Pathology of Black Creek Canal Virus Infection in Juvenile Hispid Cotton Rats (*Sigmodon hispidus*)

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Abstract

The purpose of this study was to assess the effect of inoculum dose on the pathogenesis of Black Creek Canal virus (BCCV) infection in the hispid cotton rat (*Sigmodon hispidus*), the principal host of BCCV. No sign of illness was observed in any of the 52 juvenile hispid cotton rats inoculated with 3.1, 1.1, -0.9 , or -2.9 $\log_{10}$ median infectious doses_{VeroE6} (ID_{50-VeroE6}) of BCCV and euthanized on day 9, 18, 27, or 54 postinoculation (PI). Analysis of virus assay and serological data indicated that inoculum dose could significantly affect the pathogenesis of BCCV infection in juvenile hispid cotton rats. For example, the six animals inoculated with 3.1 or 1.1 $\log_{10}$ ID_{50-VeroE6} and euthanized on day 54 PI were virus positive and antibody positive, whereas the six animals inoculated with -0.9 or -2.9 $\log_{10}$ ID_{50-VeroE6} and euthanized on day 54 PI were virus positive but antibody negative. Microscopic examination of tissues from the animals inoculated with 3.1 or 1.1 $\log_{10}$ ID_{50-VeroE6} revealed diffuse, subacute pneumonitis in the lungs of all the animals euthanized on day 18 PI or thereafter, and indicated that the severity of pneumonitis was dependent upon inoculum dose as well as duration of infection (i.e., amount of time elapsed since inoculation).

Key Words: Black Creek Canal virus—Hantavirus—hispid cotton rat—*Sigmodon hispidus*.

Introduction

HANTAVIRUS PULMONARY SYNDROME is a potentially fatal zoonosis (Koster and Levy 2006) caused by Black Creek Canal virus (BCCV), New York virus (NYV), Sin Nombre virus (SNV), and certain other members of the family *Bunyaviridae*, genus *Hantavirus* (Nichol et al. 2005), that are native to the New World. Specific rodents are the principal hosts of the hantaviruses for which natural host relationships have been well characterized. For example, the hispid cotton rat (*Sigmodon hispidus*) in southern Florida is the principal host of BCCV (Rollin et al. 1995, Glass et al. 1998), the deer mouse (*Peromyscus maniculatus*) in Canada and the western United States is the principal host of SNV (Childs et al. 1994, Drebot et al. 2001), and the white-footed mouse (*Peromyscus leucopus*) in the eastern United States is the principal host of NYV (Hjelle et al. 1995). It is generally accepted that humans usually become infected with hantaviruses by inhalation of droplets of saliva, respiratory secretions, or urine from in-

fectured rodents or inhalation of particles of dust contaminated with secretions or excretions from infected rodents.

Chronic infections in rodents are critical to long-term maintenance of hantaviruses in nature. In a previously published study (Hutchinson et al. 1998), hispid cotton rats inoculated subcutaneously with 3.0 $\log_{10}$ median cell culture infectious doses_{VeroE6} (ID_{50-VeroE6}, infectivity measured in monolayers of Vero E6 cells) at 5 weeks of age developed an IgG response to BCCV by day 14 postinoculation (PI) and were viruric through day 70 PI. The first objective of this study was to assess the effect of inoculum dose on the kinetics of infection and virus shedding in BCCV-infected hispid cotton rats.

The results of several previous laboratory studies (Lee et al. 1981, Yanagihara et al. 1985, Hutchinson et al. 1998, Botten et al. 2000) suggested that hantaviruses in their respective principal hosts are apathogenic; however, NYV and SNV have been associated with pulmonary edema and hepatitis in naturally infected white-footed mice (*P. leucopus*) and deer

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mice (*P. maniculatus*), respectively (Lyubsky et al. 1996, Netski et al. 1999). The second objective of this study was to investigate the pathogenicity of BCCV in the hispid cotton rat (*S. hispidus*) from southern Florida, where BCCV is enzootic (Glass et al. 1998).

Materials and Methods

Study design

Fifty-two hispid cotton rats from 12 litters were inoculated at the age of 21 days with 3.1, 1.1, -0.9, or -2.9 log₁₀ ID_{50-VeroE6} of BCCV and euthanized on day 9, 18, 27, or 54 PI. Each treatment (inoculum dose-time point) group comprised three or four animals, and littermates were assigned to different treatment groups. Altogether, 13 animals were inoculated with 3.1 log₁₀ ID_{50-VeroE6}, 14 animals were inoculated with 1.1 log₁₀ ID_{50-VeroE6}, 13 animals were inoculated with -0.9 log₁₀ ID_{50-VeroE6}, 12 animals were inoculated with -2.9 log₁₀ ID_{50-VeroE6}, 13 animals were euthanized on day 9 PI, 13 animals were euthanized on day 18 PI, 14 animals were euthanized on day 27 PI, and 12 animals were euthanized on day 54 PI (Table 1).

