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Remdesivir (GS-5734) protects African green monkeys from Nipah virus challenge

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Abstract

Nipah virus is an emerging pathogen in the *Paramyxoviridae* family. Upon transmission of Nipah virus from its natural reservoir, *Pteropus* spp. fruit bats, to humans, it causes respiratory and neurological disease with a case-fatality rate about 70%. Human-to-human transmission has been observed during Nipah virus outbreaks in Bangladesh and India. A therapeutic treatment for Nipah virus disease is urgently needed. Here, we tested the efficacy of remdesivir (GS-5734), a broad-acting antiviral nucleotide prodrug, against Nipah virus Bangladesh genotype in African green monkeys. Animals were inoculated with a lethal dose of Nipah virus, and a once-daily intravenous remdesivir treatment was initiated 24 hours later and continued for 12 days. Mild respiratory signs were observed in two of four treated animals, whereas all control animals developed severe respiratory disease signs. In contrast to control animals, which all succumbed to the infection, all remsdesivir-treated animals survived the lethal challenge, indicating that remdesivir represents a promising antiviral treatment for Nipah virus infection.

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SUPPLEMENTARY MATERIALS

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Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials.

INTRODUCTION

Nipah virus causes severe and often fatal respiratory and neurological disease in humans (1). The recent Nipah virus outbreak in Kerala, India, involving 23 cases and 21 deaths (2), again stressed the urgent need to develop prophylactic and therapeutic countermeasures against Nipah virus. The World Health Organization has listed Nipah virus as an emerging pathogen likely to cause major epidemics or even pandemics (3). Very few antivirals have demonstrated efficacy in animal models of Nipah virus disease. Favipiravir protected hamsters from lethal Nipah virus Malaysia infection when treatment was administered orally or subcutaneously immediately after inoculation and continued for 13 days (4). Fusion inhibitory lipopeptides were shown to reduce mortality by 33% in the African green monkey (AGM) model of Nipah virus disease when administered daily from 1 day before until 5 days after inoculation with Nipah virus Malaysia (5). To date, only monoclonal antibody m102.4 treatment has shown efficacy in nonhuman primates when administered therapeutically (6, 7). Remdesivir (GS-5734) is a nucleotide analog prodrug with broad-spectrum antiviral activity that was shown to inhibit filovirus, coronavirus, and paramyxovirus replication (8, 9). It is currently in a phase 2 clinical trial for treatment of male Ebola virus disease survivors with persistent viral RNA in semen and is being evaluated in a randomized controlled trial in the ongoing Ebola virus outbreak in the Democratic Republic of the Congo (10). In vitro, remdesivir showed potent antiviral activity against both Malaysian and Bangladesh genotypes of Nipah virus and reduced replication of Nipah virus Malaysia in primary human lung microvascular endothelial cells by more than four orders of magnitude (8), warranting further testing of the efficacy of remdesivir against Nipah virus infection in vivo. Because of the poor stability of remdesivir in rodents, the therapeutic efficacy was tested in the AGM model of lethal Nipah virus Bangladesh challenge.

RESULTS

Remdesivir treatment protects AGM from lethal Nipah virus Bangladesh disease

Two groups of four AGMs were inoculated intranasally and intratracheally with a lethal dose of Nipah virus Bangladesh. Twenty-four hours later, one group of animals was treated intravenously with remdesivir, and the other group was administered with vehicle alone. All vehicle-treated animals developed disease signs including mild respiratory signs starting about 3 to 4 days post-inoculation (dpi); this disease rapidly progressed to respiratory distress on 7 and 8 dpi (Fig. 1A). Two vehicle-treated animals were euthanized because of disease severity on 7 dpi, and the remaining two vehicle-treated animals reached humane endpoint criteria on 8 dpi (Fig. 1B). The animals treated with remdesivir all had reduced appetites starting on the day of inoculation and lasting until 12 dpi, which may have been due to the daily administration of anesthesia; two of four animals developed mild respiratory signs (abdominal breathing) that resolved 12 to 14 dpi. Clinical scores in the remdesivir-treated animals had all returned to baseline by 21 dpi, and all animals survived without further clinical signs until the end of the experiment on 92 dpi (Fig. 1, A and B). Clinical examinations were performed at several time points throughout the study, during which clinical parameters such as body weight and temperature, respiration rate, and oxygen

saturation were measured, and blood and swab samples were collected. No changes were observed in body weight or temperature during the study in vehicle-treated controls and remdesivir-treated animals. Respiration rate was increased (Fig. 1C), and oxygen saturation was decreased from baseline in vehicle-treated animals at the time of euthanasia (Fig. 1D) but not in remdesivir-treated animals throughout the study.

