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Amino Acid Substitutions in Polymerase Basic Protein 2 Gene Contribute to the Pathogenicity of the Novel A/H7N9 Influenza Virus in Mammalian Hosts

Chris Ka Pun Mok,[a,b] Horace Hok Yeung Lee,[a,b] Maxime Lestra,[b] John Malcolm Nicholls,[c] Michael Chi Wai Chan,[a] Sin Fun Sia,[a] Huachen Zhu,[a] Leo Lit Man Poon,[a] Yi Guan,[a] Joseph Sriyal Malik Peiris[a,b]

Centre of Influenza Research, School of Public Health, HKU Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong[a]; HKU-Pasteur Research Pole, School of Public Health, HKU Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong[b]; Department of Pathology, HKU Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong[c]

ABSTRACT

A novel avian-origin influenza A/H7N9 virus emerged in 2013 to cause more than 130 cases of zoonotic human disease, with an overall case fatality rate of around 30% in cases detected. It has been shown that an E-to-K amino acid change at residue 627 of polymerase basic protein 2 (PB2) occurred frequently in the H7N9 isolates obtained from humans but not in viruses isolated from poultry. Although this mutation has been reported to confer increased mammalian pathogenicity in other avian influenza subtypes, it has not been experimentally investigated in the H7N9 virus. In this study, we determined the contribution of PB2-E627K in H7N9 virus to its pathogenicity in mammalian hosts. In addition, the compensatory role of the PB2 mutations T271A, Q591K, and D701N in H7N9 virus was investigated. We characterized the activity of polymerase complexes with these PB2 mutations and found that they enhance the polymerase activity in human 293T cells. The rescued mutants enhanced growth in mammalian cells *in vitro*. Mice infected with the H7N9 mutant containing the avian signature protein PB2-627E showed a marked decrease in disease severity (weight loss) and pathology compared to mice infected with the wild-type strain (PB2-627K) or other PB2 mutants. Also, mutants with PB2-627E showed lower virus replication and proinflammatory cytokine responses in the lungs of the virus-infected mice, which may contribute to pathogenicity. Our results suggest that these amino acid substitutions contribute to mouse pathogenicity and mammalian adaptation.

IMPORTANCE

A novel avian H7N9 influenza A virus emerged in east China in 2013 to cause zoonotic human disease associated with significant mortality. It is important to understand the viral genetic markers of mammalian adaptation and disease severity in this H7N9 virus. Since many human (but not avian) H7N9 virus isolates have an amino acid substitution at position E627K in the polymerase basic protein 2 (PB2) gene, we investigated the role of this and other functionally related mutations for polymerase activity *in vitro*, virus replication competence, and pathogenicity in the mouse model. We found that E627K and functionally related mutations are associated with increased polymerase activity, increased viral replication competence, and increased disease severity in mice.

In March of 2013, a novel avian H7N9 influenza A virus causing serious human disease and death in eastern China was identified. As of 25 October, 137 human cases leading to 45 deaths have been reported (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/10u_ReportWebH7N9Number.pdf), and additional cases continue to be reported. Patients with A/H7N9 viruses typically develop a rapidly progressive viral pneumonia leading to respiratory failure and acute respiratory distress syndrome (ARDS), reminiscent of human HPAI H5N1 disease (1, 2). There is evidence that infection may also lead to mild or asymptomatic infections, and the true extent of infection is believed to be much greater, resulting in a significant "iceberg" of unrecognized infection (3). Mutations in the viral hemagglutinin allow this H7N9 virus to bind both avian-like and human-like receptors in the human respiratory tract, conferring efficient tropism for the human conducting airways and lungs, thus explaining the relative ease with which this virus crosses species from birds to humans (4–6).

Viral genetic analysis reveals that all gene segments are of avian origin, with six "internal genes" being derived from avian influenza A (H9N2) viruses, while the H7 and N9 surface proteins originate from other subtypes (1, 7, 8). Live poultry in wet markets are an important source of human infection, since most of the patients have evidence of exposure to poultry (9, 10). This is further supported by seroepidemiological data suggesting that more than 6% of exposed poultry workers are seropositive for H7N9 virus (11).

Amino acid changes in polymerase basic protein 2 (PB2) are frequently identified in H7N9 influenza viruses isolated from human patients and are believed to be adaptive changes to the mammalian host. While the avian influenza viruses usually contain glutamic acid (E) at PB2 residue 627, mutation to lysine (K) is a

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Editor: A. García-Sastre
Address correspondence to Joseph Sriyal Malik Peiris, malik@hku.hk.

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well-recognized mammalian adaptation marker and has been previously found in human isolates of highly pathogenic avian influenza (HPAI) viruses of the H5N1 and H7N7 subtypes (12, 13). It has been shown that such mutations play a crucial role in enhancing the replication efficiency and virulence of avian influenza viruses in mammals (14–16). Other amino acid substitutions in PB2, for instance, T271A, Q591K, and D701N, can partially substitute for the function of the E627K mutation in mammalian adaptation (17–19).

We previously investigated the pathogenicity of the human H7N9 viruses by carrying out experimental infection of mice with a human isolate, A/Shanghai/2/2013 (Sh2/H7N9), isolated from a fatal case of A/H7N9 disease (20). Genetic analysis showed that Sh2/H7N9 has a mutation in the PB2 gene corresponding to E627K. We found that Sh2/H7N9 is more pathogenic in mice than a duck H7N9 virus with a different gene constellation or H9N2 avian isolates, both of which contain the avian amino acid residue 627E in PB2, in terms of weight loss, virus replication, and cytokine induction. In order to understand the contribution of the E627K mutation in PB2 and other functionally related mutations to the pathogenicity of H7N9 virus infection in mammals, we generated different recombinant viruses with individual point mutations in the PB2 gene, using Sh2/H7N9 as the background. The effects of these mutations on polymerase activity, *in vitro* replication kinetics, and pathogenicity in mice were studied in detail.

