was calculated by comparing the weight of each mouse at each time point to its initial weight on day 0. The percentage of maximum body weight loss was determined by comparing the most reduced body weight of each mouse to its initial body weight. To assess virus growth in respiratory organs, 12 mice per group were infected intranasally with 10^4 or 10^5 PFU of viruses; at 1, 3, 6, and 9 dpi, three mice were euthanized and their lungs and nasal turbinates were collected, homogenized with MEM containing 0.3% BSA, and titrated in MDCK cells by plaque assays.

Our research protocol for the use of mice followed the University of Tokyo's Regulations for Animal Care and Use, which were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

Histopathological analysis. Twelve mice per group were anesthetized with isoflurane and then intranasally inoculated with 10^5 PFU (50 μ l) of virus. At 1, 3, 6, and 8 dpi, three mice were euthanized and their lungs were preserved in 10% phosphate-buffered formalin for pathological examination. The formalin-fixed lungs were processed for paraffin embedding. The paraffin-embedded tissues were cut into 5- μ m-thick sections. One section was stained by using hematoxylin-and-eosin (H&E). Additional sections were processed for immunohistological staining with an anti-influenza virus rabbit polyclonal antibody (R309; prepared in our laboratory). Specific antigen-antibody reactions were visualized by means of 3,3'-diaminobenzidine tetrahydrochloride and the Dako EnVision system (Dako Co., Ltd., Tokyo, Japan).

Virus purification and Western blotting. MDCK cells were infected with the Osaka164, Norway3487, or Osaka(3P+NP) viruses at an MOI of 5. [In "Osaka(3P+NP)," "3P" represents a complex of the three polymerases PB2, PB1, and PA.] Culture supernatants were harvested at 3, 6, 9, and 12 hpi and clarified by low-speed centrifugation (3,000 rpm, 15 min, 4°C). The supernatants were collected and ultracentrifuged through a 25% sucrose cushion (45,000 rpm, 1.5 h, 4°C), by using an SW41 Ti rotor (Beckman Coulter), and were then subjected to Western blotting.

Cells and viruses were lysed with protein sample buffer and then heated for 10 min at 95°C and immediately chilled on ice prior to SDS-PAGE. SDS-PAGE was performed on Mini protean Any kD TGX gradient gels (Bio-Rad) for 30 min at 200 V. Proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore) in

transfer buffer (100 mM Tris, 190 mM glycine, 10% [vol/vol] methanol). The membrane was rocked with Blocking One (Nacalai Tesque) for 1 h and then was incubated with an anti-influenza virus rabbit antibody (R309) and a β -actin antibody (clone AC-74; Sigma-Aldrich) for 1 h at room temperature, followed by three washes with phosphate-buffered saline (PBS) supplemented with 0.005% (vol/vol) Tween 20 (PBS-T). Finally, the membrane was incubated with ECL enhanced chemiluminescence anti-mouse IgG horseradish peroxidase-linked whole antibody from sheep (GE Healthcare) for 1 h at room temperature and then washed three times with PBS-T. The membrane was incubated with Chemilumi One Super (Nacalai Tesque), and chemical luminescent signals were visualized by means of a Versa Doc imaging system (Bio-Rad).

Statistical analysis. Differences in virus titers of supernatants, lungs, and nasal turbinates were statistically analyzed using Student's *t* test. Differences in mean maximum body weight loss in mice were also statistically analyzed by using Student's *t* test.

RESULTS

Comparison of the replicative ability and pathogenicity of A(H1N1)pdm09 viruses. As noted above, the amino acids at positions 591 and 222 of PB2 and HA, respectively, have been identified as determinants for mammalian adaptation and pathogenicity of A(H1N1)pdm09 (14, 19–21), yet our previous study suggested contributions of other viral proteins to the pathogenicity of A(H1N1)pdm09 viruses in mammals (19). We therefore compared the biological properties of five A(H1N1)pdm09 viruses isolated from patients who had no underlying risk factors for severe influenza: A/Norway/3487/2009 (Norway3487) and A/Lviv/N6/2009 (LvivN6), which were isolated from fatal cases, and A/California/04/2009 (Cal04), A/Norway/3568/2009 (Norway3568), and A/Osaka/164/2009 (Osaka164), which were isolated from mild cases. All of these viruses were generated by reverse genetics (22).

