RAPID COMMUNICATION

A Lethal Disease Model for Hantavirus Pulmonary Syndrome

J. W. Hooper,*† T. Larsen,† D. M. Custer,* and C. S. Schmaljohn*

*Virology Division and †Pathology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702

Received July 18, 2001; returned to author for revision August 1, 2001; accepted August 9, 2001

Hantaviruses are associated with two human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Development of vaccines and therapies to prevent and treat HFRS and HPS have been hampered by the absence of a practical animal model. Here we report that Andes virus (ANDV), a South American hantavirus, is highly lethal in adult Syrian hamsters. The characteristics of the disease in hamsters, including the incubation period, symptoms of rapidly progressing respiratory distress, and pathologic findings of pulmonary edema and pleural effusion, closely resemble HPS in humans. This is the first report of a lethal disease model for hantaviruses that causes HPS.

In 1993 an outbreak of a previously unrecognized disease occurred in the southwestern United States, characterized by an influenza-like prodrome, followed by rapid onset of severe respiratory distress, pulmonary edema, and shock. The case-fatality rate for the initial outbreak (24 patients, most of whom were healthy young adults) was 50% (1). Serological testing revealed that the patients had antibodies that cross-reacted with antigens from hantaviruses. Sequence analysis of RT-PCR products from autopsy specimens confirmed that the patients were infected with a previously unrecognized hantavirus (2). Virus isolates were obtained from tissues of deer mice (Peromyscus maniculatus) that had been captured in or around the dwellings of patients with severe respiratory illness in New Mexico (Muerto Canyon virus) (3) and California (Convict Creek virus) (4). These viruses were later collectively renamed Sin Nombre virus (SNV), and the disease, hantavirus pulmonary syndrome (HPS). Two years later, a series of HPS outbreaks in Chile and Argentina were reported. The causative agent, from a fatal case, was identified by genetic methods and was named Andes virus (ANDV) (5). ANDV has caused ~400 cases of HPS in Chile and Argentina and is the only hantavirus for which there is persuasive evidence of person-to-person transmission (6). Since 1993, outbreaks of HPS involving ~700 patients, caused by at least 10 different hantaviruses, have occurred throughout North, Central, and South America. Despite intensive hospital care, case-fatality rates for HPS outbreaks remain high (30–45%). There is no vaccine or effective antiviral drug to prevent or treat HPS.

Hantaviruses are members of the genus Hantavirus, family Bunyaviridae (7). These enveloped viruses have a trisegmented, negative-sense, single-stranded RNA genome. The small (S) segment encodes the nucleocapsid protein; the medium (M) segment encodes the surface glycoproteins (G1 and G2), and the large (L) segment encodes the RNA-dependent RNA polymerase. The glycoproteins, G1 and G2, but not the nucleocapsid protein, are targets of neutralizing antibodies (NAbs). Hantaviruses are carried by chronically infected rodents and are transmitted to humans who may inhale aerosolized rodent excreta.

In addition to the HPS-associated hantaviruses, there are four hantaviruses [Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV), and Puumala virus (PUUV)] associated with a disease called hemorrhagic fever with renal syndrome (HFRS). HFRS-associated hantaviruses and their hosts are found predominantly in Europe and Asia, whereas HPS-associated hantaviruses and their hosts are present only in the Americas. The initial influenza-like symptoms are similar between HFRS and HPS; however, these symptoms in HFRS are followed by renal impairment and sometimes by hemorrhagic manifestations such as petechiae. Case-fatality rates of HFRS range from 0.1–15%, depending on the type of hantavirus involved and the care the patient receives. Tens of thousands of HFRS cases are reported each year, mostly in China.

There is no practical disease model to study either HPS or HFRS and this has hampered the development of
vaccines and therapies to prevent and treat these diseases. Hantaviruses cause lethal disease in newborn rodents such as mice (8); however, the disease does not resemble the disease in humans and the age requirement for lethality makes the newborn rodent model unamenable for experiments involving active vaccination. There are reports of subtle histopathologic lesions in some of the natural hosts of certain HPS-hantaviruses; however, there are no overt symptoms of disease and no lethality (9, 10). HFRS-hantaviruses can infect several species of New and Old World monkey species and two ape species but the animals tested remained asymptomatic except for a mild proteinuria and slight rise in liver enzymes (11, 12). The mild disease, as well as economic considerations inherent to nonhuman primate studies, renders these models impractical for most studies.

