EXPERIMENTAL INFECTION OF THE SIGMODON ALSTONI COTTON RAT WITH CAÑO DELGADITO VIRUS, A SOUTH AMERICAN HANTAVIRUS

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Abstract. Forty-eight Sigmodon alstoni (Alston’s cotton rat) were inoculated with Caño Delgadito (CDG) virus to extend our knowledge and understanding of the natural host relationships of the hantaviruses indigenous to the Americas. Infectious CDG virus was recovered from oropharyngeal secretions, urine, or solid tissues of nine of 12 animals killed on day 9 post-inoculation (PI), 14 of 24 animals killed on day 18 or 27 PI, and none of 12 animals killed on day 54 PI. In addition, virus-specific RNA was detected in the kidneys of six of the 12 animals killed on day 54 PI, and adult cotton rats inoculated with the kidneys of four animals killed on day 54 PI developed antibody to CDG virus. Collectively, the results indicate that CDG virus can establish lengthy (perhaps lifelong) infections in Alston’s cotton rat and thus support the concept that S. alstoni is the principal host of CDG virus.

INTRODUCTION

Viruses in the genus Hantavirus (family Bunyaviridae) cause significant morbidity and mortality in humans worldwide. Hantaan, Seoul, Dobrava-Belgrade, and Puumula viruses are etiologic agents of hemorrhagic fever with renal syndrome in Asia, the former Soviet Union, and Europe.1 Sin Nombre is the principal host of CDG virus. Hantaan, Seoul, Dobrava-Belgrade, and Puumula viruses cause significant morbidity and mortality in humans worldwide. Hantaan, Seoul, Dobrava-Belgrade, and Puumula viruses are etiologic agents of hemorrhagic fever with renal syndrome in Asia, the former Soviet Union, and Europe.1

Specific rodents (usually one or two closely related species) are the principal hosts of the hantaviruses for which natural host relationships have been well characterized. For example, Apodemus agrarius (striped field mouse), Rattus norvegicus (Norway rat) and Rattus rattus (roof rat), Clethrionomys glareolus (bank vole), Peromyscus maniculatus (deer mouse), and Sigmodon hispidus (hispid cotton rat) are the principal hosts of Hantaan, Seoul, Puumula, Sin Nombre, and Black and Sigmodon alstoni (hispid cotton rat) are the principal hosts of Hantaan, Seoul, Puumula, Sin Nombre, and Black Creek Canal viruses, respectively.1

Humans usually become infected with hantaviruses by contact with infected rodents or infectious rodent secretion or excreta. Thus, knowledge of the natural host relationships of a hantavirus (including duration and magnitude of virus shedding in infected rodents) is essential to an accurate understanding of the epidemiology of that virus. Caño Delgadito (CDG) virus was the first hantavirus discovered in association with rodents indigenous to South America. The prototype strain of that virus was isolated from a lung of a naturally infected Alston’s cotton rat captured in Portuguesa State in late 1996 or early 1997. All of the experimental animals were antibody-negative against CDG virus prior to inoculation with the experimental virus.

MATERIALS AND METHODS

Safety. All work with infected rodents was done in a biosafety level 3 animal facility. Personal protective equipment worn by the laboratory personnel included the Breathe-Easy® powered air-purifying respiratory system with a Breathe-Easy® 10 headpiece (Racal Health and Safety, Inc., Frederick, MD), and disposable gowns, booties, and latex rubber gloves.

Study design. Forty-eight cotton rats (12 litters of four animals each) were inoculated at age 21 days with 3.1, 1.1, −0.9, or −2.9 log10 cell culture infectious dose50 (CCID50) of the CDG virus prototype strain VHV-574. One animal in each litter was assigned to each dosage level and killed on day 9, 18, 27, or 54 post-inoculation (PI). In this manner, three animals from different litters were appointed to each of the 16 dose-time treatment groups (Table 1).

Virus. The inocula were prepared from a single stock of the CDG virus prototype strain VHV-574, which originally was isolated from a lung of a naturally infected Alston’s cotton rat by cultivation in monolayer cultures of Vero E6 cells.8 The passage history and infectious titer of the virus stock were Vero E6+4 and 5.8 log10 CCID50/ml (as measured in monolayer cultures of Vero E6 cells), respectively.

Animals. The experimental animals were third or fourth generation, laboratory-reared descendants of S. alstoni captured in Portuguesa State in late 1996 or early 1997. All of the experimental animals were antibody-negative against CDG virus prior to inoculation with the experimental virus.

Inoculation, husbandry, and sampling of animals. Each animal was inoculated subcutaneously at one site over the scapular region with 0.2 ml of a suspension containing 3.1, 1.1, −0.9, or −2.9 log10 CCID50 of the experimental virus. The high-dose (3.1 log10 CCID50) inocula were prepared in cold, sterile 0.01 M phosphate-buffered saline (PBS), pH 7.4, as a 1% (v/v) suspension of the stock virus. The 1.1, −0.9, and −2.9 log10 CCID50 inocula were prepared in PBS from serial 10-fold dilutions of the high-dose inocula.

