J Med Sci 2013;33(5):271-277
http://jms.ndmctsgh.edu.tw/3305271.pdf
DOI:10.6136/JMS.2013.33(5).271

H1N1 Polymerase A Gene Enhances Replication Efficiency of Seasonal H3N2 Influenza Virus in Human Lung Cells

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The occurrence of 2009 H1N1 influenza pandemic spread rapidly worldwide and caused more than 18,000 mortalities in 4 months. Recent WHO surveillance data have shown that H3N2 virus is the predominant circulating seasonal flu virus in many countries. Earlier research suggested that the reassortment of influenza ribonucleoprotein (RNP) complex genes (PB2, PB1, PA and NP) has a tremendous impact on polymerase activity and virus replication. This study evaluated the effects of the reassortment between the H1N1 PA gene and the local H3N2 strain (A/TW3446/02; 3446) on RNP activity, growth kinetics and virulence in mice. Compared with the wild-type 3446, the reassortants of PR8 PA/3446 and 2009 pH1N1 PA/3446 grew more efficiently in human A549 cells but retained the same virulence and pathological change as 3446 in mice inoculated with high viral load.

Key words: influenza reassortment, RNP activity, PA gene

INTRODUCTION

In April 2009, a new H1N1 swine-origin influenza A virus emerged in Mexico which spread worldwide rapidly and caused more than 18,000 mortalities in 4 months.[1] The 2009 H1N1 pandemic virus (pH1N1) revealed a specific constellation of avian, human, and swine viral genes.[2,3] Co-infection with seasonal influenza A viruses and the particular combination result in reassortant viruses that acquire new characteristics of transmission, virulence, and susceptibility. It was demonstrated previously that 2009 pH1N1 elicited elevated respiratory disease relative to seasonal H1N1 viruses in ferrets.[4] The 2009 pH1N1 replicated to higher titers and more extensively in the lower respiratory tract, compared with seasonal H1N1 flu viruses.[4,5]

Influenza virus ribonucleoprotein (RNP) complex plays an important role in virus replication. RNPs comprise the viral genome, viral polymerase, and many copies of the viral nucleoprotein (NP). The influenza polymerase is a heterotrimer composed of subunits PB1, PB2 and PA. As one of the determinants of species tropism and pathogenicity, the polymerase binds NP and viral RNA to form the viral ribonucleoprotein complexes (vRNP) required for replication and transcription.[6,7] A previous study showed that replacing the PA gene with that from high pathogenic avian influenza (HPAI) H5N1 contributes to increased seasonal H3N2 polymerase activity;[8] while low pathogenic avian influenza (LPAI) H5N1 PA reassortment decreased seasonal H3N2 RNP activity.[9] In addition to seasonal H3N2, the pathogenicity of seasonal H1N1 influenza virus could be altered by reassorting with the RNP gene derived from 2009 pH1N1.[10] In particular, the PA gene of the pandemic strain plays a role in increasing the virulence of seasonal influenza virus. The PA subunit has been shown to contribute directly to the virulence in influenza viruses.[6,11-14] However, the mechanism by which the PA enhances the pathogenicity remains unclear.

Reassortment within polymerase gene segments causes changes in the pathogenicity of influenza A viruses. The reassortants between circulating human and animal influenza viruses pose serious influenza-related public health concern. In 2012, the emergence of new seasonal flu H3N2v (H3N2 variant) epidemic in the United States is a clear example. H3N2v viruses that contain the M gene from 2009 pH1N1 were first identified in US pigs in 2010 and the first clinical case was reported in

Received: June 1, 2013; Revised: July 22, 2013; Accepted: July 23, 2013
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2011.[15] Recent WHO surveillance data have shown that H3N2 virus is the predominant circulating seasonal flu virus in many countries.[16]

This study focused investigating whether the existing pathogenic H1N1 PA reassortment could alter virulence of currently circulating seasonal H3N2 influenza viruses. The activity of polymerase complexes of the reassortants containing various combinations of the PA gene from 2009 pH1N1 or PR8 strains in the H3N2 RNP backbone was made.

Results show that a reassortant seasonal H3N2 virus with the PA gene from the H1N1 viruses exhibited efficient replication, but retained the same virulence and pathological change as the parental H3N2 virus in mice.