Remdesivir treatment prevents viremia but not local virus replication

Viral loads and infectious virus titers were determined in nose and throat swabs collected during examinations. Despite the difference in clinical disease severity in remdesivir-treated animals versus vehicle-treated controls, amounts of virus shedding from the nose and throat were similar in both groups (Fig. 2A). However, all vehicle-treated animals became viremic after inoculation, whereas only one animal in the remdesivir-treated group was transiently positive for viral RNA in the blood by quantitative reverse transcription polymerase chain reaction (qRT-PCR) at 28 dpi. All other animals in the remdesivir-treated group remained negative throughout the experiment, and infectious virus could only be detected in the blood of vehicle-treated animals (Fig. 2A). Sera collected at the time of clinical examinations were used to determine immunoglobulin M (IgM) and IgG antibody titers against Nipah virus by enzyme-linked immunosorbent assay (ELISA). IgM against Nipah virus could be detected in all vehicle-treated animals at the time of euthanasia on 7 and 8 dpi; in remdesivir-treated animals, IgM was first detected on 8 dpi and plateaued about 2 weeks after inoculation (Fig. 2B). Nipah virus-specific IgG could not be detected in the vehicle-treated animals, but IgG was first detected in one remdesivir-treated animal by 11 dpi and the three remaining animals had sero-converted by 15 dpi (Fig. 2B).

At the time of euthanasia, 35 tissues were collected from each animal for virological analysis. At the time of necropsy on 7 and 8 dpi, typical viral loads in tissues collected from vehicle-treated control animals were high (Fig. 2C). Because late-onset encephalitis has been observed in survivors of Nipah virus infection (11-14), the surviving remdesivir-treated animals were monitored until 92 dpi. In these animals, viral RNA was detected in 11 tissues from three animals, with different tissues testing positive in individual animals (Fig. 2C). Although viral RNA was detected in brain tissue of one remdesivir-treated animal at 92 dpi, infectious virus could not be detected, and none of the animals showed disease signs after recovering from the initial virus challenge between 2 and 3 weeks after inoculation until the end of the study.

Histologic evidence of focal meningoencephalitis detected in one of four remdesivir-treated animals

Histologic evaluation of the brains of three remdesivir-treated animals only showed minimal evidence of focal perivascular inflammation, and no Nipah virus antigen was detected by immunohistochemistry (IHC). In the animal that had detectable Nipah virus RNA in the brain, focal, mild to moderate mononuclear meningoencephalitis was observed (Fig. 3, A and B). No clear vasculitis, viral inclusions, or syncytia formation was associated with inflammation. IHC showed Nipah virus positive labeling in neurons and granular staining within the parenchyma and rare inflammatory cells in areas of inflammation and neuronal necrosis (Fig. 3, C and D). Only rare inflammatory foci contained abundant Nipah virus

labeling (fig. S1). The animal with mild meningoencephalitis did not show respiratory signs after Nipah virus Bangladesh inoculation nor was it the animal that showed transiently positive blood in qRT-PCR on 28 dpi.

Virus neutralizing titers in serum may indicate persistent Nipah virus infection in the CNS

Virus neutralizing titers were determined in sera collected from remdesivir-treated animals at several time points after inoculation with Nipah virus Bangladesh. Low neutralizing antibody titers were detected in all four animals by 19 dpi (fig. S2). Neutralizing titers in the three animals without evidence of virus infection of the central nervous system (CNS) remained low throughout the experiment and had started to decline by the end of the experiment at 92 dpi. In the single animal with histological evidence of meningoencephalitis, neutralizing titers increased throughout the duration of the experiment and were 32-fold higher than titers in the other three animals by 92 dpi, indicating that neutralizing titers in serum may be associated with persistent Nipah virus infection.

