PATHOGENESIS OF A NORTH AMERICAN HANTAVIRUS, BLACK CREEK CANAL VIRUS, IN EXPERIMENTALLY INFECTED SIGMODOH HISPIDUS

KAREN L. HUTCHINSON, PIERRE E. ROLLIN, AND CLARENCE J. PETERS

Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract. This report describes the first detailed analysis of the replication, persistence, and excretion of a North American hantavirus in its natural rodent reservoir. Black Creek Canal virus was isolated from Sigmodon hispidus (cotton rat) shortly after the identification of a hantavirus pulmonary syndrome (HPS) case occurring in southern Florida. Six-week-old male cotton rats were inoculated subcutaneously with 1,000 tissue culture infectious doses. Viral complementary RNA (vRNA) was quantified as a means of determining the site(s) of viral activity (transcription and replication). In the first few weeks post inoculation (pi), vRNA was detectable in every tissue examined except blood. The quantities of vRNA decreased over time, and by five months pi it could be detected only in the brain. In addition to using a quantitative polymerase chain reaction (QPCR) as a means of measuring viral replication/transcription, attempts were made to reisolate virus from all tissue samples taken. Virus could be isolated from every solid tissue examined, and the titers appeared to decrease over time, similar to the QPCR results. However, in contrast to the QPCR results, infectious virus was still routinely detectable at low levels in adrenal gland, liver, kidney, and testicle 150 days pi. Although results of testing for vRNA in the blood were uniformly negative, infectious virus was detected at one week pi, reached highest titers at two weeks, and decreased dramatically by three weeks. After three weeks pi, infectious virus could only be detected sporadically in blood. Virus was isolated from urine collected during the first 70 days pi and throughout the entire study period in feces and wet bedding. These data indicate that the viral infection can be separated into an acute phase associated with high virus titers, and a chronic or persistent phase associated with lower virus titers and continued shedding of virus in excreta.

Hantaviruses are enveloped RNA viruses of the genus Hantavirus, family Bunyaviridae. Similar to other Bunyaviridae, the genome of hantaviruses consists of three negative-strand RNA segments, large (L), medium (M), and small (S), each of which is complexed with the nucleoprotein (N) to form individual nucleocapsids. Several members of the genus Hantavirus, including Han- taan (HTN), Seoul (SEO), Dobrava (DOB), and Puumala (PUU) viruses, are associated with hemorrhagic fever with renal syndrome (HFRS) of varying severity, ranging from HTN virus infection with 5–15% mortality to PUU virus infection with less than 1% mortality. These diseases are currently recognized throughout Asia, the former Soviet Union, the Balkans, and northern Europe. In 1993, a new illness associated with hantavirus infection, hantavirus pulmonary syndrome (HPS), was described. The symptoms of HPS include an abrupt onset of fever, headache, myalgias, and cough, followed by a rapid development of respiratory failure. Since the identification of Sin Nombre (SN) virus as the major etiologic agent of HPS in North America, other related hantaviruses have also been associated with HPS. Black Creek Canal (BCC), Bayou (BAY), and New York (NY) viruses are associated with HPS occurring in North America. More recently, HPS has also been identified in some South American countries, including Paraguay, Argentina, and Chile.

Each hantavirus is primarily associated with a specific rodent species; for example, HTN virus is associated with Apodemus agrarius, PUU virus with Clethrionomys glareolus, SEO virus with Rattus norvegicus, SN virus with Peromyscus maniculatus, BCC virus with Sigmodon hispidus, and NY virus with Peromyscus leucopus. Field observations and the ability to isolate and/or detect virus from wild-caught rodents even in the presence of high levels of serum antibody led to the belief that hantaviruses cause chronic asymptomatic infections in their reservoir hosts. Previous studies have shown that A. agrarius and C. glareolus infected in the laboratory harbored virus in many tissues throughout the length of the study and were capable of transmitting virus to other naive animals. In these studies, it was possible to persistently infect adult rodents, whereas other experiments found an age-related pattern of infection with SEO virus in R. norvegicus. In these experiments, newborn animals were more easily infected and transmitted virus more efficiently than adults. Taken together, the previous studies suggest that we can expect to find many similarities in the pathogenesis of each hantavirus in its reservoir host. However, the dynamics of infection and transmission may differ among the different hantavirus/reservoir pair. We present here the first experimental studies on the pathogenesis of a North American hantavirus, BCC virus, in its reservoir (S. hispidus).

