Original Research

D225G mutation in hemagglutinin of pandemic influenza H1N1 (2009) virus enhances virulence in mice

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Abstract

Although the majority of infections by the pandemic influenza H1N1 (2009) virus is mild, a higher mortality occurs in young adults with no risk factors for complications. Some of these severe cases were infected by the virus with an aspartate to glycine substitution at 225 position (D225G, H3 numbering) in the hemagglutinin (HA). Previous studies with the highly virulent 1918 pandemic H1N1 virus suggested that such substitution was associated with a dual binding specificity of the virus for both α2,3- and α2,6-linked sialic acid receptors on host cells. Thus, the D225G mutant may cause more severe disease with its increased predilection for the lower respiratory tract, where the α2,3 sialic acid receptor is more prevalent, but this hypothesis has not been investigated. We obtained a mutant virus after four sequential passages in lungs of BALB/c mice with a wild-type pandemic influenza A H1N1 (2009) virus. One plaque purified mutant virus had a single non-synonymous D225G mutation in the HA gene. This mutant was more lethal to chick embryo and produced a viral load of about two log higher than that of the wild-type parental virus during the first 24 h. A pathogenicity test showed that the 50% lethal dose in mice (LD50) was reduced from over 2×10^6 plaque-forming units (PFU) with the parental virus to just 150 PFU with the mutant virus. The survival of mice challenged with the mutant virus was significantly decreased when compared with the parental virus ($P < 0.0001$). Significantly higher viral titers and elevated proinflammatory cytokines in lung homogenates of mice infected with the mutant virus were found, which were compatible with severe histopathological changes of pneumonitis. The only consistent mutation in the genomes of viral clones obtained from dying mice was D225G substitution.

Keywords: hemagglutinin, influenza, pandemic, H1N1, D225G, D222G, sialic acid, adaptation

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Introduction

The influenza A pandemic H1N1 (2009) virus is highly infectious and was found to infect over 40% of children in a seroprevalence study recently.[1] Although most cases were mild acute upper respiratory tract infections, over 15,292 deaths were reported to the World Health Organization (WHO) by 7 February 2010.[2] In addition to extremes of age, chronic underlying medical illness or immunosuppression, clinical studies showed that pregnancy and obesity were significant predisposing factors for severe illness or mortality.[3–7] Early use of oseltamivir in mild cases can significantly reduce viral load and symptoms,[8] but the effectiveness of double-dose oseltamivir on viral load and outcome is doubtful in severe cases who presented late.[7] Although genetic analysis showed that this virus was a reassortant containing gene segments from ancestor viruses of human, swine and avian sources,[9] analyses of nasopharyngeal viral shedding and cytokine activation in human cases, and tissue tropism in cultured cells, showed that this pandemic H1N1 virus was similar to seasonal influenza virus.[10–12] *In vitro* and *in vivo* experiments showed that this virus could bind to both α2,3- and α2,6-linked sialic acid receptors and replicate in the lower respiratory tracts of infected mammals,[13,14] distinct from the seasonal H1N1 viruses that have a predilection for α2,6-linked sialic acid and mainly infect the upper

respiratory tract. These unusual viral attributes suggested that this new virus may possess some virulence characteristics similar to the highly pathogenic H5N1 or the 1918 pandemic influenza viruses that also have a predilection for both α2,3- and α2,6-linked sialic acid receptors. Histopathological studies of fatal cases of pandemic H1N1 (2009) showed some features similar to H5N1 infection.[7,15,16] Sequence analysis of this new virus found that many genetic markers associated with virulence are not present,[17] despite causing significantly higher morbidity and mortality in younger patients when compared with that of seasonal influenza viruses.[18] Since this virus probably originated from swine species through multiple reassortment events,[9] further adaptation may occur along the course of circulation in humans as in the case of a naturally occurring mutation in neuraminidase leading to oseltamivir resistance from the virus of a patient without exposure to antiviral treatment.[19] Therefore, it is conceivable that more adaptation of the receptor binding properties may occur in the pandemic (H1N1) 2009 virus comparable to that observed in the previous pandemics.[20,21] We and others have recently found that the virus from the lower respiratory tract specimens of patients with severe disease had significantly higher incidence of aspartate to glycine substitution at position 225 (D225G) (H3 numbering) in the hemagglutinin (HA).[22,23] Since a single D225G (D222G if HI numbering) amino acid mutation in the HA was found to confer dual α2,3 and α2,6 sialic acid binding specificities in the highly virulent 1918 pandemic influenza A H1N1 virus,[24] we investigated the emergence of this mutation and its significance in pathogenesis by adapting the pandemic H1N1 (2009) virus in BALB/c mice, which have predominantly α2,3 sialic acid in their airway.