To assess the growth properties of these five viruses *in vitro*, MDCK cells were infected with the viruses at a multiplicity of infection (MOI) of 0.0001. Norway3568, Norway3487, LvivN6, and Cal04 grew well, and their maximum titers (1.9×10^8 , 3.9×10^8 , and 4.2×10^8 PFU/ml at 36 hpi and 4.9×10^7 PFU/ml at 48 hpi, respectively) were significantly higher than that of Osaka164 (1.6×10^7 PFU/ml at 36 hpi) ($P < 0.01$) (Fig. 1). We also compared the replicative abilities of these viruses in the respiratory organs of mice. Mice were infected with 10^5 PFU of viruses and euthanized; their organs were collected at 3 and 6 dpi for virus titration. At 3 dpi, the virus titers in the lungs of mice infected with Norway3487 or Cal04 were significantly higher than those of mice infected with Osaka164 ($P < 0.05$), and at 6 dpi, the virus titers in the lungs of mice infected with Norway3487, LvivN6, or Cal04 were significantly higher than those of mice infected with Osaka164 ($P < 0.05$). Titers in the nasal turbinates of mice infected with LvivN6 were significantly lower than those of mice infected with other viruses at either 3 or 6 dpi ($P < 0.05$) (Fig. 2).

We next examined the pathogenicity of the viruses in mice. Four mice per group were intranasally infected with each virus at 10^4 , 10^5 , and 10^6 PFU, and morbidity and mortality were observed for 14 days. Although none of the viruses tested was lethal to mice, the degree of body weight change among the virus-infected mice varied, depending upon the infecting virus strain (Fig. 3). In mice infected with 10^6 PFU, two viruses, Norway3487 and LvivN6, caused significantly severe body weight loss (mean maximum body weight losses of $29.4\% \pm 4.0\%$ and $27.8\% \pm 4.6\%$, respectively), compared with mice infected with Cal04, Norway3568, or

FIG 1 Growth kinetics of A(H1N1)pdm09 viruses in MDCK cells. MDCK cells were infected with five A(H1N1)pdm09 viruses at an MOI of 0.0001. At the indicated times after infection, virus titers in the supernatant were determined with MDCK cells. The reported values are means $\pm$ standard deviations (SD) from three independent experiments. Asterisks indicate that the maximum titers of Norway3568, Norway3487, LvivN6, and Cal04 were significantly higher than that of Osaka164 ($P < 0.01$).

Osaka164 (body weight losses of $16.0\% \pm 1.0\%$, $16.6\% \pm 3.3\%$, or $8.1\% \pm 3.9\%$, respectively) ($P < 0.05$) (Fig. 3A). In mice infected with 10^5 or 10^4 PFU of virus, Norway3487 still caused significantly severe body weight loss ($28.9\% \pm 5.6\%$ and $27.1\% \pm 4.8\%$, respectively) ($P < 0.05$), whereas mice infected with 10^4 PFU of LvivN6 experienced no substantial body weight loss (Fig. 3B and C) compared with viruses except for Norway3487. Taken together, our results revealed that Norway3487, which was isolated from a lethal human case, exhibited the highest pathogenicity in a mouse model, whereas Osaka164 (isolated from a mild case) showed the lowest pathogenicity in this animal among the viruses tested, suggesting the close correlation between clinical severity in humans and pathogenicity in mice. These results thus suggest that this mouse model is suitable for evaluating the pathogenicity of A(H1N1)pdm09 viruses.

The HA gene of Norway3487 virus contributes to high pathogenicity in mice. There are 10 amino acid differences between the genomes of Norway3487 and Osaka164 (Table 1). To determine which viral segment(s) contributes to virulence in mice, we generated a series of reassortant viruses between Norway3487 and Osaka164 (Table 2). These reassortant viruses were named according to the origin of their Osaka164 or Norway3487 genes. For example, “Norway(PB2)” indicates a virus possessing PB2 from Norway3487 and the rest of its genes from Osaka164. We first examined the *in vitro* replicative ability of the reassortants and their parental strains using MDCK cells. Three viruses possessing the HA of Norway3487 [i.e., Norway3487, Norway(HA), and Osaka(3P+NP)], had significantly higher replicative ability than Osaka164 ($P < 0.01$), whereas the replicative ability of the remaining reassortant viruses was similar to that of Osaka164 (Fig. 4). These results indicate that the HA of Norway3487 contributes to efficient replication in MDCK cells.