DISCUSSION

No therapeutics with proven efficacy are currently available to combat Nipah virus disease in humans. Only one therapeutic has shown efficacy in a nonhuman primate model: the monoclonal antibody m102.4 (6, 7).

In our study, surviving animals were evaluated until 92 dpi, three times longer than the successful monoclonal antibody m102.4 treatment studies in AGM (6, 7). None of the treated animals developed severe respiratory disease, and all survived the acute lethal Nipah virus Bangladesh challenge. Although histologic evidence of meningoencephalitis was seen in one of four remdesivir-treated animals, remdesivir is distributed to the brain upon intravenous administration (15), and thus, remdesivir treatment may be reinitiated in cases of late-onset or relapse Nipah virus encephalitis. Intravenous remdesivir treatment was administered to a patient with acute Ebola virus meningitis that developed 9 months after the initial discharge (16), and a similar approach could potentially be used in late-onset or relapse encephalitis cases of Nipah virus infection. Remdesivir is currently in a phase 2 clinical trial in Liberia and Guinea to determine whether it can affect the persistent Ebola virus shedding in semen of Ebola male survivors (17). Likewise, treating survivors of Nipah virus infection with remdesivir could be considered to aid in completely clearing the virus, thereby preventing future relapses. If our finding of differential neutralizing antibody responses in persistently infected animals versus animals that apparently cleared the virus can be confirmed in human Nipah virus survivors, then a cohort of patients that may benefit from remdesivir treatment may be determined using a standard serological assay.

We performed this experiment only once. Another limitation of our study is the initiation of treatment relatively early after lethal Nipah virus challenge. Disease progression in humans infected with Nipah virus in Bangladesh is very rapid, with an average time from disease onset to death of 5 days (18). Moreover, it was shown that therapeutic monoclonal antibody m102.4 treatment of Nipah virus Bangladesh has to be administered earlier in infection than in Nipah virus Malaysia infection to be efficacious (7). It will be important to determine how long after lethal Nipah virus Bangladesh challenge remdesivir can be administered without

losing efficacy. However, a failure of the drug in treatment at later time points would not necessarily lower the potential utility of this drug for treatment of patients with Nipah virus infection, especially regarding its potential efficacy in postexposure prophylaxis. In addition, it could be combined with monoclonal antibody therapy to improve the treatment efficacy of both therapeutics because they have distinct mechanisms of action.

Considering the extremely high case-fatality rate of Nipah virus, the fact that very few doses of monoclonal antibody m102.4 are currently available for use in humans, and the possibility of viral escape from antibody treatment, potentially adding remdesivir to the repertoire of Nipah virus treatments would substantially improve preparedness for emergency response to future outbreaks. Single and repeated dose human safety data are already available for remdesivir, and it has been successfully and safely used for compassionate access treatment or postexposure prophylaxis in people infected with or exposed to various filoviruses (16, 19-21). These facts, combined with our findings, strongly support using remdesivir in response to the next Nipah virus outbreak, either under a compassionate use protocol or possibly a randomized clinical trial protocol. Thus, remdesivir is a promising treatment candidate for Nipah virus infection that should be further explored.

MATERIALS AND METHODS

Study design

The aim of this study was to determine the efficacy of intravenous remdesivir treatment against lethal Nipah virus Bangladesh challenge. Eight adult AGMs were randomly assigned to two groups (two males and two females per group) and challenged intranasally with 10^5 50% tissue culture infectious dose ($TCID_{50}$) (0.5 ml per nostril) and intratracheally with 10^5 $TCID_{50}$ (4 ml) of Nipah virus Bangladesh. Drug treatment was initiated 24 hours after inoculation. One group of animals received remdesivir (10 mg/kg) (Gilead Sciences) in vehicle solution [12% sulfobutylether-β-cyclodextrin in water and hydrochloric acid (pH 3.5)], whereas the other group received the same volume (2 ml/kg) of vehicle solution. These were delivered as a slow intravenous bolus injection (total dose delivered over about 5 min) administered alternatingly in the left or right cephalic and saphenous veins. Treatment was continued once daily for 12 days. After inoculation with Nipah virus, animals were monitored for signs of disease and assigned a clinical score based on an Institutional Animal Care and Use Committee (IACUC)-approved scoring sheet. Scoring was done by the same individual throughout the study; this person was blinded to assignment of the animals to the remdesivir or control group. A score (0 to 15) was assigned for general appearance, skin and fur, nose/mouth/eyes/head, respiration, feces and urine, food intake, and locomotor activity. Animals were euthanized by trained and experienced personnel when the total clinical score reached the critical number of 35 or any of the following signs were observed: impaired ambulation preventing access to food or water, severe respiratory distress (open mouth breathing with lack of activity or cyanosis), lack of mental and physical alertness, or a body temperature of ≤35°C.

