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Evaluation of Cotton Rats as a Model for Severe Acute Respiratory Syndrome

D.M. WATTS,[1] C.J. PETERS,[1] P. NEWMAN,[1] N. WANG,[1] N. YOSHIKAWA,[1] C.K. TSENG,[1] and P.R. WYDE[2]

ABSTRACT

Experimental studies were conducted to evaluate two species of cotton rats, *Sigmodon hispidus* and *Sigmodon fulviventer*, as a model for severe acute respiratory syndrome (SARS). Blood and turbinate wash samples, and lung tissue were collected from each animal at different time points after SARS coronavirus (CoV) infection for determining the growth curve of virus, if any, by the standard infectivity assay in Vero E6 cells. In addition, sections of the lung, liver, spleen, and kidney were taken and used for histology analysis. All animals were observed daily for signs of illness, and in some experiments, animals were weighed on the day when they were sacrificed. The results indicated that the cotton rat species, *S. hispidus* and *S. fulviventer*, were not a useful model for either SARS-CoV infection or disease. This observation was supported by the absence of any signs of illness, the failure to consistently demonstrate virus in the blood and tissues, and the absent of any notable histopathology. However, infected animals were capable of producing neuralizing antibodies against SARS-CoV, suggesting the seroconversion did occur. Further studies are warranted to consider other animal species in efforts to find better animal models for the evaluation of SARS-CoV vaccines and antiviral drugs. Key Words: Cotton rats—SARS—Model animal.

INTRODUCTION

SINCE THE OUTBREAK OF SEVERE acute respiratory syndrome (SARS) during 2002–2003, a global research effort has been underway to establish a suitable animal species for modeling human disease (Peiris et al. 2003). Several laboratory animal species have been shown to support the replication of the causative agent, SARS coronavirus (SARS-CoV), but disease similar to that reported in humans was not seen (Roberts et al. 2008, Tseng et al. 2007, McCray et al. 2007). Although these models have proven useful for studying the pathogenesis and for evaluating anti-viral drugs and vaccines, the idea model would be a single suitable animal species (Tseng et al. 2007). Therefore, the objective of the present study was to evaluate two species of cotton rats *(Sigmodon hispidus* and *Sigmodon fulviventer)* with the aim of finding a better model for SARS-CoV infection and human-like SARS disease. Cotton rats *(S. hispidus)* have been documented as a suitable model for other respiratory viral diseases, they can be obtained from commercial sources, and reagents are available for study of the immune response (Niewiesk and Prince 2002, Ottolini et al. 2005, Blanco et al. 2004).

METHODS

Our experimental approach during this study in evaluating cotton rats as potential models for SARS-CoV infection and disease was first to determine if either species was sus-

[1]Department of Pathology, University of Texas Medical Branch, Galveston, Texas.
[2]Department of Molecular Biology, Baylor College of Medicine, Houston, Texas.

ceptible to infection. If susceptible, a second more detailed experiment was performed to determine the clinical signs, biodistribution of virus and pathology, if any, that were associated with infection.

Animals

Male and female inbred *Sigmodon hispidus* used in the first and second experiment were obtained from a breeding colony of unknown generation at the University of Texas Medical Branch (UTMB), Galveston, Texas, and from Virion Systems, Rockville, Maryland, respectively. *Sigmodon fulviventer,* including males and females, were obtained from breeding colonies of unknown generation maintained at Virion Systems Inc. The cotton rats purchased from Virion Systems were certified to be seronegative for adventitious viruses. The animals were housed in large polycarbonate cages, fed a diet of standard rodent chow and water, and used at 6–12 weeks of age. Cotton rats in this age-range weighed approximately 100–150 g; they were matched by age and weight for use in different groups. Animals were anesthetized with isoflurane for all manipulations, and sacrificed by isoflurane asphyxiation for the collection of tissue samples. All animal experiments were approved by the UTMB Institutional Animal Care and Use Committee.

Virus and tissue cultures

The Urbani strain of SARS-CoV at the Vero 2nd passage level (kindly provided by T.G. Ksiazek, Centers for Disease Control and Prevention), was used throughout this study. Vero E6 cells (American Type Culture Collection) were used to prepare SARS-CoV virus stocks and for viral infectivity assays based on the observation of viral specific cytopathic effect as described previously (Tseng et al. 2007). Stocks were prepared by passaging the virus twice in Vero E6 cells at a multiplicity of infection of 0.001 (passage 4) and the titer was expressed as the 50% tissue culture infectious dose ($TCID_{50}$)/mL. The stock virus was aliquoted and stored at −80°C until used the experiments. In addition to using the stock virus as the inoculum for infecting animals and in the neutralization test, a separate aliquot was titrated each time blood and tissue samples were tested to serve as a control for verifying that the infectivity assay was performing properly. All experiments involving infectious virus were conducted in a Biosafety Level 3 Laboratory at UTMB.