Materials and methods

The virus stock and the mouse adapted mutant

The first isolate of pandemic influenza A H1N1 (2009) virus, A/HK/415742/09(H1N1), from a symptomatic Mexican patient who travelled to Hong Kong, was cultured and plaque purified in Madin Darby Canine Kidney (MDCK) cell monolayer.[10,25] After expansion by one more passage and determination of the titer of infectivity by plaque assays, frozen aliquots of cell culture were kept at −80°C. The highly virulent mouse adapted variant mutant A/HK/415742Md/09 was derived by multiple cycles of intranasal infection with 50 μL of lung extracts under pentobarbitone/ketamine anesthesia for groups of three mice which finally resulted in 100% fatalities as described previously.[26] Briefly, the mice were intranasally inoculated with 10^6 plaque forming units (PFU) of the virus. The lung tissues were taken from the infected mice on day 5 postinfection and homogenized in 500 μL of phosphate-buffered saline. The new mice were given 50 μL of lung homogenate by intranasal inoculation for four cycles. All three mice died during the last passage on day 4 or 5. The mouse adapted mutant was subsequently cloned by plaque purification in an MDCK cell monolayer. After titer determination, frozen aliquots of the purified virus culture were kept at −80°C till use.

Genome sequencing

Viral RNA of the wild-type and mouse adapted mutant viruses were extracted and each genome segment was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and purified by cutting and extracting the correct band on agarose gels before direct dideoxy-terminated cycle sequencing by an Applied Biosystems automated sequencer, Model ABI 3700. Genome segment-specific primers for RT-PCR were complementary to the first 16 nucleotides of each segment in combination with a primer complementary to the 12 nucleotides at the 3′ end of viral RNA. Sequencing primers (sequences available on request) were complementary to A/HK/415742/09(H1N1) and all pandemic H1N1 (2009) related viruses.

Virus growth in A549 cell line and embryonated eggs

The wild-type and mutant virus were inoculated into human bronchial carcinoma cell line A549 at one multiplicity of infection/cell and incubated at 37°C. Supernatants were collected at 12-h intervals over a 72-h period for the determination of viral load in duplicate for each sample. The virus titer yielded in the chicken egg allantoic cavity was determined by infecting nine- to 10-day-old chicken embryos with 10^5 PFU of the wild-type and mutant virus and incubated for 72 h at 34°C. Pools of three eggs each were titrated by viral RNA realtime RT-PCR assay.

Mice challenge model

BALB/c female mice, 6–8 weeks old, were kept in biosafety level-3 housing and given access to standard pellet feed and water *ad libitum*. All experimental protocols followed the standard operating procedures of the approved biosafety level-3 animal facilities and were approved by the Animal Ethics Committee.[27] Mortality of both wild-type and mutant virus was determined in mice after serial dilutions of the stocks. The median 50% lethal dose (LD50) in PFU was measured by intranasal infection of five groups of 10 mice each with serial three-fold dilutions of the mutant virus as we described previously.[27] Mortality from the mutant virus infection occurred primarily from four- to eight -days postinfection, depending on the viral load inoculated, and was therefore monitored for 21 d following the challenge. Five thousand PFU of mutant virus and two million PFU of wild-type virus were used for viral challenge in the subsequent studies on histopathology, viral load, and chemokine and cytokine levels in the lung homogenates. Infection was established by intranasal inoculation of 20 μL virus in mice anesthetized by pentobarbitone/ketamine.

