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Rapid, non-invasive imaging of alphaviral brain infection: Reducing animal numbers and morbidity to identify efficacy of potential vaccines and antivirals

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ABSTRACT

Rapid and accurate identification of disease progression are key factors in testing novel vaccines and antivirals against encephalitic alphaviruses. Typical efficacy studies utilize a large number of animals and severe morbidity or mortality as an endpoint. New technologies provide a means to reduce and refine the animal use as proposed in Hume's 3Rs (replacement, reduction, refinement) described by Russel and Burch. *In vivo* imaging systems (IVIS) and bioluminescent enzyme technologies accomplish the reduction of animal requirements while shortening the experimental time and improving the accuracy in localizing active virus replication. In the case of murine models of viral encephalitis in which central nervous system (CNS) viral invasion occurs rapidly but the disease development is relatively slow, we visualized the initial brain infection and enhance the data collection process required for efficacy studies on antivirals or vaccines that are aimed at preventing brain infection. Accordingly, we infected mice through intranasal inoculation with the genetically modified pathogen, Venezuelan equine encephalitis, which expresses a luciferase gene. In this study, we were able to identify the invasion of the CNS at least 3 days before any clinical signs of disease, allowing for reduction of animal morbidity providing a humane means of disease and vaccine research while obtaining scientific data accurately and more rapidly. Based on our data from the imaging model, we confirmed the usefulness of this technology in preclinical research by demonstrating the efficacy of Ampligen, a TLR-3 agonist, in preventing CNS invasion.

1. Introduction

Quick and accurate identification of disease progression are key factors in testing novel vaccines and antivirals. Preclinical efficacy and viral pathogenesis studies utilize a large number of animals [1–3]. These studies involve distinct endpoints, primarily mortality or largely decreased body weight to identify severe illness that is often lethal [3]. The large numbers are needed to statistically confirm viral replication sites, time points of organ infection, and overall timeline for disease progression. During many studies for viral pathogens the animals develop illnesses in which anorexia and hyperthermia or hypothermia followed by paralysis are a few of the morbidity indicators [1]. These viral studies also pose increased risk to researchers due to increasing disease development in the animals. High virus titers in infected animals, irritable animals, and organ collection all pose potentially higher risks for researchers when working with animals. New methodologies and technologies can be utilized to reduce animal numbers, animal morbidity, experimental time, and researcher exposure risk while improving our capability to localize virus replication to specific organs.

In vivo imaging systems (IVIS) generates these desirable experimental qualities utilizing either fluorescent proteins [4] or bioluminescent enzymes [5] to visualize the signal. Utilizing bioluminescent enzymes, such as firefly luciferase, provides many benefits compared to fluorescent proteins, including lower background signal in animals while providing sufficient spread within an animal for visual identification [6]. Technological developments have greatly increased the ability to detect minute levels of emitted light assisted with real-time detection of the reactive vector in living animals [7]. Previously published studies have presented the effectiveness of IVIS in studying multiple viral pathogens and their progression in animal models [8–11].

We propose the utilization of IVIS to rapidly visualize the penetration of the CNS by Venezuelan equine encephalitis virus (VEEV) in the murine model. This is an alphavirus known to cause periodic,

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large outbreaks in both humans and equines. Humans can develop severe acute, encephalitic disease followed by muscle weakness with an increased mortality rate in pediatric cases [12]. The attenuated IND vaccine strain of VEEV, TC83, was passaged 83 times and has several mutations [13,14], however, TC83 replicates to very high titers in the brains of several murine species while causing high mortality in some [2,15] especially following intranasal inoculation. Previously, the use of TC83 as a model for VEEV encephalitis has been proposed as a means to study possible antivirals against VEEV [16,17].

Our goal was to further develop this murine model for the usage in the preclinical setting for 3 equally important reasons: (1) to minimize the regulatory burden that relates to biosafety level 3 work and select agent regulations required for the experiments with wild type VEEV; (2) to shorten the experimental time needed to accurately demonstrate the brain infection as an experimental endpoint; and (3) to reduce the animal numbers and suffering that relate to studies of encephalitic diseases.

In this study, we were able to identify the invasion of the CNS by VEEV at least 3 days before any clinical signs of disease, which allows for reduction of animal morbidity providing a humane means of disease and vaccine and/or antiviral research while obtaining scientific data accurately and more rapidly. In addition to identification of viral replication we were able to accurately identify the movement of viral replication from the nasal region to the fore region of the CNS through 3-dimensional IVIS. To demonstrate the usefulness of this technology in preclinical research, we tested the efficacy of Ampligen, a TLR-3 agonist [1], in preventing CNS invasion.