MATERIALS AND METHODS

Viruses and cells. A/Shanghai/2/2013 H7N9 virus was initially isolated from a patient with a fatal infection and passaged in the allantoic cavities of embryonated eggs. Human embryonic kidney (293T), Madin-Darby canine kidney (MDCK), and human epithelial A549 cells were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum and antibiotics. The DF-1 chicken fibroblast cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics.

Plasmid construction. The plasmids with the individual genome of the influenza viruses A/Shanghai/2/2013 and A/Dk/JX/3286/2009 were constructed as previously described (21). In brief, the cDNAs of the viruses were first synthesized by reverse transcription of viral RNA with an oligonucleotide (Uni-12) complementary to the conserved 3′ end of the viral RNA. The cDNA was then amplified by PCR with gene-specific primers. The reverse transcription-PCR (RT-PCR) products of viral RNA segments were cloned into the plasmid pHW2000. The mutant PB2 construct containing specific mutations was produced by PCR amplification with the primers possessing the mutations.

Generation of recombinant viruses. The full set of eight plasmids with or without PB2 mutations cloned from the individual virus strains were transfected into the wells and cocultured with MDCK and 293T cells. After 3 days of posttransfection, the supernatants with the recombinant viruses generated were infected into embryonated eggs and incubated for three more days. The virus stock used for infection in the experiments was titrated in a plaque-forming assay on MDCK cells.

Luciferase assays of viral polymerase activity. 293T or DF-1 cell monolayers were transfected with 125 ng of luciferase reporter plasmid (pluci) and 12.5 ng internal control plasmid (phRL-CMV) together with the mix of PB2, PB1, PA, and NP plasmids in quantities of 125, 125, 125, and 250 ng, respectively. The transfected cells were incubated at 33°C, 37°C, or 39°C. After 24 h incubation, the supernatants were discarded and the cell extracts were prepared in 100 μl of lysis buffer. The luciferase levels were assayed with a luciferase assay system (Promega) and detected with a luminometer.

Cell infection. MDCK and A549 cells were seeded at 1×10^5 cells per well in 24-well tissue culture plates. The cells were infected at a multiplicity of infection (MOI) of 0.01 for the analysis of virus replication. After 1 h of virus adsorption, the virus inoculum was removed, and the cells were washed with phosphate-buffered saline (PBS) and incubated in the appropriate culture medium supplemented with 1 mg/liter and 0.2 mg/liter *N-p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin; Sigma, St. Louis, MO, USA), respectively. Samples of culture supernatants were collected for virus titration.

Experimental infection of mice. Specific-pathogen-free female BALB/c mice (6 to 8 weeks old) were infected intranasally with 1×10^5 PFU of each virus in a 25-μl volume and monitored daily for weight loss. They were sacrificed at various days postinfection for virological and cytokine assays. The lung were homogenized in 1 ml PBS. All animal procedures were carried out under institutionally approved protocols.

Quantitative analysis of cytokines. Expression levels of tumor necrosis factor alpha (TNF-α), MIP-1α, MCP-1, MCP-3, RANTES, KC, and IP-10 in the lung homogenates were quantitatively determined by flow cytometry-based immunoassay (FlowCytomix multiplex; Bender MedSystems). In brief, the lung homogenates were collected at day 3 and day 5 postinfection. A 25-μl portion of each sample was processed according to the manufacturer's protocol. The amount of cytokine (pg/ml) in the samples was determined with a BD LSRII (BD Bioscience) and was calculated by FlowCytomix Pro 2.3 software (Bender MedSystems).

Virus titration. The amount of virus in the supernatants was titrated on MDCK cells, and the titers were reported as tissue culture infectious doses ($TCID_{50}$) per 100 μl.

Histology and immunohistochemistry of mouse lung. Lung tissues from virus-infected mice were fixed in 10% neutral buffered formalin for at least 24 h before processing. The tissues were embedded in paraffin by standard tissue processing procedures, and sections were cut at 4 μm and affixed on glass slides. Standard hematoxylin-eosin staining was carried out. Immunohistochemical staining of NP antigens in the lung tissues was performed by using antibody HB65 (European Veterinary Laboratories) according to our previously published protocol (22).

Risk assessment and biosafety. Based on previously published data from other avian H9N2 influenza viruses (23), from which the polymerase genes of H7N9 are derived, risk assessment was carried out, and it was concluded that all the mutant viruses being generated by these experiments were likely to be loss-of-function mutants with regard to pathogenicity, compared to the PB2-E627K wild-type Sh2/H7N9 virus. All procedures involving recombinant H7N9 viruses were carried out in a biosafety level 3 (agriculture) facility at The University of Hong Kong.

Statistical analysis. Statistical significance of differences between experimental groups was determined by using unpaired, parametric Student's *t* test. *P* values of <0.05 were considered significant.

RESULTS

Mammalian adaptation mutations in the PB2 gene of H7N9 virus enhances polymerase activity in mini-genome reporter assays. Previous studies have shown that the amino acid substitution E627K enhances polymerase activity in mammalian cells and enhances pathogenicity of avian influenza viruses in mammals (e.g., mice), and it is believed to contribute to mammalian adaptation (14–16). The amino acid substitutions T271A, Q591K, and D710N individually can confer a similar effect on avian influenza viruses that retain 627E (17–19). We have inspected the PB2 gene sequences of the human H7N9 viruses isolated during the recent outbreak in China and found that 15 out of 16 viruses contain one or another of these adaptive mutations (PB2-E627K, 13/16; Q591K, 1/16; D701N, 1/16) (Table 1). However, none of the publicly available avian H7N9 virus sequences have any of these amino acid substitutions associated with mammalian adaptation in the PB2 gene (Table 1).

