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Oropouche virus experimental infection in the golden hamster (*Mesocrisetus auratus*)

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ABSTRACT

Oropouche virus (OROV), of the family *Bunyaviridae*, is the second most frequent arbovirus causing febrile disease in Brazil. In spite of this, little is known about pathogenesis of OROV infection. This report describes an experimental model of OROV in golden hamster (*Mesocricetus auratus*). Following subcutaneous inoculation of OROV, over 50% of the animals developed disease characterized by lethargy, ruffled fur, shivering, paralysis, and approximately one third died. Animals were sacrificed on days 1, 3, 5, 8 and 11 post-inoculation to collect tissue samples from brain, heart, liver, lung, spleen, muscle and blood for virus titration, histology and OROV immunohistochemistry. OROV was detected in high titers in blood, liver and brain, but not in the other organs. Histopathology revealed meningoencephalitis and hepatitis, with abundant OROV antigen detected in liver and brain. Diffuse galectin-3 immunostaining in brain and liver supports microglial and Kupfer cells activation. This is the first description of an experimental model for OROV infection and should be helpful to study pathogenesis and possibly to test antiviral interventions such as drugs and vaccine candidates.

1. Introduction

Oropouche virus (OROV) is an emerging zoonotic pathogen of the family *Bunyaviridae*, genus *Orthobunyavirus*, serogroup Simbu (Schmaljohn and Nichol, 2007). Viruses of the family *Bunyaviridae* have three circular nucleocapsids containing negative strand RNA and phylogenetic analysis of different OROV strains based on gene N nucleotide sequence identified three distinct genotypes (I–III) (Saeed et al., 2000). OROV enters host cells after binding to an as yet unidentified receptor, by endocytosis in clathrin-coated vesicles, and replicates in the cytoplasm (Santos et al., 2008; Schmaljohn and Nichol, 2007).

OROV is the second most frequent cause of human arboviral infection in Brazil, where over 350,000 cases of Oropouche fever have been recorded in the Amazon region (Pinheiro et al., 2004; Vasconcelos et al., 2009). In addition to the reported outbreaks, sporadic cases of OROV infection have also been published (Bernardes-Terzian et al., 2009). Undiagnosed Oropouche fever is probably frequent, especially in a region afflicted by other potentially confounding febrile tropical diseases, such as malaria and dengue, and due to poor notification the actual number of cases of OROV infection in the vastness of the Amazon basin (Pinheiro et al., 1994) is probably grossly underestimated. OROV has been detected in wild animals, mainly sloths (*Bradypus tridactylus*), and more recently was isolated from a marmoset (*Callithrix* sp.) (Nunes et al., 2005). In urban areas OROV causes outbreaks of febrile illness, transmitted by the biting midge *Culicoides paraensis*, or by other potential vectors (Pinheiro et al., 1981). ORO fever has been registered almost exclusively in the Amazon, but global warming, deforestation and redistribution of vectors and reservoirs may increase the potential of OROV to spread to other areas of the continent (Azevedo et al., 2007; Bernardes-Terzian et al., 2009; Nunes et al., 2005; Vasconcelos et al., 2001, 2009).

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OROV infection is clinically manifested by dengue-like febrile illness, with myalgia, headache, arthralgia, skin rash and malaise, which may be long-lasting and sometimes relapsing (Pinheiro et al., 1981). Patients with ORO fever usually recover after 2–3 weeks of disease without known sequelae or recorded mortality (LeDuc and Pinheiro, 1989), and autopsy data are unavailable. Therefore, despite its frequency and public health significance, little is known about pathogenesis of OROV infection. The scanty information available indicates that OROV infection is associated with viremia that declines quickly until the fifth or sixth days of illness (Pinheiro et al., 1981), and that the virus has been recovered from the *cerebral spinal fluid* (CSF) in association with clinical meningitis (Pinheiro et al., 1982).

It has been known that suckling mice and hamsters are susceptible to OROV by intracerebral (IC) inoculation (Pinheiro et al., 2004). However, to the best of our knowledge, there have been no studies using inoculation of experimental animals by subcutaneous or intradermic routes, which would more closely resemble the natural means of OROV infection. Besides helping the understanding of OROV pathogenesis, such an experimental model may turn useful for testing candidate antiviral drugs and vaccines. Hamsters have been used as experimental animals for a number of viruses, including arboviruses (Fisher et al., 2003; Sbrana et al., 2006; Tesh et al., 2001; Xiao et al., 2001a,b), but have not been adequately studied as experimental animals for OROV. An early fragmentary report restricted to the analysis of liver injury described fatally severe hepatitis with necrosis of hepatocytes and Kupffer cell hyperplasia in hamsters following intracerebral OROV inoculation (Araujo et al., 1978). Surprisingly, OROV was not detected in the animal tissues, nor were sites of viral replication determined (Araujo et al., 1978). In this study we show that hamsters inoculated subcutaneously with OROV develop a systemic infection, with remarkable neurologic manifestations and abundant viral accumulation in liver and brain.

