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NOTE

GFP-Expressing Influenza A Virus for Evaluation of the Efficacy of Antiviral Agents

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To address its value as a screening tool in the development of antiviral drugs, a recombinant influenza virus expressing green fluorescent protein (rPR8-GFP virus) was investigated *in vitro* and *in vivo*. The inhibition of viral growth by a neuraminidase inhibitor in the cells or lower respiratory tracts of mice could be visualized by the level of fluorescence. In addition, the rPR8-GFP virus exhibited high pathogenicity in mice. Taken together, these results suggest that the rPR8-GFP virus can be a useful tool for the rapid identification of antiviral drugs active against influenza viruses.

Keywords: influenza, green fluorescent protein, antiviral drug

Influenza viruses have been persistent threats to human health (Palese, 2004; Kilbourne, 2006; Neumann and Kawaoka, 2011). As the result of changes in antigenicity through antigenic drift or shift, influenza A viruses have circumvented medical interventions and have caused frequent seasonal epidemics and occasional pandemics (Guan *et al.*, 2010). Vaccines and antiviral drugs are two major options to alleviate the disease burden of influenza in humans (De Clercq, 2006; Kawaoka, 2006). In the past, M2 inhibitors, one of several categories of anti-influenza drugs, were prescribed to treat influenza infections. However, most of the currently circulating influenza viruses harbor the S31N mutation in the M2 protein, which confers viral resistance to M2 inhibitors (Cheng *et al.*, 2010). Resistance to neuraminidase (NA) inhibitors (NIs) has been shown to be associated with many mutations, which were found to arise sporadically during long-term treatment (Thorlund *et al.*, 2011). To overcome these problems, there have been many attempts to identify new treatments with high efficacy for which the incidence of resistance is low.

Although there is much known about influenza viruses as the result of numerous research studies, we still need more specific information about influenza. Recently, new approaches were developed to elucidate the mechanism of viral infection. Manicassamy *et al.* (2010) developed a fluorescently tagged influenza virus that was used to visualize the course of viral infection in living organisms and made it possible to track cells infected by influenza viruses. Using an infrared dye-conjugated antibody, Kumar *et al.* developed a high-throughput screening system to identify candidates for influenza treatment (Kumar *et al.*, 2012).

In this study, we investigated whether a recombinant influenza A virus expressing green fluorescent protein (GFP) would be useful in the screening of possible antiviral drugs. Provided by Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, New York, NY, USA), the GFP-expressing recombinant H1N1 virus (rPR8-GFP) was generated from a parental H1N1 strain (wild-type A/Puerto Rico/8/34, wtPR8) and expresses GFP at the C-terminus of the NS1 protein (Manicassamy *et al.*, 2010). To confirm the GFP expression and to evaluate the oseltamivir susceptibility of the rPR8-GFP virus, MDCK cells were infected with this virus at a multiplicity of infection of 0.01 (MOI=0.01). One hour later, 9.375 nM–0.3 μM of oseltamivir was added to the viral growth media. At 24 h post-infection (hpi), GFP expression was detected using a fluorescence microscope. The rPR8-GFP virus exhibited bright green fluorescence in infected cells and was found to be susceptible to 0.075 μM of oseltamivir (the lowest concentration that inhibited the virus), expressing a barely detectable GFP signal at this concentration (Fig. 1). Therefore, the rPR8-GFP virus might be the very tool for the rapid screening process of antiviral candidate materials. Indicated by the GFP intensity, the rPR8-GFP virus reflected the *in vitro* inhibitory efficacy of oseltamivir. The antiviral efficacy of 759 natural extracts from plants was rapidly evaluated by rPR8-GFP-based screening assay. We found 14 antiviral candidates exhibiting more than 80% reduction rates in GFP signals (Fig. 1). Before the *in vivo* evaluation of the 14 hit records, we determined the pathogenicity of the rPR8-GFP virus in a mouse model. Five BALB/c mice (female, five weeks old) were infected intranasally with different amounts of rPR8-GFP or wtPR8