TABLE 1 Summary of the PB2 mammalian signatures in the H7N9 human isolates

H7N9 virus	Residue in PB2 at amino acid position:			
	271	591	627	701
A/Fujian/1/2013	T	Q	K	D
A/Hangzhou/1/2013	T	Q	K	D
A/Hangzhou/2/2013	T	Q	K	D
A/Hangzhou/3/2013	T	Q	K	D
A/Nanchang/1/2013	T	Q	K	D
A/Nanjing/1/2013	T	K	E	D
A/Shanghai/02/2013	T	Q	K	D
A/Shanghai/4664T/2013	T	Q	K	D
A/Taiwan/S02076/2013	T	Q	K	D
A/Taiwan/T02081/2013	T	Q	E	D
A/Wuxi/1/2013	T	Q	K	D
A/Wuxi/2/2013	T	Q	K	D
A/Zhejiang/DTID-ZJU01/2013	T	Q	E	N
A/Zhejiang/HZ1/2013	T	Q	K	D
A/Shanghai/1/2013	T	Q	K	D
A/Anhui/1/2013	T	Q	K	D
Avian and environment isolates ($n = 42$)[a]	T	Q	E	D

[a] Sequences of nonhuman samples are available in PubMed.

We previously compared the pathogenicity of a human H7N9 strain (A/Shanghai/2/2013), a chicken H9N2 isolate (A/Chicken/Hong Kong/HH8/2010) which has internal genes similar to those of the human H7N9 virus, and a duck H7N9 virus (A/Duck/Jiangxi/3286/2009) which is of different genetic derivation than human H7N9 (20). We found that the human H7N9 virus (carrying PB2-627K) was more pathogenic than the two avian isolates (carrying PB2-627E). To understand the role of E627K and other PB2 mutations in host adaptation and pathogenicity of the human H7N9 viruses, we first compared the activities of the polymerase complexes of Sh2/H7N9 viruses with different amino acid substitutions in PB2 gene in mini-genome reporter assays using the human 293T cell line. Compared to the polymerase activity of the wild-type Sh2/H7N9 (PB2-627K), the lysine-glutamic acid substitution in the mutant PB2-K627E results in a lower polymerase activity at both 37°C and 33°C in mammalian cells (Fig. 1A and B). The substitutions Q591K or D701N in the PB2-K627E plasmid restored the polymerase activity at both temperatures, suggesting that these mutations can partially compensate for PB2-E627K in terms of polymerase activity. Interestingly, the T271A mutation restored the polymerase activity only at 37°C, not at 33°C.

We also tested the polymerase activity of a low-pathogenicity

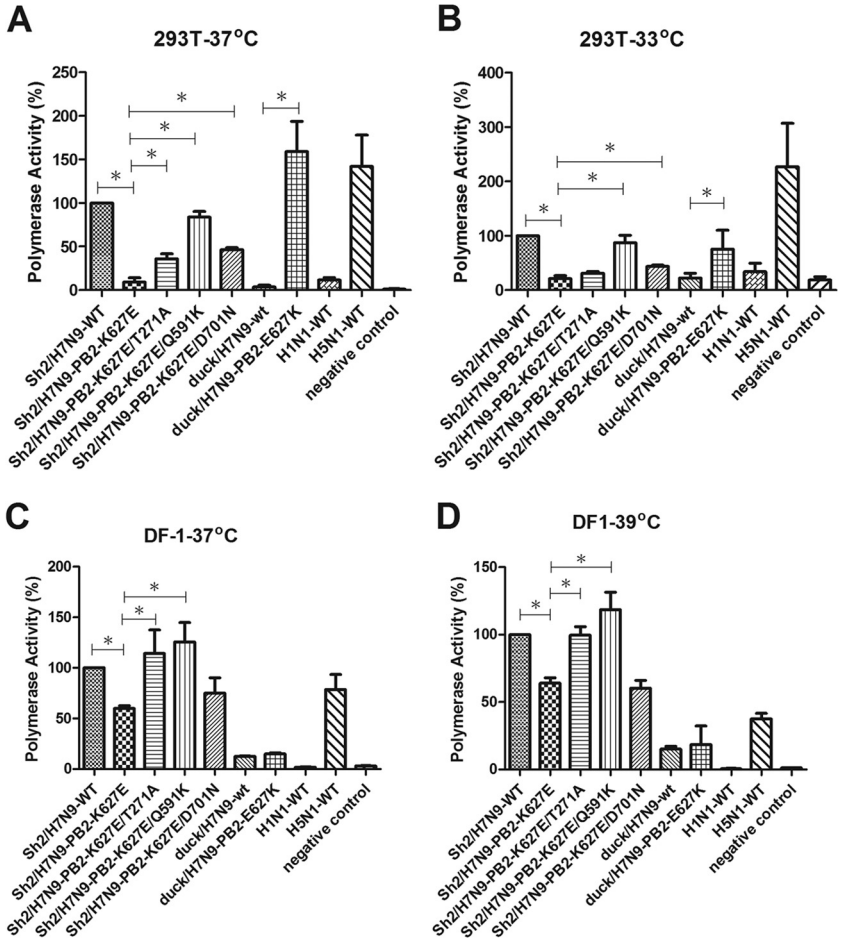

FIG 1 Polymerase activity of the Sh2/H7N9 and its PB2 mutants. 293T or DF-1 cells were transfected with plasmids containing Sh2/H7N9 PB2, PB1, PA, and NP genes plus a control luciferase reporter plasmid and a viral untranslated-region (UTR)-driven luciferase reporter plasmid. After transfection, the 293T cells were cultured at 37°C (A) and 33°C (B) and the DF-1 cells were cultured at 37°C (C) and 39°C (D) for 24 h. Luciferase activity was then assayed from the cell extracts. Results are the averages from three independent experiments. The values were statistically analyzed by two-tailed paired *t* test. *, $P < 0.05$.

avian strain (A/Duck/Jiangxi/3286/2009) (Duck/H7N9) which contains PB2-627E in the 293T cell line. The level of activity of the wild-type Duck/H7N9 virus, which is of different genetic derivation than Sh2/H7N9, is 27-fold lower than that of the wild-type Sh2/H7N9 virus. The level of polymerase activity was significantly increased when a PB2-E627K mutation was introduced into wild-type Duck/H7N9 at both 37°C and 33°C.