Safety

All work with the experimentally infected cotton rats was done inside a biosafety level 4 facility located on the Roybal Campus of the Centers for Disease Control and Prevention in Atlanta, GA.

Virus

The inocula were prepared from a single stock of BCCV strain 9408076 (passage history: Vero E6 + 4). This strain was

originally isolated from a hispid cotton rat captured in 1994 in Dade County, FL (Rollin et al. 1995). The titer of the stock virus was 5.8 log₁₀ ID_{50-VeroE6}/1.0 mL.

The high-dose inocula (3.1 log₁₀ ID_{50-VeroE6} in 0.2 mL) were prepared in cold, sterile 0.01 M phosphate-buffered saline (PBS; pH 7.40) as 1% v/v dilutions of the stock virus. The other inocula (i.e., 1.1, -0.9, and -2.9 log₁₀ ID_{50-VeroE6} in 0.2 mL) were prepared in PBS from serial 10-fold dilutions of the high-dose inocula.

Animals

The experimental animals were third- or fourth-generation, laboratory-reared descendants of hispid cotton rats captured in July 1997 in Dade County, FL. The breeding colony was established and maintained by M.L. Milazzo in an animal facility at the University of Texas Medical Branch. Tests on samples of blood, spleen, and other tissues from the wild-caught predecessors of the experimental animals were negative for evidence of BCCV infection and Tamiami virus infection (Calisher et al. 1970). Further, indirect fluorescent antibody tests (IFAT) on blood samples collected from the experimental animals immediately before inoculation with BCCV were negative for IgG to BCCV and IgG to Tamiami virus.

Inoculation, husbandry, and sampling of animals

Each animal was anesthetized with vaporized Metofane[®] (Pittman-Moore, Mundelein, IL), a blood sample was collected from a retro-orbital venous plexus by gentle puncture with a sterile microhematocrit tube, and then the entire inoculum (0.2 mL) was injected subcutaneously at a single site over the scapular region, using a 25-gauge×5/8" needle on a tuberculin syringe. The inoculated animals were housed

TABLE 1. RESULTS OF TESTS FOR HANTAVIRUS AND ANTIBODY (IgG) TO BLACK CREEK CANAL VIRUS IN 52 HISPID COTTON RATS INOCULATED WITH BLACK CREEK CANAL VIRUS, BY INOCULUM DOSE

Dose ^a	Day PI	M:F ^b	Virus ^c	Hantavirus isolated from								Antibody ^c	Antibody titers ^d
				Blood	Brain	Lung	Spleen	Kidney	Liver	OP swab	Urine		
3.1	9	1:2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/3	2/3	25–>25,600
3.1	18	1:2	3/3	3/3	3/3	3/3	3/3	3/3	0/3	3/3	2/3	3/3	1600–>25,600
3.1	27	2:2	4/4	0/4	3/4	3/4	4/4	4/4	1/4	2/4	1/4	2/4	1600–6400
3.1	54	1:2	3/3	1/3	2/3	2/3	2/3	2/3	0/3	0/3	1/3	3/3	6400
1.1	9	1:2	3/3	1/3	1/3	3/3	2/3	2/3	3/3	1/3	1/3	0/3	—
1.1	18	2:2	4/4	2/4	3/4	4/4	4/4	3/4	1/4	3/4	2/4	3/4	100–6400
1.1	27	1:3	4/4	2/4	3/4	4/4	3/4	4/4	2/4	0/4	2/4	4/4	400–6400
1.1	54	2:1	3/3	1/3	0/3	2/3	2/3	0/3	0/3	0/3	0/3	3/3	1600–6400
-0.9	9	2:2	1/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	25
-0.9	18	1:2	1/3	0/3	0/3	1/3	1/3	0/3	0/3	0/3	0/3	0/3	—
-0.9	27	2:1	3/3	2/3	1/3	1/3	2/3	1/3	0/3	0/3	0/3	0/3	—
-0.9	54	1:2	3/3	1/3	0/3	2/3	2/3	1/3	0/3	0/2	0/3	0/3	—
-2.9	9	1:2	2/3	0/3	1/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3	—
-2.9	18	2:1	1/3	1/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	1/3	25
-2.9	27	2:1	1/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	—
-2.9	54	1:2	3/3	0/3	0/3	1/3	2/3	0/3	0/3	0/2	0/3	0/3	—
Total		23:29	42/52	17/52	21/52	31/52	31/52	25/52	10/52	11/50	10/52	22/52	

^aLog₁₀ median infectious doses (ID_{50-VeroE6}).

^bNumber of male:number of female.

^cNumber of positive/number of tested.

^dRange of antibody titers in the antibody-positive animals.

