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Lethal murine infection model for human respiratory disease-associated Pteropine orthoreovirus

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ABSTRACT

Pteropine orthoreovirus (PRV) is an emerging bat-borne human pathogen causing severe respiratory illness. To date, however, the evaluation of PRV virulence has largely depended on the limited numbers of clinical cases owing to the lack of animal models. To develop an in vivo model of PRV infection, an inbred C3H mouse strain was infected intranasally with pathogenic PRV strain Miyazaki-Bali/2007. C3H mice suffered severe lung infection with significant body weight reduction and died within 7 days after intranasal infection. Infectious viruses were isolated mainly from the lungs and trachea. Histopathological examination revealed interstitial pneumonia with monocytes infiltration. Following repeated intranasal infection, mice developed antibodies to particular structural and non-structural proteins of PRV. The results of these immunological assays will help to develop laboratory protocols for sero-epidemiological studies. Our small rodent model of lethal respiratory infection will further allow investigation of the molecular mechanisms underlying the high pathogenicity of PRV.

1. Introduction

Pteropine orthoreovirus (PRV) is a member of Family *Reoviridae*, genus orthoreovirus. A prototype strain of PRV was first isolated from flying fox (*Pteropus poliocephalus*) in Nelson Bay, Australia in 1968 (Gard and Compans, 1970; Gard and Marshall, 1973). PRV had been a sole member comprising the PRV group for a considerable period until a second case of the PRV strain Pulau was reported from bat species in Malaysia in 2006 (Pritchard et al., 2006). The first human case of PRV infection by the PRV strain Melaka was reported in Malaysia in 2007 from a patient with high fever and respiratory disease (Chua et al., 2007). Since the first case of PRV infection in humans, evidence of PRV infections in humans (Cheng et al., 2009; Chua et al., 2007, 2008, 2011; Voon et al., 2015; Wong et al., 2012; Yamanaka et al., 2014) and bats (Du et al., 2010; Hu et al., 2014; Lorusso et al., 2015) in Association of Southeast Asian Nations (ASEAN) countries and China have been reported. Phylogenetic studies showed there were genetically close relationships between PRV strains of human and bat origin implying that PRVs are bat-borne zoonoses (Chua et al., 2007). In particular, one of the PRV-infected patients had a history of close contact with bats before the onset of illness (Chua et al., 2007). This epidemiological situation supports the hypothesis that patients might obtain infection from bats; however, there has been no direct evidence to show connections between bat and human regarding PRV transmission.

Sero-epidemiological studies reported that the sera from 12 of 272 patients who visited hospitals in Vietnam and 14 of 109 healthy volunteers in Malaysia were sero-positive to PRV (Chua et al., 2007; Singh et al., 2015). Furthermore, a recent epidemiological study based on PCR detected the PRV genome from 34 of 200 patients with acute upper respiratory tract infection indicating that PRV can be considered as a common pathogen in ASEAN countries causing mild respiratory infections (Voon et al., 2015). Taken together, these reports suggest that PRV has evolved to cross the species barrier between bats and humans.

Commercially small animal models including mice and Guinea pigs are commonly utilized as primary disease models for infectious diseases. Previously, studies of the pathogenicity of PRV infection in humans have been evaluated based on the limited numbers of human infection cases owing to the lack of experimental animal models. Experimental infections of PRV were limited to a primary trial using PRV strain Nelson Bay (Gard and Marshall, 1973) inoculated to suckling mice, which were killed by intra-cranial infection. The experiment was, however, obviously dissociated from spontaneous respiratory

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infection in humans as there were no reports of PRV infection in the central nervous system. However, since the first report of a human case of PRV infection, no animal models for PRV infection reflecting human respiratory infections have been developed.

Here we report a lethal lung infection model of PRV strain Miyazaki-Bali/2007 (MB) isolated from a patient with acute respiratory infection (Yamanaka et al., 2014) in adult, immunocompetent inbred mice. We identified significant differences in the susceptibility to PRV infections between mouse strains. Intranasal infection caused lethal outcome with severe pneumonia whereas oral infections was not established, suggesting that PRV is an air-borne transmissible pathogen. In addition, mice repeatedly infected with PRV developed antibodies to particular viral proteins. Such serologic information will contribute to the development of protocols for sero-epidemiological studies and sero-diagnosis. Furthermore, an animal model for pathogenic PRV infection provides a crucial opportunity to understand virus pathogenesis and to evaluate vaccines and therapeutics to combat this important pathogen.

2. Materials and methods

2.1. Cell and viruses

Mouse fibroblast L929, lung adenocarcinoma A549, and rhesus monkey kidney MA104 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) supplemented with 5% fetal bovine serum (Gibco). The quail fibrosarcoma QT6 cell line was grown in DMEM supplemented with 10% fetal calf serum. PRV strain MB (Yamanaka et al., 2014) was amplified in L929 cells and approximately 10-fold concentrated virus stock was generated using a 10% polyethylene glycol concentration method as described elsewhere (Lewis and Metcalf, 1988). Infectious virus titer was determined by a plaque assay (Kawagishi et al., 2016). Avian reovirus strain 58–132 (Takase et al., 1985), simian rotavirus strain SA11 (Taniguchi et al., 1994), and mammalian orthoreovirus prototype strain T1L were amplified in QT6, MA104, and L929 cells, respectively.