To understand the effect of the PB2 mutations of H7N9 viruses in avian hosts, the polymerase activities of the two H7N9 strains and their PB2 mutants were tested in chicken embryo fibroblast DF-1 cells (Fig. 1C and D). Although the polymerase activity of the wild-type Sh2/H7N9 is still higher than that of the mutant with PB2-K627E, the differences are less dramatic at both 37°C (1.7-fold) and 39°C (1.6-fold). While PB2-T271A and -Q591K can restore the polymerase activity when position 627 has the amino acid E, PB2-D701N shows polymerase activity similar to that of the mutant with PB2-K627E at both temperatures. Surprisingly, there was no significant difference in polymerase activity between wild-type Duck/H7N9 and its PB2-E627K mutant at the two temperatures.

Higher replication of H7N9 virus in mammalian MDCK and A549 cells is dependent on PB2 adaptation. To further understand the role of the PB2 mutations on pathogenicity in the mammalian host, recombinant viruses of wild-type Sh2/H7N9 and the PB2-K627E, PB2-K627E/T271A, PB2-K627E/Q591K, and PB2-K627E/D701N mutants were rescued using a plasmid-based reverse genetic approach. Multicycle growth kinetics of these viruses inoculated at a multiplicity of infection of 0.01 on to MDCK and A549 cells was determined at 37°C. Supernatant from the virus-infected cells was collected at various times postinfection, and the virus titers were measured by $TCID_{50}$ assay. All recombinant wild-type viruses and those with PB2 mammalian adaptations (PB2-K627E/T271A, PB2-K627E/Q591K, and PB2-K627E/D701N) replicated more efficiently than the mutant with PB2-K627E mutation in MDCK cells (Fig. 2A). Both of the recombinant viruses with PB2-K627E/T271A and PB2-K627E mutations showed lower virus replication than the wild type and the PB2-K627E/D701N mutant at all time points in human A549 cells. Interestingly, the recombinant mutant with PB2-K627E/Q591K showed a delayed replication kinetics at 24 h postinfection but replicated to titers similar to those of the wild-type virus (Fig. 2B).

The PB2 mammalian adaptation of H7N9 virus contributes to the pathogenicity in mice. To examine the pathogenicity of the recombinant viruses *in vivo*, we intranasally infected 6- to 8-week-old healthy female BALB/c mice (six mice in each group) with 1×10^5 PFU of the recombinant wild-type Sh2/H7N9 virus or its PB2 mutants. Mice inoculated with the viruses were monitored for 14 days for weight loss and mortality (Fig. 3). None of the mice died after infection of any of the recombinant viruses. As we had observed previously, the mice infected with the recombinant wild-type virus Sh2/H7N9, which contains PB2-627K, lost weight starting 2 days postinfection. These mice showed around an 18% weight loss, while mice infected with the mutant with PB2-K627E, which is typically found in avian Sh2/H7N9-like viruses, showed less than a 5% weight loss, suggesting that PB2-627K contributes to the pathogenicity of the mammal-adapted human H7N9 virus in mice. In viruses containing the avian-like amino acid residue PB2-K627E, mutants containing PB2-K627E/Q591K and PB2-K627E/D701N caused increased weight loss (10 to 13%), although it was still less severe than that caused by the recombinant wild-type Sh2/H7N9, which has PB2-627K. Interestingly, although the virus with PB2-K627E/T271A showed a compensatory increase in polymerase activity compared to the mutant with PB2-K627E at 37°C, they both caused similarly mild disease signs in the mice, as reflected by comparable levels of weight loss.