Procedures

Cotton rats were inoculated while under isoflurane anesthesia via the intranasal (I.N.) route with 100 μL that contained 10^6 $TCID_{50}$ of SARS-CoV. Two control animals in each experiment were inoculated via the same route with 100 μL each of sterile phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS) and antibiotics. The dose of virus in each experiment was confirmed by titration in Vero cells and expressed as $TCID_{50}$ per mL as described previously (Tseng et al. 2007). Four experiments were conducted, two involving two sources of *S. hispidus* and two involving *S. fulviventer*. In three experiments, animals were pre-designated for sacrifice on days 1, 2, 4, 7, or 10 post-inoculation (P.I.) to obtain selected tissue samples. On days 1 and 10 P.I., four animals were sacrificed, including one negative control and three virus-inoculated animals, and on days 2, 4, and 7 P.I., three virus-inoculated animals were sacrificed. Blood samples were obtained on day 21 from remaining animals for antibody tests based on the dilution of serum that neutralized 100% of a constant dose of SARS-CoV that produced 100% cytopathic effect in Vero cell cultures. In the first *S. fulviventer* experiment, animals were only observed for signs of illness, weighed each day, and blood samples were taken on day 21 for antibody testing.

Blood, turbinate wash samples, and tissues, including lung, heart, liver, spleen, and kidney, were collected from each animal for viral infectivity assay in Vero E6 cells (Tseng et al. 2007). Each blood and turbinate sample was diluted 1:10 in sterile PBS supplemented with 10% FCS and streptomycin and penicillin (100 mg/mL). Tissue samples were transferred to sterile vials; all samples were stored immediately at −80°C until homogenized and tested for virus. When assayed, samples were rapid thawed, and weighed, and homogenized in

sterile PBS supplemented with 10% FCS using a TissueLyser to yield 10% tissue/PBS suspensions. The suspension was clarified by centrifugation and then tested in Vero cells. The virus titer of individual samples was expressed as $TCID_{50}$ per mL of the suspension. In addition, samples of the lung, heart, liver, spleen, and kidney were taken, placed in 10% neutral buffered formalin for 72 h, and then transferred to 70% ethanol for histology analysis as described below. All animals were observed daily for signs of illness, and in some experiments, animals were weighed daily; the remaining animals were euthanized with an overdose of isoflurane via the inhalation route at the end of each experiment.

Histology and IHC

The tissues, including lung, heart, liver, spleen, and kidney, obtained from SARS-CoV inoculated and control animals were processed for histology and immunohistochemistry (IHC) according to published techniques (Tseng et al. 2007, Subbarao et al. 2004). Tissue sections were fixed in 10% buffered formalin for 72 h, transferred to 70% ethanol, and later paraffin embedded. Histopathologic evaluation was performed on deparaffinized sections stained by routine hematoxylin-and-eosin staining. IHC testing for SARS-CoV used a previously described colorimetric indirect immunoalkaline phosphatase method (Tseng et al. 2007) with a rabbit anti-SARS-CoV nucleocapsid protein antibody (Imgenex, catalog no. IMG-548). Normal mouse and goat sera were used as negative controls, and SARS-CoV antigen positive Vero cells were used as positive controls. Primary antibodies were detected with either biotinylated swine anti-rabbit immunoglobulin (DAKO catalog no. E0353) or rabbit anti-goat immunoglobulin (KPL catalog no. 16-13-06) secondary antibodies. Visualization was then achieved by incubation with streptavidin-alkaline phosphatase and naphthol-fast red substrate (DAKO) after counterstaining with Mayer's hematoxylin (Sigma-Aldrich Co., St. Louis, MO).

