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ORIGINAL ARTICLE

Pathogenesis and Chronologic Localization of the Human Influenza A (H1N1) Virus in Cotton Rats

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Abstract
Objectives: We aimed to evaluate the pathogenesis and chronologic localization of human influenza A (H1N1) virus in experimentally infected cotton rats.
Methods: The animals were intranasally inoculated with 10^7 plaque-forming units of A/Solomon Islands/3/2006 (H1N1) influenza virus and evaluated for pathogenicity for a period of 28 days. Virus replication kinetics and pathological properties were assessed chronologically. Acute antiviral responses were evaluated by mean of real-time polymerase chain reaction.
Results: Cotton rats infected with A/Solomon Islands/3/2006 virus lost weight until 6 days post-inoculation (DPI) and showed decreased activity until 3 DPI. At necropsy, focal areas of redness and consolidation of lungs were evident at 1, 2, and 3 DPI. Lung histopathology showed moderate to severe interstitial pneumonia, alveolitis and bronchiolitis. Influenza A specific viral protein was detected in bronchiolar epithelial cells, alveolar septa and pneumocytes. Influenza viruses were recovered from the lungs during the early period of infection and the titer peaked at 1 DPI. Viral proteins were detected from 4 hours to 6 hours DPI. These trends correlate with the up-regulation of mRNA expression of the IFN-α, Mx1, and Mx2 genes that play critical roles in the anti-influenza response at the early stage of infection.
Conclusion: Our results provide evidence that supports the use of cotton rats for the study of influenza virus pathogenesis and the immune response.

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1. Introduction

Influenza is a highly contagious infectious disease. Influenza viruses cause annual epidemics and occasional pandemics that have claimed the lives of millions. To develop new strategies for protection from and treatment of influenza infections, studies of influenza pathogenesis and immunity are needed.

Various animal models are available for the study of influenza virus pathogenesis, and for the development and evaluation of vaccines and antivirals. Non-human primates and mammals such as horses and pigs are natural hosts for influenza, and have been used in studies of influenza; however, their high cost, large size and the limited number of available reagents preclude their routine use in the laboratory [1]. Ferrets (*Mustela putorius furo*) are a widely used small animal model for studies of influenza virus pathogenicity. They are susceptible to natural infection with influenza and develop acute respiratory disease and pathological lung lesions similar to humans [2,3]. However, ferrets are outbred and, for the study of immunity, reagents are limited. In contrast, mice are inbred and there is an abundance of reagents to characterize their immune responses. Importantly, mice are not naturally infected with seasonal influenza viruses without prior adaptation.

The cotton rat (*Sigmodon hispidus*) has been proposed as an animal model to study the pathogenesis of respiratory viruses including influenza [4–8]. *S. hispidus* is inbred and susceptible to infection with unadapted human influenza strains, and heterosubtypic immunity to influenza can be invoked. With the development of species-specific reagents, the mechanisms of influenza pathogenesis and immunity have been investigated in cotton rats [4,9]. Previous studies have shown that cotton rats can be used as an animal model for influenza; however, a chronological study of influenza virus localization in cotton rats is lacking. In this study, we validated the use of cotton rats as an influenza virus animal model and characterized the pathogenesis and chronologic localization of human influenza A (H1N1) virus in the cotton rat by immunohistochemical analysis and virus titration.

Interferons (IFNs) are induced upon viral infection and they play a critical role in the first line of defense against invading pathogens. The myxovirus-resistance proteins (Mx), which belong to the superfamily of dynamin-like large GTPases, have been identified in a wide variety of vertebrates. They are mainly induced by type I IFNs, and exhibit antiviral activity [10]. The cDNAs for the cotton rat homologs of IFN-α, Mx1 and Mx2 genes were cloned and the mRNA expression levels of these genes were assessed during influenza virus infection by reverse transcriptase-polymerase chain reaction (RT-PCR) [9,11]. These approaches allow for only a semiquantitative analysis of mRNA expression by densitometry. Therefore, in the present study, we used a real-time quantitative RT-PCR assay to evaluate the mRNA expression of these genes in cotton rats infected with A (H1N1) influenza virus and investigated the relationship between the expression levels and viral replication and clearance.