Experiments were conducted in triplicate of five mice challenged by wild-type or mutant virus. Five mice in each group were killed on day 5 postchallenge. Lung homogenate, lung, brain, kidney, liver and spleen tissue samples were collected from these mice, normal uninfected mice and the surviving mice for histopathological, immunological and virological assays.

Viral titer and viral load

Titers of released virus in lung homogenates were determined by plaque assays, whereas viral RNA in lung tissues was quantified by realtime RT-PCR as reported previously.[11] Briefly, total RNA in lung homogenates was extracted using a RNeasy Mini kit (Qiagen, Germantown, MD, USA). Viral cDNA was synthesized by Superscript RT II (Invitrogen Corp., Carlsbad, CA, USA) using Uni12 primer (AGC AAA AGC). Realtime PCR was performed on LightCycler 480 system (Roche Applied Sciences, Upper Bavaria, Germany) using SYBR Green I Master (Roche) with gene specific primer pair (forward primer: GAT ACA CCA GTC CAC GAT TG; reverse primer: ACC ATC CAT CTA CCA TCC C) targeting H1 gene. The pcDNA3.1 plasmid, which contains the cloned H1 gene of the virus, was applied as standard. The stability of the mutant virus was monitored by RT-PCR and sequencing of the 380 bp H1 fragment using PCR primers spanning the 225 position. The forward 5′-GTT CAT GGC CCA ATC ATG AC-3′ and reverse 5′-GAT TTC CAG TTG CTT CGA ATG-3′ primers were used. Sequence readouts with forward and reverse primers were examined to confirm polymorphisms at the 225 position in the HA gene.

Enzyme immunoassay for cytokines and chemokines

Levels of proinflammatory cytokines and chemokines, interleukin (IL)-1B, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α and MIP-2 in lung homogenates were tested by ELISA kits (R&D Systems) using the protocol as described,[27] with modifications according to the instructions of the kit suppliers.

Histopathological examination

The lung, brain, spleen, kidney and liver tissues of challenged mice were immediately fixed in 10% buffered formalin and embedded in paraffin wax. Sections of 4–6 μm thickness were mounted on slides. Histopathological changes were examined by hematoxylin and eosin (H&E) staining under a light microscope as described.[27]

Statistical analysis

Survival of mice was analyzed by the Kaplan–Meier method and log-rank test using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA), whereas the profiles of viral load, cytokine and chemokine were calculated by Student's *t*-test with Stata statistical software. Results were considered significant at $P < 0.05$.

Results

The highly virulent mouse adapted mutant A/HK/415742Md/09 was derived after five cycles of lung to lung passage. The mutant virus caused 100% fatalities in mice within seven days postinoculation. Observation of symptoms showed that the fur of the infected mice started to ruffle within three days and respiratory rate increased within four days postinfection. Virus from fatal mice was purified by plaque assay in which inoculation with 10^6 diluted virus yielded 10^2 plaques with the size of about 2 mm after three days of culture in MDCK cell monolayer. To identify mutations in the viral genome that may arise during the lung to lung passage in mice, whole genome sequencing and analysis of the parental and two clones of the plaque-purified adapted virus was performed (GeneBank Accession numbers GU931801–GU931816, HM100229–HM100236). Compared with the parental virus, there was one non-synonymous mutation, D225G, in the HA of the adapted virus mutant A/HK/415742Md/09, which was used in all subsequent experiments (Table 1). We have compared all the proteins between the mutant virus and wild-type virus to confirm that the virus has only a single mutation of D225G in HA. As for another mutant clone 415742M with similar LD50 to 415742Md which also had D225G substitution, changes were found at amino acid position 40 with leucine to arginine mutation in the neuraminidase gene, position 230 with arginine to lysine mutation in the PA gene and synonymous mutation at position 363 in the PB1 gene. All clones from mice which died after inoculation with adapted mutant virus had the D225G substitution. These results suggested that the D225G substitution in HA is the only consistent mutation and might be responsible for the enhanced virulence of the virus in mice.