RESULTS

Experiment 1

Sigmodon hispidus from the UTMB colony was derived from a Florida rat. As a pilot experiment to assess the susceptibility of this species to SARS-CoV infection, a total of 16 animals were inoculated via the intranasal route with 100 μL that contained 10^6 tissue culture $dose_{50}$ ($TCID_{50}$) of SARS-CoV, and two control animals were inoculated with saline supplemented with 10% FCS and antibiotics (Table 1). Six of the animals, including the two controls, died from unknown causes during this experiment. The possibility that they died from SARS-CoV was excluded by the failure to detect virus in necropsied tissues using the Vero cell assay. In addition, a similar pattern of mortality was observed in the UTMB cotton rat colony. Of the 10 animals that survived until day 21 P.I., none showed signs of illness and

Table 1. Summary of Experimental Studies to Evaluate *Sigmodon hispidus* and *Sigmodon fulviventer* as a Model for SARS Disease

Experiment	*Species (n)*[a]	*Signs*[b]	*Weight loss*[c]	*Virus isolation*[d]	*IHC*[e]	*Pathology*[f]	*Antibody*[g]
1	S. hispidus (16)	None	Not done	Not done	Neg	Not done	Not done
2	S. hispidus (15)	None	None	Positive	Neg	None	Negative
3	S. fulviventer (12)	None	Not done	Negative	Neg	Not done	1/80 to 1/360
4	S. fulviventer (20)	None	None	Negative	Neg	Positive	1/40 to 1/160

[a]Experiment number/species (number of animals).
[b]Signs of illness.
[c]Not weighted; weighed daily and no loss seen.
[d]10% of organ homogenates tested at sensitivity of 100 $TCID_{50}$.
[e]Immunohistochemical, neg = no viral antigen detected.
[f]Histopathological examination detected no lesions in *S. hispidus* and inconsistent mild lesions in *S. fulviventer* lung compared to controls.
[g]Serum collected on days 21–22 post-inoculation had neutralization titers as indicated.

blood samples taken on day 21 were negative for SARS-CoV antibody. No further experiments were performed with cotton rats from the UTMB colony because of the questionable health status of the colony.

Experiment 2

Of 15 commercially procured *S. hispidus* inoculated with 10^6 $TCID_{50}$ of SARS-CoV, none showed any overt signs of illness or lost weight (Table 1). Virus was recovered from nasal wash samples obtained from each of three animals on day 1, and from three of four animals on day 2. P.I. titers for day 1 animals were 2.5×10^3, 1×10^3 and 2.5×10^3, and for day 2, ranged from 1×10^2 to 2.5×10^4. None of the nasal wash samples were positive on days 4–10 P.I. Virus was also detected in the lungs of one of three animals on day 1 P.I. (titer of 1.5×10^2). All lung samples taken from four animals on days 2, 4, 7, and 10 were negative for virus. Virus was not detected in blood samples, nor was there any evidence of virus by the Vero cell assay or the IHC staining in tissues derived from the lung, heart, liver, spleen, and kidney. Specimens from controls animals, including the lung did not show any pathology, nor was there any evidence of viral specific staining in any of the tissues. Although virus was recovered from nasal wash samples and from the lung of one animal, it is likely that the low titers represented residual virus inoculum because of the failure to demonstrate detectable virus in any of the tissues. In addition, that the animals did not become infected was supported by the absence of SARS-CoV neutralizing in blood samples taken from five animals on day 22 P.I. These findings indicated that *S. hispidus* was not suitable as a model for SARS.

Experiment 3

As a pilot experiment to assess the susceptibility of *Sigmodon fulviventer* to SARS-CoV infection, 12 animals were inoculated IN with 10^6 $TCID_{50}$ of SARS-CoV, and two control animals were inoculated with PBS 10% FCS and antibiotics (Table 1). None of the animals developed signs of illness over a 21-day observation period. However, on day 21 P.I., blood samples from all 10 animals tested had detectable antibody that ranged from a titer of 1/80 to 1/360, and the two controls were negative for antibody. Thus, the positive serological data indicated that *S fulviventer* was susceptible to SARS-CoV infection. Therefore, a second experiment was performed to evaluate *S fulviventer* further.