2. Materials and Methods

2.1. Animals

Young adult (6–9 weeks old) cotton rats (*S. hispidus*) were obtained from an inbred colony maintained at Virion Systems, Inc. (Rockville, MD, USA). The animals were housed in standard polycarbonate rat cages and fed a diet of rodent chow and water. They weighed approximately 90 g at the start of the experiment. The animals were seronegative for adventitious respiratory viruses and other common rodent pathogens. All of the rats were serologically negative for currently circulating seasonal influenza viruses as determined by hemagglutination inhibition (HI) assays.

2.2. Virus

The A/Solomon Islands/3/2006 (A/Sol) strain of influenza A (H1N1) virus was grown in eggs on the 10^{th} day post-fertilization to a titer of 10^8 plaque-forming units (PFU)/mL. The virus was stored at −80°C and thawed immediately before use. The virus was not adapted to replicate in cotton rats by serial passages.

2.3. Experimental design

Four cotton rats were randomly allocated to each group, and baseline body temperature and body weight were measured before inoculation. Animals were anesthetized with isoflurane and then intranasally inoculated with 10^7 PFU of virus in a volume of 0.1 mL per 100 g body weight. Cotton rats inoculated with uninfected allantoic fluid (mock) were used as controls for the experiments. Cotton rats were observed daily for mortality, weight loss and body temperature change. They were sacrificed by CO_2 asphyxiation at designated times (4 hours post-inoculation (HPI) and 1, 2, 3, 4, 5, 6, 7, 14, 21 and 28 days post-inoculation (DPI)). To evaluate the serum antibody titer, cotton rats were bled from the retro-orbital venous plexus under anesthesia. To determine the replication and systemic spread of the virus, four infected and two mock-infected cotton rats were euthanized at designated times. At necropsy, tissue samples were collected aseptically, immediately frozen on dry ice and stored at −80°C until use. All experimental animal work was carried out in BSL2+ animal facilities, following approval of the animal safety protocols by the Institutional Animal Care and Use Committee.

2.3.1. Virus titration in tissues

For the virus titration, tissue samples were thawed and homogenized in 1 mL of cold phosphate buffered

saline (PBS) supplemented with penicillin and streptomycin and clarified by centrifugation (2500 g) at 4°C. Virus quantities in the lungs, nose, brain, spleen, kidneys, liver and heart were measured by plaque assays in Madin-Darby Canine Kidney cells. The lowest level that this assay could reliably detect was 1.7 $\log_{10}$ PFU.

2.3.2. HI assays

Pre- and post-exposure sera were collected at designated times from cotton rats and H1-specific antibodies were evaluated by HI assays using 0.75% guinea pig red blood cells (RBCs). Serum samples were treated with a receptor-destroying enzyme (RDE) from *Vibrio cholerae* (Denka Seiken Co., Ltd., Tokyo, Japan). Briefly, one part serum was diluted with three parts enzyme and the mixture was incubated overnight at 37°C. After heating at 56°C for 30 minutes the samples were diluted 1:10 by the addition of six volumes of PBS. The HI assay was performed in 96-well polystyrene plates by two-fold serial dilutions of sera in PBS followed by the addition of 4 HA units of inactivated A/Sol virus to each well. After 30 minutes incubation at room temperature, 0.75% guinea pig RBCs were added to each well and the mixture was incubated for 60 minutes. HI titers were determined by analysis of the RBC pellets at the bottom of the wells.

2.3.3. Histopathological analysis of cotton rat lungs

For the histopathological analysis, tissues from four cotton rats were collected and fixed in 10% neutral-buffered formaldehyde. The tissues were routinely processed and embedded in paraffin wax. They were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for light microscopy. Lungs were evaluated for four indicators of pulmonary inflammatory changes: peribronchiolitis (inflammatory cells surrounding a bronchiole), epithelial injury (death and detachment of bronchiolar epithelial cells), interstitial pneumonia (thickening of alveolar walls due to inflammatory cell infiltration) and alveolitis (inflammatory cells within alveolar spaces). Slides were scored blind on a 0−3 severity scale, with validation of the scoring performed by two pathologists as previously described [7,12].