The animals were housed individually in microisolators and strict barrier care was practiced throughout the study to prevent virus transmission between animals. On day 9, 18, 27, or 54 PI, the animals were subdued in an atmosphere of CO2 and killed with chloroform. Immediately thereafter, cardiac blood, oropharyngeal (OP) secretions, and urine, feces, brain, lung, spleen, kidney, and liver were collected and stored at

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−80°C. Urine was collected by cystocentesis and stored in 0.3 ml of PBS containing 10% (v/v) heat-inactivated (56°C for 30 minutes) fetal bovine serum (FBS). (The volume of urine in the bladders ranged from 0.00 ml to 0.15 ml. If the bladder was empty, the bladder lumen was rinsed with 0.3 ml of PBS−FBS.) The OP secretions were collected with a sterile cotton swab wetted with PBS−FBS, then expressed from the swab by agitation in a vial containing 0.3 ml of PBS−FBS. Feces were collected through an incision along the anti-mesenteric border of the descending colon.

Assay for infectious virus. The infectivity of each high-dose (3.1 log10 CIID50) inoculum was determined by titration using monolayer cultures of Vero E6 cells in 24-well plastic plates (each well = 1.78 cm²). Serial 10-fold dilutions of the inoculum were prepared in PBS and four cultures each were inoculated with 50 µL of each dilution. The inoculated cell cultures were incubated at 37°C for 60 minutes, overlaid with 1.5 ml of a maintenance medium, and then incubated at 37°C for 60 minutes, overlaid with 0.2 ml of each homogenate, and blood samples collected from the cultures on day 13 PI were tested for antibody using an indirect fluorescent antibody test (IFAT). Subsequently, virus isolation was attempted on the cell culture material (passage history: Vero E6+1) using Vero E6 cell monolayers in 12-well plastic plates (each well = 3.14 cm²). In that situation, the inoculum was 0.2 ml of undiluted E6+1 material, the monolayer was maintained under 2.5 ml of fluid maintenance medium, and the IFAT cell spots were prepared on day 13 PI.

The kidneys of the 12 animals killed on day 54 PI were tested for infectious hantavirus by inoculation of 12 adult S. alstoni. The inocula were 10% (w/v) crude tissue homogenates. One adult S. alstoni was inoculated subcutaneously with 0.2 ml of each homogenate, and blood samples collected from the adult animals on day 28 PI were tested for antibody against CDG virus by using the enzyme-linked immunosorbent assay (ELISA) described in this report.

Assay for CDG virus-specific RNA. Kidneys from 27 of the 48 experimental animals were tested for CDG virus-specific RNA using a heminested reverse transcriptase−polymerase chain reaction (RT−PCR) assay. The 27 animals included three inoculated with 3.1 log10 CIID50 and killed on day 18 PI, three inoculated with −0.9 log10 CIID50 and killed on day 18 PI, three each inoculated with 3.1, 1.1, or −2.9 log10 CIID50 and killed on day 54 PI, and 12 inoculated with −2.9 log10 CIID50. Total RNA was extracted from 30−40 mg of each kidney using TRIzol® Reagent (Life Technologies, Inc., Grand Island, NY). The RNA extracts were purified by using the RNeasy® Mini Kit (Qiagen, Inc., Valencia, CA). Reverse transcription and PCR amplification of a 590-nucleotide fragment of the CDG virus small genome segment was attempted on 10% of each purified RNA extract, using the Access RT−PCR Assay (Promega, Inc., Madison, WI) in conjunction with oligonucleotides 5′-GCTGTAATGAGCAACCTCAAA-
GA-3' and 5'-GGTGTGATTTCATCACAGCTTTCAT-3'. The PCR amplification of a 443-nucleotide fragment was attempted on 10% of each RT-PCR, using Taq DNA polymerase (Promega, Inc.) in conjunction with oligonucleotides 5'-CGGTGCGTGTGTCAGCAGTGA-3' and 5'-GGGTGTGATTTCATCACAGCTTTCAT-3'. The control negatives for the RT-PCR assay were RNase-free water and RNA extracted from a kidney of a cotton rat that was free of hantavirus infection. The control negatives for the heminested PCR assay were 10% of the negative control reaction mixtures from the RT-PCR assay. The RT-PCR and second-round PCR products were subjected to agarose gel electrophoresis, and the agarose gels were stained with ethidium bromide and examined for bands of the expected size (590 and 443 base pairs [bp], respectively).