To examine the pathogenicity of the reassortant viruses in mice, we intranasally inoculated mice with 10^4 to 10^6 PFU of viruses and observed their mortality and morbidity for 14 days. The viruses possessing the HA of Norway3487 [i.e., Norway3487, Nor-

FIG 2 Virus titers in the respiratory tract of mice infected with A(H1N1)pdm09 viruses. Mice were intranasally inoculated with 10^5 PFU of each A(H1N1)pdm09 virus. Tissues were collected from mice ($n = 3$) on the indicated days postinfection (dpi), and titers were determined in MDCK cells. Asterisks signify significant differences in virus titers ($P < 0.05$).

way(HA), and Osaka(3P+NP)] caused more severe body weight loss than did Osaka164 (Table 2), whereas the other reassortant viruses caused body weight loss similar to that caused by Osaka164, suggesting that the HA gene has an important role in the increased pathogenicity of Norway3487 in mice.

The PB2 and PB1 genes of Osaka164 cause increased pathogenicity in mice in concert with the HA, NA, and NS genes of Norway3487. Intriguingly, as shown in Table 2, Osaka(3P+NP) was lethal to mice, with a 50% mouse lethal dose (MLD_{50}) of $10^{5.0}$ PFU, yet the parental pathogenic strain Norway3487 did not kill mice even when the mice were infected with the highest dose tested (i.e., 10^6 PFU/mouse). Since there are no amino acid differences in the PA, NP, M1, or M2 of Osaka164 and Norway3487, these results suggest that the expression of the PB2 and PB1 genes from Osaka164 and that of the HA, NA, and NS genes from Norway3487 enhance virulence in mice to a greater extent than the expression of these genes in the parental Norway3487 virus (Table 2). To better understand the difference in virulence in mice infected with Osaka164, Norway3487, and Osaka(3P+NP), we examined virus replication in the respiratory organs of mice. Mice were infected with 10^4 PFU of Osaka164, Norway3487, and Osaka(3P+NP), and virus titers were determined in the lungs and nasal turbinates at 1, 3, 6, and 9 dpi. For Osaka164, the virus titers in the lungs of the infected mice were lower than those of mice infected with other viruses, and no or low virus titers were recovered from the lungs or nasal turbinates, respectively, of the infected mice at 9 dpi (Table 3). On the other hand, although virulence differed between Osaka(3P+NP) and Norway3487, the virus growth properties in the lungs and nasal turbinates of infected mice were similar between these two viruses (Fig. 5 and Table 3).

We next analyzed the histopathology in the lungs of the virus-infected mice (Fig. 6). At 1 and 3 dpi, we did not observe any substantial histopathological differences in the lungs of the mice

FIG 3 Body weight changes in infected mice. Four mice per group were intranasally inoculated with 10^6 (A), 10^5 (B), or 10^4 (C) PFU (each in 50 μ l) of Cal04, Norway3568, Norway3487, Osaka164, or LvivN6. Body weights were monitored daily. The values represent average scores of overall body weight loss compared with initial body weight $\pm$ standard deviations (SD) from four mice.

infected with these viruses. However, differences between the Osaka(3P+NP) and other virus groups were observed at 6 dpi (Fig. 6); that is, the accumulation of neutrophils was more prominent in the Osaka(3P+NP)-infected mice (Fig. 6A and B) than in the other groups, whereas the lungs of mice infected with Norway3487 or Osaka164 virus exhibited lesions that included mono-nuclear cells, neutrophils, and regenerated epithelial cells (Fig. 6E

and F, Norway3487, and I and J, Osaka164), indicating evidence of recovery from bronchopneumonia (Fig. 6). These data suggest that the Osaka(3P+NP) virus induced a longer active inflammatory process than did Norway3487 or Osaka164 virus. Of note,

TABLE 1 Amino acid differences between Osaka164 and Norway3487^a

Protein	Position	Amino acid in:	
		Osaka164	Norway3487
PB2	340	K	N
	649	I	V
PB1	667	T	I
HA	125 ^b	N	D
	203	S	T
	222	D	G
NA	248 ^c	N	D
NS1	103	F	L
	123	I	V
NS2	63	K	E

^a There were no amino acid differences in the PA, NP, PB1-F2, M1, and M2 proteins.

^b H1 numbering.

^c N1 numbering.