2.3.4. Immunohistochemical detection of influenza antigen

The expression of influenza virus antigens was evaluated by immunohistochemistry. Cotton rat tissues were deparaffinized with xylene and rehydrated through graded alcohols. Endogenous alkaline phosphatase was quenched with 20% glacial acetic acid solution for 10 minutes at room temperature. All slides were treated with 100 μg/mL of proteinase K (Gibco BRL, Grand Island, NY, USA) for 20 minutes at 37°C and then incubated with normal goat serum for 15 minutes at room temperature to saturate non-specific protein binding sites. A monoclonal antibody (Novus Biologicals, Inc., Littleton, CO, USA) against the nucleoprotein of influenza A virus was applied and the slides were incubated for 60 minutes at 37°C in a humidity chamber. After three washes with 0.1% Tween 20 in PBS (PBST), sections were flooded and incubated for 60 minutes at 37°C with biotinylated goat anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1:250 in PBST. After three washes with 0.1% PBST, the sections were incubated with alkaline phosphatase-conjugated Streptavidin (Merck KGaA, Darmstadt, Germany) diluted 1:250 in PBST for 25 minutes at 37°C. They were then equilibrated with Tris buffer (pH 8.2) for 5 minutes at room temperature. The colorimetric reaction was produced by immersing the sections in a solution of Fast Red (Roche Diagnostics, Mannheim, Germany) for 10 minutes at room temperature. The sections were lightly counterstained with Mayer's hematoxylin.

2.4. Expression of IFN-α and Mx genes in cotton rat lungs

To evaluate the chronologic change in expression of the IFN-α, Mx1 and Mx2 mRNAs in influenza A (H1N1) virus infected cotton rats, relative quantitative real-time RT-PCR was performed in triplicate. The primer sequences and the GenBank IDs of the target genes are summarized in Table 1.

Total RNA was extracted from lung tissue homogenates using MagNA Pure LC (Roche) according to the manufacturer's instructions. The RNA was used as a template for the preparation of cDNA by reverse

Table 1. Nucleotide sequences of the primers used in real-time polymerase chain reaction

Target gene	Primer name	Primer sequence (5′−3′)	Amplicon length (bp)	GenBank ID
IFN-α	IFN-α-F	CCCAGCAGCTCCAGAAGG	228	AF421386
	IFN-α-R	TCACAGTCCCCAGCGAGTC		
Mx1	Mx1-F	CCGGGAGCTGCCAGATTTTGT	186	DQ218274
	Mx1-R	GACTTGGCAGCACTGGAGAGGTTA		
Mx2	Mx2-F	GCGGGTTGCCTTTTCCAGTGTA	188	DQ218273
	Mx2-R	GCGAGGCTCCCATGTAAAGTTCTG		
β-actin	β-actin-F	CTGGCTGGCCGGGACCTGAC	225	AF421789
	β-actin-R	TCGCTCGTTGCCAATAGTGATGAC		

IFN = interferons; Mx = myxovirus-resistance proteins.

transcription using random primers and SuperScript III RT enzyme (Invitrogen, CA, USA). PCR amplification and analysis were achieved using an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA, USA) with software version 2.3. All reactions were performed with the SYBR Green PCR Master Mix (Applied Biosystems) using a 10 μL reaction volume in 384-well plates. For quantification, 2 μL cDNA was added to each well before the plate was sealed, centrifuged and placed in the PCR machine. Amplification conditions consisted of an initial pre-incubation at 95°C for 10 minutes, followed by amplification of the target DNA for 40 cycles (95°C for 15 seconds and 60°C for 1 minute). Real-time PCR reactions were analyzed and the results were normalized to the housekeeping gene beta (β)-actin mRNA level in each sample. A no-template control without genetic material was included to assess possible contamination and non-specific reactions.

3. Results

3.1. Clinical evaluation of influenza virus-infected cotton rats

Cotton rats were observed on a daily basis to assess their body weight and temperature changes, and to record the presence of clinical signs of infection. A/Sol-infected cotton rats showed ruffled hair and decreased activity from 1 DPI to 3 DPI. All of the cotton rats lost a small amount of weight for 6 days following infection; their weights normalized by 10 days after infection (Figure 1A). Body temperature dropped at 1 and 2 DPI and then returned to normal baseline measures (Figure 1B). The mean maximum weight loss was 5.0% at 6 DPI and the mean peak drop in body temperature was 1.3°C at 2 DPI.