2. Materials and methods

2.1. Virus

The strain BeAn 19991 of OROV was kindly provided by Luiz Tadeu M. Figueiredo (University of São Paulo School of Medicine, Ribeirão Preto, SP, Brazil). OROV stock was obtained after five intracerebral passages in Swiss newborn mice sacrificed 2–3 days post-inoculation. Brains were suctioned, pooled, suspended in minimal essential medium (MEM) with 3% heat-inactivated fetal bovine serum (FBS) and frozen at −70 °C. Later the pool was thawed, cleared by centrifugation at 5445 × *g* at 4 °C for 10 min, filtered through 0.22 μm syringe-mounted filters and titrated by detection of cytopathic effect (CPE) in 10-fold serial dilutions inoculated in quadruplicate monolayers of Vero cell cultures. A suspension of brains obtained from noninfected animals was prepared in the same way and inoculated in the same cultures to control for toxicity. The OROV titer obtained was $10^{7.25}$ $TCID_{50}$/mL (50% tissue culture infectious dose per mL).

2.2. Animal model

Three-week-old Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Central Animal Facility of the University of São Paulo School of Medicine, Ribeirão Preto, Brazil and kept in accordance with the guidelines of the University of São Paulo Ethics Committee for Animal Experimental Research. To determine the median lethal dose (LD_{50}) four groups of 13 hamsters were inoculated subcutaneously in the anterior surface of the right thigh with 100 μL of serial 10-fold dilutions of OROV starting from $10^{7.25}$ $TCID_{50}$/100 μL down to $10^{4.25}$ $TCID_{50}$/100 μL. Animals were observed twice daily for 4 weeks and the LD_{50} (Reed and Muench, 1938) was determined to be equivalent to $10^{5.6}$ $TCID_{50}$. Subsequently, 18 more animals were inoculated subcutaneously with 4 LD_{50} of OROV in 100 μL and three sham-inoculated control animals received equivalent volume of brain suspension obtained from non infected swiss suckling mice, processed in the same way. The animals were examined twice daily for clinical and behavioral abnormalities, with measurement of weight and rectal temperature. Next 47 animals were inoculated with 4 LD_{50} of OROV, 11 sham-inoculated control animals received equivalent volume of brain suspension obtained from non infected swiss suckling mice and on days 1, 3, 5 and 8 post-infection they were anesthetized with 0.2 mL of 50 mg/mL ketamine and 0.02 mL of 50 mg/mL xylazine intramuscular (IM), exsanguinated by cardiac needle puncture, dissected under sterile conditions to obtain tissue samples for analysis. Anticoagulated blood was cleared by centrifugation and the plasma was filtered and frozen for later virus titration. Tissue samples from brain, liver, spleen, kidney, heart, lung and skeletal muscle of the left hind limb were obtained and split in two halves: one was weighed, manually homogeneized in 1 mL of MEM with 3% FBS and stored at −70 °C for later virus titration; and the other was fixed in 3.7% neutral buffered formalin for 20 h, dehydrated by immersion in a series of ethanol dilutions in water (80%, 90%, 95%) for 2 h each, then in absolute ethanol overnight, and finally embedded in paraffin. For virus titration, samples were thawed, cleared by centrifugation at 5445 × *g* at 4 °C for 10 min and filtered through 0.22 μm syringe-mounted filters. Virus titers were determined at three independent times for each tissue sample by inoculation of 10-fold serial dilutions of plasma or tissue homogenates onto quadruplicate monolayers of Vero cells grown in 96-well plates. The presence of CPE was evaluated on the third day of incubation at 37 °C in 5% CO_2, and titers were expressed in $TCID_{50}$/mL (Reed and Muench, 1938). The CPE observed at the end-point dilution was always confirmed by indirect immunofluorescence (IF) carried out on scraped cells spotted on glass slides fixed with cold acetone for 10 min and dried at room temperature. For IF, slides were incubated with anti-OROV polyclonal mouse antiserum (a gift from Luiz Tadeu M. Figueiredo, FMRP-USP, Ribeirao Preto, Brazil) diluted 1:100 in phosphate-buffered saline (PBS) pH 7.4 (1×) (Gibco, Los Angeles, CA), then washed in PBS for 5 min and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Chemicon, Temecula, CA) diluted 1:50 in PBS with 0.05% Evans blue (Gibco, Los Angeles, CA).

2.3. Histopathology and immunohistochemistry

Five-micrometer thick formalin-fixed, paraffin-embedded tissue sections were stained by hematoxylin–eosin (H&E) and immunohistochemistry. The immunohistochemical stains were performed using the avidin–biotin–peroxidase technique by an automated staining process (Ventana Nexes IHC stainer, Tucson, AZ, USA). The following antibodies were applied: anti-OROV mouse polyclonal antibody (diluted 1:100), or anti-galectin-3 (M3/38 hybridoma, ATCC TIB 166), diluted 1:100. Negative controls were run concomitantly by omission of primary antibody in all analyses. Slides were counterstained with Harris hematoxylin and mounted under coverslip with Entellan (Merck, Darmstadt, Germany).

3. Results

3.1. Clinical findings

In the observational experiment, 10 of 18 (55%) hamsters inoculated with 4 LD_{50} of OROV developed illness three or more