The viral load assay showed that replication kinetics of the mutant was similar to the parental virus in the human lung cell line, A549, whereas the viral load of the mutant was about two log higher than that of the parental virus in the allantoic fluid of embryonated chicken eggs during the first 24 h (Figure 1a). The mutant virus also showed higher virulence in chick embryos. As shown in Figure 1b, a significantly higher percentage of chick embryos was killed by the mutant virus by 48 and 72 h ($P < 0.002$), whereas 50% of those inoculated by the wild-type virus still survived at this juncture, respectively.

In the BALB/c mice challenge experiment, the parental virus resulted in no lethal effect in mice even at an inoculum of 2×10^6 PFU, whereas an inoculum of 5000 PFU of the mutant virus killed all mice on day 6 postinfection ($P < 0.0001$) (Figure 2). There was a graded dose response of fatality and the mean survival time with the mutant virus. The LD50 of the mutant virus was determined to be about 150 PFU, while the survival increased with the decrease of the inoculum from 5000 to 160 PFU ($P < 0.0001$). Even when the survival of mice challenged with 2×10^6 PFU of parental virus and 160 PFU of mutant virus was compared, the survival of mice challenged by the mutant virus was still significantly lower than that of the parental virus ($P = 0.004$). Reversion of the mutant virus to the wild-type genotype at 225 position was not found by RT-PCR sequencing of the H1 fragment in the lung homogenates of mice infected by the mutant virus. The results confirmed that one non-synonymous mutation of D225G in the HA of the pandemic H1N1 (2009) virus has markedly increased its virulence in the mouse model.

To further understand the underlying mechanism for the increased virulence of this mutant virus in mice, we

Table 1 Amino acid sequences related to virus binding in different strains of influenza A virus H1N1 HA were compared and a mutant of D225G was found in A/HK/01/2009 mutant strain

	98
aca042009	AGWILGNPECESLSTASSWSYIVETPSSDNGTCYPGDFIDYEELREQLSSVSSFERFEIFPK
apr81934	AGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEELREQLSSVSSFERFEIFPK
asc11918	AGWLLGNPECDLLLTASSWSYIVETSNSENGTCYPGDFIDYEELREQLSSVSSFEKFEIFPK
AWSN1933t	TGWLLGNPECDSLLPARSWSYIVETPNSENGACYPGDFIDYEELREQLSSVSSLERFEIFPK
sw-ha	AGWILGNPECESLSTASSWSYIVETSSSDNGTCYPGDFIDYEELREQLSSVSSFERFEIFPK
A/HK/01/2009-WT	AGWILGNPECESLSTASSWSYIVETSSSDNGTCYPGDFIDYEELREQLSSVSSFERFEIFPK
A/HK/01/2009-Mut	AGWILGNPECESLSTASSWSYIVETSSSDNGTCYPGDFIDYEELREQLSSVSSFERFEIFPK
	136 **153**
aca042009	TSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKGNSYPKLSKSYINDKGKEVLVLW
apr81934	ESSWPNHNTN-GVTAACSHEGKSSFYRNLLWLTEKEGSYPKLKNSYVNKKGKEVLVLW
asc11918	TSSWPNHETTKGVTAACSYAGASSFYRNLLWLTKKGSSYPKLSKSYVNNKGKEVLVLW
AWSN1933t	ESSWPNHTFN-GVTVSCSHRGKSSFYRNLLWLTKKGDSYPKLTNSYVNNKGKEVLVLW
sw-ha	TSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKENSYPKLSKSYINDKGKEVLVLW
A/HK/01/2009-WT	TSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKENSYPKLSKSYINDKGKEVLVLW
A/HK/01/2009-Mut	TSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKENSYPKLSKSYINDKGKEVLVLW
	183 **190** **194** **222** **225–228**
aca042009	GIHHPSTSADQQSLYQNADTYVFVGSSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPG
apr81934	GIHHPPNSKEQQNIYQNENAYVSVVTSNYNRRFTPEIAERPKVRDQAGRMNYYWTLLKPG
asc11918	GVHHPPTGTDQQSLYQNADAYVSVGSSKYNRRFTPEIAARPKVRDQAGRMNYYWTLLEPG
AWSN1933t	GVHHPSSSDEQQSLYSNGNAYVSVASSNYNRRFTPEIAARPKVKDQHGRMNYYWTLLEPG
sw-ha	GIHHPSTSADQQSLYQNADAYVFVGSSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPG
A/HK/01/2009-WT	GIHHPSTSADQQSLYQNADAYVFVGSSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPG
A/HK/01/2009-Mut	GIHHPSTSADQQSLYQNADAYVFVGSSRYSKKFKPEIAIRPKVR**G**QEGRMNYYWTLVEPG*