OP, oropharyngeal; PI, postinoculation.

individually in microisolators in a Maxi-Mizer Caging System (Thoren Caging Systems, Hazelton, PA) and observed at least once daily for signs of illness. Strict barrier care was practiced throughout the study to prevent virus transmission between animals.

The virus-inoculated animals and four sham-inoculated (control) animals were euthanized by exposure to a lethal dose of vaporized Metofane (Pittman-Moore). An oropharyngeal (OP) swab and samples of cardiac blood, urine, brain, lung, spleen, kidney, and liver were collected from each animal at death. The secretions on the OP swab were stored in 0.3 mL of PBS–fetal bovine serum (FBS) in a 1.5 mL cryovial. Urine was collected by cystocentesis and stored in 0.3 mL of PBS–FBS in a 1.5 mL cryovial. The volume of urine ranged from 0.00 to 0.15 mL; if empty, the bladder lumen was rinsed with 0.3 mL of PBS–FBS. The samples from the OP swabs and samples of cardiac blood, urine, and solid tissues were stored at -70°C , samples of serum from cardiac blood were stored at -20°C , and the carcasses of the virus- and sham-inoculated cotton rats were fixed by immersion in 10 volumes of 10% w/v neutral-buffered formalin for 72 h and then stored in 10 volumes of 70% v/v ethanol.

Virus assay

The titers of the high-dose inocula (i.e., the 1% v/v suspensions of the stock virus) were measured in monolayers of Vero E6 cells in 24-well plastic plates, each well 1.78-cm^2 . Serial 10-fold dilutions of each high-dose inoculum were prepared in PBS, each dilution was tested in quadruplicate (i.e., four monolayers), and the inoculated monolayers were maintained under a fluid overlay at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell spots were prepared from the monolayers on day 11 PI, rendered noninfectious by irradiation (5×10^6 rads, Co^{60} source), fixed in cold acetone, and then tested for cell-associated hantavirus antigen, using an IFAT. The primary and secondary antibodies in the IFAT were a hyperimmune mouse ascitic fluid raised against a recombinant SNV nucleocapsid protein antigen (CDC-SPB #703110, P.E. Rollin) and a fluorescein isothiocyanate-conjugated goat antibody raised against mouse (*Mus musculus*) IgG (Cappel Labs, West Chester, PA), respectively. The titer of each high-dose inoculum was calculated by using a method described previously (Reed and Muench 1938).

Virus isolation was attempted on the samples from the OP swabs, 10% v/v suspensions of blood in PBS–FBS, urine in PBS–FBS, and crude 10% w/v homogenates of solid tissues in sterile PBS as described previously (Fulhorst et al. 2002). Briefly, 0.25 mL of a sample was adsorbed onto a confluent monolayer of Vero E6 cells grown in a 12.5-cm^2 plastic cell culture flask, the inoculated monolayer was maintained under 5.0 mL of a fluid maintenance medium at 37°C in a humidified atmosphere of 5% CO_2 in air, half of the overlay was replaced with fresh maintenance medium on day 6 PI, approximately 50% of the Vero E6 cells in the monolayer were blind-passaged in 5.0 mL of growth medium in a sterile 12.5-cm^2 plastic culture flask on day 13 PI, the growth medium on the new monolayer was replaced with 5.0 mL of fresh maintenance medium on day 19 PI, and cell spots prepared from the original monolayer on day 13 PI and cell spots prepared from the second (i.e., blind-passaged) monolayer on day 26 PI were tested for hantavirus antigen, using the IFAT described

above. Only samples that were antigen positive on day 26 PI were treated as culture positive in the analyses that included virus assay data.

Genetic characterization of viruses

Samples of kidney from two virus-positive animals (both infected by inoculation with $3.1 \log_{10} \text{ID}_{50}\text{-VeroE6}$, one each euthanized on day 9 and 18 PI) were tested for BCCV RNA to provide an assurance that the hantavirus isolated from the experimentally infected animals was from the stock virus. Total RNA was isolated from 30 to 40 mg of each kidney, using TRIzol® Reagent (Life Technologies, Grand Island, NY). Reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a 261-nt fragment of the BCCV small genomic segment was attempted on 10% of each sample of total RNA, using the Access RT-PCR Assay (Promega, Madison, WI) in conjunction with oligonucleotides 5'-ACAGCAGCTAGTGGCAGCTAGAC-3' and 5'-AGCACATTGCCATACCTAAGTGAG-3'. The negative controls for the RT-PCR assay were RNase-free water and RNA isolated from a kidney of an uninfected hispid cotton rat. The PCR products of the expected size were purified from gel slices, and both strands of each gel-purified PCR product were sequenced directly, using the dye termination cycle sequencing technique (Applied Biosystems, Foster City, CA) in conjunction with the oligonucleotides that were used in the RT-PCR assay.