Clinical examinations were performed on 0, 1, 3, 5, 8, 15, 19, 23, 28, 35, 42, 50, 63, and 92 dpi. On examination days, clinical parameters such as bodyweight, respiration rate, and

oxygen saturation (SPO_2), as well as a blood sample and oral and nasal swabs, were collected. Swabs were collected in 1 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) with penicillin (50 U/ml) and streptomycin (50 μg/ml; Gibco), and the blood was collected in EDTA tubes (BD); 140 μl was directly transferred into lysis buffer for RNA extraction, and the remainder was frozen at −80°C until virus titration. Upon euthanasia, necropsies were performed, and samples of the following tissues were collected: conjunctiva, tonsil, pharynx, nasal mucosa, trachea, right and left bronchus, all lung lobes, bronchial lymph node (LN), heart, liver, spleen, kidney, adrenal gland, pancreas, jejunum, colon transversum, brain (frontal, cerebellum, and stem), pituitary gland, cervical spinal cord, mandibular LN, salivary gland, inguinal LN, axillary LN, mesenteric LN, urinary bladder, testes/ovary, and femoral bone marrow. Primary data are reported in data file S1.

Ethics and biosafety statement

All animal experiments were approved by the IACUC of Rocky Mountain Laboratories (protocol no. 2017-018), National Institutes of Health and carried out by a certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility, according to the institution's guidelines for animal use, and followed the guidelines and basic principles in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. AGMs (PrimGen) were housed in adjacent individual primate cages, allowing social interactions in a climate-controlled room with a fixed light-dark cycle (12-hour light/12-hour dark). Animals were monitored at least twice daily throughout the experiment; this was increased to a minimum of four times per 24 hours when severe disease started to develop. Commercial monkey chow, treats, and fruits were provided twice daily by trained personnel. Water was available ad libitum. Environmental enrichment consisted of a variety of human interaction, commercial toys, videos, and music. Humane endpoint criteria, specified and approved by the IACUC, were applied to determine when animals should be humanely euthanized.

Sample inactivation was performed according to standard operating procedures for removal of specimens from high containment approved by the Institutional Biosafety Committee (22).

Histopathology and IHC

All tissues collected in 10% neutral-buffered formalin during necropsy were fixed for ≥7 days and removed from BSL4 according to approved standard operating procedure before being embedded in paraffin, sectioned to 3 μm, and stained with H&E for evaluation of histological lesions. To detect Nipah virus by IHC, tissues were stained with a mouse polyclonal antibody against whole Nipah virus as a primary antibody [Centers for Disease Control and Prevention (CDC)], as described elsewhere (23). Briefly, tissues were stained with a 1:2000 dilution of anti-Nipah virus antibody, followed by incubation with a secondary alkaline phosphatase-linked antimouse antibody and counterstaining with hematoxylin.

Virus and cells

Nipah virus (strain Bangladesh/200401066) was isolated from a throat swab collected from a patient with Nipah virus disease in January 2004 in Bangladesh. The virus isolate was propagated in Vero C1008 cells in DMEM supplemented with 10% fetal calf serum, 1 mM L-glutamine (Lonza), penicillin (50 U/ml), and streptomycin (50 μg/ml).

Quantitative reverse transcription polymerase chain reaction

RNA was extracted from swab samples in DMEM and from EDTA blood samples using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. RNA was extracted from tissues using the RNeasy Kit (Qiagen). Tissues (30 mg) were homogenized in RLT buffer, and RNA was extracted, according to the manufacturer's instructions. Five microliters of RNA was used in a one-step real-time RT-PCR targeting the nucleoprotein (NP) gene using the Rotor-Gene Probe Kit (Qiagen), according to the manufacturer's instructions (primer and probe sequences are available upon request). In each run, standard dilutions of a titered virus stock were run in parallel to calculate $TCID_{50}$ equivalents in the samples.