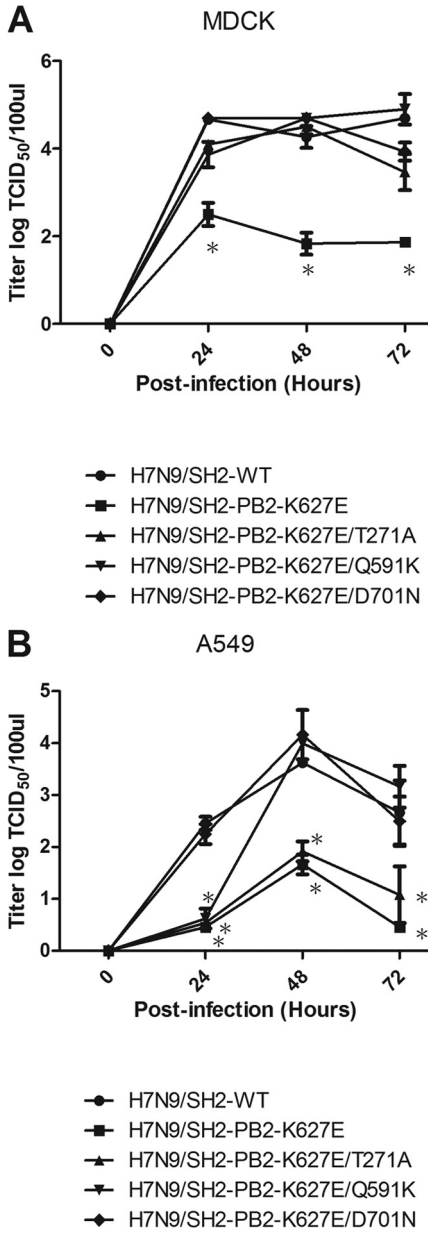

FIG 2 Replication kinetics of the H7N9 variants on MDCK and A549 cells. MDCK (A) and A549 (B) cells were infected with the indicated viruses at an MOI of 0.01 and cultured at 37°C in the presence of TPCK-trypsin at 1 μg/ml and 0.2 μg/ml, respectively. Culture supernatants were harvested at the indicated times, and virus titers were determined by $TCID_{50}$ assay. Results are the averages from three independent experiments. The viral titers of PB2 mutants were compared to the those of the recombinant wild-type virus using the two-tailed paired *t* test. *, $P < 0.05$.

Next, the severity of the inflammation, virus titers, and the cytokine induction in the lungs of the mice infected by recombinant viruses PB2-627K (wild type), PB2-K627E (avian consensus), PB2-K627E/D701N, PB2-K627E/Q591K, and PB2-K627E/

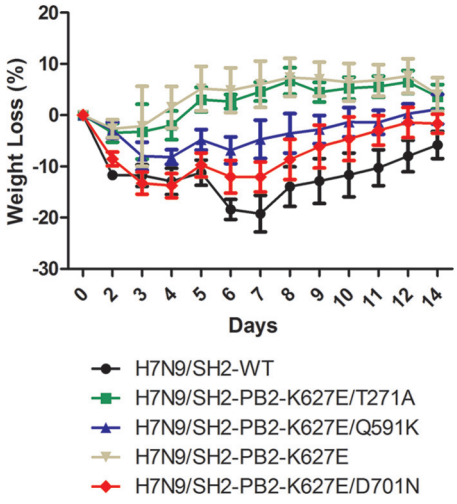

FIG 3 Weight change in mice infected with the Sh2/H7N9 virus and its PB2 mutants. Female BALB/c mice were infected intranasally with 1×10^5 PFU of the indicated viruses. The virus-infected mice were monitored for 14 days, and their weights were determined daily. Results are means ± standard deviations (SD) for six infected mice. The values were statistically analyzed by two-tailed paired *t* test. *, $P < 0.05$.

T271A were compared. The mice infected with recombinant wild-type Sh2/H7N9 having PB2-627K showed the most severe degree of inflammation, with perivascular, peribronchial, and parenchymal infiltration of inflammatory cells being seen (Fig. 4A). Influenza virus nucleoprotein-positive cells were identified in these areas of the lung (Fig. 5). Mice infected with the PB2-K627E/D701N and PB2-K627E/Q591K viruses had less inflammation than the wild-type-infected mice (Fig. 4C and D). The mice infected with PB2-K627E and PB2-K627E/T271A viruses showed similar but the least degree of inflammation (Fig. 4B and E). Only limited viral antigens were identified in the lungs of mice infected with different recombinant viruses (Fig. 5). This indicates that the presence of viral nucleoprotein as detected by immunohistochemistry does not correlate with the disease severity and viral titer detected by traditional methods, such as $TCID_{50}$ assay. However, our results clearly demonstrated that PB2 is the controller of inflammation in areas of infection.

We then determined the levels of virus replication in the lungs of the virus-infected mice. Mice were infected intranasally with a dose of 1×10^5 PFU viruses (Fig. 6). Mice infected by the mammal-adapted virus Sh2/H7N9 (PB2-627K) or the mutants with PB2-627E/701N and PB2-627E/Q591K had virus titers of around $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$/100 μl at 3 to 5 days postinfection. There was no detectable virus in the lungs of mice infected with PB2-E627 virus at day 3 postinfection, and even at day 5 postinfection, the level of virus in the lung remained significantly lower than that seen in mice infected with wild-type and all other recombinant mutants. The virus titer in the lungs of mice infected with PB2-K627E/T271A virus is statistically significantly lower than the titer at day 3 ($P < 0.05$). All the viruses were cleared in the lungs of the mice infected by the three groups of viruses at day 8 postinfection (data not shown).

The PB2 mutations of H7N9 virus increase the cytokine expression in mice. It has been suggested that cytokine dysregulation contributes to the pathogenesis of highly pathogenic avian influenza (HPAI) H5N1 virus in humans, as shown by the clinical studies as well as those in *in vivo* (mice and ferret) or *in vitro* models. We previously showed that mice infected by the Sh2/H7N9 virus exhibited higher levels of proinflammatory cytokines than the low-pathogenicity Duck/H7N9 and H9N2 viruses, although not to the same degree as HPAI H5N1 virus. To better understand the viral determinants of pathogenesis of Sh2/H7N9-