The nucleotide sequence of the RT-PCR product generated from the kidney of each of four animals was determined to provide an assurance that the hantavirus recovered from the experimental animals was the CDG virus prototype strain VHV-574. The four animals included two inoculated with 3.1 log_{10} CCID_{50} (one each killed on day 18 and 54 PI), one inoculated with 1.1 log_{10} CCID_{50} and killed on day 54 PI, and one inoculated with 0.9 log_{10} CCID_{50} and killed on day 18 PI. DNA fragments of the correct size (590-bp) were purified from agarose gel slices, using the QIAquick® Gel Extraction Kit (Qiagen, Inc.). The purified DNA fragments were sequenced directly using the dye termination technique (Applied Biosystems, Inc., Foster City, CA) in conjunction with oligonucleotides 5'-GCT-GTAATGAGCAACCTCAAAGA-3' and 5'-GGTGTGATTTCATCACAGCTTTCAT-3'. The nucleotide sequences of the four fragments were compared to the sequence of the homologous region of the small genomic segment of the CDG virus prototype strain VHV-574 (GenBank Accession No. AF000140).

**Assay for CDG virus antibody.** Sera from the experimental animals were tested for antibody against the CDG virus prototype strain VHV-574, using an ELISA previously described. The test antigen was a lysate of Vero E6 cells infected with the experimental virus. The control (comparison) antigen was prepared from uninfected Vero E6 cells in a manner that quantitatively was identical to that used to prepare the test antigen. Serial four-fold dilutions (from 1:80 through 1:1,310,720) of each serum sample were tested against both antigens. Cotton rat antibody bound to antigen was detected by using a mixture of goat anti-rat IgG peroxidase conjugate and goat anti-*Peromyscus leucopus* IgG peroxidase conjugate in conjunction with the ABTS (2, 2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Optical densities (ODs) at 405 nm (reference = 490 nm) were measured with a Dynatech MRX II microplate reader (Dynatech Industries, Inc., McLean, VA). The adjusted OD (AOD) of a serum-antigen reaction was the OD of the well coated with the test antigen minus the OD of the corresponding well coated with the comparison (uninfected Vero E6 cell lysate) antigen. All tests were done in duplicate and the reactivity of a sample was measured as the sum of the mean AODs for the series of four-fold dilutions (from 1:80 through 1:1,310,720). A serum was considered to be positive if the mean AOD at 1:80 was ≥ 0.200 and the sum of the mean AODs from 1:80 through 1:5,120 was ≥ 0.750. The titer of a positive sample was the reciprocal of the highest dilution for which the AOD was ≥ 0.200.

**RESULTS**

The median infectious titer of the high-dose inocula was 3.1 log_{10} CCID_{50} (range = 3.0-3.1 log_{10} CCID_{50}). Thus, each animal was inoculated with approximately 3.1, 1.1, −0.9, or −2.9 log_{10} CCID_{50} in a volume of 0.2 ml.

Signs of severe illness were not observed in any of the 48 experimental animals. However, 12 of the 27 animals inoculated with 3.1, 1.1, or −0.9 log_{10} CCID_{50} and killed on day 9, 18, or 27 PI; none of the nine animals inoculated with 3.1, 1.1, or −0.9 log_{10} CCID_{50} and killed on day 54 PI; and none of the 12 animals inoculated with −2.9 log_{10} CCID_{50} (Table 1). The virus-positive specimens included 2 bloods, 21 spleens, 20 kidneys, 18 lungs, 9 livers, 4 brains, 11 OP secretions (throat swabs), and 14 urines.

Antibody against CDG virus was found in 32 of the 36 animals inoculated with 3.1, 1.1, or −0.9 log_{10} CCID_{50}, and in two of the 12 animals inoculated with −2.9 log_{10} CCID_{50} (Table 1). The antibody titers in the positive animals ranged from 80 to 327,680 and peaked at day 18 or 27 PI.

The RT-PCR and/or second-round PCR products of the expected size (590 bp and 443 bp, respectively) were generated from the kidneys of six of the 12 animals killed on day 54 PI and four other animals (Table 2). The nucleotide sequences of the four RT-PCR products selected for characterization were identical to the nucleotide sequence of the homologous region of the CDG virus prototype strain VHV-574, indicating that CDG virus was in fact the hantavirus recovered from the experimental animals.

The 12 animals inoculated with −0.9 log_{10} CCID_{50} and two of the 12 animals inoculated with −2.9 log_{10} CCID_{50} became infected with CDG virus, as measured by recovery of virus or seroconversion (Table 3). Thus, the infectivity of the virus stock in the experimental animals was 7.9 log_{10} ID_{50}/ml, more than 100-fold greater than the infectivity of the same stock in monolayer cultures of Vero E6 cells (i.e., 5.8 log_{10} CCID_{50}/ml).