3.2. Gross lung lesions and lung histopathology

At necropsy, marked congestion was observed at 4 HPI and continued until 3 DPI. The lung lesions were predominantly observed in the dorsal portion of the cranial and middle lobes and were characterized by multifocal tan-mottled areas with irregular and indistinct borders. No gross lesions were observed in any other organs. For the histopathological analysis, all tissues were processed routinely and stained with H&E. At 1 DPI, the bronchiolar lumens were filled with many mononuclear cells, a small number of neutrophils, and sloughing epithelial cells (Figure 2B). Peak epithelial damage was evident at 1 DPI. Lesions at 3 and 4 DPI were similar, but more severe than those observed at 1 DPI. Prominent alveolar thickening with extensive inflammatory infiltrates and alveolitis were detected at 3

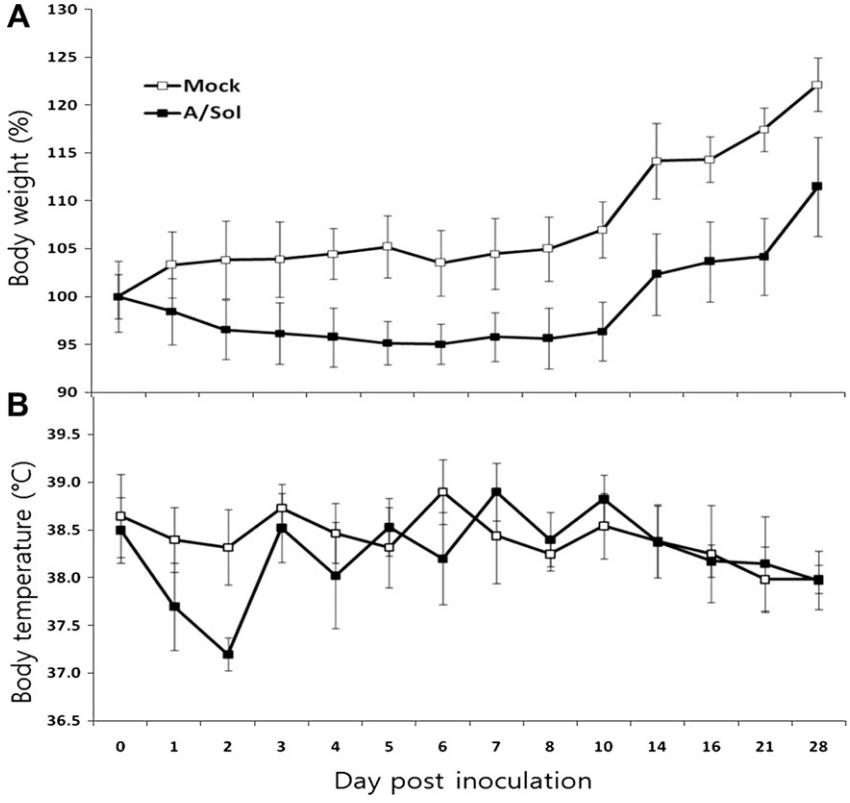

Figure 1. Body weight and body temperature changes in cotton rats inoculated with A/Solomon Islands/3/2007 virus. Four animals in each group were inoculated intranasally with 10^7 PFU of virus (A/Sol) and allantoic fluid (mock) and (A) body weights and (B) body temperatures were monitored.