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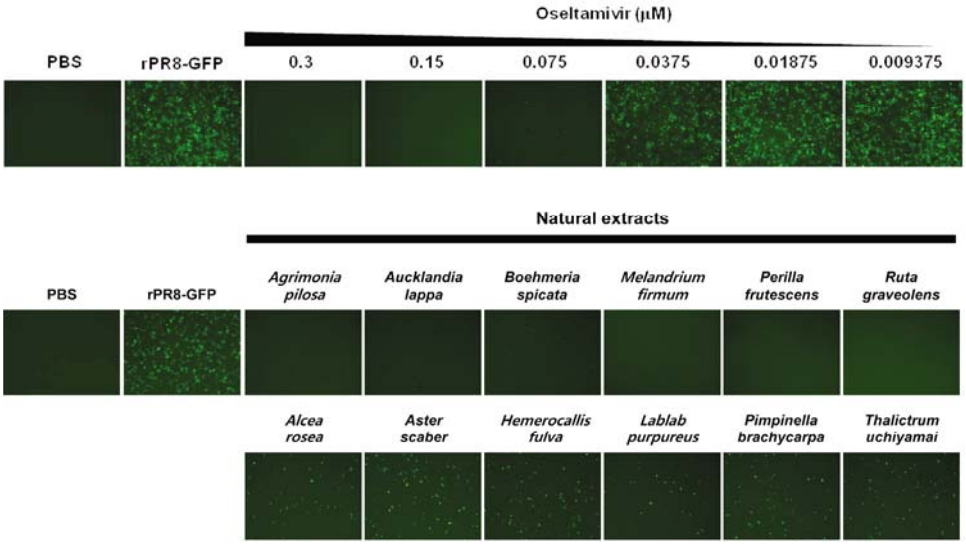

Fig. 1. Evaluation of the efficacy of oseltamivir and natural extracts by infection of the rPR8-GFP virus in MDCK cells. MDCK cells were infected with rPR8-GFP at a multiplicity of infection of 0.01 (MOI=0.01). After one hour of infection, 9.375 nM–0.3 μM of oseltamivir or 10 μg of each natural extract was added into the viral culture media. At 24 h post-infection (hpi), the GFP signals were detected using a fluorescence microscope (40× magnification). As a control, one well of MDCK cells was mock-infected with PBS.

virus in 50 μl. The body weight changes and survival rates of the mice were recorded for 14 days post-infection (dpi). Mice exhibiting the loss of more than 25% of their initial body weights were scored as experimental deaths and were euthanized humanely. The mice infected with the mouse-adapted wtPR8 virus, which is lethal in mice (Garcia-Sastre *et al.*, 1998), experienced massive weight loss and succumbed to death even when infected with only 10^2 plaque-forming units (PFU) (Figs. 2A and 2B). Even though it had not been subjected to prior adapation, the rPR8-GFP virus resulted in severe weight loss and 100% mortality in mice infected with 10^6 or 10^7 PFU (Figs. 2C and 2D). However, mice infected with 10^5 PFU of the rPR8-GFP virus slowly gained body weight after 7 dpi. Based on these survival results, the 50% mouse lethal dose (MLD_{50}) of the wtPR8 and rPR8-GFP viruses were determined to be $10^{1.5}$ and $10^{5.5}$ PFU, respectively (Reed and Muench, 1938). Although attenuated compared with wtPR8, the rPR8-GFP virus was sufficiently pathogenic to kill mice without adaptation. These results demonstrate that the rPR8-GFP virus is suitable for use in challenge experiments in a mouse model.

Next, we investigated whether the rPR8-GFP virus could invade the lower respiratory tracts of mice. According to recently published papers that discussed the pathogenesis of a reverse-genetically rescued 1918 virus and of 2009 pandemic H1N1 clinical cases, severe outcomes of influenza infections mainly resulted from fatal pulmonary damage (Kobasa *et al.*, 2007; Soto-Abraham *et al.*, 2009). If this rPR8-GFP virus could infect the lungs of mice and if an antiviral drug could counteract the rPR8-GFP infection, this system might be an efficient tool to identify potent drugs because the fluorescence signals of the infected lungs reflect the severity of infection. We therefore infected six BALB/c mice (female, five weeks old) intranasally with 10^5 PFU in 50 μl and sacrificed three mice at 3 and 6 dpi to determine the viral titers in the lungs. As shown in Fig. 3, the rPR8-GFP virus infected the lungs of mice and replicated to $10^{5.47}$ PFU/ml/g (5.47±0.054 PFU/ml/g in log scale) at 3