Virus titrations

Virus titrations were performed by endpoint titration in Vero C1008 cells. Cells were inoculated with 10-fold serial dilutions of blood, swabs samples, or tissue homogenates. One hour after inoculation of cells, the inoculum was removed and replaced with 100 μl of DMEM (Sigma-Aldrich) supplemented with 2% fetal bovine serum (HyClone) [1 mM L-glutamine (Lonza), penicillin (50 U/ml), and streptomycin (50 μg/ml); Thermo Fisher Scientific]. Five days after inoculation, cytopathic effect was scored, and the $TCID_{50}$ was calculated.

Enzyme-linked immunosorbent assay

Serum samples were tested with an in-house IgM capture assay for detection of Nipah virus IgM antibodies and with an in-house indirect assay for detection of Nipah virus IgG antibodies (24). Nipah virus Malaysia antigen was used in both assays. Briefly, for the IgM capture ELISA, plates were coated overnight with anti-IgM antibody (1:1000 dilution), followed by incubation with study samples at fourfold dilutions for 1 hour. Whole Nipah virus antigen (1:4000 dilution) or negative control antigen (1:4000 dilution) was then added to wells and incubated for 1 hour, followed by mouse anti-Nipah virus antibodies (1:4000 dilution) for 1 hour and a secondary antimouse antibody conjugated with horseradish peroxidase for 1 hour. For the IgG ELISA, plates were coated with whole Nipah virus antigen (1:4000 dilution) or negative control antigen (1:4000 dilution), followed by incubation with study samples at fourfold dilutions for 1 hour. Protein A/G conjugated with horseradish peroxidase (1:5000 dilution) was then added and incubated for 1 hour.

Virus neutralization assay

Twofold serial dilutions of heat-inactivated (30 min, 56°C) sera were prepared in DMEM containing 2% fetal calf serum, 1 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml), after which 200 $TCID_{50}$ of Nipah virus Bangladesh was added. After 1 hour of

incubation at 37°C, this mix was added to Vero C1008 cells. At 5 dpi, wells were scored for cytopathic effect. The virus neutralization titers were expressed as the reciprocal value of the highest dilution of the serum that still inhibited Nipah virus Bangladesh replication.