To evaluate the efficacy of candidate hantavirus vaccines, investigators have used infection models rather than disease models. For example, we have used a hamster infection model to study the immunogenicity and protective efficacy of DNA vaccines against the HFRS-associated hantaviruses (13). In this system, vaccinated hamsters are challenged intramuscularly (im) with a defined dose of hantavirus and 28 days later, serological assays are performed to detect evidence of infection. An infection model is used because HFRS-hantaviruses do not cause disease in hamsters even at doses as high as 20,000 plaque-forming units (PFU).

Results and Discussion. Andes virus is lethal in adult hamsters. To prepare for future studies evaluating vaccines against HPS-hantaviruses, we sought to determine the dose infecting 50% of the hamsters (ID$_{50}$) for the two hantaviruses that are responsible for the overwhelming majority of HPS cases in North and South America, SNV and ANDV, respectively. Groups of six to eight hamsters were injected im with serial 10-fold dilutions (from 20,000 to 2 PFU) of ANDV or SNV. To rule out the possibility that seroconversion was caused by input virus, rather than an active infection, eight hamsters were injected with 20,000 PFU of $\gamma$-irradiated ($3\times10^6$ rad) SNV or ANDV. Our initial plan was to obtain blood 28 days after challenge and then perform serological assays to detect evidence of infection and calculate ID$_{50}$ values. Unexpectedly, hamsters injected with ANDV began to die 11 days postinfection (Fig. 1a). There were no overt clinical changes until less than 24 h before the hamsters succumbed to the viral infection. During the hours before death, the hamsters became moribund and appeared to be in respiratory distress, as indicated by severe dyspnea. Death occurred within 5–18 h after initial signs of distress. Hamsters injected with the highest doses of virus died the earliest (all hamsters injected with $\geq200$ PFU died within 2 weeks of challenge). One hamster injected with 2 PFU of virus died 24 days after challenge. From these data, the lethal dose required to kill 50% of the hamsters (LD$_{50}$) was calculated to be 8 PFU. Infection was not uniformly fatal because two hamsters that survived the challenge showed serological evidence of infection as measured by antinucleocapsid ELISA and a plaque-reduction neutralization test (PRNT). The antinucleocapsid endpoint titers were 12,800 and 6400 and the PRNT$_{50}$ titers were 640 and 320, respectively. From these data, the ANDV ID$_{50}$ was calculated to be 3 PFU. All of the hamsters injected with the irradiated ANDV survived and remained seronegative by both ELISA and PRNT. Thus, our experiment to determine the ANDV ID$_{50}$ resulted in the discovery that ANDV is highly lethal in hamsters. In subsequent experiments, we found that...
Lethality in hamsters is not a general property of HPS-associated hantviruses because the SNV-challenged hamsters showed no symptoms of disease even when injected with a dose as high as $2 \times 10^4$ PFU (Fig. 1a). To look for evidence of infection, the SNV-infected hamsters were bled on day 28 as originally planned and antinucleocapsid ELISA and PRNT antibody titers were elicited in hamsters injected with 2 PFU of virus. The serology data for the groups challenged with the highest and lowest doses of SNV are shown (Fig. 1b). Antibody responses did not depend on the concentration of the input virus because equivalent antinucleocapsid ELISA and PRNT antibody titers were elicited in hamsters injected with $2 \times 10^4$ or 2 PFU of SNV. Thus, SNV readily infected hamsters (ID$_{50} < 2$), but did not cause disease. The molecular basis for the different pathogenesis (in hamsters) exhibited by these two closely related (6) hantviruses, as well as the pathogenesis of other ANDV variants and the other HPS-associated hantviruses, remains to be determined.

To rule out the possibility that a contaminating pathogen, rather than ANDV, was responsible for the observed lethality in hamsters, we prepared a twice-plaque-purified viral stock and compared it with the original virus. Ten hamsters were injected with 2000 PFU of twice-plaque-purified ANDV and 10 hamsters were injected with 2000 PFU of the original ANDV stock. All of the hamsters in both groups died after 10–14 days. The mean time-to-death for the twice-plaque-purified virus, day 11, was slightly earlier than the original virus, day 13 ($P = 0.001$). This difference may be an artifact of small sample size. Alternatively, it could indicate that the original viral stock contains populations of ANDV with different levels of virulence and the twice-plaque-purified virus is at the more virulent end of the spectrum. The lethality of the twice-plaque-purified ANDV indicated it was highly unlikely that a contaminant in the original stock was killing the animals.