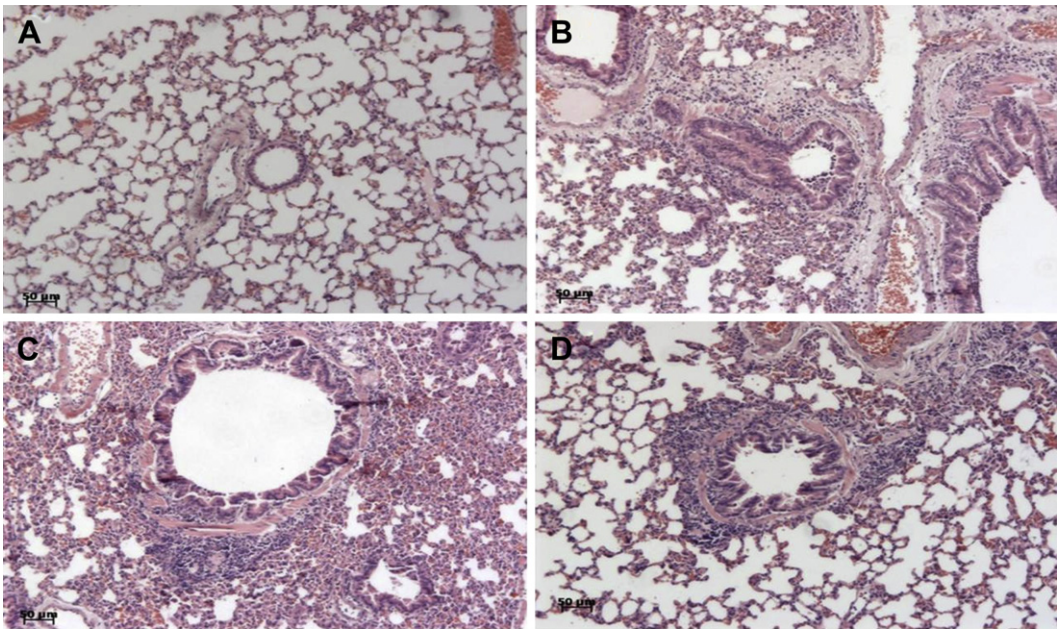

Figure 2. Representative histopathologic results in tissues from cotton rats inoculated with the A/Solomon Islands/3/2006 virus. Formalin-fixed lung sections were stained with hematoxylin and eosin (H&E). (A) Mock-infected cotton rat lung at 3 DPI. (B) Lung tissue at 1 DPI shows moderate peribronchiolar and interstitial inflammation that infiltrates the bronchiolar lumen. (C) Lung tissue at 3 DPI shows marked peribronchiolar, alveolar and interstitial inflammation. (D) Lung tissue at 7 DPI shows marked peribronchiolitis and resolution of alveoli. Sections are all at 100× magnification.

and 4 DPI (Figure 2C). Peribronchiolitis was most prominent at 3 DPI and was also detected at 28 DPI (Figure 2D). At 14 DPI, microscopic lesions had resolved. Interlobular septa were slightly distended by small numbers of neutrophils and mononuclear cells. Pathological scores for these findings are shown in Figure 3.

3.3. Immunohistochemical detection of influenza virus antigen

When influenza A viral antigen expression was investigated, bronchiolar epithelial cells and desquamated cells in the bronchiolar lumen were found to be infected by A/Sol virus. Pneumocytes and cells in the thickened alveolar wall were stained with an influenza

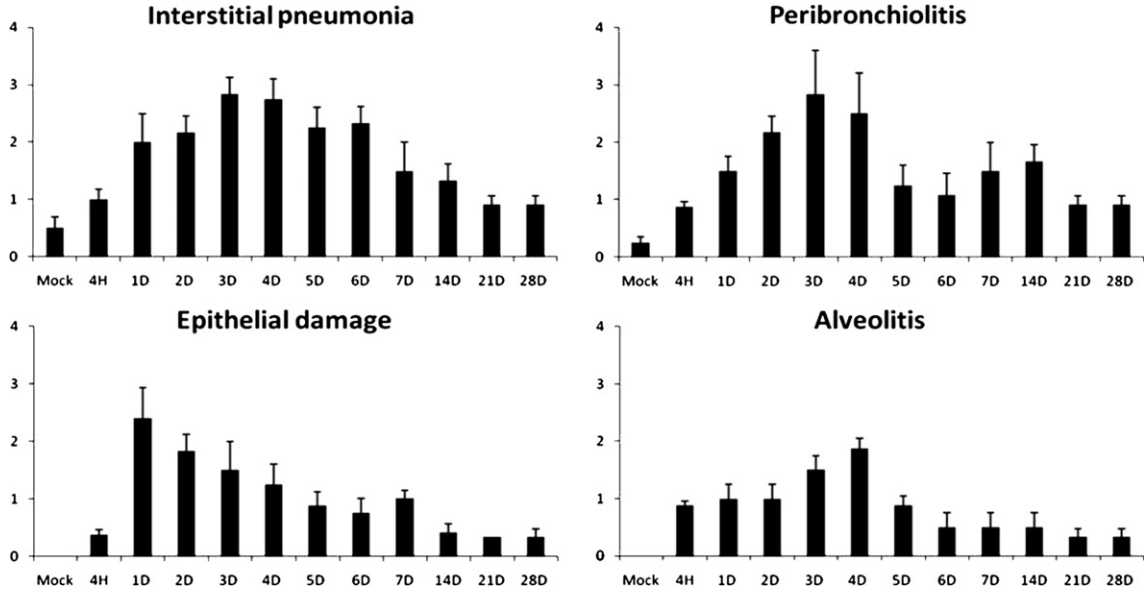

Figure 3. Quantification of histopathologic changes in the lungs of A/Solomon Islands/3/2006 inoculated cotton rats. The changes were scored on a scale of 0 (normal) to 3 (severe) depending on the severity of lesions. Each bar denotes the mean pathology score ± SD for four animals.