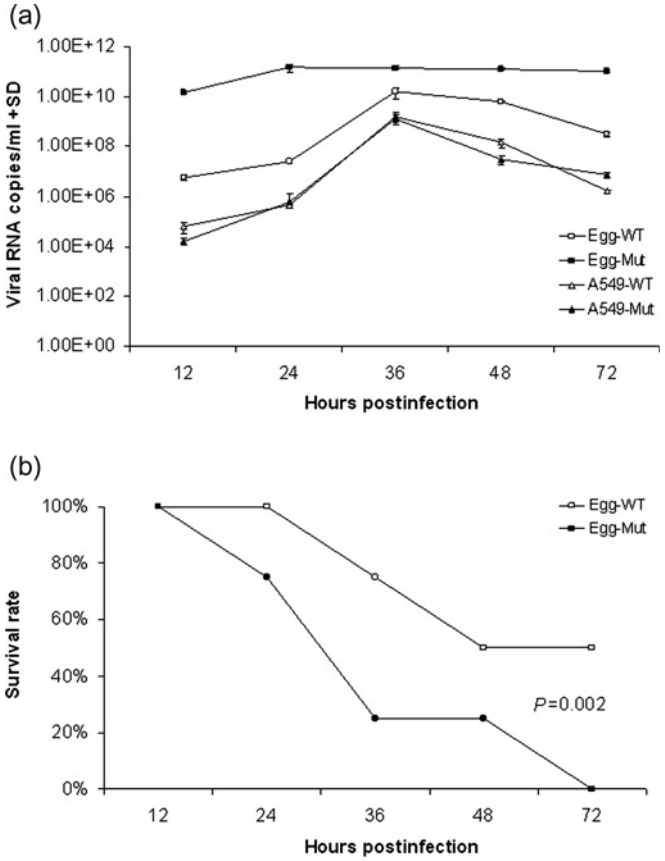

Figure 1 Viral growth in chick embryo and human cell line A549. (a) Viral load profile in allantoic fluid of embryonated eggs (Egg) and cell culture supernatant of A549 cell line (A549) at indicated time points postinfection by pandemic H1N1 (2009) wild-type (WT) and mouse adapted mutant virus (Mut). (b) Survival rate of chick embryo ($n = 12$ per group) infected by wild-type (Egg-WT) or mutant (Egg-Mut) virus at indicated time points

compared the virological, pathological and inflammatory responses in mice challenged with the mutant and the parental virus. When the lung homogenates of the challenged mice were examined for virus replication, about one log higher viral load as determined by RT-PCR (Figure 3a) or plaque assays (Figure 3b) were found in mice infected by the mutant virus than the parental virus, although the inoculum of the mutant virus is about 2.4 log lower than that of the parental virus. Histopathological examination of lung tissues collected on day 5 postinfection showed only mild interstitial inflammatory infiltration in those