Statistical analyses

There were no statistical analyses used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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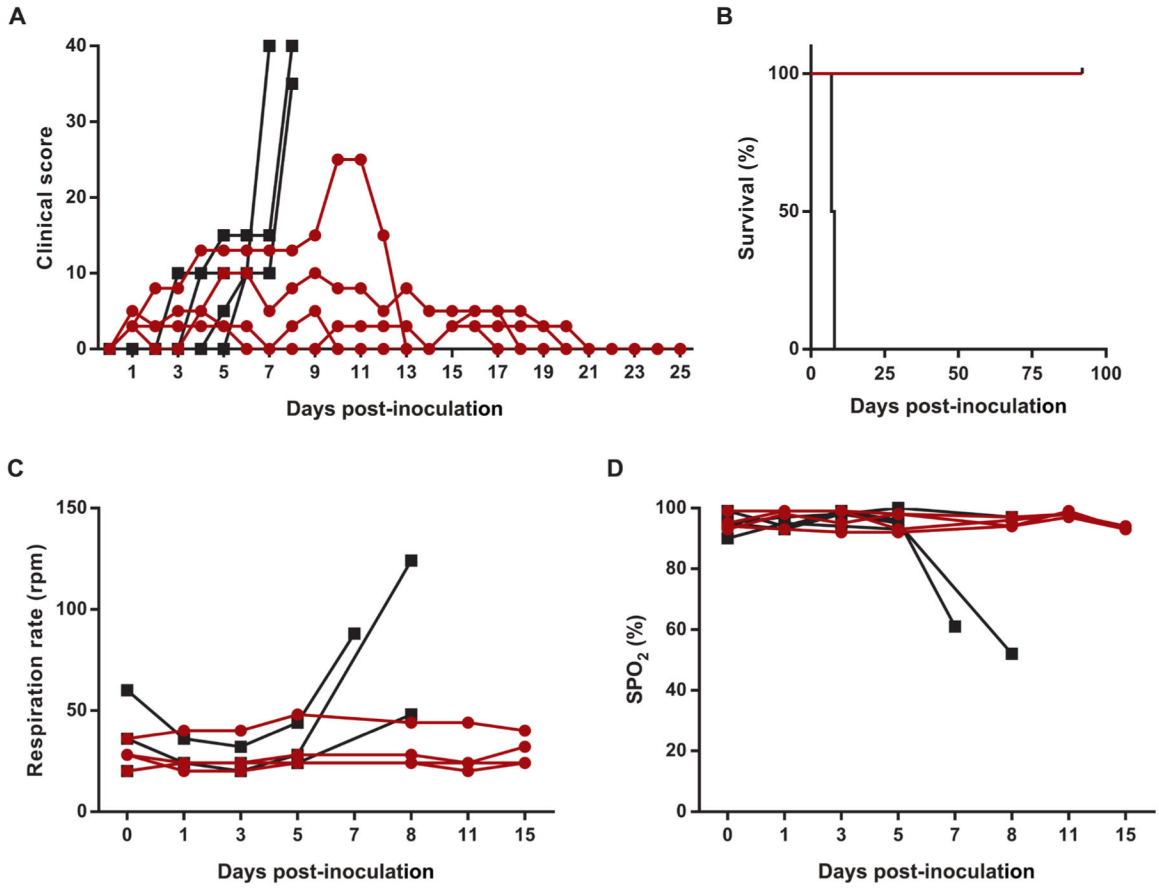

Fig. 1. Clinical signs in AGMs inoculated with a lethal dose of Nipah virus Bangladesh and treated with remdesivir.

Two groups of four AGMs were inoculated intranasally and intratracheally with 10^5 $TCID_{50}$ of Nipah virus Bangladesh. At 1 dpi, the groups were treated intravenously with remdesivir (10 mg/kg, red circles) or vehicle solution (2 ml/kg, black squares); treatment was continued for 12 days. After inoculation, the animals were observed twice daily for clinical signs of disease and scored using a predetermined clinical scoring system (**A**). Survival after inoculation and treatment is indicated in (**B**). At regular time points after inoculation, clinical examinations were performed, during which respiration rate (**C**) and oxygen saturation (SPO_2) (**D**) were determined.