A specific nucleoprotein antibody (Figure 4). A strong immunohistochemical signal was observed in the nucleus of bronchiolar epithelial cells and pneumocytes, but rarely in the cytoplasm. The viral protein was detected in the lungs as early as 4 HPI and was detected up to 6 DPI; it was not found after this. From 1 DPI to 3 DPI, the positive signal was very strong, and it was extensively detected in cotton rat lungs (Figure 4C); after 3 DPI the intensity of the positive signal gradually reduced. Influenza A virus-specific antigen was confined to the respiratory tract and was not detected in any other extrapulmonary tissues or in the mock-infected cotton rat tissues.

3.4. Replication and clearance of influenza virus from cotton rat organs

Following the intranasal inoculation of cotton rats with 10^7 PFU of A/Sol virus, replication and clearance of the virus in the respiratory tracts were evaluated. Four animals were sacrificed for virus titration at each time point starting at 4 HPI. A high titer of influenza A (H1N1) virus was detected in the lungs of cotton rats at 4 HPI ($10^{5.3}$ PFU/g). The titer rose to $10^{6.7}$ PFU/g at 1 DPI and dropped to $10^{4.3}$ PFU/g at 2 DPI and to $10^{2.4}$ PFU/g at 3 DPI. At 4 DPI, a low titer of the virus was detected in only one of the four animals. Virus replication in the lung peaked on the 1st day of infection and rapidly dropped to undetectable levels by 4 DPI. At 1 DPI, $10^{4.4}$ PFU/g of virus was detected in the nose; however, virus replication in the nose was not observed at any other time (Table 2). No virus was detected in the liver, kidney, spleen, heart or brain tissues of A/Sol-infected cotton rats.

3.5. Serological analysis

For the evaluation of serum neutralizing antibodies against A/Sol virus, we collected sera daily by the 7th day after infection and thereafter at weekly intervals for 28 days. HI assays showed that antibody titers were elevated to 1:640 to 1:1,280 at 21 and 28 DPI. The antibody was first detected at 7 DPI (1:40). Mock-infected cotton rats had undetectable titers (<1:10) during the whole experimental period (Figure 5).

3.6. Expression of the mRNA of IFN-α and Mx genes in cotton rat lungs

We examined host gene expression for 28 days during the course of influenza A (H1N1) virus infection. For quality assessment of the real-time PCR assay, the housekeeping β-actin and target gene cDNA amplification products were separated in agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen). Duplicate amplification products corresponding to the β-actin control gene and to the IFN-α, Mx1, and Mx2 genes were visualized at 225, 228, 186, and 188 bp, respectively (data not shown). The expression of the Mx1 and Mx2 mRNAs was highly up-regulated in the early period of infection and peaked at 2 DPI. The two Mx