Syrian hamsters, similar to humans, appear to be especially vulnerable to lethal ANDV-associated disease. To determine whether other rodent species were susceptible to ANDV, we injected 2000 PFU of the twice-plaque-purified ANDV into ten 8- to 10-week-old female BALB/c mice (NCI, Frederick, MD) by the same method used to inject hamsters. The mice showed no signs of disease, but 9 of 10 were infected as indicated by a positive NAb response 35 days after virus injection (PRNT$_{80\%}$ GMT = 139).

Postmortem examination of hamsters that died as a result of ANDV infection revealed a large volume (3–5 ml) of a semiopaque yellow effusion present within the thoracic cavity (Figs. 2a and 2b). The lungs were variably collapsed in some hamsters. The livers were consistently congested and slightly enlarged in all hamsters. There were no other visible signs of disease.

Histologically, the lung, liver, and spleen had the most significant and consistent changes. All hamsters had a mild to moderate subacute interstitial pneumonia with intraalveolar edema, protein, and fibrin that multifocally filled alveolar spaces and expanded alveolar septal walls (Figs. 2c and 2d). The inflammatory component consisted primarily of macrophages and lymphocytes with lesser numbers of neutrophils within septal walls and surrounded the larger blood vessels with an increased number of alveolar macrophages in alveolar spaces. The edema was often profound around the large blood vessels with markedly dilated lymphatics and expansion of the perivascular interstitium. The lungs were mildly to moderately congested and mesothelial cells were often hypertrophied. There was a mild to moderate subacute hepatitis in all hamsters. Randomly scattered throughout the parenchyma were low numbers of hyperchromatic hepatocytes with irregular cellular borders containing karyorrhectic debris. Increased numbers of lymphocytes were occasionally present surrounding larger vessels. The splenic red pulp of all hamsters was expanded by abundant numbers of large round cells with an increased cytoplasmic to nuclear ratio. The morphology of these cells is consistent with that of previously reported in human cases; however, the histogenesis has not been fully elucidated. There were a variety of other lesions present in this initial group of hamsters. Some of these changes were considered background lesions arising from autolysis after death; others warrant further investigation.

**FIG. 2.** ANDV causes HPS in hamsters. (a) Diaphragm, hamster 210. The arrows outline a prominent fluid line delineated on the diaphragm. Note the congested and slightly enlarged liver. (b) Thoracic cavity, hamster 210. Illustration of the abundant fluid present in the thoracic cavity of hamsters exposed to ANDV. Note the atelectasis of the right lung. (c) Quality control hamster. Normal lung. H&E (X400). (d) Lung, hamster 164. Interstitial pneumonia with prominent intraalveolar edema and fibrin. Note the increased thickness of the alveolar septal walls relative to the normal lung. H&E (X400). (e) Lung, hamster 162. Lung tissue demonstrating antigen staining of endothelial cells in the septal walls and an alveolar macrophage (arrow). EnVision-AP system, anti-SNV rabbit antibody. HistoMark RED chromagen with hematoxylin counterstain (X600). (f) Lung, hamster 164. Blood vessel demonstrating viral antigen within endothelial cells. Vectastain ABC-AP system, anti-ANDV human serum. HistoMark RED chromagen with hematoxylin counterstain (X1000). (g) Liver, hamster 158. Note the diffuse and often punctate staining pattern within hepatocytes. EnVision-AP system, anti-SNV rabbit antibody. HistoMark RED chromagen with Hematoxylin counterstain (X400). (h) Spleen, hamster 162. Antigen-positive cells in the marginal zone. Note the peripheralized staining pattern in some cells (arrow). Vectastain ABC-AP system, anti-ANDV human serum. HistoMark RED chromagen with hematoxylin counterstain (X600).
Immunohistochemistry was performed on the lung, liver, and spleen. Endothelial cells lining blood vessels and alveolar septa and lesser numbers of alveolar macrophages were positive for viral antigen (Figs. 2e and 2f). Midzonal hepatocytes were abundantly stained with antigen (Fig. 2g). Viral antigen in the spleen was present in red pulp macrophages, endothelial cells, and in the marginal zone peripheral to follicular areas. The positive cells in the marginal zone (see Fig. 2h) are consistent with those of antigen-presenting cells; however, immunoidentification of these cells is not yet complete. Side-by-side comparison demonstrated a similar staining pattern for both anti-ANDV human antibody and anti-SNV rabbit polyclonal antiserum, although the anti-SNV serum generally elicited more robust staining.

