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A pharmacologically immunosuppressed mouse model for assessing influenza B virus pathogenicity and oseltamivir treatment

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ABSTRACT

Immunocompromised patients are highly susceptible to influenza virus infections. Although neuraminidase inhibitor (NAI) therapy has proved effective in these patients, the treatment regimens require optimization, which can be partly addressed via animal models. Here, we describe a pharmacologically immunosuppressed mouse model for studying the pathogenesis of influenza B viruses and evaluating the efficacy of antiviral treatment. We modeled clinical regimens for dexamethasone and cyclophosphamide to immunosuppress BALB/c mice that were then inoculated with B/Phuket/3073/2013 (Yamagata lineage) or B/Brisbane/60/2008 (BR/08, Victoria lineage) virus. Although both viruses caused morbidity and mortality in immunosuppressed mice, BR/08 was more virulent, consistently inducing greater morbidity and 100% lethality in mice inoculated with at least 10^3 $TCID_{50}$/mouse. The replication of both viruses was prolonged in the lungs of immunosuppressed mice, but the extent of pulmonary inflammation in these mice was markedly less than that in immunocompetent animals. Most of the examined cytokines, including IFN-γ, IL-1β, and RANTES, were significantly decreased in the lungs of immunosuppressed mice, as compared to immunocompetent animals, until at least 10 days post-infection. Treatment with the NAI oseltamivir for 8 or 16 days increased the mean survival time and reduced virus spread in the lungs of immunosuppressed mice challenged with a lethal dose of BR/08 but did not completely provide protection or decrease the virus titers. Our data suggests that the synergy of the viral load and aberrant immune responses is a key contributor to the severity of infection, as well as the limited efficacy of oseltamivir, which in immunosuppressed mice curtails virus release without clearing infected cells.

1. Introduction

Influenza B viruses cause significant disease burden during annual epidemics but remain less studied than influenza A viruses (Paul Glezen et al., 2013; Tafalla et al., 2016). Laboratory-confirmed influenza B viruses account for approximately 22% of influenza cases in the United States on average, but this proportion is highly variable depending on the season (Barr and Jelley, 2012; Blanton et al., 2017; Shang et al., 2016; WHO, 2017). Influenza B virus infections are clinically indistinguishable from influenza A virus infections, but the associated complications may differ (Koutsakos et al., 2016) and may include encephalitis, encephalopathy, gastrointestinal symptoms, or myositis (Burnham et al., 2013; Paddock et al., 2012). Influenza B viruses can be prevalent in children with cancer or other immunosuppressive conditions and can contribute to their deaths (Carr et al., 2011; CDC, 2017b; McCullers and Hayden, 2012; Peltola et al., 2003; Silvennoinen et al., 2011).

Influenza A and B viruses cause prolonged viral shedding and considerable morbidity and mortality in immunocompromised patients (Gooskens et al., 2009; Ljungman et al., 1993; Prasad and Spradbrow, 1977; Whimbey and Bodey, 1992), in whom progression of infection to the lower respiratory tract may induce severe disease. Influenza vaccines are often poorly immunogenic and unlikely to be fully protective in immunocompromised patients (Yousuf et al., 1997). Therefore, neuraminidase (NA) inhibitors (NAIs), the only class of drugs available for influenza prophylaxis and therapy, are particularly important in these patients, although there is a high risk of NAI resistance developing (Carr et al., 2011; Gubareva et al., 1998; van der Vries et al., 2013). Antiviral therapy reportedly improves clinical outcomes in immunocompromised patients (Boudreault et al., 2011; Lee and Ison, 2012), but current strategies require optimization (Ison, 2015; Whitley and Monto, 2006).

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Preclinical studies in animal models can provide valuable information for optimizing treatment protocols for immunocompromised patients. There are multiple methods for modeling immunosuppression in mice (Bosma and Carroll, 1991; Mostafa et al., 2016; Wahlberg et al., 1992). One approach that mimics the treatment of hematologic malignant neoplasms involves using cyclophosphamide (CP) and dexamethasone (DEX), which are widely used clinically as monotherapies or in combination (Kaufman et al., 2009; Michallet et al., 2011). CP and DEX have different mechanisms of action and complementary immunosuppressive effects. CP is an alkylating agent, has myelosuppressive activity, and interferes with DNA replication, leading to apoptosis (Colvin, 2003). CP is used to treat aplastic anemia (Brodsky et al., 1996) and to prevent graft-versus-host disease in recipients of allogeneic hematopoietic stem cell transplants (Luznik et al., 2010) and rejection of organ transplants (Cooksley et al., 2005). DEX is a potent corticosteroid that causes apoptosis of lymphocytes (Estlin et al., 2000) and is given as pulse therapy for acute lymphoblastic leukemia, lymphoma, Hodgkin disease (Pui, 2006), and other inflammatory diseases (Li et al., 2017).

Data on influenza B viruses in immunosuppressed models is lacking. Here, we used pharmacologically immunosuppressed mice to characterize two influenza B viruses representing the Victoria and Yamagata lineages. We then evaluated the NAI oseltamivir in this model system, focusing on prolonged treatment.