2. Materials and methods

2.1. Cells and viruses

Baby hamster kidney (BHK-21) and Vero cells (American Tissue Culture Collection, Manassas, VA) were maintained in minimal essential medium (MEM) supplemented with 10% FBS, L-glutamine and vitamins.

VEEV TC83 viral stock was produced in Vero E6 cells and stored at −80 °C in 1-mL aliquots until use. All work with infectious virus was performed at UTMB Bio-Safety Level 2 (BSL-2) in accordance with institutional health and safety guidelines.

2.2. Construction of VEE recombinant virus

DNA work was accomplished using standardized cloning techniques [18] with commercially available enzymes. The competent *Escherichia coli* strain of cells DH5α (Invitrogen) was used for all cloning and maintenance of the recombinant constructs. Platinum *Pfx* and *Taq* (Invitrogen) polymerases were utilized for all polymerase chain reactions (PCR). Sequencing of all cDNA fragments and plasmids was completed at the UTMB sequencing core facility.

The TC83 VEEV vector, two helper plasmids, and the firefly luciferase vector (provided by Dr. Frolov, University of Alabama) used to package TC83 into infectious virions, are all described elsewhere [19,20].

2.3. Animals

Six to eight week female ICR mice were purchased from Charles River (Wilmington, MA) and housed in the Galveston National Laboratory ABSL-2 facility. All animals were given a minimum of 2 days to become acclimated to the environment before any study manipulations. All animal studies were approved by the UTMB Institutional Animal Care and Use committee (IACUC) and carried out according to NIH guidelines.

2.4. Telemetry

All animals were implanted subcutaneously with a BMDS IPTT-300 transponder (chip) purchased from Bio Medic Data Systems, Inc., with a trocar needle assembly. The animals were monitored for signs of infection or migration of the transponder for at least 24 h prior to further progression into the study. All chips were scanned using the DAS-6007 transponder reader (Bio Medic Data Systems, Inc.) and temperature data downloading was performed in accordance with the manufacturer's protocol.

2.5. In vivo imaging

ICR mice were used for all imaging experiments. Mice were shaved prior to inoculation to maximize detection of the bioluminescent signal. All mice were inoculated through intranasal exposure of 4×10^6–1×10^7 pfu in 40 μL volumes. Prior to imaging mice were given luciferin through either intraperitoneal injection (10 μL/g body weight of a solution containing 15 mg/mL Luciferin) or intranasal inoculation (10–20 μL/nare of a solution of 3 mg/mL of Luciferin). *In vivo* images were acquired with the IVIS charge-coupled-device camera system and analyzed with the LivingImage 3.0 and 4.0 software package. Exposure times used were 1–5 s per image. Three dimensional imaging was accomplished utilizing the LivingImage 4.0 software package. Default wavelength and auto exposure detection software defaults were selected through the Wizard bioluminescent selection tool in which 5 still images were completed at increasing wavelength filters (560, 580, 600, 620, 640 nm). Surface topography was generated automatically through the software with minimal user changes and final DLIT reconstruction and organ fitting was accomplished utilizing a non-linear transformation.

2.6. Infectious virus titration in organs

To quantify VEEV replication within the CNS, specimens were dissected at necropsy and homogenized in MEM containing 1% penicillin-streptomycin solution (50,000 units of penicillin and 50,000 μg of Streptomycin in 500 mL media volumes). Suspensions were clarified by centrifugation and the supernatants were harvested and frozen at −80 °C until analysis was performed. The titer of infectious virus was determined using a plaque assay in Vero cells.

3. Results

3.1. Rescue of TC83-Luciferase

To generate the engineered TC83-Luciferase virus, we utilized a previously designed VEEV rescue system [19] based upon the recombinant TC83 vaccine strain [21]. A firefly luciferase gene was cloned into the TC83 cDNA plasmid directly downstream of the sub-genomic promoter (Fig. 1). The recombinant virus was rescued in Vero cells at a comparable titer to wild type TC83. Plaque size analysis showed some differences from wild type TC83 virus [19]. Luminometer detection confirmed luciferase activity *in vitro* following virus infection and replication in Vero cells. Visualization of bioluminescent signal from cells infected with TC83-Luciferase confirmed our successful rescue of an infectious recombinant TC83 virus which we can further utilize for *in vivo* studies.