To evaluate infectious virus in the organs of hamsters that succumbed to ANDV infection, plaque assays were performed on tissues collected at necropsy. Infectious virus was detected in the lungs, liver, kidney, spleen, heart, and small intestine, but not from the large intestine or brain (Fig. 3a). The highest concentration of virus was found in the liver. One liver sample (hamster 206) had a
viral concentration of $3.1 \times 10^8$ PFU/ml, which is a concentration 10–100 times greater than routinely attained in Vero E6 cell culture supernatants. To confirm that the tissue-isolated virus was in fact ANDV, we performed PRNT using human convalescent sera from HPS patients from Argentina. Virus isolated from the liver was completely neutralized by HPS patient serum diluted 1:1280 and the PRNT80% was 5120. Thus, the organs of hamsters that died as a result of infection not only contained ANDV antigen, but also contained high concentrations of infectious virus.

The large volume of pleural effusion that filled the thoracic cavity of the infected hamsters did not contain infectious ANDV at the time of necropsy (Fig. 3a). We tested the pleural effusion for NAb by PRNT from four different hamsters and found all were positive (PRNT80% ranged from 20 to 640, GMT = 160). To monitor the relative levels of serum viremia and NAbs during the course of infection, we collected serum samples at days 0, 5, 8, 12, and 14 after challenge and performed plaque assays and PRNT (Fig. 3b). Infectious virus was first detected in the serum on day 8, but was absent on day 12 or 14. In contrast, NAb was not detected until day 12 or 14. These data suggest that the hamster’s immune system responded to the infection and was capable of neutralizing a transient serum viremia. However, the immune response failed to eliminate infectious virus from the organs (Fig. 3a) and, in most cases, was insufficient to protect against lethal disease. Liver, lung, and spleen tissue homogenates from hamster 164 did not contain infectious virus, but tissue samples from the same organs did contain viral antigen (Fig. 2f). This could indicate that the immune response in this hamster had neutralized the ANDV not only in the serum but also in the organs. An alternative trivial explanation is that the virus in these samples from hamster 164 might have been degraded and inactivated by proteases released after the hamster died, or during sample preparation.

To determine whether or not previous infection with heterotypic HFRS or HPS hantaviruses could confer protection against a lethal ANDV infection, groups of seven to eight hamsters [14- to 16-week-old female Syrian hamsters (Charles River Laboratories, Inc., Wilmington, MA)] were injected im (caudal thigh) with $1000$ ID50 of HTNV, SEOV, DOBV, PUUV, or SNV, which was 2000 PFU for HTNV, SEOV, DOBV, and SNV; 20,000 PFU of PUUV (unpublished data). After 4 weeks; the hamsters were challenged im with 2000 PFU of ANDV. Three hamsters that had survived ANDV infection in an earlier experiment and three naive hamsters were also challenged. Serum samples were collected from all hamsters before ANDV challenge and then from survivors 4 weeks after challenge. All of the hamsters survived except the naive hamsters and two of eight DOBV-infected hamsters (Fig. 4). These data indicated that previous infection with a heterotypic hantavirus elicited protective immunity against ANDV and parenthetically reaffirmed that the hamster-lethal agent was a hantavirus. Whether this protective immunity is directed toward the glycoproteins, nucleocapsid protein, a combination of both proteins, or neither (i.e., innate response) remains to be determined.

To begin to dissect the mechanism of the protective immunity elicited by prior hantavirus infection, we performed PRNT assays on hamster sera collected before

FIG. 4. Previous immunity to homo- or heterotypic hantaviruses protects against an otherwise lethal challenge with ANDV. Serum samples from naive hamsters, hamsters that had survived a previous ANDV challenge, or hamsters previously (28 days) infected with a heterotypic hantavirus were collected and tested for NAb by PRNT. Homotypic NAb titers, as well as ANDV cross-NAb titers, were determined. Hamsters were then challenged with 2000 PFU of ANDV (im). After 28 days, postchallenge serum samples were collected from survivors and ANDV NAb titers were determined. The ANDV-specific NAb PRNT80% titer before and after ANDV challenge, and the homotypic NAb PRNT80% titer before ANDV challenge are shown. Sterile immunity was defined as the absence of ANDV-specific NAb after ANDV challenge. Identification numbers of hamsters that exhibited sterile immunity are bold. A dagger (†) represents death of the hamster, which occurred 10–15 days after challenge.
and after ANDV challenge. Although all of the hamsters had homotypic PRNT titers before challenge, none of the sera cross-neutralized ANDV (Fig. 4), indicating that pre-existing cross-neutralizing antibodies were not contributing to the protective effect. However, all of the protected hamsters had relatively high homotypic PRNT titers before ANDV challenge, and the unprotected hamsters had relatively low homotypic PRNT titers (DOBV-preinfected hamster 2143 and 2147 had PRNT<sub>100</sub>% of <20 and 40, respectively), suggesting that a homotypic PRNT titer is a surrogate marker of cross-protective immunity. Several hamsters demonstrated ANDV NAb titers after challenge, indicating the animals were infected, but not killed, by ANDV. Interestingly, five of eight hamsters previously infected with HTNV, three of eight previously infected with PUUV, and two of eight previously infected with SNV were completely protected against ANDV infection as defined by the complete absence of an ANDV-NAb response after ANDV challenge.