Antibody assay

Serum samples from the cotton rats were rendered noninfectious by gamma irradiation (5×10^6 rads, Co^{60} source) and then tested for antibody to BCCV, using an IFAT. Serial fourfold dilutions (from 1:25 through 1:25,600) of each sample were tested against acetone-fixed cell spots that contained BCCV-infected Vero E6 cells mixed 1:1 with uninfected Vero E6 cells. Antibody (IgG) bound to antigen was revealed by using a fluorescein isothiocyanate-conjugated goat antibody raised against white-footed mouse (*P. leucopus*) IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The antibody titer of a positive sample was the reciprocal of the highest dilution that produced a specific pattern of fluorescence in the cytoplasm of approximately 50% of the cells in duplicate cell spots.

Histopathology

Formalin-fixed samples of brain, heart, lung, thymus, spleen, liver, kidney, and salivary gland from 12 animals inoculated with $3.1 \log_{10} \text{ID}_{50}\text{-VeroE6}$, lung from 12 animals inoculated with $1.1 \log_{10} \text{ID}_{50}\text{-VeroE6}$, and brain, heart, lung, thymus, spleen, liver, kidney, and salivary gland from two sham-inoculated (uninfected) hispid cotton rats were embedded in paraffin, sectioned at $4 \mu\text{m}$, deparaffinized, rehydrated through graded alcohol incubations, stained with hematoxylin and eosin, and then examined by light microscopy at low ($\times 100$), medium ($\times 200$), and high ($\times 400$) powers. The prevalence of mononuclear leukocytes (PML) in each of 10 randomly selected medium-power fields ($\times 200$) in each tissue section was scored: 1 ($>1\%$ but $<25\%$), 2 ($>25\%$ but $<50\%$), and 3 ($\geq 50\%$). The median score for the 10 randomly selected high-power fields was used for analysis.

Immunohistochemistry assay for hantavirus antigen

Thin ($4\ \mu\text{m}$) sections of formalin-fixed brain, heart, lung, thymus, spleen, liver, kidney, and salivary gland from 12 animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ were tested for hantavirus antigen, using an immunohistochemistry (IHC) assay. The tissue sections were deparaffinized and rehydrated through graded alcohol incubations and then treated with DAKO[®] Target Retrieval Solution (Dako, Carpinteria, CA) and incubated at 90°C for 20 min to effect antigen retrieval. The primary antibody was a 1:800 v:v dilution of an anti-BCCV immune rabbit serum (CDC-SPB #SPR464, P.E. Rollin). (The working dilution of the immune rabbit serum was determined beforehand in a series of titration experiments.) Nonspecific binding of rabbit IgG was minimized by treating the tissue sections with goat serum for 20 min before application of the primary antibody. The secondary antibody was a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Goat IgG bound to tissue-associated rabbit IgG was detected by using the LSAB2a Streptavidin-Biotin System (Dako). Endogenous peroxidase activity was minimized by pretreatment of the tissue sections with 3% hydrogen peroxide, binding of endogenous avidin and biotin was blocked by using the DAKO Blocking Kit (Dako) according to the manufacturer's instructions, and the chromogen and counterstain were diaminobenzidine and hematoxylin, respectively. All steps in the staining process were done at room temperature in an Autostainer Universal Staining System (Dako). The negative controls included tissue sections from BCCV-infected cotton rats stained with normal (nonimmune) rabbit serum and tissue sections from sham-inoculated (uninfected) cotton rats stained with the anti-BCCV immune rabbit serum. The prevalence of antigen-positive cells in each of 10 randomly selected medium-power ($\times 200$) fields in each tissue section was scored: 1 ($\geq 1\%$ but $< 25\%$), 2 ($\geq 25\%$ but $< 50\%$), and 3 ($\geq 50\%$). The median score for the 10 randomly selected high-power fields was used for analysis.

Results

The median titer of the high-dose inocula (i.e., $1\% \text{ v/v}$ suspensions of the stock virus) was $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ (range: $3.1\text{--}3.5\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$). Thus, each animal was inoculated with approximately 3.1 , 1.1 , -0.9 , or $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ in a volume of $0.2\ \text{mL}$.

No sign of illness was observed in any of the 52 virus-inoculated animals or 4 sham-inoculated animals. Further, the brains, viscera, thoracic cavities, and abdominal cavities of the virus-inoculated animals and sham-inoculated animals were macroscopically unremarkable at necropsy.

A total of 416 specimens from 52 animals inoculated with BCCV were assayed for hantavirus, using monolayer cultures of Vero E6 cells. Hantavirus antigen was found in cell spots from 122 (29.3%) of 416 cultures harvested on day 13 PI and 156 (37.7%) of 414 cultures harvested on day 26 PI. Only one specimen (an OP swab from an animal inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 18 PI) was antigen positive on day 13 PI and antigen negative on day 26 PI. The assays on OP swabs from two animals were discarded because the cultures were heavily contaminated with fungus. Overall, the results of the IFAT from the cell spots prepared on day 26 PI increased the number of culture-positive animals by 6: 1 inoculated with $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, 2 inocu-

lated with $-0.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, and 3 inoculated with $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$.