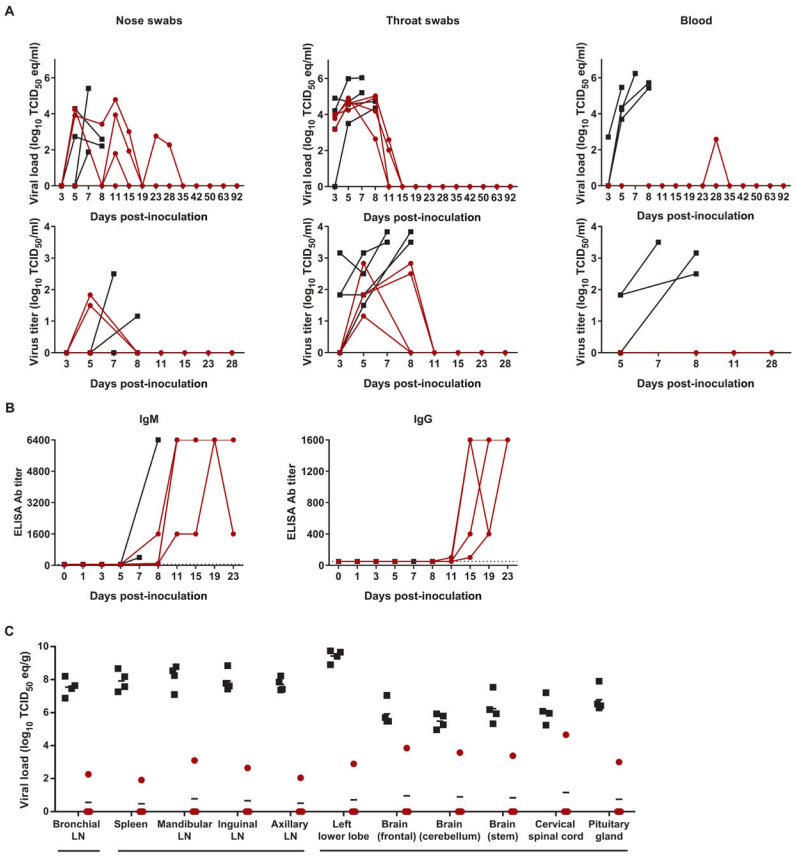

Fig. 2. Virus replication and serology in AGMs inoculated with a lethal dose of Nipah virus Bangladesh and treated with remdesivir.

Two groups of four AGMs were inoculated intranasally and intratracheally with 10^5 $TCID_{50}$ of Nipah virus Bangladesh. At 1 dpi, groups of four animals were treated intravenously with remdesivir (10 mg/kg, red circles) or vehicle solution (2 ml/kg, black squares); treatment was continued for 12 days. During clinical examinations, nose swabs, throat swabs, blood, and serum were collected, and the presence of viral RNA was determined by qRT-PCR [(**A**), top panels], and infectious virus was determined by virus titration [(A), bottom] in swabs and blood. The presence of IgM and IgG antibodies against Nipah virus was determined using ELISA (**B**); dotted line indicates lower limit of detection. At the time of necropsy, tissues were collected, and the presence of viral RNA was determined. Nipah virus RNA-positive tissues present in remdesivir-treated animals euthanized at 92 dpi are shown and compared to the same tissues collected from vehicle-treated animals on 7 and 8 dpi (**C**); underlined tissues were collected from the same animal. Ab, antibody.

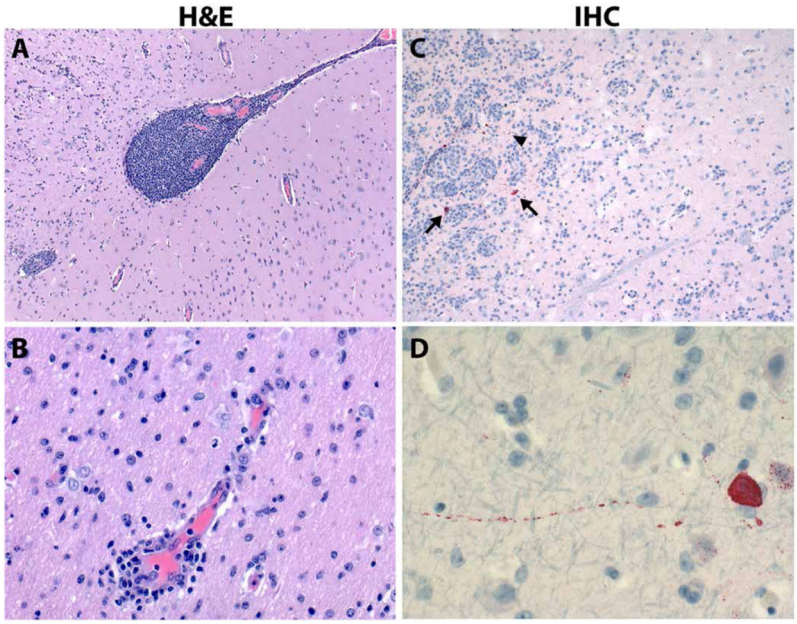

Fig. 3. Presence of Nipah virus in the cerebrum of one AGM inoculated with Nipah virus Bangladesh and treated with remdesivir.

At 92 dpi, all surviving animals were euthanized, and brain tissue was collected for histopathologic analysis. (**A**) Mononuclear perivascular cuffing and edema [hematoxylin and eosin (H&E), cerebrum, ×5]. (**B**) Neuronal necrosis, gliosis, and mononuclear perivascular cuffing (H&E, cerebrum, 20×). (**C**) Representative scattered granular staining in areas of parenchymal inflammation (arrowhead) with occasional intracellular neuronal staining (arrows) (IHC assay targeting whole Nipah virus antigen, cerebrum, ×20). Targeted antigen in red. (**D**) Staining of neuronal cell body and processes (IHC assay, cerebrum, ×40).