This is the first report of any hantavirus causing lethal disease in an adult nonhuman animal. Not only is ANDV lethal in hamsters, but also the disease resembles HPS in humans (14). Similarities between the ANDV-associated disease in hamsters and HPS in humans are as follows: the clinical symptoms, including severe dyspnea, rapid progression from first symptoms to death [<1 day in hamsters vs 4 days in humans infected with SNV (14)], fluid in the pleural cavity; the histopathology in the lungs and spleen; and the incubation period of 10–24 days in hamsters vs 12–27 days in humans (15). Differences between the hamster and human disease include significant histopathology in the liver and readily detectable infectious virus in hamsters, which are not generally present in HPS patients. This model can be used to investigate the molecular basis of hantavirus disease and might also contribute to our understanding of other diseases that involve vascular leakage. In addition, because this is a lethal disease model, it will be possible to evaluate the protective efficacy of candidate hantavirus vaccines and antiviral drugs in a way never before possible. Our observation that a previous infection with hetero- or homotypic hantaviruses protected against ANDV holds well for development of effective HFRS and HPS vaccines.

Methods. Viruses and cells. ANDV, strain Chile-9717869, HTNV, strain 76-118 (16), SEOV, strain SR-11 (17), DOBV (18), and PUUV, strain K27 (19), and SNV, strain CC107 (4) were propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586). The original ANDV, strain Chile-9717869, stock was kindly provided by T. Ksiazek (Special Pathogens Branch, Center for Disease Control, Atlanta, GA), who isolated the virus from the kidneys and lungs of Oligoryzomys longicaudatus captured by J. Mills (Center for Disease Control, Atlanta) during an outbreak in Chile (20). The original stock had been passaged four times in Vero E6 cells. We prepared a twice-plaque-purified ANDV stock as follows. A hantavirus plaque assay was performed on the original ANDV stock that had been passaged an additional time in Vero E6 cells. A well-isolated plaque was picked with a glass pastu-pipet and transferred to 0.5 ml cEMEM. After several hours at 4°C, the plaque-pick was subjected to three rapid freeze-thaws. This once-plaque-purified virus was then titrated by plaque assay and the plaque-picking procedure was repeated. The entire 0.5 ml of twice-plaque-picked virus was then used to infect a T-25 flask containing Vero E6 cells. After 12 days, the overlying medium was collected. This passage 1 (P1) stock was then used to infect Vero E6 monolayers in four T-150 flasks (multiplicity of infection was ~0.05). After 6 days, the overlying medium was collected, pooled, and this passage 2 (P2) stock was used in challenge experiments.

Intramuscular Injection of Virus into Hamsters. Fourteen- to sixteen-week-old female Syrian hamsters (Charles River) were injected intramuscularly (caudal thigh) with 0.2 ml of virus diluted in phosphate-buffered saline, pH 7.4. Injected hamsters were placed in isolator units (one to four hamsters per cage) within a biosafety level 4 (BSL-4) suite. Hamsters were observed two to three times daily.

ELISA. Antinucleocapsid ELISA were performed as described previously (13). The antigen was a histidine-tagged truncated PUUV nucleocapsid fusion protein expressed from pPUUSXdelta (provided by F. Elgh, SMI, Sweden) in Escherichia coli BL21 (DE3) (Novagen, Madison, WI) and affinity purified in Ni-NTA columns (Qiagen, Valencia, CA).