MATERIALS AND METHODS

Cells and viruses. HEK293T cells and MDCK cells were maintained in DMEM supplemented with 10% FBS. A549 cells were maintained in minimal essential medium (MEM) supplemented with 10% FBS. All cells were cultured at 37°C with 5% CO_2. The whole genome of seasonal H3N2 strain, A/TW3446/02 (3446), was isolated in Chang Gung Memorial Hospital, and the PA gene of PR8 (A/PR/8/34; PR8; H1N1) and 2009 pH1N1 (A/TW126/09; SOIV; H1N1) strains were cloned into the pHW2000 vector as described previously.[10]

R**everse genetics.** The wild-type virus and the PR8 and 2009 pH1N1 PA-reassortant viruses were rescued using reverse genetics system reported by Hoffmann *et al*.[17] The rescued viral genome was confirmed by sequencing. The stock viruses were propagated in MDCK cells followed by plaque assay.

Plaque assay. Confluent MDCK cells in six-well plates were washed with PBS, and serial dilutions of the virus were adsorbed onto cells for 1 h at 37°C. Unabsorbed viruses were removed by washing the cells with PBS. The washed cells were then overlaid with 2 ml of overlay DMEM (without FBS) that had been supplemented with 0.3% agarose. After incubation for 48 h at 37°C, the cells were fixed with 10% (v/v) formalin for 1 h. Following formalin removal, the cells were stained with 0.5% crystal violet and the plaques were visualized. The visible plaques were counted, and the titers of virus (in PFU/ml) were determined. The numbers and sizes of plaques were determined from at least three independent experiments.

Chloramphenicol acetyl transferase assay for viral polymerase activities. 293T cells were co-transfected with 0.5 μg of the pPolI-CAT-RT plasmid and 0.5 μg of each of the different combinations of RNP protein expression plasmids (pcDNA3.1-PB2, pcDNA3.1-PB1, pcDNA3.1-PA, pcDNA3.1-NP). At 24 h post transfection, the cells were lysed in a 250-μl lysis buffer, and the cell extracts were analyzed for Chloramphenicol Acetyl Transferase (CAT) expression using a CAT ELISA kit (Roche) according to the manufacturer's instructions. All experiments were performed in triplicate.

Mouse experiments. Animal experiments were performed in an animal BSL2 laboratory at the National Defense Medical Center Institutes of Preventive Medicine (IPM) under an animal study protocol approved by IACUC of IPM. The wild-type C57BL/6 mice were purchased from BioLasco (Taiwan). Mice anesthetized intramuscularly with Zoletil 50 (25 mg/kg) and inoculated intranasally with 5×10^5 PFU of viruses in 50-μl phosphate-buffered saline (PBS). The infected mice were weighted for 14 days and observed daily for illness or death.

RESULTS

H1N1 PA gene reassortment altered seasonal H3N2 polymerase activity

To investigate the effects of reassortment of the H1N1 PA gene on the polymerase activity in the seasonal H3N2 virus, H3N2 (A/TW3446/02) RNP was used as a backbone, and the reassortants were generated by replacing the PA gene with that from the 2009 pH1N1 and PR8 strains, respectively. The plasmids of PB2, PB1, PA, NP and virus-like reporter gene CAT were co-transfected into 293T cells, and CAT protein expression levels were measured at 24 h post transfection. The RNP complex of the reassortant viruses with not only 2009 pH1N1 PA but also PR8 PA revealed higher activity *in vitro* than the wild-type 3446 virus (7.6 folds and 6.5 folds, respectively.), suggesting that H1N1 PA is suitable for the RNP complex of 3446 strain (Fig. 1A).