Antibody (IgG) against CDG virus was detected in four of the 12 adult animals inoculated with the kidneys of the 12 animals killed on day 54 PI. The antibody titers in the antibody-positive animals ranged from 320 through 1,280 (mode

### Table 2

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<th>Dose†</th>
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* No. positive/no. tested; † = none tested.
† 3.1, 1.1, −0.9, or −2.9 log_{10} cell culture infectious dose_{50} (CCID_{50}).
‡ The RNA-negative animal was culture-negative, antibody-positive.
§ The RNA/positive animal was culture-negative, antibody-positive.
DISCUSSION

The present study provides strong evidence that virus recovery by cultivation in Vero E6 cells is not a particularly sensitive method for the detection of infectious CDG virus in rodent tissues, urine, or OP secretions. Thus, the failure to recover virus from some animals, particularly the infected (i.e., antibody-positive or RNA-positive) animals killed on day 54 PI, could be because the titers of infectious virus in those animals were too low to be detected by cultivation in Vero E6 cells.

A hallmark of the hantaviruses is their ability to establish chronic infections in their respective principal rodent hosts.1 The infections (seroconversions) in the animals inoculated with CDG virus can establish chronic (perhaps lifelong) infections in Alston’s cotton rats and thus support the concept that S. alstoni is the principal host of CDG virus.

In general, the results of the present study resemble those of laboratory studies on Black Creek Canal viral infection in S. hispidus, Hantaan viral infection in A. agrarius, Seoul viral infection in R. norvegicus, Puumula viral infection in C. glareolus, and Sin Nombre viral infection in P. maniculatus.11–15 The similarities include transient viremia, virus persistence in solid tissues (especially lung or kidney) in the face of a vigorous antibody response, and prolonged shedding of virus in excreta or secreta. The recovery of CDG virus-specific RNA from animals killed on day 54 PI indicates that the kidney is a site for viral persistence and suggests that S. alstoni can develop persistent viriceric infections.

Horizontal virus transmission appears to be the dominant mode of intra-specific hantavirus transfer in naturally infected rodent populations.1 A rodent’s first encounter with rodents other than its mother and littermates likely occurs soon after it is weaned. Thus, newly weaned animals probably constitute a substantial segment of a rodent population that is susceptible to infection with a hantavirus. That assessment was the basis for using 21-day-old (newly weaned) animals in the present study. Whether experimental CDG viral infection in adult cotton rats differs from the same in 21-day-old cotton rats remains to be determined.

Differences among the virus profiles of experimental animals in different dose-time treatment groups suggest that inoculum dose can affect the kinetics of CDG viral infection in S. alstoni. For example, infectious virus was recovered from the brains of the three animals inoculated with 1.1 log$_{10}$ CCID$_{50}$ and killed on day 18 PI, but was not recovered from the brains of the 18 animals inoculated with 3.1 or −0.9 log$_{10}$ CCID$_{50}$ and killed on day 9, 18, or 27 PI. Similarly, CDG virus was recovered from the kidneys of five of the six animals inoculated with 1.1 or −0.9 log$_{10}$ CCID$_{50}$ and killed on day 27 PI, but was not recovered from the kidneys of the three animals inoculated with 3.1 log$_{10}$ CCID$_{50}$ and killed on day 27.

The results of a recent study suggested that adaptation of Puumula virus to growth in cell culture can result in a reduction in the infectivity of that virus in its principal host, C. glareolus.16 Whether adaptation of CDG virus to growth in Vero E6 cells can affect the infectivity of CDG virus in S. alstoni or, more importantly, the duration of infection or magnitude and duration of virus shedding in infected animals remains to be determined.

It generally is accepted that hantaviral infections in their principal rodent hosts are asymptomatic. However, this assertion appears to be based largely on studies that did not include detailed pathologic examinations of infected animals. The results of recent studies on naturally infected animals suggested that Sin Nombre and New York-1 viruses can cause histologic lesions in the lungs and other organs of P. maniculatus and Peromyscus leucopus (white-footed mouse), respectively.17,18 In the present study, 12 animals killed on day 18 PI or thereafter appeared lethargic for 1–2 days between days 10 and 15 PI. The 12 animals included five virus-positive animals killed on day 18 PI, four virus-positive animals killed on day 27 PI, two virus-positive animals killed on day 54 PI, and one antibody-positive, virus-negative animal killed on day 54 PI. The recovery of infectious virus from 11 of the 12 affected animals suggests that CDG virus can cause clinically detectable disease in Alston’s cotton rats.

Taken together, the recovery of virus from animals killed on day 9, 18, or 27 PI, and the failure to recover virus from the infected animals killed on day 54 PI indicate that virus titers in the acute phase of infection are higher than virus titers in the chronic phase of infection. This suggests that newly infected cotton rats pose a greater risk of infection to humans than chronically infected cotton rats.

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