PRNT. PRNT were performed essentially as previously described (13). Heat-inactivated (56°C, 30 min) serum samples were diluted in Eagle’s minimal essential medium with Earle’s salts (EMEM) containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES pH 7.4; and antibiotics [penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml)] (cEMEM) and then combined with an equal volume (111 μl) of cEMEM containing ~75 PFU of virus and 10% guinea pig complement (Cat. No. ACL-4051, Accurate Chemical and Scientific Corporation, Westbury, NY). This mixture was incubated overnight at 4°C and then a plaque assay was performed as described, using 7-day-old Vero E6 monolayers in sixwell plates. ANDV, HTNV, and SEOV PRNT were stained with neutral red (GIBCO BRL) after 7 days and DOBV, PUUV, and SNV PRNT were stained after 9 days. Plaques were counted 2 days (37°C) after staining.

Plaque Assay. The hantavirus plaque assay was performed as follows. Samples diluted in cEMEM were added, 200 μl per well, to sixwell plates (Costar) containing 7-day-old Vero E6 monolayers. After a 1-h absorption at 37°C, 3 ml overlay medium [Earle’s basal minimal
essential medium, 10 mM HEPES, 0.6% agarose (Sea Kem ME agarose, FMC Bioproducts, Rockland, ME), 8 mM L-glutamine, antibiotics containing 10% FBS and 1× nonessential amino acid mixture (GIBCO) was added to each well. Plates were incubated at 37°C in a CO2 incubator for 7 days (ANDV, HTNV, SEOV) or 9 days (SNV, PUUV, DOBV) and then stained by adding 2 ml/well of overlay medium containing 5% FBS and 5% neutral red solution (GIBCO). Plaques were counted after 2 days at 37°C.

Preparation of Tissue for Histology. Tissues fixed in neutral buffered Formalin were processed and embedded in paraffin according to established protocols (21). Immunolocalization of ANDV in hamster lungs was performed with an alkaline phosphatase procedure (EnVision System; DAKO Corp., Carpinteria, CA or Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA) according to manufacturer’s directions. The primary antibody was an ANDV-specific pool of human sera obtained from HPS cases in Argentina or hyperimmune rabbit sera produced against SNV nucleocapsid. Negative controls included replicate paraffin sections incubated with non-immune human or rabbit serum in place of the primary antibody and naive hamster tissue exposed to the primary antibody and to negative serum. Tissue sections were pretreated in citrate buffer heated to 97°C in a water bath for 30 min. Before adding the primary antibody, we exposed the tissues to a serum-free protein block (DAKO) for 20 min. Both primary antibodies were incubated on the tissue sections for 3 h or overnight at 4°C. A biotinylated goat anti-human IgG/IgM/IgA (Kirkegaard and Perry Laboratories) served as the secondary antibody for the Vector Laboratories system. Goat anti-rabbit and goat anti-mouse antibodies conjugated to an alkaline phosphatase-labeled dextran polymer provided with the EnVision kit served as the secondary antibody for that system. The secondary antibodies were incubated for 1 h (Vector Laboratories) or 30 min (EnVision Kit) at room temperature. Sections were incubated for 50 min in the dark with the chromogenic substrate 6-bromo-2-hydroxy-3-napthoic acid and levamisole to inhibit endogenous alkaline phosphatase (HistoMark RED; Kirkegaard and Perry Labs.). Immunostained sections were counterstained with hematoxylin, dehydrated, and cover slips applied with Permount (Fisher Scientific).

Preparation of Tissue for Virus Titration. Approximately 250 mg of tissue was placed in a microfuge tube containing 0.5 ml cEMEM. Samples were ground by hand with a disposable pestle, subjected to three rapid freeze-thaws, and spun in a microfuge at maximum speed for 5 s to pellet debris. Titers of infectious virus in the supernatants were determined by plaque assay. Viral titers are given as PFU per ml of tissue homogenate.

ACKNOWLEDGMENTS

We thank D. Scott for assistance with the necropsies and critical manuscript review; S. Dunio and A. Schmaljohn for critical manuscript review; and N. Davis, J. Brubaker, L. Miller, R. Mooxley, J. Britton, and B. Thompson for expert technical assistance. We also thank T. Kiasek for providing the ANDV isolate. F. Elgh for providing pFUUSXdelta. C. Rossi for providing anti-SNV nucleocapsid rabbit serum, and P. Padula (Instituto Nacional de Enfermedades Infecciosas, Buenos Aires, Argentina) for providing serum samples from HPS patients in Argentina. All experiments involving the use of ANDV or SNV in animals were performed in USAMRIID’s BSL-4 laboratory and all other work involving infectious hantaviruses was performed in a BSL-3 laboratory at USAMRIID. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

REFERENCES