MATERIALS AND METHODS

Virus. The BCC virus used in this study was originally isolated from the lung of an infected cotton rat (S. hispidus) trapped near the residence of a HPS case in southern Florida. After its initial isolation, the virus was passaged three times in Vero E6 (E6) cells in an attempt to increase the viral titer. Infectious virus titers were obtained by infecting E6 cells with \log_{10} dilutions of virus and using an indirect fluorescent antibody (IFA) procedure to determine the tissue culture infectious dose (TCID).

Animal inoculations and sample collection. Male four-week-old cotton rats (S. hispidus) were obtained from Virion Systems Inc. (Rockville, MD). After the animals had recovered from the stress of shipment (one week), they were transferred into the biosafety level 4 (BSL 4) laboratory where they were allowed an additional week to acclimate to their
new surroundings. They were individually anesthetized with metofane, weighed, prebled from the retro-orbital plexus, and inoculated subcutaneously with 1,000 TCIDs of BCC virus diluted in 0.5 ml of sterile saline. Negative controls were treated exactly the same except they were inoculated with 0.5 ml of sterile saline. Due to the extremely aggressive behavior of male cotton rats, the animals were housed in individual cages covered with polyester filler bonnets and placed inside a Laminar Flow isolator (Lab Products, Maywood, NJ). All animals were bled weekly for the first four weeks postinoculation (pi). Feces and wet bedding, in a location away from the water bottle, were collected for viral quantification. On days 7, 14, 21, 50, 70, 115, and 150 pi, three animals were bled by cardiac puncture and killed, and the brains, salivary glands, thyroid glands, lungs, hearts, livers, spleens, adrenal glands, kidneys, and testicles were removed and individually frozen at −70°C. Urine was also collected directly from the bladder with a sterile syringe to measure virus in the urine.

**Virus isolation and titration.** The frozen tissues were thawed once, homogenized in an equal volume of minimal essential medium containing Earle’s salts, 2% fetal bovine serum (FBS), 0.29 mg/ml of L-glutamine, gentamicin (0.2 mg/ml), and amphotericin B (MEM-M). Homogenates were diluted to 10% in MEM-M. Two hundred microliters were used to determine infectious virus titers. Serial 10-fold dilutions of the 10% tissue suspensions were made in MEM-M and 0.5 ml of the dilutions were inoculated onto E6 monolayers in 25-cm² plastic tissue culture flasks. Virus was allowed to adsorb to the cells for 1 hr, followed by the addition of 5.0 ml of MEM-M. Cells were incubated at 37°C in 5% CO₂. Medium was changed on day 7. On day 14, 0.2 ml of cell supernatant was removed from the tissue culture flasks and used to infect cell monolayers on 16-well chamber slides (Nunc Inc., Naperville, IL). The medium was changed on days 3 and 9. On day 14, the cell monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.2, fixed in cold acetone, air-dried, gamma irradiated (1 × 10⁶ rads), and tested for the presence of BCC antigen by an IFAT test (IFAT), using an anti BCC virus primary antibody, which had been prepared by inoculation of adult cotton rats with purified BCC virus. The sera did not react with uninfected E6 cells nor with samples from uninfected rats. A goat anti–*Peromyscus leucopus* (a sigmodontine rodent) IgG fluorescein isothiocyanate conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody. The virus titers are presented as the TCID/ml of tissue homogenate and are the geometric means of all positive samples.

**Purification and quantification of RNA.** The slow and rather inefficient growth of the HPS-causing hantaviruses in tissue culture cells precludes the use of more conventional methods of measuring replication. We therefore used a quantitative polymerase chain reaction (QPCR) to measure viral RNA as an indicator of viral activity (transcription and replication). The cDNA synthesis step was carried out using a genomic sense primer specific for nucleocapsid viral complementary (vcRNA) (mRNA plus antigenomic RNA). Since vcRNA is not packaged into mature virions (Hutchinson KL, unpublished data), we were confident that the QPCR method was an accurate and sensitive method for identifying the sites of viral activity.