Replication ability of reassortant seasonal H3N2 viruses with H1N1 PA gene in human lung cells

The data of the polymerase activity assays showed that H1N1 PA played an important role in the 3446 RNP activity in A549 cells. To determine whether the replication ability of those viruses was also enhanced, recombinant viruses by reverse genetics with the different PA gene combinations were generated using 3446 as a genetic backbone to replace the PA gene derived from 2009 pH1N1 and PR8 strains. The growth kinetics of these viruses was compared with that of wild-type 3446

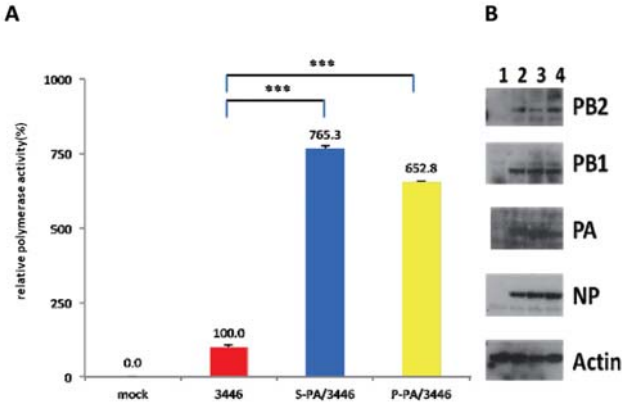

Fig. 1 Heterologus strains of influenza virus PA subunits enhance the activity of a seasonal H3N2 influenza virus polymerase.

Expression plasmids for seasonal H3N2 (3446) RNP genes were cotransfected with viral-like CAT reporter RNA into 293T cells. CAT expression level was detected and the percentage difference in reporter protein expression was evaluated as the indicator of RNP activities. (A) The reassortant was generated by replacement of 2009 pH1N1 PA gene (S-PA, blue bar) or PR8 PA gene (P-PA, yellow bar). (B) The expression of polymerase subunits and NP in the transfected cells was confirmed by western blotting using primary antibodies to detect PB2, PB1, PA and NP proteins in the cell lysates. Lane 1: mock, 2: S-PA/3446, 3: P-PA/3446, 4:3446. Data represent the mean±SD. Similar results were obtained from three separate experiments. ***$p < 0.001$ (⊢⊣ two-tailed t test).

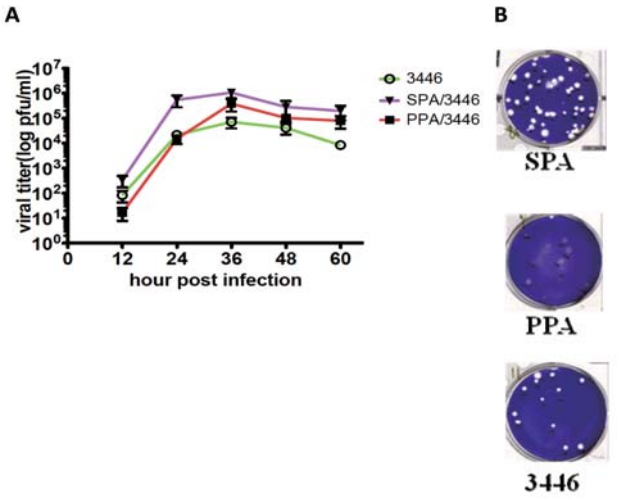

Fig. 2 Multicycle replication kinetics of recombinant viruses in infected A549 cells and plaque morphology.

Chimeric H3N2 viruses containing PR8 PA (P-PA/3446; red line) or 2009 pH1N1 PA (S-PA/3446; purple line) and wild-type H3N2 virus (3446; green line) were rescued by reverse genetics. Multistep growth curves were employed to monitor the viral growth kinetics of human A549 cells infected at a multiplicity of infection of 0.001. At 12, 24, 36, 48, and 60 h p.i., the supernatant was collected for viral titration. The titer was determined by plaque assay on MDCK cells (A). The plaque morphology was visualized at 48 h p.i. (B).

in A549 cells. It was found that the reassortant viruses containing 2009 pH1N1 PA or PR8 PA has higher viral titer than the wild-type 3446 virus in A549 cells. The 2009 pH1N1 PA reassortant virus (SPA/3446) replicated well, reaching a maximum titer of 1.07×10^6 PFU/ml at 36 h post-infection (p.i.), which was 10-fold higher than that of the wild-type 3446 virus (~7×10^4 PFU/ml at 36 h p.i.). In addition, PR8 PA reassortant virus (PPA/3446) reaching the maximum titer of 4×10^5 PFU/ml at 36 h p.i., which is higher than the wild-type 3446 virus (Fig. 2A), suggests that the PA gene makes a critical contribution to the increased replication efficiency of H1N1 PA reassorted seasonal H3N2 viruses in human lung cells. Moreover, both SPA/3446 and PPA/3446 reassortant viruses revealed larger plaque size morphology on MDCK cells compared with that displayed by the wild-type 3446 virus (Fig. 2B).