TABLE 2 Evaluation of virulence in mice infected with reassortants between Osaka164 and Norway3487^a

Virus	MLD ₅₀ ^b	% of body wt loss (mean $\pm$ SD) ^c
Osaka164	$>10^{6.5}$	8.1 $\pm$ 3.9
Norway3487	$>10^{6.5}$	29.4 $\pm$ 4.0*
Norway(3P+NP) ^d	$>10^{6.5}$	9.1 $\pm$ 2.6
Osaka(3P+NP)	10^5	34.9*
Norway(PB2)	$>10^{6.5}$	13.2 $\pm$ 2.6
Norway(PB1)	$>10^{6.5}$	10.0 $\pm$ 2.8
Norway(PA)	$>10^{6.5}$	11.8 $\pm$ 1.1
Norway(NP)	$>10^{6.5}$	8.3 $\pm$ 3.1
Norway(HA)	$>10^{6.5}$	22.6 $\pm$ 3.4*
Norway(NA)	$>10^{6.5}$	9.7 $\pm$ 2.4
Norway(M)	$>10^{6.5}$	14.8 $\pm$ 4.1
Norway(NS)	$>10^{6.5}$	15.8 $\pm$ 4.3

^a Mice were infected with 10^4 , 10^5 , or 10^6 PFU of reassortant viruses that combined the genes of the Norway3487 and Osaka164 viruses, generated by reverse genetics, with those of the parental Norway3487 and Osaka164 viruses.

^b MLD₅₀, 50% mouse lethal dose.

^c The mean maximum body weight losses $\pm$ standard deviations (SD) shown in the table are for mice that were infected with 10^5 PFU. Asterisks indicate that the mean maximum body weight loss was significantly severe in mice infected with the reassortant virus compared with that in mice infected with Osaka164 ($P < 0.05$).

^d 3P, three-polymerase complex (i.e., PB2, PB1, and PA).

FIG 4 Growth kinetics of reassortant viruses in MDCK cells. MDCK cells were infected with reassortants between Osaka164 and Norway3487 at an MOI of 0.0001. At the indicated times after infection, virus titers in the supernatants were determined with MDCK cells. The reported values are means $\pm$ standard deviations (SD) from three independent experiments. Asterisks indicate that the maximum titers of Norway(HA), Osaka(3P+NP), and Norway3487 were significantly higher than that of Osaka164 ($P < 0.01$).

although there was no significant difference between the virus titers in the lungs of Norway3487 and Osaka(3P+NP) at 9 dpi (Table 3), viral antigen-positive cells in the lungs of mice infected with Osaka(3P+NP) were much more prevalent than those in the lungs of mice infected with Norway3487 at 8 dpi [Fig. 6C and D, Osaka(3P+NP), G and H, Norway3487, and K and L, Osaka164]. In addition, our *in vitro* study demonstrated that higher levels of M1 protein were expressed in MDCK cells infected with Osaka(3P+NP) than in those infected with Osaka164 or Norway3487 at 9 and 12 h postinfection ($P < 0.05$), yet there were no significant differences observed in viral protein levels in the culture supernatant of the cells infected with these viruses (Fig. 7). These results indicate that enhanced and/or sustained viral protein expression may contribute to pathogenicity in mice.

Amino acid substitutions in PB2 and PB1, in concert with HA, NA, and NS of Norway3487, contribute to high virulence in mice. There are only three amino acid differences between Osaka(3P+NP) and Norway3487: two in PB2 and one in PB1. To identify the molecular determinants responsible for the high virulence of Osaka(3P+NP) in mice, we tested three reassortant viruses, Osaka(PB2), Osaka(PB1), and Osaka(PB2/PB1), which possess PB2, PB1, or both genes from Osaka164, respectively, in the genetic background of Norway3487 (Fig. 5). We found that Osaka(PB2/PB1) was more pathogenic than Osaka(PB2) and Osaka(PB1): the MLD₅₀ values for Osaka(PB2/PB1), Osaka(PB2), and Osaka(PB1) were $10^{4.3}$, $10^{5.5}$, and $10^{5.7}$ PFU, respectively (Fig. 5).