The RNA was purified using the acid-guanidinium isothiocyanate-phenol chloroform method as previously described. Viral nucleocapsid vcRNA was quantified using an electrochemiluminescence detection system, QPCR System 5000 (ABI/Perkin-Elmer Corporation, Norwalk, CT) as reported for the closely related hantavirus SN. Since the PCR primer set developed for the QPCR of SN virus nucleocapsid RNA also amplified BCC virus RNA, the same primers and T7 transcripts used for SN virus were used in this study. After amplification, the PCR product was detected and quantified by hybridization to an electrochemiluminescence [tris(2,2'-bipyridine)-ruthenium(II) chelate] (TBR) labeled probe (5’-(TBR)-AAA CCT GAT CCA ACA GGG ATT GAI C-3’). The hybridized product was captured on streptavidin-coated magnetic beads. The bead-bound product was collected by an external magnet, washed to remove any unincorporated TBR-labeled probe, and stimulated by an electrochemical reaction to a high-energy state. Light, released upon relaxation to the ground state, was detected using a photomultiplier tube and converted to a digital output. The amount of PCR product was quantified directly by the integrated relative luminosity. Serial dilutions of RNA transcripts synthesized in vitro were amplified to form a standard curve, allowing interpolation of the amount of viral RNA present in the samples. Each RNA sample was run in triplicate reactions, and each reaction was measured in triplicate by the QPCR machine. The results are presented as the geometric means of all the positive replicates for a given date.

The QPCR of viral genomic RNA was carried out as above with the exception that a + sense primer specific for the genomic S segment RNA was used in the cDNA synthesis step.

**Seroology.** Sera collected throughout this experiment were screened for the presence of IgG against BCC virus by an IFAT and by an ELISA. In the IFAT procedure, confluent monolayers of E6 cells grown on T75 tissue culture flasks were infected with BCC virus. Cells were harvested by scraping the monolayers 14 days pi, cells were washed with sterile PBS, resuspended in 90% FBS, and gamma irradiated with 5 × 10⁶ rads. Irradiated cells were stored at −70°C. Cells were thawed, washed with PBS, and spotted onto Teflon-coated, 12-well slides. The slides were air-dried and fixed in cold acetone. Sera were diluted four-fold (1:25–1:6,400) in PBS, pH 7.2, and placed in individual wells of the slides. Slides were incubated for 1 hr at 37°C in an humidified chamber and then washed twice for 10 min each with PBS, pH 7.2. Fluorescein isothiocyanate–conjugated goat anti–*Peromyscus leucopus* IgG was added to the slides, which were then incubated for an additional hour at 37°C in an humidified chamber. Slides were washed as above and mounted in 10% glycerol in PBS, pH 7.2. Slides were examined using a Axioskop microscope (Zeiss, Oberkochen, Germany). Samples were considered positive if 30% or more of the cells were fluorescent. Negative controls included sera from un inoculated animals as well as uninfected E6 cells incubated with a known positive serum.

The ELISA for viral IgG was performed as previously
described.\textsuperscript{21} Polystyrene chloride microtiter plates were coated overnight at 4°C with antigen (Ag) derived from a basic buffer-detergent extract of E6 cells infected with BCC virus (BCC Ag) or uninfected E6 cells (E6 Ag), both of which had been subjected to gamma irradiation (5 × 10^6 rads). Whole blood specimens were serially diluted in 5% skim milk in PBS-Tween and allowed to react with the Ag-coated wells. A mixture of goat anti-Rattus and goat anti-\textit{Peromyscus} IgG conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories) was used to detect bound IgG. Optical densities (ODs) were measured at 410 nm, and adjusted OD values were obtained by using the equation: OD of BCC-Ag-coated wells minus OD E6-Ag-coated wells.

\textbf{Biosafety.} All animal work and subsequent work with infectious virus was carried out inside a BSL 4 laboratory. Blood samples and chamber slides were double bagged in heat-sealed bags and passed to the outside through a tank of 3% Lysol® (Reckitt and Colman, Inc., Montvale, NJ). Before being opened, the bags were placed in a container of dry ice and treated with gamma irradiation (5 × 10^6 or 1 × 10^7 rads, respectively).