Experiment 4

A total of 20 *S. fulviventer* were inoculated with 10^6 $TCID_{50}$ of SARS-CoV, and two control animals were inoculated with saline supplemented with 10% FCS and antibiotics (Table 1). Signs of illness were not observed for any of the animals and none lost weight during the experiment. Attempts to detect virus in the nasal wash and blood samples taken from each animal using Vero E6 tissue cultures were unsuccessful. Although the lung histology revealed a very mild interstitial pneumonia in four of six animals sacrificed on days 4 and 7 P.I., all lung tissue samples, as well as tissue samples derived from the heart, liver spleen and kidney of the animals were negative for virus by IHC staining and by assay in Vero cells. The reason for the mild pathology in infected animals is unknown since specimens from controls animals, including the lung, did not show either pathology or viral antigens in any of the tissues. However, blood from each of five animals taken on day 22 P.I. were positive for neutralizing antibody with the titers ranging from 1:40 to 1:160, and the samples from the control animals were antibody negative. These findings confirmed our previous observation that *S. fulviventer* appeared susceptible to infection with SARS-CoV infection but it must have been mild and transient

DISCUSSION

Efforts to identify an animal species for modeling human SARS disease have employed many of the common laboratory animals ranging from mice to non-human primates (Roberts et al. 2008). Some animals (e.g., the hamster, aged mice, transgenic mice bearing SARS-CoV hACE2 receptor, and mice infected with serially passaged adapted virus) have more re-

cently yielded promising results as disease models (Roberts et al. 2008, Tseng et al. 2007, McCray et al. 2007).

In our first experiment, neutralizing antibody was not detected in *S. hispidus* on day 21 post-exposure to 10^6 $TCID_{50}$ of SARS-CoV. Since the health status of these animals was suspect and there was no evidence of infection, no more experiments were performed with animals derived from the UTMB colony. Therefore, a second experiment was performed with commercially purchased *S. hispidus*. Likewise, neutralizing antibody was not detected on day 21 post-exposure to 10^6 $TCID_{50}$ of SARS-CoV, thus indicating that this species was not susceptible to infection using this dose of virus. In contrast, in two subsequent experiments, *S. fulviventer* became infected following exposure to the same dose of virus as indicated by the detection of antibody in animals on day 21 post-exposure to virus. Even though infection was demonstrated, none of the animals showed signs of illness, nor was there any conclusive evident of viral replication in selected tissues. The reason for the difference in susceptibility of the two species to SARS-CoV is unknown. The sex and age of the two species was the same. While the generation of the animals could have differed, the possible influence, if any, on susceptibility is unknown.

The failure of infected *S. fulviventer* to lose weight as possible evidence of clinical illness following exposure to 10^6 $TCID_{50}$ of SARS-CoV contradicted reported observations that lethally infected transgenic mice lost as much as 40% of body weight following infection with a similar dose of the same virus strain (Tseng et al. 2007). In addition, in our more recent studies to characterize other strains of transgenic mice that did not develop a fatal infection, weight loss was a consistent indicator of SARS-CoV infection (Tseng et al., 2007). Others have reported weight loss to be a clinical outcome of SARS-CoV in infected inbred mice (Roberts et al. 2008). Although weight loss is a reliable indicator of SARS-CoV infection in some mice, these results may not be applicable to cotton rats. For example, cotton rats are excellent models of respiratory viruses, such as respiratory syncytial virus (RSV) and human metapneumovirus (HMPV), but infection with these viruses does not result in a loss of weight. Thus, weight loss must be interpreted with caution because of the possibility of the response variation among mammalian species.

Although *S. fulviventer* became infected, evidence of SARS-CoV replication was not detected in selected tissues obtained during the first week following exposure to the virus. The detection of virus early after exposure was believed to reflect residual viral inoculum because of the sporadic pattern and low titers of the virus. However, low-level viral replication cannot be excluded because the detection level of the cell culture assay is about 100 TCID50. Nevertheless, because of the lack of any clinical signs of illness and the absence of any confirmed viral induced pathology in the tissues, *S. fulviventer* was not considered to warrant any further evaluations as a potential model for SARS-CoV infection. Overall, our efforts involving both *S. hispidus* and *S. fulviventer* did not yield a useful model for either infection or disease. This observation was supported by the absence of any signs of illness, the failure to demonstrate virus in the tissues consistently, and the absence of any confirmed virus induced histopathology. In addition, SARS-CoV antibody was not detected in *S. hispidus* as evidence that this species was not susceptible to infection. However, more recent data indicate that other strains of SARS-CoV, serially passaged virus, or the use of aged animals offer alternative approaches for providing better animal models to evaluate SARS-CoV vaccines and antiviral drugs (Roberts et al. 2008).

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Address reprint requests to:
Dr. Douglas M. Watts
University of Texas Medical Branch
Department of Pathology
301 University Boulevard
Galveston, TX 77555-0609

E-mail: dowatts@utmb.edu