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A replication-incompetent influenza virus bearing the HN glycoprotein of human parainfluenza virus as a bivalent vaccine

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ABSTRACT

Influenza virus and human parainfluenza virus (HPIV) are major etiologic agents of acute respiratory illness in young children. Inactivated and live attenuated influenza vaccines are approved in several countries, yet no vaccine is licensed for HPIV. We previously showed that a replication-incompetent PB2-knockout (PB2-KO) virus that possesses a reporter gene in the coding region of the PB2 segment can serve as a platform for a bivalent vaccine. To develop a bivalent vaccine against influenza and parainfluenza virus, here, we generated a PB2-KO virus possessing the hemagglutinin-neuraminidase (HN) glycoprotein of HPIV type 3 (HPIV3), a major surface antigen of HPIV, in its PB2 segment. We confirmed that this virus replicated only in PB2-expressing cells and expressed HN. We then examined the efficacy of this virus as a bivalent vaccine in a hamster model. High levels of virus-specific IgG antibodies in sera and IgA, IgG, and IgM antibodies in bronchoalveolar lavage fluids against both influenza virus and HPIV3 were detected from hamsters immunized with this virus. The neutralizing capability of these serum antibodies was also confirmed. Moreover, the immunized hamsters were completely protected from virus challenge with influenza virus or HPIV3. These results indicate that PB2-KO virus expressing the HN of HPIV3 has the potential to be a novel bivalent vaccine against influenza and human parainfluenza viruses.

1. Introduction

Over the last 60 years, annual vaccination has been the most effective strategy for the prevention and control of influenza virus infection [1,2]. Inactivated influenza vaccines are licensed in many countries, and in some countries live attenuated influenza vaccines are also licensed. The former provide protection with minimal reactogenicity but a short duration of effect [1,3]. The latter, by contrast, can confer longer protection and higher efficacy particularly in young children but have the potential to cause minor symptoms of respiratory illness [4]. In the US, a live attenuated influenza vaccine is currently licensed only for healthy, non-pregnant persons ranging from 2 to 49 years of age [1,2]. Hence, a novel influenza vaccine that has minimal reactogenicity with higher efficacy is desirable.

Human parainfluenza virus (HPIV) causes serious lower respiratory tract diseases in young children. It is one of the most common causes of hospitalization for fever and/or acute respiratory illness in children under 5 years of age, and in particular, in infants aged 0–5 months [5,6]. Among the four types of HPIV, a vaccine for children against HPIV type 3 (HPIV3) is most desirable as HPIV3 contributes to more than half of all of the HPIV hospitalizations annually [6,7].

The quest for an HPIV vaccine began soon after HPIV was first isolated in the 1960s. Hemagglutinin-neuraminidase (HN) glycoprotein, one of the major surface antigens of HPIV, has been targeted for triggering immunity against HPIV because of its high immunogenicity [8]. Despite efforts to develop an effective HPIV vaccine, no such vaccine had yet been licensed [7]. Considering that the hospitalization rate for influenza virus is also highest among infants aged 0–5 months, a combined vaccine of HPIV and influenza virus would decrease the burden on this population [9].

Previously, we genetically engineered a replication-incompetent PB2-knockout (PB2-KO) influenza virus that harbors a foreign gene [10], and demonstrated its potential in a mouse model to serve as a platform for bivalent vaccines [11]. In the next

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step toward the development of a bivalent vaccine for influenza and parainfluenza, here we generated a PB2-KO virus expressing the HN of HPIV3 (HPIV3-HN), and tested its efficacy as a bivalent vaccine in a hamster model.

2. Materials and methods

2.1. Cells

Human embryonic kidney 293 (HEK293) and HEK293T (a derivative of the HEK293 cell line into which the gene for the simian virus 40T antigen has been inserted) cells were maintained in Dulbecco's modified Eagle medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Life Technologies, Carlsbad, CA). Madin–Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) (Life Technologies) supplemented with 5% newborn calf serum (NCS) (Sigma–Aldrich). AX4 cells, which are an MDCK-derived cell line with enhanced expression of the human α-2,6-sialyltransferase, the human-type receptors for influenza virus [12], were maintained in 5% NCS–MEM supplemented with puromycin (2 μg/ml, Nacalai Tesque, Kyoto, Japan). AX4/PB2 cells (AX4 cells stably expressing the PB2 protein derived from A/Puerto Rico/8/34 [PR8]) were maintained in 5% NCS–MEM supplemented with puromycin (2 μg/ml, Nacalai Tesque) and blasticidin (10 μg/ml, Life Technologies) [10]. Rhesus monkey kidney epithelial (LLC-MK2) cells were maintained in medium 199 (Life Technologies) supplemented with 1% horse serum (ATCC, Manassas, VA). All cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