\section*{RESULTS}

\textbf{Measurement of viral replication using QPCR.} Initially, BCC virus appeared to replicate systemically in the cotton rat, since the levels of S segment vcRNA were high in every tissue examined except blood. The levels of vcRNA were highest the first two weeks pi, with the exception of the salivary gland in which the RNA level peaked at seven days pi and rapidly decreased but remained detectable, at low levels, for 115 days. Viral replication persisted in every solid tissue examined for the first 50 days of the study, after which vcRNA levels in the testes became undetectable. After 70 days pi, vcRNA levels were still high in the brains; still detectable, albeit in greatly reduced amounts, in the salivary glands, hearts, spleens, and kidneys; but could no longer be detected in the thyroid glands, lungs, livers, or adrenal glands. Interestingly, at 150 days pi vcRNA levels were still high in two of three brains examined but could not be detected in any other tissue (Figure 1). We were unable to detect vcRNA in any of the blood samples (Figure 1).

\textbf{Presence of infectious virus.} Despite our inability to detect vcRNA in the blood, infectious virus was present. There was a short period of viremia peaking on day 14, in which virus was isolated from 100% of the rodents (Figure 2). Virus titers decreased very rapidly and by 21 days pi virus could be detected only at low titers in 20% of the animals. Although we were unable to detect virus in the blood, between 50 and 115 days pi, we were able to isolate virus from one rodent on day 150 (Figure 2).

Similar to the QPCR data, the virus titers also peaked within the first 21 days, and infectious virus was recovered from every tissue examined (Figure 2). Between 21 and 50 days pi, there was a dramatic reduction in infectious virus titers in all tissues examined. However, the production of infectious virus persisted, at low titers, for the duration of the study in most organs, most notably the brains, salivary glands, lungs, livers, kidneys, and testicles (Figure 2). The persistence of infectious virus through 150 days pi was unexpected since we were able to detect vcRNA only in the brains (compare day 150 in Figures 1 and 2). At no time were there any obvious signs of infection (no weight loss, no increase in mortality, no ruffling of the fur, nor any apparent abnormalities of motor function or behavior) in any of the rodents.

\textbf{Correlation between amount of viral RNA and amount of infectious virus.} To assess if QPCR could detect periods of viremia and to determine how closely the QPCR data correlated with the virus isolation data, we quantified viral genomic RNA in the blood. As shown in Figure 3, not only can the QPCR method be used to determine viremia, but the quantitation results mirror those found by virus isolation.

\textbf{Recovery of infectious virus from feces, urine, and bedding.} Our attempts to house more than one male cotton rat in a cage were unsuccessful because of the aggressive and cannibalistic nature of male cotton rats; therefore, the direct examination of viral transmission in this study was not feasible. We did, however, document shedding of virus in the feces and urine. Infectious virus could be detected in urine obtained from the bladder beginning on day 7. The titer increased from 100 TCID/ml on day 7 to a minimum of 10,000 TCID/ml on day 21 and remained high until decreasing to lower levels after day 50 (Figure 4). Infectious virus, TCID titer > 200, could also be detected in the feces and wet bedding throughout the study period. While vcRNA could be detected on days 21 and 50 pi in urine obtained directly from the bladder, it was not detectable at any time in the feces.

\textbf{Antibody response.} Antibodies were first detectable by using the IFAT two weeks pi and rapidly increased thereafter. The production of antibodies as measured by ELISA was delayed by 1–2 weeks. However, high titers were reached by 50 days pi, and remained at high levels in all animals (Figure 5).

\section*{DISCUSSION}

Rodents serve as the natural reservoirs for hantaviruses, and each different hantavirus is primarily associated with a single rodent species. One of the hallmarks of hantavirus association with its rodent reservoir is the establishment of a chronic, asymptomatic infection in the host animal. Since human infections are believed to occur through close contact with infected rodents or their excreta, an understanding of the dynamics of hantavirus infection in and shedding from rodents is necessary for a thorough understanding of the epidemiology and pathogenic mechanisms of HPS.