Pathogenicity test in mice

The virulence of SPA/3446 and PPA/3446 reassortants and wild-type 3446 viruses in mice were further examined. Wild-type C57BL/6 mice (B6) were inoculated intranasally with the highest obtainable titer of each virus (5×10^5 PFU), and morbidity and mortality were monitored daily for 2 weeks. Neither the 3446 virus nor the H1N1 PA reassortants cause any significant disease symptoms and death in wild-type B6 mice (Fig. 3A, B). Histological analyses revealed no inflammation in lung sections of mice infected with wild-type 3446 and H1N1 PA reassortants. On immunohistochemistry (IHC) analysis, the brown spots represented influenza virus antigen. Wild-type 3446 and H1N1 PA reassortants were all located in the bronchioles but not alveoli (Fig. 4A-C). Taken together, these data suggested that the H1N1 PA reassorted H3N2 viruses have the same pathogenicity in mice.

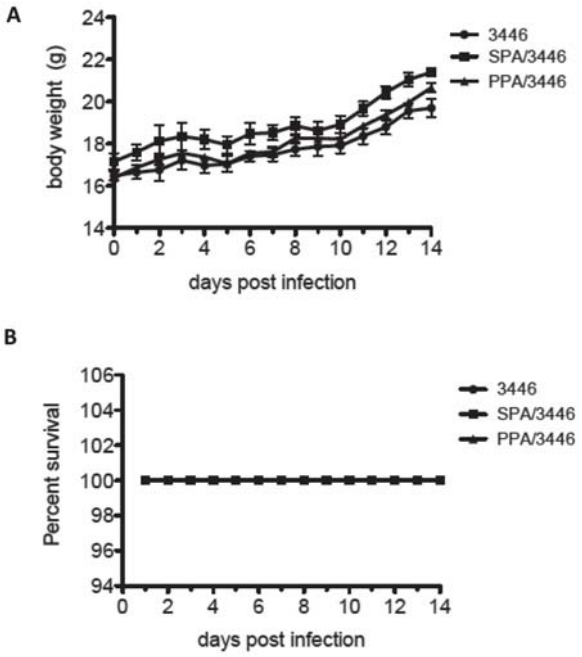

Fig. 3 Recombinant viruses do not affect pathogenecity in normal wild-type (WT) B6 mice.
Groups of three wild-type B6 mice were inoculated intranasally with 5×10^5 plaque-forming units (PFU) of recombinant H3N2 viruses in a volume of 50 μl. Both survival curves (A) and body weight changes (B) of the infected mice were monitored 14 days.