2.2. Viruses and plasmid-driven reverse genetics

The wild-type HPIV3 (strain C243) obtained from ATCC was propagated in LLC-MK2 cells. The wild-type influenza virus (PR8) and the PR8-based PB2-KO virus expressing HPIV3-HN (strain C243) from its PB2 gene (designated as PR8/PB2-HPIV3HN) used in this study were engineered by using reverse genetics as previously described [13]. We also engineered a PB2-KO virus expressing enhanced GFP (EGFP) from its PB2 gene (designated as PR8/PB2-EGFP) as a control for the PB2-KO virus vector. Briefly, for the expression of viral RNA (vRNA), plasmids containing the cloned cDNAs of the PR8 genes between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmids) were constructed. Plasmids [pPolIPB2(120)HPIV3HN(336)] and [pPolIPB2(120)EGFP(336)] were constructed to replace the PolI plasmid encoding the PB2 segment with a segment encoding the PR8-derived 3′ PB2 noncoding region, 120 nucleotides that corresponded to the PB2-coding sequence at the 3′ end of the vRNA followed by the HPIV3HN-coding or EGFP-coding sequence, 336 nucleotides that corresponded to the PB2-coding sequence at the 5′ end of the vRNA, and finally the 5′ PB2 noncoding region [14]. To generate the PR8/PB2-HPIV3HN or PR8/PB2-EGFP virus, pPolIPB2(120)HPIV3HN(336) or pPolIPB2(120)EGFP(336), respectively, and the remaining 7 PolI plasmids of PR8 were cotransfected into HEK293T cells along with eukaryotic protein expression plasmids for PB2, PB1, PA, and NP derived from PR8 by using the TransIT 293 transfection reagent (Mirus Bio Corp., Madison, WI), following the manufacturer's instructions. At 48 h post-transfection, the supernatants containing the PR8/PB2-HPIV3HN or PR8/PB2-EGFP virus was harvested and inoculated into AX4/PB2 cells to make stock viruses.

2.3. Detection of the HN glycoprotein expressed from PR8/PB2-HPIV3HN

AX4 and AX4/PB2 cells grown in 12-well plates (Asahi Techno Glass, Shizuoka, Japan) were inoculated with PR8/PB2-HPIV3HN or PR8/PB2-EGFP virus, and incubated for 48 h prior to being subjected to the immunofluorescence assay (IFA). Cells were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde (Wako, Ltd., Osaka, Japan) and permeabilized with 0.1% Triton X-100 (Nacalai Tesque). They were then incubated with a goat anti-HPIV3 polyclonal antibody (ab28584, Abcam, Cambridge, UK) and a mouse anti-influenza NP monoclonal antibody (clone 2S-347/3). All cells were then incubated with an Alexa Fluor 488-labeled donkey anti-goat secondary antibody (Life Technology), an Alexa Fluor 549-labeled chicken anti-mouse secondary antibody (Life Technology), and Hoechst 33342 (Life Technologies) for the detection of influenza PR8-NP, HPIV3-HN, and nuclei, respectively.

2.4. Growth kinetics and virus titration

To determine virus growth rates, triplicate wells of confluent AX4 or AX4/PB2 cells were infected with PR8/HPIV3HN, PR8/PB2-EGFP or wild-type PR8 at a multiplicity of infection (MOI) of 0.001. Supernatants were collected every 12 h for 3 days and subject to plaque assays in AX4 or AX4/PB2 cells.

2.5. Genetic stability of the HN gene in PR8/PB2-HPIV3HN virus

PR8/PB2-HPIV3HN virus was passaged 5 times by inoculating virus into AX4/PB2 cells at an MOI of 0.001 and incubating for 3 days. Supernatant of the fifth passage was subject to a plaque assay and IFA as described above to determine the number of plaques that retain the HN gene.

2.6. Immunization and protection tests

Four-week-old female Syrian hamsters ($n = 3$, Japan SLC, Inc., Shizuoka, Japan) were anesthetized with Ketamine and Xylazine via intraperitoneal injection, and then intranasally inoculated with 200 μl/hamster of PBS, PR8/PB2-EGFP virus (10^9 plaque-forming units [PFU]/hamster), or PR8/PB2-HPIV3HN virus (10^9 PFU/hamster) three times at 2-week intervals. The PB2-KO viruses used for these immunizations were prepared by ultracentrifugation of viral supernatants in a Type19 Beckman rotor (18,000 × *g*, 2 h, 4 °C) through a 20% sucrose cushion.

Two weeks after the final immunization, hamsters were intranasally challenged with 10^6 PFU/hamster of PR8 virus or 10^4 PFU/hamster of HPIV3 (strain C243), and body weight was monitored. We first tested two inoculation volumes of virus, 50 and 200 μl/animal, and found that the latter was better for robust infection. We then determined the optimum amount of virus to use for the inoculum; for A/PR/8/34 virus, preliminary experiments with 10^6 PFU/animal resulted in consistent infection. For HPIV3, among the three virus doses (10^4, 10^5 and 10^6 PFU/anima) tested, we found that virus was present longer in the lungs of animals infected with 10^4 PFU/animal than in those infected with higher doses. We therefore used the virus doses of 10^6 PFU/200 μl/animal for influenza virus and 10^4 PFU/200 μl/animal for parainfluenza virus.

On days 3, 6 (and 9 for the HPIV3-challenged group) post-challenge, nasal turbinates, tracheae, and lungs of hamsters ($n = 3$) were collected after euthanasia, and homogenized. Virus titers were determined by using plaque assays with AX4 cells for PR8 virus and LLC-MK2 cells for HPIV3.