To obtain insight into the dynamics of hantavirus infections occurring in the natural rodent population, it was important for us to design our experimental studies to correspond as much as possible to the data available on infections occurring in the field. In the wild, the prevalence of hantavirus antibodies is associated with increasing mass (age) in \textit{P. maniculatus},\textsuperscript{22, 21} \textit{Sigmodon hispidus} (Glass GE, unpublished data), and \textit{R. norvegicus}.\textsuperscript{24, 25} Unlike \textit{C. glareolus}\textsuperscript{26} and \textit{R. norvegicus},\textsuperscript{24, 25} in which prevalence is similar between the sexes, in \textit{S. hispidus} prevalence is much more prevalent among males than females (Glass GE, unpublished data), as is the case with \textit{P. maniculatus} and \textit{Reithrodontomys megalotis}.\textsuperscript{22} One possible explanation for the higher prevalence of infected males could be that the females are less suscep-
tible to infection. Another possibility is that because female cotton rats are much less aggressive than males, they would be less likely to become infected, assuming transmission occurs through fighting and wounding, as has been suggested for *R. norvegicus.*

One of the first questions we wished to address was where does the virus replicate and do the sites of virus replication change with time? We have been unable to plaque any of the North American hantaviruses directly from animal tissues. This was true even when animals were inoculated with virus previously adapted to growth in tissue culture cells. Because of the difficulty of using more conventional methods to examine viral replication, we chose a QPCR approach that would allow us to measure viral transcription/replication by quantifying vcRNA. As early as seven days pi, vcRNA could be detected in every tissue examined (Figure 1), suggesting that the viral replication, at least in an acute infection, occurs systemically. It is interesting that vcRNA could be easily detected in the testicles during the first 50 days pi, suggesting a possibility for venereal transmission.

While we were confident that our QPCR data reflected where the virus is actively replicating, this method cannot be used to distinguish between a productive infection resulting in infectious virus and an abortive infection resulting...
FIGURE 2. Recovery of infectious virus from inoculated *Sigmodon hispidus*. Virus titers were measured as tissue culture infectious dose/ml of tissue homogenate. Results are median of positive samples. The numbers above each bar denote no. positive/no. tested. Due to limited space in tissue culture incubators, tissue homogenates were not diluted past 1:10^6; therefore, values of 10^6 should be read as ≥ 10^6.

in the accumulation of viral RNA with little or no infectious virus being produced. Since reisolation of BCC virus was possible, we determined how the quantification of vcRNA compared with the production of infectious virus. This information would be exceedingly useful when examining other hantavirus/rodent infections in which it may not be possible to reisolate virus.

Infectious virus was readily detected in blood samples taken early after inoculation, but vcRNA was not found indicating that circulating virus was not cell-associated nor did it contain significant quantities of non-virion sense RNA. Viremia began by day 7 (15 of 19 positive) and peaked by day 14 when 100% (16 of 16) of the animals were positive for virus in the blood (Figure 2). The levels of virus rapidly decreased such that by day 21, circulating virus was present only in 20% (3 of 15) of the animals. No infectious virus was recovered from blood samples taken 50–115 days pi. Exceptionally, infectious virus (100 TCID/ml) was present in the blood of one rat 150 days pi, suggesting sporadic periods of viremia may occur (Figure 2).

As can be seen in Figure 2, there are some similarities between the amount of vcRNA in different tissues and the ability to recover infectious virus. Virus was isolated from every tissue type examined and the titers were highest during
Figure 3. Correlation between the quantity of genomic small (S) segment RNA and ability to reisolate virus from whole blood. Each point corresponds to the geometric mean of all positive samples.

Figure 4. Presence of virus in urine. Urine was taken directly from bladders of killed animals and pooled. Virus titers were measured as tissue culture infectious dose/ml of urine. Due to limited space in tissue culture incubators, urine samples were not diluted past $10^4$; therefore, values of $10^4$ should be read as $\geq 10^4$.

Figure 5. Antibody response as measured by ELISA. Results are the median values of all positive animals.