2. Materials and methods

2.1. Viruses, cells, and compound

The human influenza B/Phuket/3073/2013 (PH/13) and B/Brisbane/60/2008 (BR/08) viruses (unadapted for replication in mice) were obtained from the Centers for Disease Control and Prevention, grown in 10-day-old embryonated chicken eggs for 72 h at 33 °C, and the 50% tissue culture infectious dose ($TCID_{50}$/mL) was determined in Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA). Oseltamivir phosphate (oseltamivir) [ethyl (3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] was dissolved in distilled water.

2.2. Immunosuppressive mouse model

Animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital (St. Jude) in compliance with National Institutes of Health guidelines and the Animal Welfare Act. Female 6-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were treated with DEX + CP (Sigma-Aldrich, St. Louis, MO). DEX (10 mg/kg/day in 0.2 mL) was administered to mice daily for 10 days intraperitoneally (ip), starting 1 day before inoculation with influenza B virus (−1 day post-infection [dpi]). CP (150 mg/kg/day in 0.3 mL) was administered ip as two doses at −1 and 5 dpi (Mostafa et al., 2016). The timing of virus infection in relation to immunosuppression was chosen to extend window of virus spread and clearance.

2.3. Pathogenicity of influenza B viruses in mice

Mice were anesthetized with isoflurane and inoculated intranasally with influenza B virus (10^2–10^5 $TCID_{50}$/mouse in 30 μL). Mice (n = 5/group/virus dose) were monitored daily for disease signs, weight loss, and survival (weight loss exceeding 25% was the endpoint for mortality). At 3, 6, and 10 dpi, lungs and nasal turbinates were collected from additional animals (n = 3/time point), and virus titers were determined by $TCID_{50}$ assay in MDCK cells.

2.4. Differential leukocyte counts and flow cytometry

Mice (n = 5/group) were anesthetized, and blood samples were collected in 10% EDTA (disodium) by retro-orbital bleeding at −1, 3, 6, 10, 13, 17, and 24 dpi. Cells were enumerated and analyzed for differential leukocyte counts with a FORCYTE hematology analyzer (Oxford Science, Oxford, CT). For flow cytometry analysis of blood, cells were blocked with mouse BD Fc block (BD Biosciences, Franklin Lakes, NJ) in staining medium (0.5% FBS in PBS) for 10 min at 4 °C, stained with CD3-APC, CD4-FITC, CD8-PE, CD19-PE-Cy7, and CD49b-APC-Cy7 antibodies (BD Biosciences) for 30 min at 4 °C. Cellular populations were gated and quantified by FlowJo software (v. 7.6.1).

2.5. Cytokine and chemokine analysis

At 3, 6, and 10 dpi, the concentrations of each of 25 cytokines and chemokines were measured in lung homogenates (n = 3/group) with a MYCTOMAG-70K-PMX MILLIPLEX® MAP mouse cytokine/chemokine panel (Millipore). The plates were read on a Luminex 100/200 analyzer using the xPonent software.

2.6. Lung histopathology and immunohistochemistry

Lungs were collected at 6 or 16 dpi (n = 2/group), infused with 10% neutral-buffered formalin (Thermo Scientific), and stained with hematoxylin and eosin or subjected to immunohistochemical staining with anti-HA goat antiserum (B/Florida/04/2006, Yamagata lineage). The extent of virus infection and lung lesions was quantified as the percentage of the total alveolar lung field with active or inactive infection by using Aperio ImageScope software (Marathe et al., 2016).

2.7. Oseltamivir efficacy

Mice were anesthetized and inoculated intranasally with 5 MLD_{50}/30 μL/mouse of BR/08 (2.5×10^5 $TCID_{50}$). Oseltamivir treatment (20 mg/kg/day by twice-daily oral gavage) was initiated 1 h after inoculation with BR/08 and continued for 8 (immunocompetent) or for 8 or 16 days (immunosuppressed). Control (inoculated, untreated) mice received sterile water. Mice (n = 5/group) were observed daily for disease signs, weight loss, and survival (weight loss of > 25%). Additional groups of control and oseltamivir-treated mice (n = 3/group) were euthanized at 6 and 10 dpi, and virus titers were determined by $TCID_{50}$ assay in MDCK cells.

2.8. Sequence analysis

Viral RNA was isolated from mouse lung homogenates at 6 and 9 dpi with an RNeasy Mini Kit (Qiagen). Samples were reverse transcribed and analyzed by PCR using primers specific for the NA gene (Tewawong et al., 2016). PCR products were purified with a QIAquick PCR Purification Kit (Qiagen). Sanger sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude. DNA sequences were completed and edited with the Lasergene sequence analysis software (DNASTAR, Madison, WI).

2.9. Serologic assay

Sera were collected by retro-orbital bleeding at 22 dpi, treated with receptor-destroying enzyme (Denko-Seiken, San Jose, CA), heat-inactivated at 56 °C for 1 h, and tested by hemagglutination inhibition (HI) assay with 0.5% turkey red blood cells (Rockland Immunochemicals).

2.10. Statistical analysis

Virus titers, cell counts, and cytokine/chemokine levels were compared by one-way ANOVA with Bonferroni's post-test (GraphPad Prism 5.0 software). The Kaplan-Meier method was used to estimate the probability of survival, and the log-rank test was used to compare