To further identify the amino acid that determines the high virulence of Osaka(3P+NP) in mice, we generated six mutant Norway3487 viruses possessing single or double point mutations at positions 340 and 649 of PB2 and position 667 of PB1 (Fig. 8) and examined their properties. We did not find any appreciable differences in the replicative abilities of all of these viruses in MDCK cells (data not shown) or in mice (Fig. 8 and Table 3). Furthermore, we did not find any significant difference in the polymerase activities in the minigenome assay (data not shown). Nonetheless, among the six viruses tested, Norway PB2 V649I/PB1 I667T and Norway PB2 N340K/PB1 I667T caused the highest

mortality rates in mice, with MLD₅₀ values of $10^{4.5}$ PFU and $10^{4.8}$ PFU, respectively (Fig. 8). The viruses with single point mutations in their PB2 or PB1 genes on the Norway3487 backbone were less pathogenic than Norway PB2 V649I/PB1 I667T or Norway PB2

TABLE 3 Virus titers in the respiratory tract of mice infected with reassortant viruses^a

Virus	dpi	Virus titer (log ₁₀ PFU/g [mean $\pm$ SD]) in:	
		Nasal turbinate	Lung
Osaka164	1	4.0 $\pm$ 0.6	6.9 $\pm$ 0.4
	3	6.0 $\pm$ 0.1	8.1 $\pm$ 0.5
	6	6.2 $\pm$ 0.2	7.1 $\pm$ 0.3
	9	4.2 $\pm$ 1.2	— ^b
Norway3487	1	5.4 $\pm$ 0.2	8.0 $\pm$ 0.2
	3	7.1 $\pm$ 0.3	8.5 $\pm$ 0.4
	6	6.3 $\pm$ 0.4	7.7 $\pm$ 0.2
	9	5.1 $\pm$ 1.1	4.8 $\pm$ 1.0
Osaka(3P+NP)	1	5.1 $\pm$ 0.4	7.9 $\pm$ 0.4
	3	6.3 $\pm$ 0.3	8.8 $\pm$ 0.3
	6	6.3 $\pm$ 0.6	7.6 $\pm$ 0.3
	9	5.4 $\pm$ 1.4	4.2 $\pm$ 0.7
Osaka(PB2)	1	4.7 $\pm$ 0.4	7.4 $\pm$ 0.2
	3	5.7 $\pm$ 0.7	8.3 $\pm$ 0.2
	6	5.3 $\pm$ 0.8	7.0 $\pm$ 0.2
	9	5.1 $\pm$ 1.7	4.0 $\pm$ 0.7
Osaka(PB1)	1	4.5 $\pm$ 0.4	7.3 $\pm$ 0.1
	3	7.2 $\pm$ 0.3	8.2 $\pm$ 0.3
	6	6.6 $\pm$ 0.3	7.3 $\pm$ 0.1
	9	5.3 $\pm$ 0.8	4.3 $\pm$ 0.4
Osaka(PB2/PB1)	1	4.8 $\pm$ 0.4	6.8 $\pm$ 0.4
	3	7.3 $\pm$ 0.4	8.4 $\pm$ 0.1
	6	6.7 $\pm$ 0.2	7.4 $\pm$ 0.1
	9	4.9 $\pm$ 0.2	4.7 $\pm$ 0.3
Norway PB2 N340K	1	4.4 $\pm$ 0.1	6.4 $\pm$ 0.1
	3	5.9 $\pm$ 0.8	8.2 $\pm$ 0.1
	6	6.2 $\pm$ 0.1	7.6 $\pm$ 0.3
	9	5.2 $\pm$ 0.5	4.6 $\pm$ 0.9
Norway PB2 V649I	1	5.1 $\pm$ 0.5	6.9 $\pm$ 0.1
	3	5.8 $\pm$ 1.5	8.1 $\pm$ 0.1
	6	5.6 $\pm$ 1.2	7.7 $\pm$ 0.1
	9	3.9 $\pm$ 0.8	6.0 $\pm$ 0.2
Norway PB2 N340K/V649I	1	4.1 $\pm$ 0.2	6.6 $\pm$ 0.4
	3	6.6 $\pm$ 0.6	8.5 $\pm$ 0.3
	6	5.6 $\pm$ 1.4	7.6 $\pm$ 0.1
	9	5.1 $\pm$ 1.8	5.1 $\pm$ 0.7
Norway PB2 N340K/PB1 I667T	1	5.0 $\pm$ 0.7	7.0 $\pm$ 0.1
	3	6.7 $\pm$ 1.1	8.3 $\pm$ 0.2
	6	6.6 $\pm$ 0.9	8.0 $\pm$ 0.2
	9	3.6 $\pm$ 0.7	4.9 $\pm$ 0.6
Norway PB2 V649I/PB1 I667T	1	4.2 $\pm$ 0.2	6.1 $\pm$ 0.3
	3	5.1 $\pm$ 0.8	7.9 $\pm$ 0.2
	6	5.5 $\pm$ 1.0	7.6 $\pm$ 0.3
	9	3.5 $\pm$ 0.7	3.8 $\pm$ 1.0
Norway PB2 N340K/V649I/PB1 I667T	1	4.5 $\pm$ 0.6	7.3 $\pm$ 0.3
	3	7.1 $\pm$ 0.3	8.1 $\pm$ 0.1
	6	6.1 $\pm$ 0.2	7.8 $\pm$ 0.1
	9	4.3 $\pm$ 0.7	4.3 $\pm$ 0.2