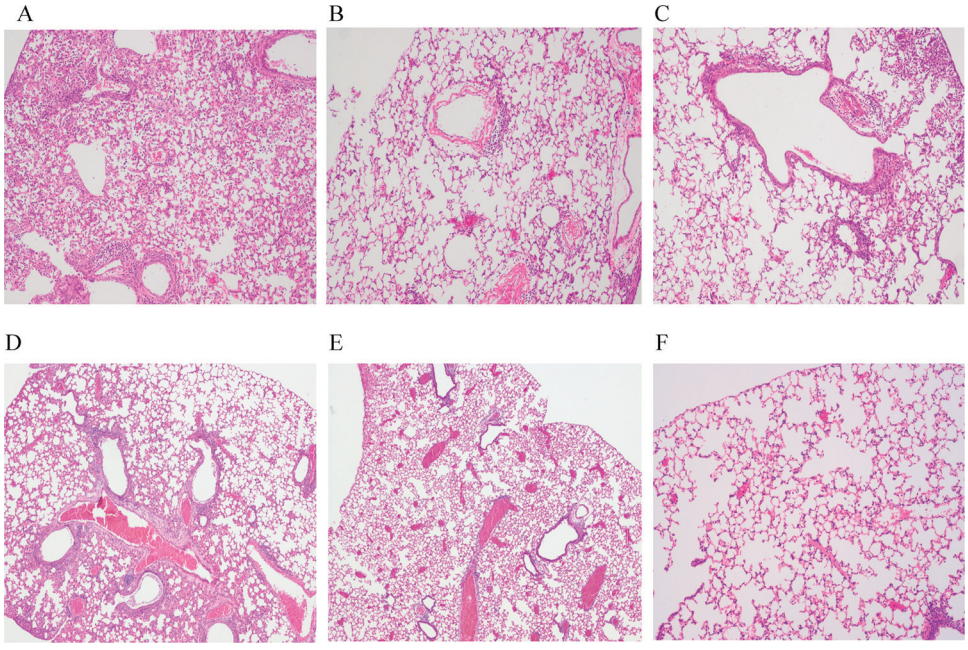

FIG 4 Histology of the mice infected with the Sh2/H7N9 and its PB2 mutants. The histology of lung sections was determined in samples stained by hematoxylin-eosin from mice infected with Sh2/H7N9 (A), Sh2/H7N9-PB2-K627E (B), Sh2/H7N9-PB2-K627E/D701N (C), Sh2/H7N9-PB2-K627E/Q591K (D), or Sh2/H7N9-PB2-K627E/T271A (E) or mock infected (F) at 5 days postinfection. Magnification, ×10.

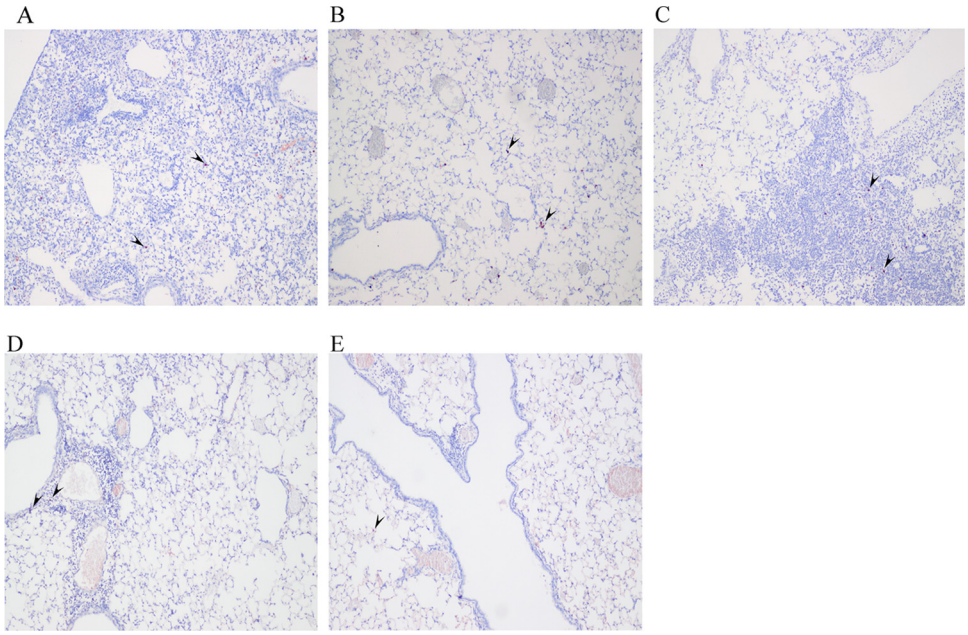

FIG 5 Immunohistochemistry of mice infected with the Sh2/H7N9 virus and its PB2 mutants. Immunohistochemical staining of NP antigens in the lung sections were determined from the samples of the mice infected with Sh2/H7N9 (A), Sh2/H7N9-PB2-K627E (B), Sh2/H7N9-PB2-K627E/D701N (C), Sh2/H7N9-PB2-K627E/Q591K (D), or Sh2/H7N9-PB2-K627E/T271A (E) at 5 days postinfection. Arrowheads indicate influenza virus nucleoprotein-positive cells. Magnification, ×10.

like viruses, we compared the induction of proinflammatory cytokines in the lungs in mice infected with wild-type Sh2/H7N9 (PB2-E627K) and with different PB2 mutant viruses. Mice infected by the five groups of viruses had higher levels of proinflammatory cytokines in the lungs than the uninfected controls. Compared to the lungs from mice infected with the PB2-K627E mutant, lungs from the mice infected with the wild-type Sh2/H7N9-E627K, PB2-K627E/D701N, PB2-K627E/Q591K, and PB2-K627E/T271A viruses exhibited significantly higher levels of the proinflammatory cytokines TNF-α, MIP-1α, RANTES, MCP-1, MCP-3, and KC at day 5 postinfection (Fig. 7). Only wild-type Sh2/H7N9-E627K and PB2-K627E/D701N triggered higher levels of IP-10 at day 5 than other mutants. Our results suggest that mammalian adaptations in PB2 of H7N9 virus enhance proinflammatory cytokine induction in mice.