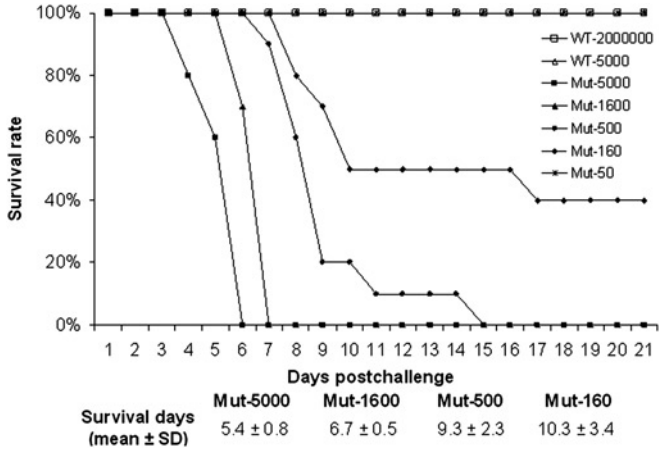

Figure 2 Mice survival rate and days after intranasal inoculation with pandemic H1N1 (2009) wild-type (WT) and mouse adapted mutant (Mut) virus. The mice (10/group) were intranasally inoculated with WT virus of two million (WT-2000000) and 5000 (WT-5000) PFU, or mutant virus of 5000 (Mut-5000), 1600 (Mut-1600), 500 (Mut-500), 160 (Mut-160) and 50 (Mut-50) PFU, and monitored for 21d. Survival days (mean ± SD) of mice infected with different doses of mutant virus are also shown. PFU, plaque forming units; SD, standard deviation

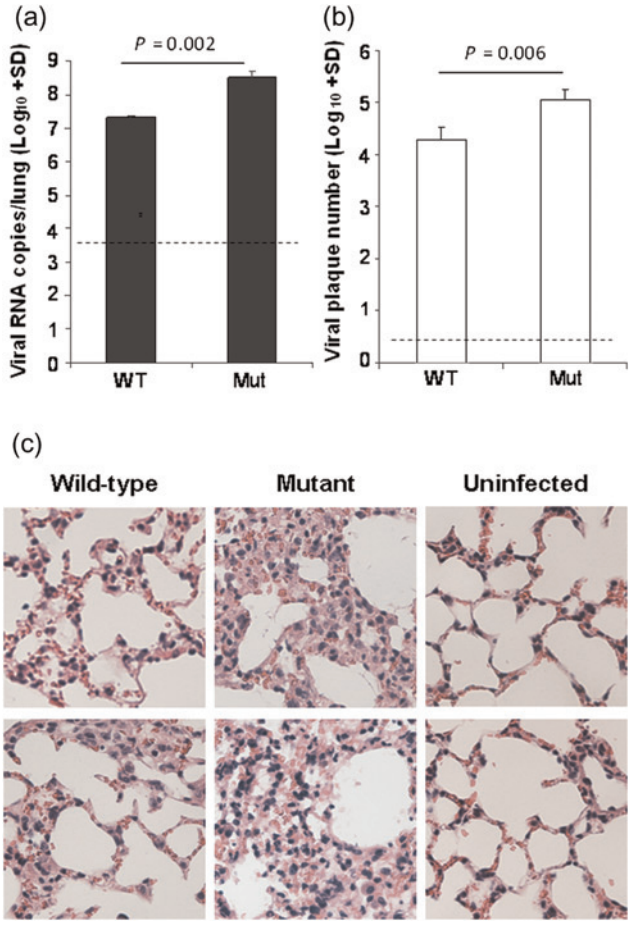

Figure 3 Virological and histological findings in mice inoculated with 5000 plaque forming units (PFU) of mutant virus (Mut) or two million PFU of wild-type virus (WT) on day 5 postchallenge. (a) Viral load detected by realtime reverse transcription-polymerase chain reaction (RT-PCR) and (b) viral titer determined by plaque assay in lung homogenates of mice infected by pandemic H1N1 (2009) WT and mouse adapted Mut. The dotted line indicates detection limit in the experiment and the *P* value between two groups is also indicated. (c) Histopathological changes in lung sections of infected and uninfected mice were examined under light microscopy with hematoxylin–eosin stain (magnification ×200)