Hantavirus was isolated from all of the animals inoculated with 3.1 or $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, 8 (61.5%) of the 13 animals inoculated with $-0.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, and 7 (58.3%) of the 12 animals inoculated with $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ (Table 1). The culture-positive samples from the 27 animals inoculated with 3.1 or $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ included blood ($n = 13$), brain ($n = 18$), lung ($n = 24$), kidney ($n = 21$), spleen ($n = 23$), liver ($n = 10$), OP swabs ($n = 11$), and urine ($n = 10$). The culture-positive samples from the 15 culture-positive animals inoculated with -0.9 or $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ included blood ($n = 4$), brain ($n = 3$), lung ($n = 7$), spleen ($n = 8$), and kidney ($n = 4$). A majority (64.7%) of the 17 culture-positive blood samples, all of the culture-positive OP swabs, and 9 (90.0%) of the 10 culture-positive urine samples were from animals inoculated with 3.1 or $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 9, 18, or 27 PI. The sequences of the PCR products generated from the kidneys of the animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ were identical to the homologous sequence of the stock virus.

Antibody (IgG) to BCCV was found in 10 (76.9%) of the 13 animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, 10 (71.4%) of the 14 animals inoculated with $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, 1 (7.7%) of the 13 animals inoculated with $-0.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$, and 1 (8.3%) of the 12 animals inoculated with $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ (Table 1). Eight (61.5%) of the 13 animals inoculated with 3.1 or $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 9 or 18 PI, 2 (40.0%) of the five culture-positive animals inoculated with -0.9 or $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 9 or 18 PI, and none of the six culture-positive animals inoculated with -0.9 or $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 54 PI were antibody positive to BCCV. The endpoint antibody titers in the antibody-positive animals inoculated with 3.1 or $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ ranged from 25 to $> 25,600$, and the endpoint antibody titers in the antibody-positive animals inoculated with -0.9 or $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ were 25.

By inoculum dose, the prevalence of infected (antibody and/or culture positive) animals ranged from 100% in the 13 animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and 100% in the 14 animals inoculated with $1.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ to 58.3% in the 12 animals inoculated with $-2.9\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$. Accordingly, the titer of the stock virus in the animals in this study was greater than $8.7\ \log_{10}\ \text{ID}_{50}\text{-Shis}/1.0\ \text{mL}$ (*S. hispidus* [Shis]).

The most striking histological abnormalities in the animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ were found in the lungs of the animals euthanized on day 18 (i.e., Shis-3.1-18a, Shis-3.1-18b, and Shis-3.1-18c) and lungs of the animals euthanized on day 27 PI (i.e., Shis-3.1-27a, Shis-3.1-27b, and Shis-3.1-27d) (Table 2). Tissues from the fourth animal inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ and euthanized on day 27 PI (i.e., Shis-3.1-27c) were not examined microscopically. Diffuse pneumonitis (PML score = 2), focal interstitial edema, and focal alveolar edema were found in Shis-3.1-18a (Fig. 1B and 1C), Shis-3.1-18b, and Shis-3.1-18c. Diffuse pneumonitis (PML score = 2) without pulmonary edema was found in Shis-3.1-27a (Fig. 1D), Shis-3.1-27b, and Shis-3.1-27d. Microscopic examination of the lungs of the other animals inoculated with $3.1\ \log_{10}\ \text{ID}_{50}\text{-VeroE6}$ revealed diffuse pneumonitis (PML score = 1) without edema in one of the three animals euthanized on day 9 PI (Shis-3.1-9b) and diffuse pneumonitis (PML score = 1) in all three animals euthanized on day 54 PI (Shis-3.1-54a, Shis-3.1-54b, and

TABLE 2. INFLAMMATION IN TISSUES OF HISPID COTTON RATS INOCULATED WITH 3.1 log₁₀ MEDIAN INFECTIOUS DOSES_{VERO E6} OF BLACK CREEK CANAL VIRUS, BY DAY POSTINOCULATION

Tissue ^a	Day PI ^b				Total
	9	18	27	54	
Brain	0/2	0/3	2/3 (1)	0/2	2/10
Lung	0/3	3/3 (2)	3/3 (2)	3/3 (1)	9/12
Liver	1/3 (1)	0/3	2/3 (1)	1/3 (1)	4/12
Kidney	0/3	0/3	1/3 (1)	1/3 (1)	2/12
Salivary gland	0/3	0/3	2/3 (1)	1/3 (1)	3/12

^aThe hearts, thymuses, and spleens of the animals were histologically unremarkable. Focal interstitial and alveolar edema as well as diffuse, subacute pneumonitis were found in the lungs of the three animals euthanized on day 18 PI.