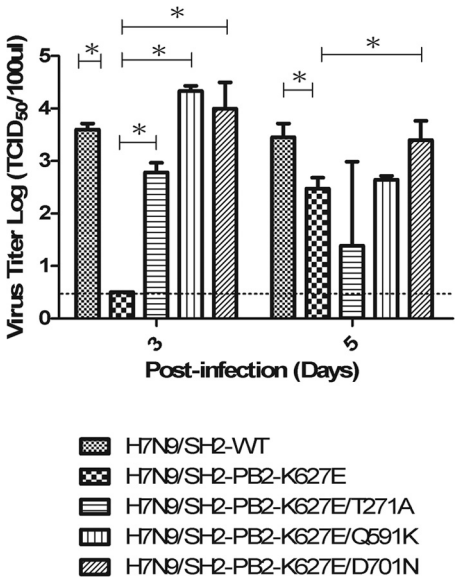

FIG 6 Lung virus titers of mice infected with Sh2/H7N9 and its PB2 mutants. BALB/c mice were infected with the indicated viruses at 1×10^5 PFU. Infected mice were sacrificed on 3 and 5 days postinfection, and virus titers in lung homogenates were measured in MDCK cells. Results from each group and each time point are expressed as means ± SD from four infected mice. The dotted line represents the detection limit at $10^{-0.5}$ $TCID_{50}$/100 μl. The values were statistically compared with those for the PB2-K627E mutant using the two-tailed paired *t* test. *, $P < 0.05$.

DISCUSSION

The H7N9 viruses isolated from humans in China in 2013 frequently have mutations in the PB2 gene that have previously been associated with mammalian adaptation, while the viruses obtained from poultry and associated with zoonotic transmission events do not (8, 24). The role of these PB2 mutations in H7N9 has not been investigated previously. In this study, we systematically investigated the effects of the PB2 mutations Q591K, E627K, and D701N, which have been detected in human isolates of H7N9 (Table 1), and T271A, which is a mammalian adaptation and has been previously reported to be functionally relevant in avian or swine virus isolates (17, 25). We have determined their contribution to polymerase activity in mini-genome reporter assays and to pathogenicity in mice. Sh2/H7N9 virus with PB2-K627E (found in avian viruses) showed lower polymerase activity at both 33°C and 37°C in mammalian cells and had reduced pathogenicity in experimentally infected mice compared with the recombinant wild-type strain that had the mammalian adaptation PB2-627K commonly found in human isolates. The Q591K, D701N, and T271A changes in PB2 appear to partially compensate for the lack of the PB2-E627K adaptation in polymerase activity and viral rep-