^a Mice were intranasally inoculated with 10^4 PFU of Osaka164, Norway3487, or Osaka(3P+NP). Tissues were collected from mice ($n = 3$) on the indicated days postinfection (dpi), and titers were determined in MDCK cells.

^b —, titer of <3.0 log₁₀ PFU/g.

FIG 5 Characterization of reassortant viruses in mice. Mice were intranasally inoculated with 10^4 PFU of Osaka164, Norway3487, and reassortant viruses. Three mice per group were euthanized at 3 dpi, and virus titers in the lungs (■) and nasal turbinates (□) were determined by using plaque assays in MDCK cells. The results are expressed as mean titers $\pm$ standard deviations (SD). Asterisks indicate that virus titers in the nasal turbinates of mice infected with Norway3487 or reassortant viruses were significantly higher than those in the respiratory tract of mice infected with Osaka164 ($P < 0.05$). MLD₅₀ values were calculated by observing mice infected with 10^4 , 10^5 , or 10^6 PFU of parental or reassortant viruses. The mean maximum body weight losses (%) $\pm$ standard deviations (SD) shown are for mice that were infected with 10^5 PFU. Asterisks indicate that the mean maximum body weight loss was significantly severe in mice infected with the reassortant virus, compared with that in mice infected with Osaka164 ($P < 0.05$). The minus sign indicates that the maximum body weight loss could not be measured because all of the infected mice died.

FIG 7 Viral protein expression levels in cell lysates and culture supernatants of cells infected with viruses. MDCK cells were infected with Osaka164, Norway3487, or Osaka(3P+NP) viruses at an MOI of 5, and samples were collected at 3, 6, 9, and 12 hpi. Cell lysates (Lysates) and concentrated culture supernatants (Sup) were subjected to Western blotting to detect M1 protein. The expression levels of M1 in the Osaka(3P+NP)-infected cells were significantly higher than those in the Osaka164- or Norway3487-infected cells at 9 and 12 h postinfection ($P < 0.05$), whereas no significant differences in the levels of released virus particles were observed among these virus groups.

N340K/PB1 I667T, indicating that these mutations (PB2 N340K or V649I and PB1 I667T), in concert with HA, NA, and NS derived from Norway3487, contribute to high virulence in mice.

DISCUSSION

Here, we demonstrated that HA is responsible for the increased pathogenicity of Norway3487, which was isolated from a fatal case

FIG 6 Pathological findings in mice infected with Osaka164, Norway3487, or Osaka(3P+NP). The images represent the lungs of mice infected with Osaka(3P+NP) (A to D), Norway3487 (E to H), or Osaka164 (I to L). (A, E, and I) Images of the lungs at low magnification are shown. The blue circles indicate the lesions. (B) Magnified view of the boxed area in panel A. Infiltration of neutrophils was obvious at 6 dpi and is indicated by red arrowheads. (F and J) Magnified views of the boxed areas in panels E and I. The lungs of mice infected with Norway3487 (F) or Osaka164 (J) exhibited lesions that included mononuclear cells, neutrophils, and regenerated epithelial cells. (C, G, and K) Immunostaining of lungs infected with Osaka(3P+NP) (C and D), Norway3487 (G and H), and Osaka164 (I and L) at 8 dpi at low magnification is shown. (D, H, and L) Magnified views of the boxed areas in panels C, G, and K, respectively. (D) Many viral antigen-positive cells (brown pigment) can be seen in the Osaka(3P+NP)-infected lungs, whereas few viral antigen-positive cells were present in lungs infected with Norway3487 (H) or Osaka164 (L).