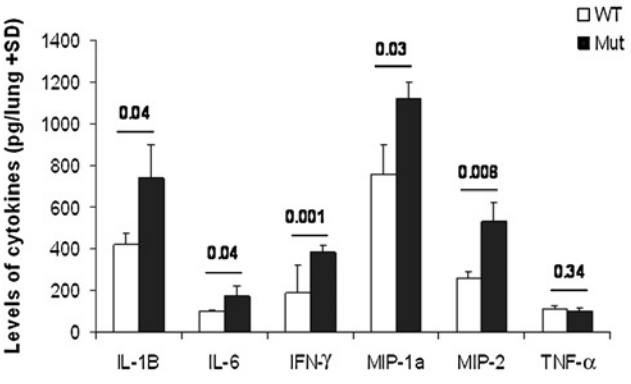

Figure 4 Proinflammatory cytokine and chemokine profiles of mice inoculated with 5000 plaque forming units (PFU) of mutant virus (Mut) or two million PFU of wild-type virus (WT) at day 5 postchallenge. Levels of interleukin (IL)-1B, IL-6, interferon-γ, tumor necrosis factor-α, macrophage inflammatory protein (MIP)-1α and MIP-2 in lung homogenates of mice infected by WT or Mut virus were tested by enzyme-linked immunosorbent assay and compared between two groups. Their *P* values are also shown

mice infected by the parental virus, whereas severe alveolar damage, pneumonitis with thickened interstitium and heavy interstitial inflammatory infiltration were found in those infected by the mutant virus (Figure 3c). In contrast to that observed in infected mice with highly pathogenic H5N1 virus, the mutant virus was not found to be spread to brain or cause pathological changes in brain tissues as examined by both realtime RT-PCR and H&E staining in tissues collected from infected mice on day 5 postinfection (data not shown). Furthermore, significantly higher levels of proinflammatory cytokines and chemokines, including IL-1B, IL-6, IFN-γ, MIP-1α and MIP-2, were found in the lung homogenates of mice infected by mutant virus than that of the wild-type virus on day 5 postinfection (Figure 4). IFN-α levels in lung homogenates were found to be similar in mice infected by mutant or parental virus. These results indicated that the D225G mutant virus could replicate more efficiently and cause more severe inflammation in the lungs of the infected mice.

Discussion

The genetic basis for the virulence of the present pandemic H1N1 (2009) virus is still not clear. Virulence determinants identified in the H5N1 virus, such as E627K at PB2, N66S in PB1-F2, PDZ domain in NS1 and multibasic HA cleavage motif, were not generally found in the pandemic H1N1 (2009) virus.[17] Although the majority of patients with severe disease had risk factors relating to immunosuppression, about 20% of severe cases are healthy immunocompetent adults with no risk factors for severe complications from influenza. Our recent study found that patients with severe disease had a higher incidence of D225G mutation in the virus from their endotracheal specimens,[23] and we speculated that such mutation may endow the virus to infect the lower respiratory tract. Similar mutation in the H1N1 (1918) virus was found to be associated with its predilection for binding to α2,3 sialic acid receptors that are more abundant in the terminal bronchiole, type 2 pneumocytes and alveolar macrophages of humans.[24] To understand the role of such mutation in the pathogenesis of the pandemic H1N1 (2009) virus, we infected BALB/c mice with this virus by lung to lung adaptation. BALB/c mice were known to contain predominantly α2,3 sialic acid in their respiratory tract, and lung adaptation would serve as a selection force for the mutant viruses that favorably bind to such receptors.[28] We found that just four cycles of lung passage were sufficient to select a highly pathogenic mutant virus. Previous mouse adaptation studies with seasonal influenza viruses often required 10–20 passages before the LD50 can be reduced from more than 7 to 2 log. More interestingly, only one consistent nonsynonymous mutation of D225G at HA was found in this study, although concomitant multiple mutations in HA and other viral proteins were reported in previous mouse adaptation studies with human and avian influenza viruses.[29,30] Mouse adaptation of the human H3N2 isolate A/HK/1/68 showed 11 mutations spanning eight viral proteins (HA, NA, NP, PB2, PA, M1, M2 and NS1).