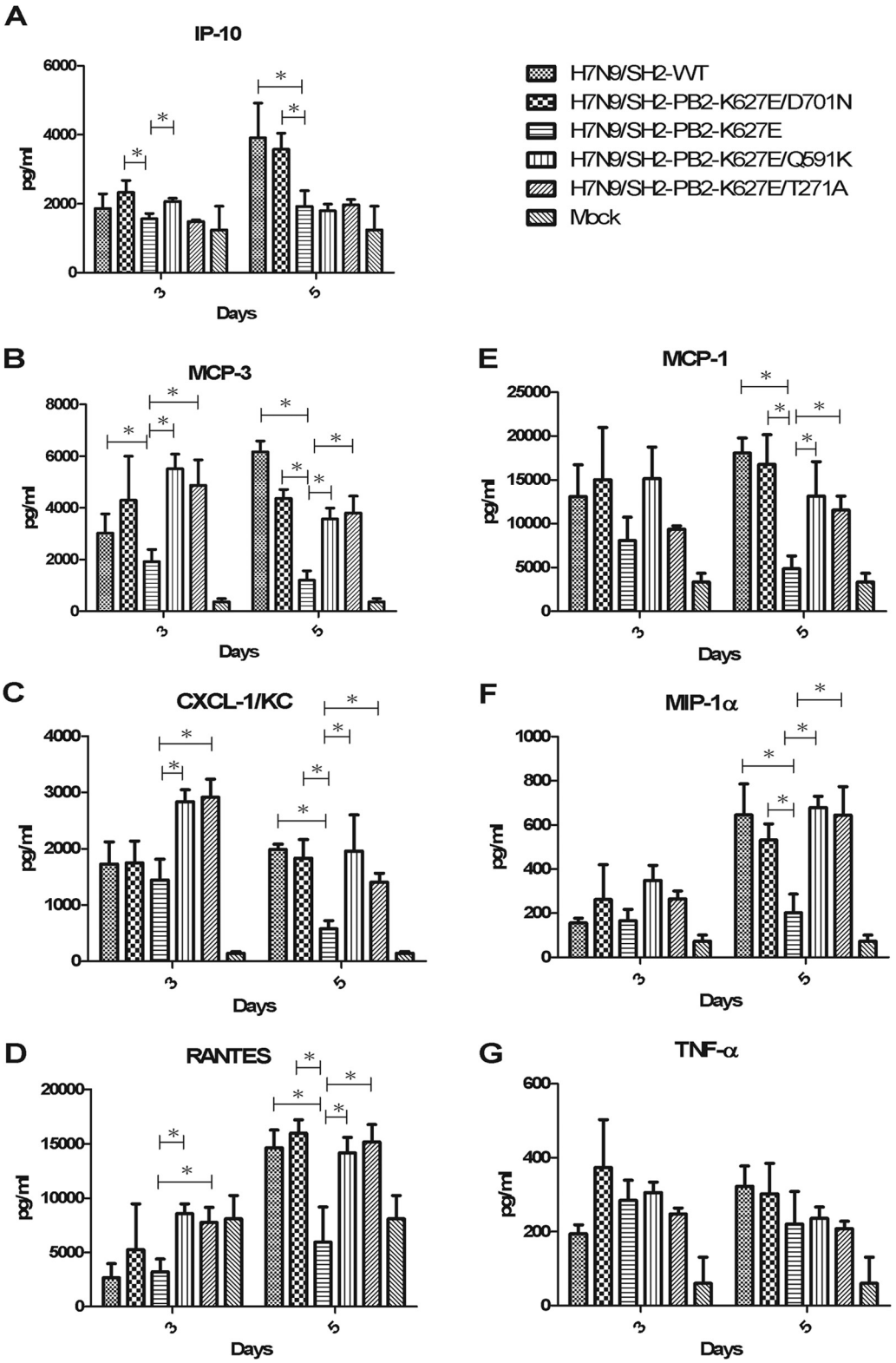

FIG 7 Cytokine responses in the lungs of mice infected with Sh2/H7N9 and its PB2 mutants. Cytokine levels (A) IP-10, (B) MCP-3, (C) KC, (D) RANTES, (E) MCP-1, (F) MIP-1α, and (G) TNF-α from virus-infected lungs (4 mice per virus group, days 3 and 5 postinoculation) were measured individually by the FlowCytomix system (eBioscience). Results are means ± SD for four infected mice ($n = 4$). The values were statistically compared using the two-tailed, paired *t* test. *, $P < 0.05$.

lication competence in MDCK cells. However, PB2-T271A does not confer increased pathogenicity in mice, as assessed by weight loss and lung histology. Our results are consistent with a previous study that found that the PB2-T271A mutation increases virus replication without increased weight loss in the mice infected by recombinant virus which contains the RNP genome of the avian influenza strain. On the other hand, the mutations Q591K and D701N as well as E627K contribute to increased weight loss in mice, in the order PB2-E627K > PB2-D701N > PB2-Q591K > PB2-T271A. Viral replication titers and proinflammatory cytokine levels in the lungs of mice infected with the PB2-E627K or -D701N virus are comparable and significantly higher than those in mice infected with the PB2-627E virus.

It is well known that PB2-E627K enhances the polymerase activity in mammalian cells. In our study, we found that PB2-627K also showed higher polymerase activity in the avian DF-1 cells at both 37°C and 39°C than PB2-627E, while T271A, Q591K, and D701N all restored polymerase activity to levels comparable to that of PB2-E627K. Similar observations were found in a study showing that the polymerase activities of the clade 2.2 H5N1 virus containing PB2-627K are higher than those of virus with PB2-627E in both 293T and DF-1 cells (26). While the results of the polymerase activity correlate with the replication level in mammalian cells, the H5N1 virus which contains PB2-627K showed replication similar to that of the virus with PB2-627E in avian cells. The results suggest that avian influenza virus with PB-627K is not likely to be favored among the virus populations in the avian host, even if it contains a higher polymerase activity than the virus which contain PB2-627E. Nevertheless, no H7N9 viruses which contain PB2-627K have been identified in avian hosts so far. Interestingly, in DF-1 cells, the PB2-E627K mutation does not enhance polymerase activity of the Duck/H7N9 virus, which is genetically distinct from the recently emerged zoonotic H7N9 virus. However, the biological significance of the levels of polymerase activity in the two H7N9 lineages in avian cells needs to be investigated further.

The internal genes (encoding PB1, PB2, PA, and NP) of the human H7N9 viruses and the clade 0 H5N1 viruses (such as A/HongKong/483/1997) are derived from H9N2 viruses, but contemporary lineages of H5N1 viruses may have internal genes derived from other sources. While avian isolates of both H7N9 and clade 0 H5N1 viruses have PB2-627E, some human isolates of both H7N9 and clade 0 H5N1 viruses carry PB2-E627K. It is thus interesting to compare the effects of such mutations in the two viral backgrounds. The PB2-E627K mutation in the human H7N9 and H5N1 viruses both increase the polymerase activity and virus replication *in vitro* using mammalian cells (Fig. 2) (27), suggesting that there is a common role for the mutation in the polymerase complex derived from H9N2 viruses. However, we previously showed that the pathogenicities of the two viruses in BALB/c mice are largely different. The 483/H5N1 virus causes very high lethality in mice together with more virus replication and cytokine induction in the lung compared to the SH2/H7N9 virus (20). The difference may possibly be explained by the hemagglutinin of the H5N1 virus, which has the multibasic cleavage site associated with highly pathogenic avian influenza viruses, while the H7N9 virus does not. Nevertheless, the pathogenicities of both 483/H5N1 and Sh2/H7N9 viruses decrease dramatically if the K at position 627 of PB2 is replaced with E (14).

The mechanism by which the PB2 mutations contribute to enhanced polymerase activity is still not fully understood. Mehle and Doudna suggested that a dominant inhibitory activity in human cells restricts the function of polymerases containing PB2-627E, while the polymerase complex of influenza virus with PB2-627K works more efficiently (28). On the other hand, Moncorgé et al. showed that a positive factor is responsible for the replication advantage of PB2-627K virus in mammalian cells (29). Hudjetz and Gabriel recently showed that human importin-α isoforms 1 and 7 are the positive regulators of polymerase complex with PB2-627K but not PB2-627E (30). Structural analysis indicated that there is a change of surface charge around residue 627 which may block or favor particular protein-protein interactions and affect the polymerase functions (18, 21). Whether these explanations are applicable to the H7N9 virus is still unclear.

In summary, our results explain the rapid acquisition of mutations in the PB2 gene at position 627, 701, or 591 in mammalian isolates of H7N9 viruses. This is more likely to occur after the avian virus has infected the mammalian host, although it remains possible that such mutations may also spontaneously appear within poultry and get selected for preferential transmission to humans. Similar adaptive changes have been reported to occur human virus isolates of other avian viruses (e.g., clade 0 or clade 1 H5N1 viruses) as they adapt in humans (31). In contrast, PB2-E627K changes are fixed within avian viruses of the clade 2.2 lineage that are now endemic in Egypt, with evidence of reduced severity of human disease caused by this lineage (32).

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