^bNumber of animals with inflammation/number of animals examined. The number in parentheses is the median score for the prevalence of mononuclear cells in 10 medium-power (×200) fields in each tissue section in which inflammation was observed: 1 (≥1% but <25%) and 2 (≥25% but <50%).

Shis-3.1-54c). The cellular infiltrates in the lungs of Shis-3.1-18a, Shis-3.1-18b, and Shis-3.1-18c included peribronchial, perivascular, and interstitial aggregates of mononuclear cells with the appearance of small lymphocytes, large (reactive) lymphocytes, and activated macrophages. The cellular infiltrates in the lungs of the other affected animals were similar in appearance to the cellular infiltrates in the lungs of the animals euthanized on day 18 PI but restricted to peribronchial and perivascular aggregates. The cellular infiltrates in some of the animals also included small numbers of plasma cells and an occasional polymorphonuclear cell. The lungs of Shis-3.1-9a and Shis-3.1-9c (both inoculated with 3.1 log₁₀ ID_{50-VERO E6} and euthanized on day 9 PI) and the lungs of the sham-inoculated animals were microscopically unremarkable.

Other notable microscopic abnormalities in the animals inoculated with 3.1 log₁₀ ID_{50-VERO E6} included portal triaditis (PML score = 1) in Shis-3.1-9b, Shis-3.1-27a, Shis-3.1-27d, and Shis-3.1-54c (Table 2). The cellular infiltrates in the livers of these four animals consisted of focal aggregates of mononuclear cells with the appearance of small and large lymphocytes. The livers of the other animals inoculated with 3.1 log₁₀ ID_{50-VERO E6} and the sham-inoculated animals were microscopically unremarkable.

Microscopic examination of representative sections of lung from the 12 animals inoculated with 1.1 log₁₀ ID_{50-VERO E6} revealed diffuse pneumonitis (PML score = 1) in the 3 animals euthanized on day 18 PI and diffuse pneumonitis (PML score = 1) in the 6 animals euthanized on day 27 or 54 PI (Table 3). The cellular infiltrates in the lungs of the affected animals included peribronchial and perivascular aggregates of mononuclear cells with the appearance of small lymphocytes, large (reactive) lymphocytes, and activated macrophages. The lungs of the three animals inoculated with 1.1 log₁₀ ID_{50-VERO E6} and euthanized on day 9 PI were histologically unremarkable.

Hantavirus antigen was found in samples of lung and other solid tissues from 11 of 12 animals inoculated with 3.1 log₁₀ ID_{50-VERO E6} (Table 4). Antigen was found in endothelial cells in large vessels in lung, alveolar wall capillaries, interstitial capillaries in heart, brain, and salivary gland, and glomerular

TABLE 3. PREVALENCE OF PNEUMONITIS IN HISPID COTTON RATS INOCULATED WITH BLACK CREEK CANAL VIRUS, BY INOCULUM DOSE AND DAY POSTINOCULATION

Dose ^a	Day PI ^b				Total
	9	18	27	54	
3.1	1/3	3/3 (2)	3/3 (2)	3/3 (1)	10/12
1.1	0/3	3/3 (1)	3/3 (1)	3/3 (1)	9/12

^a3.1 log₁₀ or 1.1 log₁₀ median infectious doses_{VERO E6} (ID_{50-VERO E6}, infectivity measured in Vero E6 cells).

^bNumber of animals with diffuse, subacute pneumonitis/total number of animals examined. The number in parentheses is the median score for the prevalence of mononuclear leukocytes in each of 10 randomly selected medium-power fields (×200): 1 (≥1% but <25%) and 2 (≥25% but <50%). Focal interstitial and alveolar edema were found in the three animals inoculated with 3.1 log₁₀ ID_{50-VERO E6} and euthanized on day 18 PI, none of the seven other animals inoculated with 3.1 log₁₀ ID_{50-VERO E6}, and none of the animals inoculated with 1.1 log₁₀ ID_{50-VERO E6}.

capillaries in kidney. Antigen was also found in endothelial cells lining hepatic sinusoids, macrophages and plasma cells in spleen and thymus, and occasional glial cells in brain. The most extensive staining of antigen (IHC assay score = 3) was in the samples of lung from the three animals euthanized on day 9 PI. The most intensive staining of antigen was in endothelial cells in alveolar wall capillaries in these three animals (i.e., Shis-3.1-9a, Shis-3.1-9b, and Shis-3.1-9c).

Discussion

Horizontal transmission appears to be the dominant mode of intraspecific hantavirus transmission in naturally infected rodent populations (Meyer and Schmaljohn 2000). A hispid cotton rat's first encounter with conspecifics other than its mother and littermates likely occurs soon after it is weaned.