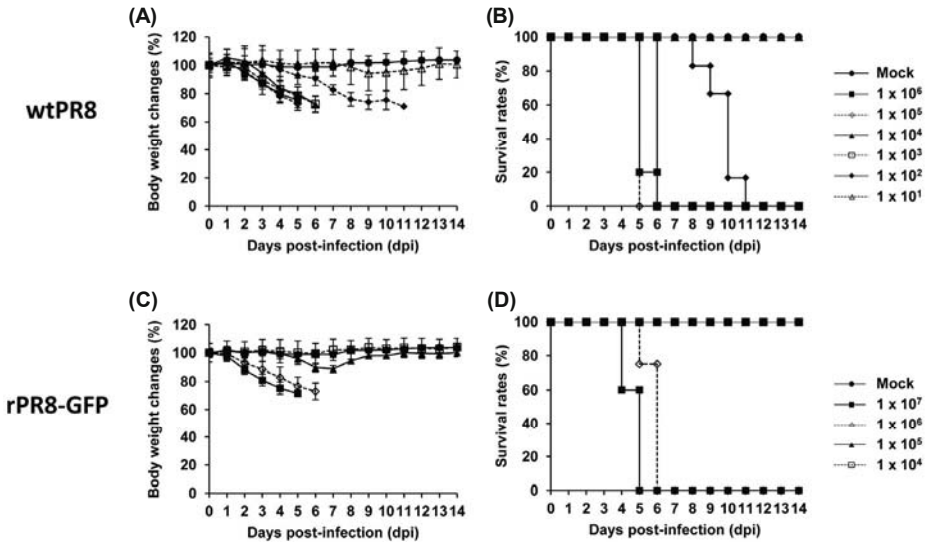

Fig. 2. Body weight changes and survival rates of BALB/c mice infected with the wtPR8 or rPR8-GFP virus. To analyze the pathogenicity of the parental H1N1 virus (A/Puerto Rico/8/34; wtPR8) (A and B) and the rPR8-GFP virus (C and D), five BALB/c mice (female, five weeks old) were infected intranasally with different titers (plaque-forming units, PFU) of each virus in 50 μl. The body weight changes (A and C) and survival rates (B and D) of the mice were recorded for 14 days post-infection (dpi). Mice in the mock group were infected with 50 μl of PBS and used as a control.

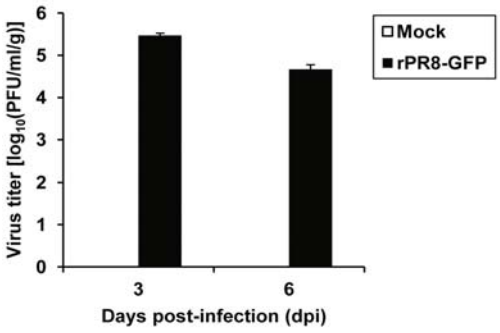

Fig. 3. Replication of the rPR8-GFP virus in the lungs of the infected mice. To assess whether the rPR8-GFP virus could replicate in the lower respiratory tracts of mouse, six BALB/c mice (female, five weeks old) were infected intranasally with 10^5 PFU in 50 μl. Three mice were euthanized at 3 or 6 dpi, and the lungs were collected for the analysis of viral replication. Mice in the mock group were mock-infected with 50 μl of PBS and used as a control. The viral titers were determined by plaque assays in MDCK cells.

dpi and to $10^{4.67}$ PFU/ml/g (4.67±0.105 PFU/ml/g in log scale) at 6 dpi. Unlike seasonal influenza viruses, which caused only mild inflammatory diseases restricted to the upper respiratory tract, the rPR8-GFP virus invaded the lower respiratory tracts of mice, resulting in considerable rates of viral replication. This result might also explain why infected mice exhibited severe weight loss and high mortality (Figs. 2C and 2D). Taken together, our results suggest that the rPR8-GFP virus can be as effective as mouse-adapted strains in the lung pathogenesis model in mice.