2.2. Fluorescent-focus assay (FFA)

Viruses were inoculated into A549 cells. After absorbance at 37 °C for 1 h, culture medium was replaced with fresh medium and incubated at 37 °C. At 16 h post infection, cells were fixed with 100% ethanol and infected cells were visualized by immunostaining using antiserum against PRV strain MB σC at a dilution of 1:2000, incubated at 37 °C for 60 min, followed by Alexa Flour 488 conjugated anti-mouse IgG second antibody at a dilution of 1:3000 (Invitrogen).

2.3. Antibodies

Polyclonal rabbit anti-σC antiserum was prepared (Sigma Aldrich) against purified His-tagged PRV strain MB σC protein obtained in bacterial culture. In brief, the strain MB σC gene was inserted into the pTrcHis-A plasmid (Thermo Fisher Scientific), which enabled expression of a 6 × His tagged recombinant protein. The plasmid construct was used for transformation of *Escherichia coli* strain BL21 (Thermo Fisher Scientific). Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. The bacterial pellet was lysed by Bugbuster® (Millipore) and target proteins were purified using a His select nickel affinity gel (Sigma Aldrich) according to manufacturer instruction.

2.4. Experimental infection of animals

We purchased four-week old, male C3H/HeNCrl (C3H) mice from Charles River Laboratories and four-week old, male Jcl:ICR (ICR), Balb/cAjcl (Balb/c), and C57BL/6JJcl (C57BL) mice from Japan CLEA. Animals were inoculated intranasally or orally with 20 or 100 µl virus, respectively. To obtain the Kaplan-Meier survival curve, animals were observed up to 22 dpi. Body weight was recorded every 1–2 days. Surviving C3H mice after intranasal infection of PRV strain MB (2×10^5 PFU) were mixed and again infected intranasally with 2×10^6 PFU, up to a total 2 or 4 infections to obtain anti-sera.

To define the extent of viral replication in mouse organs, animals were sacrificed at 2, 4, and 6 days after intranasal infection. Organs were disrupted by repeated freeze-thaw cycles (2 times) followed by homogenization using a bead homogenizer (BeadSmash 12, WAKEN BTECH Co. Ltd.). Serum was separated from the whole blood by clotting at 4 °C and centrifugation at 3000 × *g*. Virus titers in organs and sera were determined by plaque assay. For pathological study, C3H mice infected intranasally with 2×10^5 PFU of PRV strain MB were sacrificed at day 4. Organs were fixed in 10% buffered formaldehyde and processed for pathological examination including HE staining and IHC analysis using σC-specific antiserum.

The study was approved by the Animal Research Committee of the Research Institute for Microbial Diseases, Osaka University. The experiment was conducted following the guidelines for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

2.5. Cytokine detection

Inflammatory cytokine IP-10 levels in blood were examined using a Quantikine ELISA kit (R&D Systems) according to manufacturer instruction.

2.6. Viral neutralization (NT) assay

Neutralizing antibody titers of the C3H sera were examined by the neutralization assay. In brief, serially diluted sera (1:20 to 1:5120) in DMEM)) were prepared in a 96-well microplate. An equal volume of virus fluid (50–100 FFU/25 µl) was added and incubated at 37 °C for 60 min in a 5% CO_2 incubator. As a positive control, viruses were mixed with an equal volume of DMEM. After incubation, 50 µl neutralized viruses were inoculated onto a monolayer of A549 in a 96-well microplate. After incubation for 16 h, cells were fixed with absolute ethanol and cells expressing viral antigens were detected using murine anti-PRV strain MB σC antibody (Kawagishi et al., 2016) and goat anti-mouse IgG antibody Alexa488 conjugate. The NT titer was indicated by the reciprocal value of the maximum dilution at which the number of foci showed 50% reduction compared with the positive control.

NT assays against MRV, RV, and ARV were performed similarly although virus titration was measured by plaque assay. Briefly, MRV, RV, and ARV incubated with serially diluted sera were inoculated to L929, MA104, or QT6 cells, respectively, and overlaid with agarose gel. Plaque numbers were compared to control samples and the neutralization titer of 50% reduction was calculated. NT titers were expressed as the mean (95% confidence interval (CI)). 95% CI was calculated according to the following formula:

$$95\%\mathrm{CI} = \mathrm{means} \pm 1.96 \times (\mathrm{SD}/\sqrt{n})$$

where SD is standard deviation and n is sample number.

2.7. Plasmid construction

For immunological assay, expression plasmid vectors for N-terminal FLAG-tagged PRV proteins were constructed. Genes encoding each viral protein, including λA, λB, λC, µA, µB, µNS, σA, σB, σC, σNS, p17, and p10, were amplified by RT-PCR using specific primers based on sequences deposited in the GenBank (accession numbers: AB908278 to AB908287). Amplified PCR products fused to the FLAG epitope tag at the N-terminus were cloned into the pEF plasmid vector (Mizushima and Nagata, 1990). All plasmids were confirmed by DNA sequencing. Primer sequences used for plasmid construction are available upon