TABLE 4. PREVALENCE OF HANTAVIRUS ANTIGEN-POSITIVE CELLS IN TISSUES OF COTTON RATS INOCULATED WITH 3.1 log₁₀ ID_{50-VERO E6} OF BLACK CREEK CANAL VIRUS, BY DAY POSTINOCULATION

Tissue ^a	Day PI ^b				Total
	9	18	27	54	
Lung	3/3 (3)	3/3 (1)	2/3 (1)	0/3	8/12
Thymus	1/3 (1)	2/3 (1)	2/3 (1)	1/3 (1)	6/12
Heart	2/3 (1, 2)	3/3 (1)	2/3 (1)	0/2	7/11
Brain	2/2 (1)	3/3 (1)	2/3 (1)	1/2 (1)	8/10
Salivary gland	2/3 (1)	0/3	2/3 (1)	0/3	4/12
Liver	1/3 (2)	2/3 (1)	2/3 (1)	0/3	5/12
Kidney	2/3 (2)	1/3 (1)	2/3 (1)	0/3	5/12
Spleen	2/3 (1)	3/3 (1)	1/3 (1)	0/3	6/12
Total	3/3	3/3	3/3	2/3	11/12

^aHantavirus antigen was not found in the samples of pancreas, adrenal gland, ovary, or gastrointestinal tract tissues from the 12 animals inoculated with 3.1 log₁₀ ID_{50-VERO E6} of Black Creek Canal virus.

^bNumber of hantavirus antigen positive/number of examined. The numbers in parentheses are the median scores for the prevalence of antigen-positive cells in 10 medium-power (×200) fields in each antigen-positive tissue section: 1 (≥1% but <25%), 2 (≥25% but <50%), and 3 (≥50%).

FIG. 1. High-power (400 $\times$) images of lung tissue from four hispid cotton rats infected with Black Creek Canal virus and an uninfected hispid cotton rat. The infected animals, that is, cotton rats 3.1-9b, 3.1-18a, 3.1-27a, and 3.1-54b, were inoculated subcutaneously with 3.1 log₁₀ median infectious doses (ID_{50-VeroE6}) of virus and killed on day 9, 18, 27, or 54 postinoculation, respectively. (A) 3.1-9b, focal interstitial pneumonitis. (B, C) 3.1-18a, moderate interstitial pneumonitis with (B) perivascular cuffing of inflammatory cells and (C) focal alveolar edema. (D) 3.1-27a, marked perivascular and interstitial inflammation. (E) 3.1-54b, focal (peribronchiolar) inflammation. (F) The uninfected (sham-inoculated) hispid cotton rat. The tissues were sectioned at 4 μ m and then stained with hematoxylin and eosin.

This assumption was the basis for using 21-day-old (recently weaned) animals in this study. We note that hispid cotton rats can be weaned at 15 days of age with no apparent effect on growth rate (Meyer and Meyer 1944, Keys 1958) and that the hispid cotton rats in this study were weaned at 17 days of age.

The results from the animals inoculated with 3.1 log₁₀ ID_{50-VeroE6} of BCCV in this study are highly similar to the results of a previously published study in which 5-week-old hispid cotton rats were inoculated subcutaneously with 3.0 log₁₀ ID_{50-VeroE6} of BCCV (Hutchinson et al. 1998) and laboratory studies on the pathogenesis of other hantaviruses in their respective principal hosts (Lee et al. 1981, Yanagihara

et al. 1985, Botten et al. 2000, Fulhorst et al. 2002). The similarities include transient viremia, disseminated infection, early and oftentimes vigorous antibody (IgG) response to BCCV, chronic viruria, and absence of clinically apparent disease.

Collectively, the results of this study indicate that inoculum dose can significantly affect the pathogenesis of BCCV infection in juvenile hispid cotton rats. For example, the six animals inoculated with 3.1 or 1.1 log₁₀ ID_{50-VeroE6} and euthanized on day 54 PI were culture positive and antibody positive, whereas the six animals inoculated with -0.9 or -2.9 log₁₀ ID_{50-VeroE6} and euthanized on day 54 PI were culture positive but antibody negative.

The absence of a measurable antibody (IgG) response to BCCV in the animals inoculated with -0.9 or $-2.9 \log_{10}$ ID_{50-VeroE6} and euthanized on day 54 PI suggests that the levels of virus (antigen) in these animals were too low to stimulate a humoral antibody (IgG) response during the 54-day study period. Perhaps these animals would have developed measurable levels of IgG to BCCV at some point after day 54 PI.