FIG 8 Characterization of mutant viruses in mice. Mice were intranasally inoculated with 10^4 PFU of Norway3487 and point mutant viruses. Three mice per group were euthanized at 3 dpi, and virus titers in the lungs (■) and nasal turbinates (□) were determined by using plaque assays in MDCK cells. The results are expressed as mean titers $\pm$ standard deviations (SD). MLD₅₀ values were calculated by observing mice infected with 10^4 , 10^5 , or 10^6 PFU of Norway3487 or the point mutant viruses. The mean maximum body weight losses (%) $\pm$ standard deviations (SD) shown are for mice that were infected with 10^5 PFU.

and exhibited high virulence in mice. HA is critical for the host range restriction and pathogenicity of influenza viruses (3, 23–26). Generally, binding specificities to avian- or human-type receptors are determined by specific amino acids in HA (27, 28). For A(H1N1)pdm09 viruses, we and others have demonstrated that an Asp-to-Gly change at position 222 (H1 numbering) of HA, which confers avian-type receptor-binding specificity (15–19), results in enhanced virulence in mouse and nonhuman primate models (19–21, 34). In agreement with these previous findings (19–21), we found that Norway3487, which was the most pathogenic of the viruses tested in mice, possesses Gly at position 222 of HA (Table 1), whereas Osaka164 does not, thereby explaining the difference in virulence in mice between these two viruses. In addition, we tested the effect of the NS gene, which encodes the interferon (IFN) antagonist NS1 protein, on the interferon response because there are two amino acid differences between the NS1 of Norway3487 and that of Osaka164. The levels of IFN- β mRNA were similar among Norway3487-, Osaka164-, and Norway(NS)-infected cells (data not shown), suggesting that Norway3487 NS1 does not contribute to or offers a limited contribution to the pathogenicity of viruses.

Interestingly, we found that a reassortant virus, Osaka(3P+NP), possessing the polymerase complex of Osaka164 and its HA, NA, and NS genes from Norway3487, was more pathogenic in mice than was the parental Norway3487 virus (Table 2). Although there was no significant difference between the virus titers in the lungs of Norway3487 and Osaka(3P+NP) at 9 dpi, viral antigen-positive cells in the lungs of mice infected with Osaka(3P+NP) were more prevalent than in mice infected with the parental Norway3487 and Osaka164 viruses (Fig. 6). Our *in vitro* study demonstrated that the expression levels of viral protein in the Osaka(3P+NP)-infected cells were significantly higher than those in the Norway3487- or Osaka164-infected cells (Fig. 7), and this feature may contribute to the high prevalence of viral antigen-positive cells in the Osaka(3P+NP)-infected mouse lungs (Fig. 6). Therefore, it is highly possible that the increased and/or sustained

expression of viral proteins in the lungs of the infected mice may contribute to enhanced immune responses (e.g., prolonged neutrophil accumulation and inflammatory responses) in the lungs, resulting in higher pathogenicity.

We further identified that Lys or Ile at position 340 or 649 of PB2, respectively, and Thr at position 667 of PB1 contribute to this high virulence of Osaka(3P+NP) in mice even though there was no marked difference in replication efficiencies among the three viruses (Fig. 8). Positions 667 in PB1 and 649 in PB2 are located in the interaction site between PB2 and PB1 (29–31), and position 340 in PB2 is located in the cap binding domain (31, 32). Additionally, viruses that possess both PB2-649I and PB1-667T are classified in clade 4 of the A(H1N1)pdm09 viruses (33). Of note, no viruses possessing only one of these two mutations has yet been found, indicating that these mutations might cooperate and provide an advantage in terms of stability or survival for viruses over the presence of either mutation alone. Since these amino acid differences had no effect on viral replicative ability *in vitro* or *in vivo* (Fig. 5, Fig. 8, and Table 3), we can hypothesize that the increased virulence of Osaka(3P+NP) might be attributed to other events, such as aberrant immune responses to infection with this virus. Further study is needed to assess the mechanistic role of these amino acids in the pathogenicity of A(H1N1)pdm09 viruses.

While most individuals infected with A(H1N1)pdm09 virus do not experience severe symptoms and recover without the need for hospitalization, this study demonstrates the risk of enhanced virulence as a result of reassortment. It is therefore imperative that we continue to monitor A(H1N1)pdm09 viruses to manage the risk associated with the emergence of more pathogenic strains.

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