The wtPR8 virus is susceptible to oseltamivir phosphate *in vivo* (Yen *et al*., 2007). We therefore determined whether the rPR8-GFP infection could be treated with oseltamivir phosphate in a mouse model. To determine whether the resultant fluorescence signals in infected mice were correlated with the oseltamivir phosphate treatment, six BALB/c mice (female, five weeks old) per group were infected intranasally with 10^5 PFU in 50 μl. For two days after the infection, a group of infected mice was orally treated twice per day with 25 mg/kg of oseltamivir phosphate (virus/Osel group), whereas the other infected mice were treated with PBS (virus/PBS group). Mice in a control group were mock-infected and treated with PBS (PBS/PBS group). At 3 and 6 dpi, three mice per group were sacrificed, and the lungs of the mice were removed for the detection of GFP signals using an IVIS-200 series system (Caliper Life Science, USA). Initially, we attempted to use non-invasive monitoring and imaged the thoracic region of living mice because the lungs were the target organ of interest. However, the fluorescence intensity was too low to be read, and the fur exhibited non-specific fluorescence. We then euthanized the mice and removed their thoracic cage to provide an open space for the measurement of the fluorescence emitted by the infected lungs. However, the lungs shrunk immediately after the incision, and the surfaces of the lungs were hidden around the heart. Thus, we had to remove the lungs from the mice for imaging. The lungs of the control mice in the PBS/PBS group were imaged first to establish the background level of fluorescence. In the infection-only group (virus/PBS group), the lungs of mice exhibited a high level of fluorescence at 3 dpi, and the signals continued until 6 dpi (Fig. 4). These results were consistent with the viral replication observed in the lungs of infected mice (Fig. 3). In addition, the correlation between the level of rPR8-GFP virus replication and the fluorescence signals of the infected lungs in this model was established.

To assess the potential use of the rPR8-GFP virus in the screening of antiviral drugs, we also investigated whether the rPR8-GFP infection could be treated with oseltamivir phosphate. After treatment with oseltamivir phosphate, the lungs of the rPR8-GFP-infected mice (virus/Osel group) exhibited no fluorescence, similar to the control mice (Fig. 4). Using this model, we were able to easily determine that only two days of oseltamivir treatment was able to effectively inhibit the replication of the rPR8-GFP virus. To find a new candidate agent for the influenza treatment, we will further evaluate the *in vivo* efficacy of the 14 selected natural extracts against influenza using this model.

We found that the rPR8-GFP virus might be a useful indicator for the *in vitro* and *in vivo* assessment of anti-influenza agents. As compared with classical methods, the inhibitory efficacy of candidate materials could be easily determined by measuring the intensity of the fluorescence signals. Therefore, a GFP-expressing influenza virus might accelerate the development of antiviral drugs. Additionally, a GFP-expressing influenza virus can be used to rapidly evaluate vaccine efficacy, neutralizing antibody responses, and viral pathogenesis through the comparison of GFP signals. In summary, our work demonstrates that a GFP-expressing virus might be a powerful tool for the rapid development of medical interventions targeting influenza A virus.

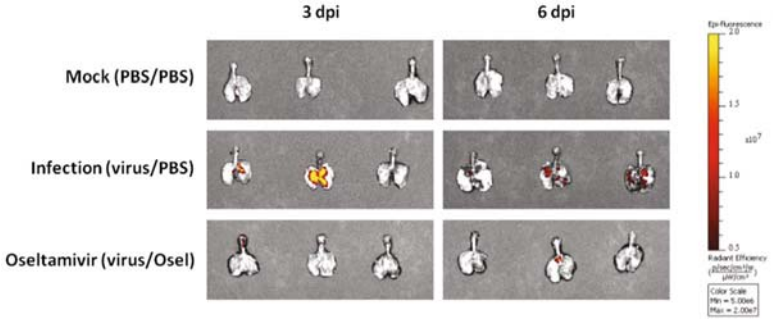

Fig. 4. Imaging of the lungs of infected mice. After being infected intranasally with 10^5 PFU of the rPR8-GFP virus in 50 μl, three groups of BALB/c mice (female, five weeks old) were treated with PBS (PBS/PBS group and virus/PBS group) or oseltamivir phosphate (virus/Osel group) for two days. At 3 and 6 dpi, three mice per group were euthanized, and the lungs were prepared for imaging.

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