The majority of published studies on the ecology of hantaviruses have utilized antibody (IgG) data to estimate the incidence and/or prevalence of infections in rodent populations. The results of this study indicate that inoculum dose can affect the length of the period between exposure and seroconversion in hispid cotton rats and suggest that inoculum dose can affect the proportion of infected hispid cotton rats that develop a measurable IgG response to BCCV. As such, the incidence of seroconversions may significantly underestimate the incidence of infections in a rodent population, especially when the first-capture and follow-up blood samples are separated by short periods (i.e., a few months). Further, seroprevalence may significantly underestimate the prevalence of infected animals in a rodent population.

The results of this study indicate that Vero E6 cells grown in monolayers are not particularly susceptible to BCCV infection when compared to juvenile hispid cotton rats. (Recall that the titers of the stock virus in Vero E6 cells and juvenile hispid cotton rats in this study were $5.8 \log_{10}$ ID_{50-VeroE6}/1.0 mL and $8.7 \log_{10}$ ID_{50-Shis}/1.0 mL, respectively). Hypothetically, some of the culture-negative specimens in this study were virus positive, but the titers of BCCV in these specimens were simply too low to detect by cultivation in monolayers of Vero E6 cells. An appropriate test of this hypothesis would entail exposure of juvenile hispid cotton rats to culture-negative specimens from BCCV-infected hispid cotton rats.

The majority of the culture-positive OP swabs and culture-positive urine samples were from animals inoculated with 3.1 or $1.1 \log_{10}$ ID_{50-VeroE6} and euthanized on day 9 or 18 PI, suggesting that the amount of virus shed in secretions and excretions is related to duration of infection. The failure to isolate virus from the OP swabs and urine samples from the animals inoculated with -0.9 or $-2.9 \log_{10}$ ID_{50-VeroE6} could be interpreted as evidence that the amount of virus shed in secretions and excretions is related to inoculum dose as well as duration of infection. Alternatively, the failure to isolate virus from the OP swabs and urine samples from the animals inoculated with -0.9 or $-2.9 \log_{10}$ ID_{50-VeroE6} could signify that virus shedding in these animals was very short-lived or never occurred during the 54-day study period.

As stated previously, chronic infections in rodents appear to be critical to long-term maintenance of hantaviruses in nature. The isolation of hantavirus from all 12 virus-inoculated animals euthanized on day 54 PI indicates that BCCV infections in juvenile hispid cotton rats can be chronic, regardless of inoculum dose. Whether age of rodent at onset of infection, route of exposure, and/or physiological status and other host factors can significantly affect the duration of infection, duration of virus shedding, or magnitude of virus shedding in BCCV-infected hispid cotton rats has not been investigated.

The results of the IHC assays for hantavirus antigen in tissues from the animals inoculated with $3.1 \log_{10}$ ID_{50-VeroE6} indicate that BCCV infections in juvenile hispid cotton rats

can be widely disseminated. The isolation of hantavirus from samples of brain, lung, spleen, and kidney from other animals in this study suggests that BCCV infections in juvenile hispid cotton rats infected by exposure to low doses of virus (i.e., $\leq 1.1 \log_{10}$ ID_{50-VeroE6}) can be systemic too.

The results of this study indicate that BCCV in experimentally infected hispid cotton rats can cause diffuse pneumonitis and that the severity of the pulmonary pathology is dependent upon duration of infection as well as inoculum dose. Note that the severity of the pneumonitis in the animals inoculated with $3.1 \log_{10}$ ID_{50-VeroE6} peaked between days 9 and 54 PI and that the pulmonary pathology in the animals inoculated with $3.1 \log_{10}$ ID_{50-VeroE6} and euthanized on day 18 or 27 PI was more severe than the pneumonitis in the animals inoculated with $1.1 \log_{10}$ ID_{50-VeroE6} and euthanized on day 9, 18, 27, or 54 PI. Further work is needed to determine whether BCCV in naturally infected hispid cotton rats is pathogenic, and, if so, whether BCCV has a negative effect on the fitness of infected hispid cotton rats compared to uninfected counterparts (Kallio et al. 2007).

A previously published study (Lundkvist et al. 1997) demonstrated that adaptation of a rodent-associated hantavirus (i.e., Puumala virus) to growth in Vero E6 cells can reduce the infectivity of the virus in its principal host. Hypothetically, $3.1 \log_{10}$ ID_{50-VeroE6} and even lower doses of wild-type BCCV from naturally infected hispid cotton rats are more pathogenic than $3.1 \log_{10}$ ID_{50-VeroE6} of BCCV adapted to growth in Vero E6 cells.

The results of previously published laboratory studies suggested that SNV and other New World hantaviruses are apathogenic in their respective principal hosts; however, these studies either did not entail microscopic examination of tissues from the experimentally infected rodents (Hutchinson et al. 1998, Fulhorst et al. 2002) or were restricted to very low inoculum doses (Botten et al. 2000, 2003). Hypothetically, these hantaviruses are pathogenic in their respective principal hosts and infection can negatively affect the fitness of naturally infected rodents.

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Disclosure Statement

No competing financial interests exist.

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