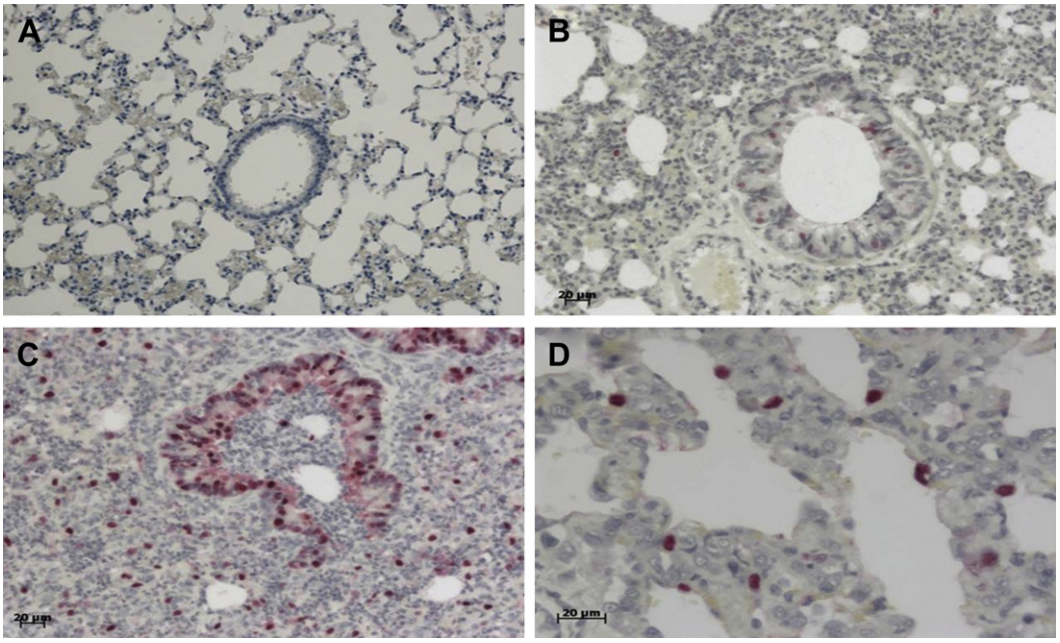

Figure 4. Representative immunohistochemical results in tissues from cotton rats inoculated with A/Solomon Islands/3/2006 virus. Formalin-fixed lung sections were processed for hematoxylin and eosin (H&E) and immunohistochemical staining. (A) Mock-infected cotton rat lung tissue at 1 DPI shows no positive signal. (B) Viral antigen was detected in the bronchial epithelial cells at 4 HPI. (C) Extensive viral antigen was detected in the bronchial epithelial cells and cells in the interstitium at 1 DPI. (D) Viral antigen was detected in pneumocytes and interstitial cells in the thickened alveolar wall at 3 DPI. Sections (A)–(C) are at ×200 magnification; (D) is at 400× magnification.

Table 2. Viral titers in the lung and nose of cotton rats inoculated with A/Solomon Islands/3/2006 virus

	Viral titer (mean $\log_{10}$ PFU ± SD/ml)[a]						
	Day post-inoculation						
Organ	4 hr	1	2	3	4	5	6–28
Lung	5.3 ± 0.1	6.7 ± 0.1	4.3 ± 0.2	2.4 ± 0.5	4.4	–[b]	–
Nose	–	4.4 ± 0.1	–	–	–	–	–

[a]*For viral titration, four cotton rats were euthanized at the designated times. When viruses were recovered from all three rats, mean titers are presented. When viruses were not recovered from all three animals, individual titers are shown;* [b]*Virus not detected (detection limit: 1.7 $\log_{10}$ PFU/g of tissue).*
PFU = plaque-forming units; SD = standard deviation.

genes were expressed at low levels after 3 DPI (Figure 6). Expression of the IFN-α mRNA was detected in the lung tissue of infected cotton rats from 4 HPI to 28 DPI. Increased IFN-α mRNA expression was detected from 4 HPI to 5 DPI with a peak at 1 DPI. The β-actin gene was used as a control gene, because it is not involved in the regulation of cytokine expression. The mRNA expression of lung tissue β-actin was similar for both A/Sol and mock-infected control cotton rats, indicating that the observed changes in the cytokine mRNA levels were likely related to changes in the expression of the cytokines.

4. Discussion

Cotton rats are small, inbred, easy to handle, and relatively inexpensive to maintain. They have been suggested as an animal model for the study of human influenza viruses without the need for prior adaptation [4,7,13]. Influenza virus-infected cotton rats show signs of illness, such as an increased breathing rate and weight loss. The virus replicates in the upper and lower respiratory tract, where a robust cytokine response was observed [7]. The objectives of this study were to confirm that the cotton rat can be used as an influenza virus animal model and to characterize the pathogenesis and chronological localization of human influenza A (H1N1) virus in cotton rats. Our results demonstrate that cotton rats are susceptible to human influenza A virus infection and develop illness. Animals infected with A/Sol virus showed a drop in body temperature at 1 and 2 DPI and a slight weight loss during the 6 days after infection. Infected animals developed gross and microscopic pulmonary lesions without prior host adaptation of the virus. Bronchiolar epithelium is a primary target tissue of influenza virus, and infection of lung tissue is characterized by peribronchiolitis, epithelial necrosis, moderate to severe interstitial pneumonia and alveolitis. In a previous study, cotton rats infected with 10^7 $TCID_{50}$ of A/Wuhan/359/95 (H3N2) virus showed viral replication in the lungs with a peak titer within the 1st day of

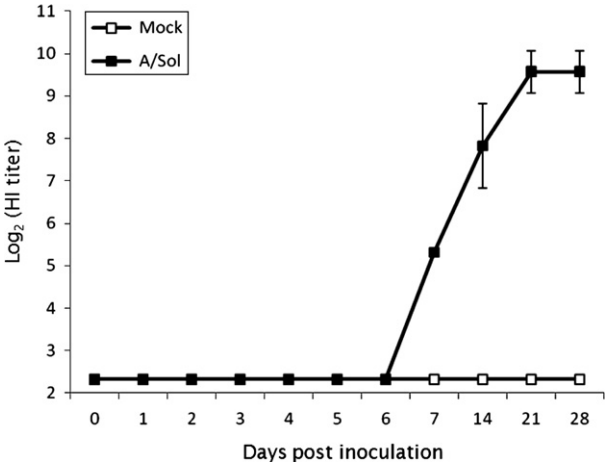

Figure 5. Serum antibody titers of cotton rats inoculated with Mock or A/Solomon Islands/3/2006 virus. Sera were taken from four cotton rats at designated time from 1 to 28 DPI. Antibody titers were determined by hemagglutination inhibition (HI) assays using 0.75% guinea pig erythrocytes. Titers are shown as $\log_2$ (HI titer) ± SD values.