The first 21 days pi. Since there was also a high titer of virus circulating in the blood during the first 21 days pi, it was not possible to differentiate between virus circulating through the tissues and virus coming from the different tissues. At day 21 and beyond, when little or no virus was isolated from blood, it was possible to make this distinction. A dramatic decrease was observed in the amount of infectious virus recovered on day 50, at which time infectious virus was either undetectable or detectable at very low levels (Figure 2). This decrease in virus titers corresponds to the presence of high titers of circulating ELISA antibodies directed against BCC virus (Figure 5). It is possible that the cell-mediated immune response against the virus is also occurring during this period. At 70 days pi, the amount of virus recovered increased in most tissues and often persisted throughout the study period. From these data we believe the infection of cotton rats with BCC can be divided into an acute phase that ends between 21 and 50 days pi, and a chronic or persistent phase that begins between 50 and 70 days pi.

In view of the fact that we were unable to detect vcRNA in any tissue except the brain on day 150, we were surprised to isolate virus from most tissues (Figure 2). In designing the QPCR protocol, we chose conditions that would allow us to quantify a large range of RNA amounts and not conditions that would give maximum sensitivity. Therefore, while it is possible to use a QPCR method to measure viral replication, the absence of detectable RNA, using these PCR conditions, does not necessarily mean the virus has been cleared. We are currently trying newer QPCR procedures in an attempt to increase sensitivity.

After determining that we could use the QPCR method as a means of measuring viral replication, we documented that if a primer specific for viral genomic RNA was used in the synthesis of cDNA, it could also measure viremia. Additionally, the quantification of viral genomic RNA correlated very well with the production of infectious virus. As can clearly be seen from the results presented in Figure 3, the amount of viral genomic RNA, as measured by QPCR, can be used directly to predict the amount of infectious virus present.

While we were unable in these studies to directly examine viral transmission, we were able to detect virus being shed in the urine and feces. The presence of virus in the urine was determined directly (Figure 4) and indirectly by attempting to reisolate virus from wet bedding. Infectious virus was readily detected in urine taken directly from the bladder during the first 70 days pi, the only period tested. It was also possible to detect vcRNA in the urine taken directly from the bladders on days 21 and 50 pi, suggesting that at least some of the virus was cell associated. Virus was also sporadically isolated from wet bedding and consistently from dried feces, strongly suggesting that the virus was somewhat stable in the environment. In future studies we hope to be able to examine animal-to-animal transmission of BCC virus by pairing males and females of the same age and size. These studies should enable us to examine vertical as well as horizontal transmission, and to determine if excretion, virus titers, and/or transmission is affected by the stress of breeding.

We used two independent techniques (IFA and ELISA) to measure antibody production in these animals. The animals maintained high antibody titers throughout the study period (Figure 5).

In general, the results presented here are in close agreement with earlier studies on HTN virus infections of A. agrarius and PUU virus infections of C. glareolus. Notable differences are that the viremia lasted for a longer period than has been seen in the other studies, and in the PUU virus/C. glareolus studies virus could not be detected in the kidneys.

There is some indication in experimental infections of R.
norvegicus with SEO virus that the susceptibility to infection, especially persistent infections, may decrease with the age of the rats. While we do not dispute that newborn animals may be more susceptible to experimental infections, all the available data on infection of wild rodents would indicate that most rodent infections occur after weaning, as has been shown for PUU virus infections in C. glareolus. SEO virus in R. norvegicus, SN virus in P. maniculatus, and BCC virus in S. hispidus (Glass GE, unpublished data). The inability to obtain persistent infections of adult rats inoculated with SEO virus could likely be due to either the strain of virus used or the passage history of the inoculum. We have previously found that the passage history of SN virus could affect whether the infections were transient or persistent (Hutchinson KL and others, unpublished data).

These data clearly demonstrate that the BCC virus/S. hispidus pairing is a viable system in which to study a North American hantavirus in its natural reservoir. While it has been known for some time that hantaviruses cause chronic/persistent infections in rodents, there is currently no information available on the mechanisms used in the induction of viral persistence. We have recently begun to examine the immune response in these rodents in an effort to define some of the mechanisms used by BCC virus to induce chronic/persistent infections.

References