In particular, substitutions at the 701 position in PB2 were also shown to affect virulence in the avian H5N1 and H7N7.[31,32] Passaging the human H1N1 isolate A/Puerto Rico/8/34 in mice revealed the importance of substitutions in PB2 (I504V and I550L), PB1 (L208R) and PA (E349G).[33] Mouse adaptation studies with human H1N1 or H3N2 viruses suggested that the loss of potential glycosylation sites in HA may lead to resistance to inhibition by mannose binding proteins or altered receptor specificity,[30] which appeared to be consistently associated with increased virulence in mice, although the exact mechanism is uncertain. It was reported that multiple mutations in the PB2 gene can achieve a similar effect on viral pathogenicity as a virus having a lysine at the 627 position in the H5N1 virus.[34] In another study, T97I substitution in the PA gene was found to play a role for transforming an avirulent avian H5N2 strain into a virulent virus after passaging in mice.[35] Apart from mouse adaptation experiments, other methods have also been used to determine critical substitutions in the viral genome for virulence. Amino acid substitutions found in HA, PB2, PA and NA of an avian H7N7 influenza virus isolated from the fatal case in the Netherlands were found to be responsible for enhanced viral replication and virulence in animal models.[36] In another study where the NS gene segment of the H5N1 virus was introduced into H7N1 virus backbone using reverse genetics technology, the reassortant virus demonstrated greater viral replication in both cell lines and in lungs of mice, and resulted in death in mice.[37]

The LD50 of the same strain of the pandemic H1N1 (2009) virus has been reported. In one report, the virus A/California/04/2009 was found to replicate efficiently in the lungs of infected BALB/c mice, but exhibited only transient weight loss with no fatality when an inoculum of over one 10^6 PFU was used,[38] whereas another report estimated that the LD50 of the same virus in BALB/c mice was 5.8 million PFU.[39] Our results suggested that the pandemic (H1N1) 2009 virus can replicate to a high titer in the lungs of infected mice, but did not exhibit highly pathogenic features in mice without prior adaptation. The results from glycan binding assays were also conflicting as one study showed that the pandemic (H1N1) 2009 virus A/California/04/2009 binds only to α2,6 sialic acid,[38] while another study showed that the same virus binds to α2,6, and also binds considerably to α2,3 sialic acid receptors.[13] These inconsistencies may have resulted from the selection of sialyl probes used in the two studies. By analysis of HA–sialic acid interaction using computer-generated models, it was found that lysine at positions 133, 145 and 225 forms a positively charged lysine fence, which has favorable binding to both α2,3 and α2,6 sialic acid.[14] In the mouse adapted mutant virus described here, the D225G mutation may confer significantly increased affinity of binding to α2,3 sialic acid receptors and result in more efficient viral replication in the lungs, which explains its high lethality and proinflammatory pathology in mice. Consistent with the results of animal experiments, viral loads in cell culture supernatant of human cell line, A549, and that of allantoic fluid in chick embryo suggested that the mutant virus is able to achieve a higher viral load than the parental virus in chick embryos. These findings support the hypothesis that the D225G mutant virus has a higher predilection for α2,3 sialic acids, which are abundantly expressed in cells of both chicken embryos cells and BALB/c mouse. Further studies should be performed to demonstrate whether the D225G mutant of this pandemic H1N1 virus has more affinity of binding to 2,3 sialic acid, and whether the mutant virus has any changes in its transmissibility in a ferret or guinea pig model. In conclusion, this is the first study that corroborated the clinical findings that a single D225G mutation in HA of the pandemic H1N1 (2009) virus is sufficient to confer adaptation and enhance virulence in mice. The mutant virus grows significantly faster in and is more lethal to chick embryo than the parental virus. Furthermore, the increased virulence of this mutant virus was clearly demonstrated by both a higher viral load and the concomitantly elevated levels of proinflammatory cytokines and chemokines in the lungs of infected mice. A kinetics study of lung viral load, cytokine level and pathology may improve the understanding of the link between infection and mortality.

Author contributions: K-YY and BZ conceived and supervised the study; K-HC, HC, AJXZ, JZ, CCSC, VKMP, KZ and VHCL carried out the wet laboratory and P3 animal studies; D-YJ, PCYW, JFWC, KKWT and HC analyzed and interpreted the data; and K-YY, KKWT and BZ wrote the manuscript.

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