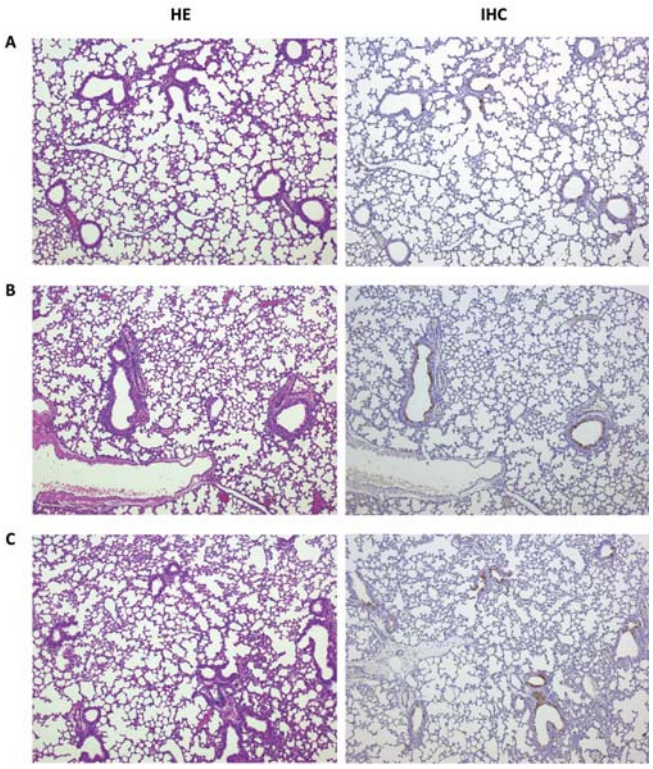

Fig. 4 Histopathology and immunohistochemistry in lungs of mice inoculated with recombinant viruses.
The left lung of mice infected with 3446 and H1N1 PA /3446 H3N2 viruses (5×10^5 PFU/mouse) was collected on 3 d p.i. using the standard protocol. Tissue samples were embedded in paraffin, and then cut into 5-μm sections for antigen detection using IHC staining and pathological examination using HE staining. (A) 3446; (B) P-PA/3446; and (C) S-PA/3446.

DISCUSSION

Reassortment is a critical mechanism for the emergence of novel pandemic strains of influenza viruses, such as the 2009 H1N1 pandemic virus . The 2009 H1N1 pandemic virus (pH1N1) revealed a particular combination of RNP genes, which were derived from avian-like PB2, PA, human-like PB1, and classical swine-like NP.[18] Although H3N2 is the major seasonal human influenza virus currently circulating in many countries, the 2009 H1N1 pandemic virus is still circulating in the world. The reassortment between the existing pathogenic strain and seasonal influenza viruses is a global public health concern.

This study focused on whether the PA gene derived from H1N1 can affect the pathogenicity of the current circulating seasonal H3N2 influenza virus. It was found that the H1N1 PA reassortment increased the RNP activity of seasonal H3N2 virus and replication efficiency in cell culture. Recent studies have suggested that the PA gene derived from the H5N1 viruses plays an important role in affecting the RNP activity of seasonal H3N2 viruses. The HPAI H5N1 PA gene increased the H3N2 RNP activity, while the LPAI H5N1 did not.[6,7] Previous research showed that the PA from the 2009 H1N1 pandemic virus increased seasonal H1N1 polymerase activity.[8] This study found that the H1N1 PA is incidentally compatible with a seasonal H3N2 backbone, which is of a lineage from different that of seasonal H1N1 (Table 1). The H1N1 PA reassorted seasonal H3N2 viruses were also found to replicate better than the parental strains in human A549 cells. However, no significant changes in pathogenicity of the reassortant viruses were found in the mice model. It is likely that the dose inoculated was not lethal, and that the highest virus yields were obtained (10^7 PFU/ml) in MDCK cells is also a limitation for this

Table 1 Alignment of PA protein from 2009 H1N1 pandemic (126), PR8, and H3N2 (3446) viruses.

study. Recombinant viruses infected only the epithelial cells of bronchioles but not the alveolar epithelium of mice and did not cause severe inflammation in lungs, possibly to limited dosage by intranasal inoculation. Therefore, these results cannot reflect the actual differences in virulence among recombinant viruses. In addition, seasonal human H3N2 viruses are not mouse-adapted strains, and therefore replicate poorly in the lungs of wild-type mice, resulting in early clearance of the virus during infection.[19,20] In contrast, the PR8 virus is the well-known mouse-adapted laboratory strain that can cause severe flu syndrome, even death. Therefore, the differences in pathogenicity between the H1N1 PA reassortant and the parental H3N2 viruses may not be easily distinguished in wild-type C57BL/6 mice with normal immunity. Innate immunity has recently been shown to be an important component in the host defenses against influenza infection.[21-23] Further study is needed to determine whether the enhancement of RNP activity by PA reassortment can affect pathogenicity in innate immunity-deficient mice.

ACKNOWLEDGMENTS

The authors thank Prof. Shin-Ru Shih (Chang Gung University) for kindly providing the viral RNA (vRNA) of A/TW3446/02(H3N2) and the two plasmids expressing PA of 2009 pH1N1(A/TW126/09) and A/PR/8/34 (H1N1).

DISCLOSURE

All authors declare that this study has no conflict of interest.

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