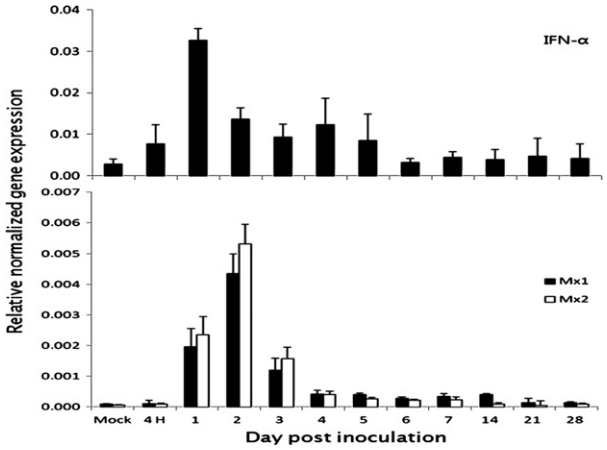

Figure 6. Quantification of the m RNA expression of the IFN-α and Mx genes in A/Solomon Islands/3/2006 infected cotton rat lung tissue. The expression of the IFN-α and Mx1 and Mx2 genes was evaluated chronologically after infection with the virus. Real-time PCR expression levels were normalized using β-actin gene expression as a control. The expression levels are shown as Mean ± SD of the $-\Delta\Delta C_T$ value from three replicates. IFN = interferons; Mx = myxovirus-resistance proteins.

infection and a rapid drop in titer to undetectable levels by 3 DPI. In the present study, influenza virus replicated in cotton rat lungs with a peak titer at 1 DPI and a subsequent decrease to undetectable level after 4 DPI (Table 2). Our results support the previous kinetic study of virus replication in cotton rat lungs.

As far as we know, our study is the first to reveal the site of virus replication and the chronological expression pattern of an influenza viral protein in the cotton rat. Using immunohistochemical analysis, we detected influenza virus antigen in the lungs of the cotton rat from 4 HPI to 6 DPI. The major replication sites were bronchiolar epithelial cells, inflammatory cells in the thickened alveolar septa and pneumocytes. The intensity of virus replication and viral protein expression were concordant with a peak viral titer at 1 DPI.

Expression of the Mx proteins is mainly regulated by IFN-α/β (type I IFNs) [14,15]. Two cotton rat Mx genes have been cloned. They encode nuclear Mx1 and cytoplasmic Mx2 proteins. Induction of these two genes was demonstrated in the lungs of influenza virus-infected cotton rats. The present study revealed a clear correlation between the mRNA expression levels of the IFN-α and Mx genes. Expression of the IFN-α and Mx genes was markedly up-regulated at the mRNA level in lung tissue from cotton rats that were experimentally infected with human influenza A (H1N1) virus. The mRNA levels of the IFN-α and Mx genes coincided with virus replication, suggesting that the early host response effectively controls influenza virus replication in this model. Laboratory mouse strains lack Mx gene expression [16]. Therefore, influenza virus replication in the lungs of mice is prolonged compared with its replication in ferrets, cotton rats and humans. Our data indicate that Mx1 and Mx2 were up-regulated at the early stage of infection. However, only nuclear Mx1 is known to inhibit the replication of influenza virus. In a previous study, Mx2 exhibited antiviral activity against the bunyavirus (Rift Valley fever virus) and the rhabdovirus (vesicular stomatitis virus) that is known to replicate in the cytoplasm of infected cells [17]. Influenza A virus antigen was primarily detected in the nucleus of the infected cells of cotton rat lungs, it replicated during the early period of infection and cleared at 3 DPI. Expression of the Mx1 gene peaked at 2 DPI. Mx1 might inhibit replication of the virus in the nucleus and contribute to viral clearance from the lung. In conclusion, our results confirm that cotton rats can be used for the study of influenza virus pathogenesis and the immune response.

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